QUANTITATION OF GROUP A ROTAVIRUS BY REAL TIME POLYMERASE CHAIN REACTION IN CHILDREN WITH VARYING SEVERITY OF GASTROENTERITIS

DISSERTATION SUBMITTED AS PART OF FULFILMENT FOR THE M.D. (BRANCH-IV MICROBIOLOGY) DEGREE EXAMINATION OF THE TAMILNADU DR.M.G.R.MEDICAL UNIVERSITY, TO BE HELD IN APRIL 2014

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CERTIFICATE

This is to certify that the dissertation entitled, 'Quantitation of group A rotavirus by real time polymerase chain reaction in children with varying severity of gastroenteritis' is the bonafide work of Dr. Susmitha Karunasree Perumalla towards the M.D (Branch - IV Microbiology) Degree examination of the Tamil Nadu Dr.M.G.R Medical University, to be conducted in April 2014.

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INTRODUCTION
Rotaviral gastroenteritis is one of the leading causes of mortality and morbidity in Indian children below 5 years of age. India spends about 2 to 3.4 billion rupees annually on the treatment of these cases (1). One in approximately every 260 children below five years of age die of rotavirus diarrhea every year (2).
Faeco oral route is the most important mode of transmission and hence improvement in sanitation and general hygiene should ideally prevent transmission. However, even in the developed countries, this could not be achieved. It is difficult to prevent feco-orally transmitted diseases in developing countries without multiple preventive measures, including vaccination. The World Health Organization (WHO) has
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INTRODUCTION

Rotaviral gastroenteritis is one of the leading causes of mortality and morbidity in Indian children below 5 years of age. India spends about 2 to 3.4 billion rupees annually on the treatment of these cases (1). One in approximately every 260 children below five years of age die of rotavirus diarrhea every year (2).

Faeco oral route is the most important mode of transmission and hence improvement in sanitation and general hygiene should ideally prevent transmission. However, even in the developed countries, this could not be achieved. It is difficult to prevent feco-orally transmitted diseases in developing countries without multiple preventive measures, including vaccination. The World Health Organization (WHO) has recommended incorporation of vaccines against rotavirus diarrhea in the national immunization schedules of developing countries with high diarrheal mortality (3). Though vaccines are available, they have to be in a reasonable price range to be affordable for the poorer countries which have the majority of the mortality and severe disease burden. To cater to this need in India, various indigenous vaccines are being developed.

Rotavirus is a double stranded RNA virus belonging to the family Reoviridae. The genome is segmented. The virus is a triple layered particle with outer, middle and inner layers. Different serogroups are identified based on the antigenic specificity of the middle layer of the virus and electrophoretic mobility of the 11 RNA segments of the viral genome. Out of the seven different serogroups (A – G) identified, groups A, B and C are associated with humans and group A viruses are most commonly

associated with severe life threatening disease worldwide (1). Among the serogroups, there are genotypes which are designated G and P types based on the antigenic determinants of the outer layer. Distribution of the genotypes varies from place to place and more than one genotype may exist in a given geographical region.

Gastroenteritis with its causes, pathophysiology and clinical features is a well defined entity. The choice of variables in objectively defining the severity of gastroenteritis are varied. Defining severity objectively helps in assessment of the effect of therapeutic and preventive intervention. In assessing severe disease, a number of scoring systems have been developed, based on objective signs and symptoms reported by mothers. The most widely used scoring system for clinical assessment of severity is the 20 point Vesikari scoring system (4) with seven different components determining if an individual has mild, moderate, severe or very severe gastroenteritis.

Rapid development of molecular techniques helped not only in detection but also in quantitation of the virus. Virus quantitation in stool is a marker for viral replication since gut is the primary site of viral replication. Real-time polymerase chain reaction (RT-PCR) is a simple, very sensitive and less time consuming test in comparison to conventional PCR assays in detecting very small amounts of nucleic acid with less chance for cross contamination. It enables us to view the increase in the amount of DNA as it is amplified in real time. It has been estimated that real-time PCR resulted in 186% increase in detection of the virus as compared to electron microscopy (5).

Quantitation using real time polymerase chain reaction, has helped in studying the associations of viral load with various aspects of pathogenesis and outcome of infection. In a previous study on rotavirus infection and gastroenteritis, it was found

that children with more severe diarrhea had greater viral loads (6). Although any gene of the virus could be amplified for quantitation, generally if the purpose is to distinguish between related viruses, a variable region of the genome is used and if the purpose is to quantify the amount of virus, a conserved region is used for amplification. Among the relatively conserved viral genes, VP6 is a suitable target for amplification and encodes the protein used for grouping rotaviruses. VP6 is a structural protein of rotavirus which forms the middle coat of the triple layered particle. It is highly antigenic and is employed in several commercial kits available for detection of rotavirus through enzyme immune assays.

This study estimated the viral load in stool samples from children with gastroenteritis in Vellore by quantitative real time reverse transcriptase PCR of the VP6 gene using Taqman probes.

AIM OF THE STUDY

To study the correlation between Group A rotavirus load and severity of gastroenteritis.

OBJECTIVES OF THE STUDY

To correlate viral load by real-time PCR in children with mild, moderate and severe rotavirus gastroenteritis assessed by the 20 point Vesikari scoring for rotavirus diarrhea.

Hypothesis

Clinical severity of rotavirus gastroenteritis will correlate with the amount of virus excreted.

JUSTIFICATION FOR THE STUDY

The study was conducted to examine viral excretion from children with rotaviral gastroenteritis in stool samples with defined Vesikari scores. Vaccines are being developed indigenously and there will be a need to test them for their ability to prevent severe rotavirus diarrhoea. In order to provide an additional capacity to test severe disease, we intended to estimate the range of viral excretion in children with Vesikari scores for mild (score 1-5), moderate (score 6-10), severe (score 11-15) and if possible, very severe disease (score 16-20). With the ongoing clinical trials on vaccines, estimation of viral load in stools excreted will be an important tool in studying vaccine efficacy.

REVIEW OF LITERATURE

1. ROTAVIRUS DISEASE BURDEN

1.1 Global scenario

Globally, diarrhoea is the second leading cause of death among children under five years of age (7,8). One of every nine deaths in this age group is because of diarrhea (7). Worldwide statistics in 2008 reveal that approximately 4,53,000 deaths due to diarrhea in the under five age group are because of rotavirus infection i.e. about 37 percent of all diarrheal deaths and five percent of all deaths in the <5year age group are because of rotavirus diarrhea (2). When categorised into mild, moderate and severe diarrhea based on the care to be given, i.e. home care, hospital/ clinic visit and hospital admission respectively, rotavirus diarrhea has been estimated to result in approximately 111 million episodes requiring home care, 25 million cases requiring out-patient visits to a hospital and 2 million cases requiring hospitalization in the under five age category (9).

By the time children reach 5 years of age, almost all of them would have an episode of rotaviral diarrhea. One in five children will visit the out-patient clinic and one in 60 children will be admitted to a hospital (9). About 98% of rotavirus diarrheal deaths occur in the developing countries. The five major countries contributing to more than half of rotavirus diarrheal deaths are Ethiopia, Democratic Republic of Congo, Nigeria, India and Pakistan (10). Nearly 95% of all children will have evidence of prior infection (11).

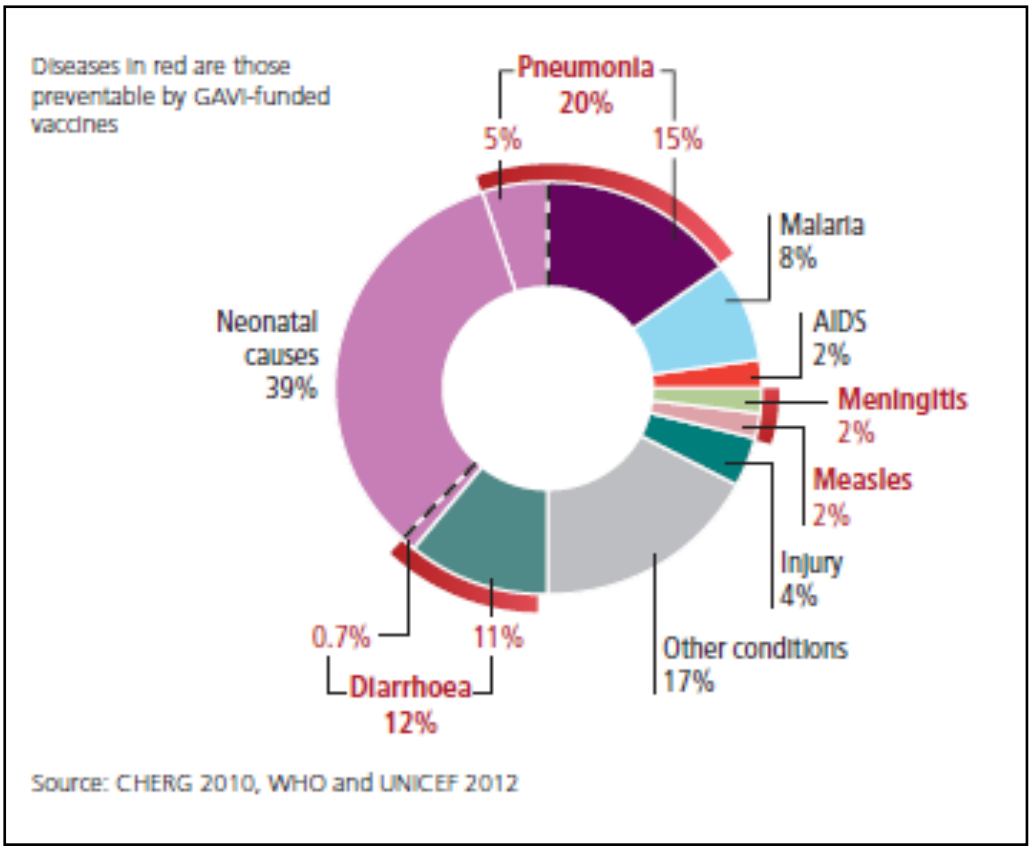


Figure 1. Causes of death in children in the <5 year age group

1.2 Disease burden: India

An estimate of disease burden revealed that about 122,000-153,000 rotavirus diarrhea deaths are from India (1). In other words, India contributes to over 20% of the rotavirus diarrheal deaths globally (12). In addition, 457,000–884,000 hospital admissions, and over 2 million out-patient consultations in children <5 years of age occur annually in India because of rotaviral diarrhea (1). This poses a heavy economic burden on a developing nation.

1.3 Economic burden

The total medical costs can be broadly divided into direct and indirect costs. Direct costs are direct medical expenses incurred which pertain to hospitalization and treatment. Direct non-medical costs pertain to transportation to and from the hospital, amount spent by the family to stay with the sick child at the hospital, etc. Indirect costs include loss of wages incurred by the parents,etc. The expenditure incurred differs with the level of care i.e. primary, secondary or tertiary level hospitals.

Table 1. Economic burden of rotaviral diarrhea in children < 5 years of age in Vellore, India (13)

Level of care	Direct medical cost per hospitalization	Direct non medical costs per hospitalization
Primary	Rs. 1833.28	Rs. 45.72
Secondary	Rs. 4289.56	Rs. 332.46
Tertiary	Rs. 3530.81	Rs. 46.20

Thus the burden of direct medical costs due to rotaviral gastroenteritis in India in children <5 years of age ranges from Rs. 1.8 to 3.2 billion annually. The indirect costs account for a 64 million rupees in the outpatient visit setting (1). India spends Rs. 71.93 – 126.17 per child per year for treating rotaviral gastroenteritis (1).

2. ROTAVIRUS: HISTORY

In 1963, Adams and Kraft, using the electron microscope described murine rotavirus as the causative agent of epizoonotic diarrhea in infant mice (EDIM) (14). Following this, rotaviruses were also isolated from many other animals with gastroenteritis.

In 1973, viral particles were seen by electron microscopy of biopsies from duodenal specimens from children with gastroenteritis. They were about 70nm in diameter (15). Thereafter, they were observed in stool samples of children with diarrhea (16–18). Because of their morphological appearance, they were designated Rotaviruses (in Latin where 'rota' means 'wheel'). They were soon identified as causative agents in gastroenteritis in children worldwide.

2.1. REOVIRIDAE FAMILY

Rotavirus belongs to Reoviridae family. The Reoviridae family comprises of nine genera. The viruses exhibit a wide host range, which include plants, invertebrates and vertebrate hosts. The most common genera that are important in human diseases are Orthoreovirus, Orbivirus, Rotavirus, Coltivirus, and the Seadornavirus. Their genome is made up of double stranded RNA surrounded by a non-enveloped capsid. The size of the virions is approximately 60 to 80 nm and the RNA exists in 10 to 12 segments, which can reassort giving rise to novel viruses.

2.2. STRUCTURE OF ROTAVIRUS

The virus is 70 nm in size excluding the spikes. Unique features of this virus are a segmented genome and the presence of RNA dependent RNA polymerase. The protein capsid is icosahedral in symmetry and is comprised of three layers; an outer, intermediate and an inner layer. Spikes made of protein project from the surface of the outer layer. With the spikes, it is about 100 nm in size. The RNA is double stranded and exists as 11 segments. The virus is a triple layered particle (TLP). The three layers consist of an inner layer, a middle coat and an outer capsid. These three layers enclose the tightly packed segmented double stranded RNA along with the VP1 polymerase and VP3 capping enzyme complexes. Each gene segment has atleast one open reading frame and encode the structural and non structural proteins. All gene segments except segment 11 are monocistronic. The innermost layer is a protein shell made up of 120 copies of VP2 forming an icosahedral lattice.

The middle layer consists of 780 copies of VP6. This layer exists as trimeric pillars in an icosahedral lattice. Though it is not exposed on the viral surface, VP6 is the main target for the majority of antibodies elicited in rotavirus infection. Its function is to coordinate the entry into the cell through interactions with the outer proteins VP7 and VP4. The outer most layer is a thin glycoprotein layer formed by 780 copies of VP7. Sixty spikes made up of VP4 project from the surface of the outer layer of the virion. Aqueous pores perforate the middle and outer layers. The structural proteins of the outer layer, VP4 and VP7 elicit neutralizing antibodies. VP4 gets cleaved into VP8* and VP5* which enhances cell infectivity and penetration into the host cell. VP7 mediates calcium dependant uncoating of the virus and along with VP4 (19), help in

translocating the double layered sub viral particle across the host cell membrane into the cytoplasm (20).

The virus is hardy and is not easily inactivated by treatment with various organic and inorganic chemicals like ether, fluorocarbons, and chlorine at concentrations used for sewage effluent and potable water treatment. They survive for weeks in drinking and recreational waters. They have the ability to retain their infectivity for several days on inanimate surfaces under favourable conditions of humidity (21). They are inactivated by antiseptics containing relatively higher alcohol concentrations(>40%), >20000 ppm of free chlorine or iodophores with >10000 ppm iodine and calcium chelators (22).

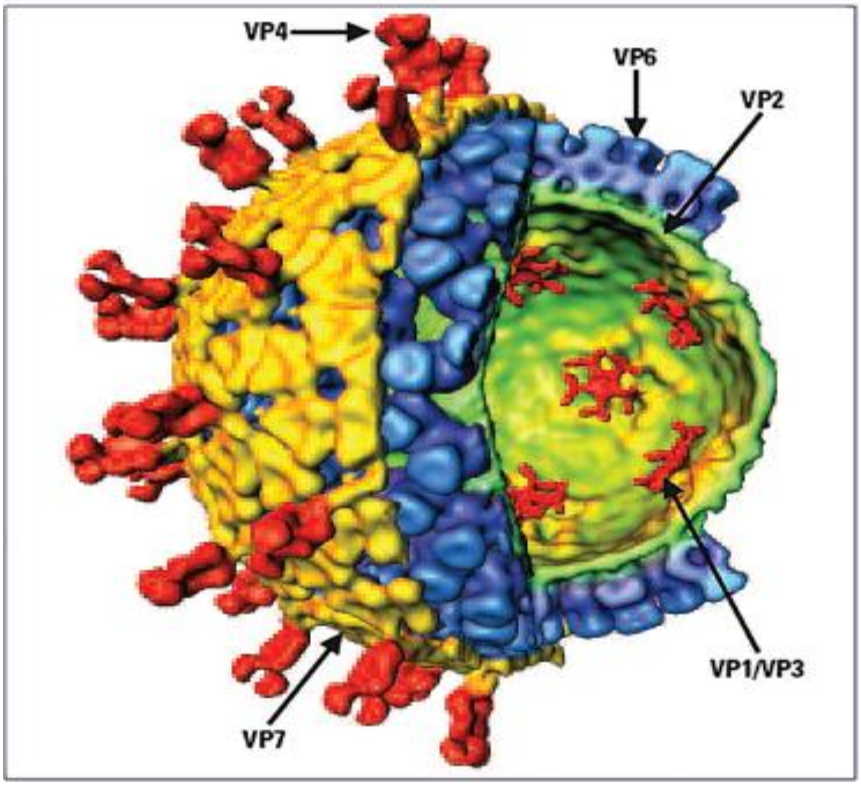


Figure 2. Structure of Rotavirus

2.3. PATHOGENESIS

The most common route of transmission of rotavirus is the faeco-oral route. The manifestation of clinical infection depend on the viral and host factors. The single most important host factor affecting the outcome of infection is the age of the individual. Thus early neonatal period is spared owing to the transplacentally acquired maternal antibodies. Adults rarely present with life threatening diarrhea unless it is caused by an unusual strain or with a very high infective dose.

Virus factors involve the proteins encoded by various genes. The genes that are shown to be associated with virulence are 3, 4, 5, 9 and 10. Gene 10 encodes the non structural protein 4 which functions as an enterotoxin. It also regulates viral replication and calcium balance in the enterocyte environment. As compared to bacterial diarrhea, virus induced diarrhea shows little inflammation. Once ingested, the virus not destroyed by the stomach acidity gets attached to the small intestinal epithelial cells. An interaction between the virus and a series of sialylated and non sialylated receptor molecules on the host surface brings about binding of the virus to the host cell. Studies in animal models show that intestinal villous epithelium is the site of attachment whereas the crypt epithelium is relatively spared (23).

The pathophysiology is multi-factorial. The postulated mechanisms of pathogenesis involve a malabsorption component resulting from damage to the absorptive intestinal villi caused by the virus. There is also a down regulation of absorptive enzymes produced by the enterocyte. Rotavirus causes an increase in intracellular calcium thus disturbing the intracellular homeostasis. NSP4 plays a key role in the increase in

intracellular calcium ions. It causes the release of Ca2+ from the storage sites like the endoplasmic reticulum. NSP4 from prior infected cells after binding to specific receptors on adjacent enterocytes result in phospholipase C- inositol 1,3,5 triphosphate mediated cascade of events ultimately causing an increase in intracellular Ca2+.

NSP4 can directly act on crypt cells or directly stimulate enteric nervous system, which increases Ca2+ in the cells. When NSP4 acts directly on the crypt cell, there is activation of Cl^- transporter contributing to secretory diarrhea because there is a concomitant increase in water secretion. Microvillar cytoskeletal made up of Ca2+ sensitive F-actin, villin and tubulin proteins undergoes disruption. NSP4 also acts on the tight junctions, which maintain epithelial barrier decreasing the intracellular resistance and cause paracellular leakage. The degree of damage to the mucosa correlates with the severity of diarrhea (24).

The other mechanism involves an alteration in Na+/K+ equilibrium as a result of increased plasma membrane permeability. There is an increased intracellular Na+ and a decreased intracellular K+ (25). This imbalance may cause decreased NaCl absorption and solute absorption, which takes place alongside Na+ and hence results in water secretion into the lumen of the gut.

Sodium glucose transport protein 1 (SGLT1) which is responsible for sodium reabsorption by the enterocytes is competitively inhibited by NSP4 (26). The enteric nervous system is situated immediately beneath the villous epithelium receives stimuli from the damaged cells. Also the prostaglandins and the chemokines released during epithelial damage stimulate the enteric nervous system which in turn trigger the crypt cells to pour out the secretions into the gut lumen.

Immunofluorescent staining and light microscopic examination of duodenal biopsy specimens from children presenting with severe diarrhea showed patchy rotavirus antigen distribution over the villous epithelium and short blunt villi with crypt hypertrophy respectively (24,27).

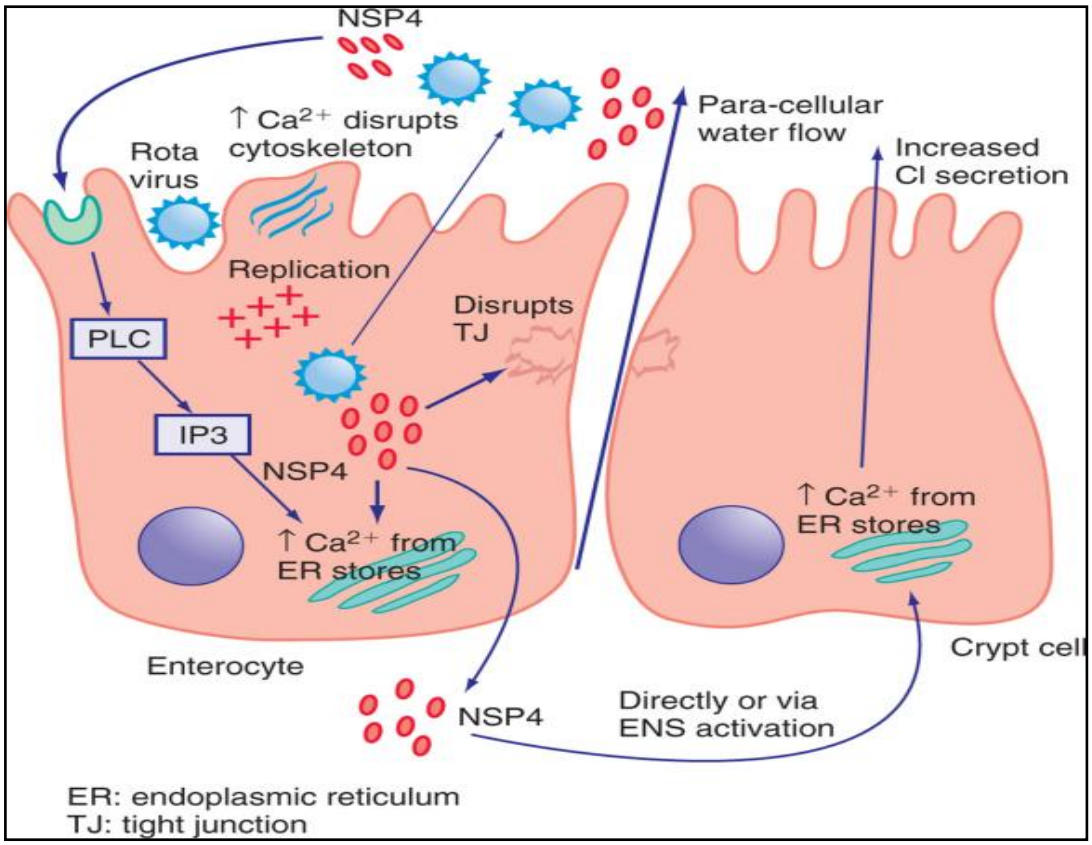


Figure 3. Pathogenesis of rotavirus diarrhea

2.4. IMMUNITY IN ROTAVIRAL INFECTIONS

Protective immunity is mediated by both the cellular and humoral immune system. Serological response to the first infection is believed to be homotypic i.e. directed towards specific viral proteins. The subsequent infections elicit a heterotypic serological response which is broader (28). In the case of most immunocompromised patients, severe diarrhea is rarely a consequence of natural infection but viral shedding takes place over a prolonged duration.

Severe diarrhea is however seen in severely immunocompromised individuals (i.e. congenital immunodeficiency states, solid organ or bone marrow transplant patients) (29). Studies have shown that in children, soon after natural infection, rotavirus specific serum IgA correlates with intestinal IgA levels and it can be used as a correlate for protection, and in vaccine trials, circulating IgA antibody levels in serum have been considered the best marker for vaccine take (30).

Rotavirus antigenemia was observed in children with rotavirus diarrhea and this showed correlation with viral load detected in the stool samples. This however did not translate into any increase in extra intestinal manifestations of the infection (31). Viremia was seen in 90% of antigenemic children in a study. The presence of viremia can be explanatory of the extra intestinal manifestations of the infection (32).

3. CLINICAL FEATURES

The clinical spectrum is varied and ranges from asymptomatic subclinical infection to severe life threatening diarrhea resulting in death. The incubation period lasts for 1 – 3 days and symptoms that follow may be of abrupt onset. There are however no striking clinical or epidemiological features which can differentiate a child with rotaviral diarrhea from a child with diarrhea attributable to any other etiology.

Infections in neonates may be asymptomatic because of the presence of circulating maternal antibodies (33,34). In addition, the immature alimentary tract and also the phenomeneon of reassortment occurring in the rotavirus genome giving rise to unique strains with a possibly different virulence mechanisms may be responsible for the asymptomatic nature of most of the neonatal infections. It was also noticed that the first episodes of rotaviral infection, past the neonatal period (around 3 to 24 months of age) may present with more vomiting than watery diarrhea, along with fever. Even beyond this period, vomiting frequently precedes diarrheal onset and may persist for 2 to 3 days. About a third of patients present with fever. Loose and watery stools are typical leading to life-threatening dehydration. The dehydration associated with this virus is comparatively more severe than that associated with other common gut pathogens(35,36). Diarrhea may continue for 4 to 5 days. Dehydration and the resulting electrolyte imbalance leads to cardiovascular failure which is the most common cause of death (37). Virus can be detected in infected stools using antigenic tests for about 4 to 10 days after the onset of symptoms. Using more sensitive methods like reverse transcriptase PCR, the detection can extend to 57 days though

the child may not be shedding infectious particles at this point (38). Usually there are no RBC or leukocytes in the stool (39).

In children with immunocompromised states, rotavirus infection manifests as chronic diarrhea. Neurological symptoms and the subsequent detection of rotavirus in cerebro spinal fluid of children have been reported. Various other conditions like aseptic meningitis, otitis media and respiratory illnesses which are extra intestinal infections have also been reported. Detection of rotavirus antigenemia and viremia in children with gastroenteritis is in support of extra intestinal manifestations. The virus has also been seen in a variety of conditions like sudden infant death syndrome, intussusception and necrotizing enterocolitis, but these findings may be co-incidental and not causal (40,41).

4. EPIDEMIOLOGY

Six to twelve months of age is the most vulnerable period for severe gastroenteritis (42). The age at which severe gastroenteritis usually happens is proportional to the waning of maternally derived antibodies, the maturation of the child's GI tract and the active acquisition of immunity due to natural infection (43). Though the rate of occurrence of rotaviral infection is similar in both developed and developing countries, mortality is higher in the latter. Malnutrition, lack of access to timely medical care, coinfections with other intestinal pathogens may be some of the contributing factors (44). The hardy nature of the virus, its relatively high concentrations of 10^{11} particles/mL in stool (45) and low minimum infective dose of 10 focus forming units (46) are also responsible for the high incidence rates. The peak incidence in temperate regions is in the cooler months (47). Cooler and drier months

are preferred in the tropical areas though there is no clear demarcation in the seasonal appearance in the tropics. The drier climate may be preferred for its transmission because of lower relative humidity which inactivates the virus (21).

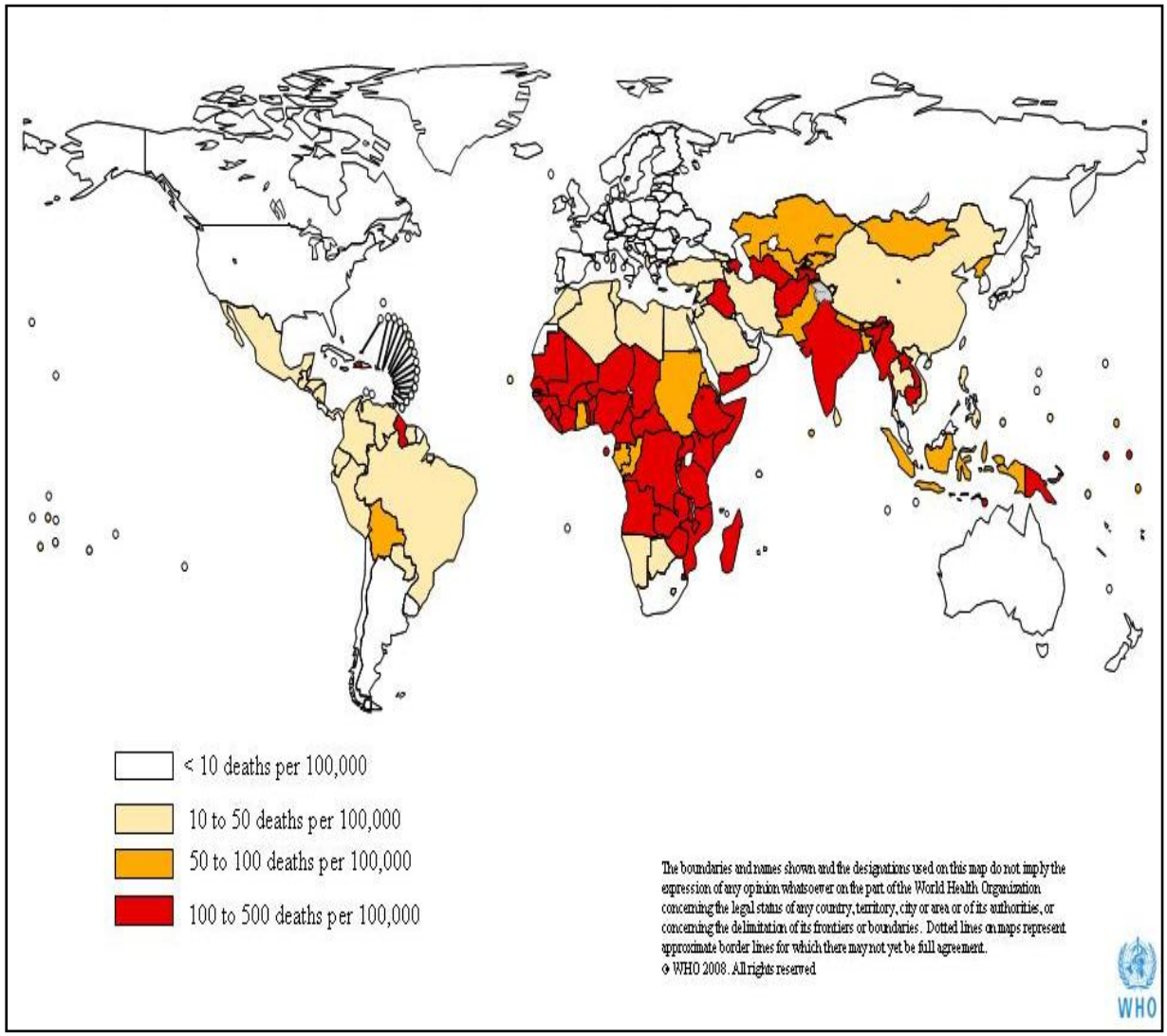


Figure 4. Mortality due to rotavirus in the <5 year age group

4.1 SEROTYPES/ GENOTYPES

There is a high level of antigenic and genomic variability in circulating human rotaviruses. Both antigenic determinants and genomic information have been used as the basis for classification. Serogroup is the broadest unit of classification, and is based on the VP6 protein. Seven groups (A-G) are identified, among which group A rotaviruses are found to be the commonest, causing life threatening diarrhea in children all over the world. The determination of serogroups and these subgroups was carried out by using hyperimmunized serum from animals (32). The VP6 antigen can be detected by serological tests like immunofluorescence, enzyme immunoassays and methods like immune electron microscopy. Genetic reassortment can take place between rotaviruses of the same group but does not occur among different serogroups. Groups A, B, and C rotaviruses are seen in humans and animals. Groups D, E, F, and G have been reported only in animals so far. Group A rotaviruses are implicated in endemic diarrhea of children. Group B and group C infect all age groups. The adult diarrhea rotavirus (ADRV) belongs to Group B and it was responsible for massive outbreaks of adult gastroenteritis in China (49). Group C is associated with mild diarrhea in both adults and children (50,51).

Serogroup A is further divided into various subgroups. They are subgroups I, II, I+II, and non-I non-II based on monoclonal antibody recognition of VP6 antigenic determinants (52). The initial serological classification within group Agave a binomial nomenclature comprising of a ‘G’ serotype and a ‘P’ serotype for each virus strain. Thetwo proteins, VP7 and VP4, of the outer layer and the spikes, respectively, induce antibody responses which are neutralizing and the genetic variability involved in

encoding them is the basis for G and P type classification (53). Around 14 G serotypes (G1 to G14) and atleast 20 P serotypes (P[1] to P[20]) are identified so far and of these, 10 G types and about 5 P types are found in humans infected with rotaviruses (53).

In the case of G type, there is a one to one correspondence between the protein and gene, such that serotypes, based on monoclonal antibodies, and genotypes have the same nomenclature. This is however not the case with P serotypes where there are a lot of cross reactions within the P serotypes, and a P serotype can include more than a single genotype.

Rotavirus strains may exhibit a degree of confinement to a particular geographic region or variations with climatic conditions. Factors like genetic reassortment between animal and human strains, antigenic drift and introduction of animal rotaviruses into the circulating pool of human strains ensure a continuous phenomenon of genetic diversity. Hence surveillance becomes imperative in determining whether new strains are emerging (54). Several G and P types may be circulating in a given diarrheal season. Though there are dominant G and P types with geographical confinement, they may be replaced by other strains during a particular rotaviral gastroenteritis season (55). Till 1990, the four most common combinations of G and P types seen were G1P[8], G2P[4], G3P[8] and G4P[8]. With increased strain surveillance, new GP combinations are being found. Currently, the most commonly occurring G serotypes are G1 to G4 and G9. The commonest P genotypes are P[4], P[6] and P[8] and their corresponding serotypes are 1B, 2A and 1A respectively (56). The five most prevalent GP combinations are G1P[8], G2P[4], G3P[8], G4P[8] and

G9P[8]. Uncommon strains are also found. Regional serotypes of importance are G8P[6] in Africa and G5P[8] in Brazil (48). The G9 strains were first identified in children with gastroenteritis in Philadelphia. There are some reports of increased severity of gastroenteritis in Latin American countries (57), and children <6 years showed increased risk of infection by the new strain (58). Currently G9P8 strains are one of the most commonly circulating strains worldwide. Children can have multiple episodes of diarrhea caused by different G and P types. The protective efficacy of the prior natural rotavirus infection over the subsequent infections ranged from 46% to 100%. Generally it is thought that the initial infections conferred homotypic protection which later broadened to a heterotypic protection. A more recent study conducted by Kang et al, where 373 children were followed up from birth for a three year period showed that although there was protection, there was no evidence of homotypic protection alone offered by the initial infections (59).

4.2 GENOTYPES IN INDIA

Strains belonging to different genotypes are reported from different parts of the country. G1, G2, G4, G9 and G12 are among the commonest VP7 genotypes and P[4], P[6], P[8] and P[11] are the common P types reported from all over India. With the introduction of molecular biology based typing methods, detection of so far untypeable strains is being done and thus the unusual strains are increasingly being detected. Many of them are believed to be of animal origin or reassortants between animal and human strains because of their identity with the corresponding genes in the rotaviruses infecting animals.

G6 strain and G8 strain showed more than 95% identity with VP7 gene of the bovine rotavirus strains. The G6 strain was described from Pune (60) and G8 from Vellore and Mysore (61,62). The VP7 gene of G3P[8] shares 100% identity with the VP7 of simian rotaviruses and the P[8] component has 99% identity with the P[8] of human rotaviruses. This was described among the tribal residents of Western India (63). G9P[19] is a porcine–human reassortant and is described from Manipur (64). Another genotype which is a bovine – human reassortant is the G10P[11] genotype described from Vellore (65). G12 strains which were first identified in Philippines were increasingly reported from India. The G12 genotype is seen in combination with many P types. Among hospitalized children, data from Vellore during the period 2002- 2005 reveal that the most predominant VP7 types were G1 followed by G9 and then G2 (65). A reversal of the above order was observed in studies conducted in the same region during 2005-2007 where G2 was the most common G type followed by G9 and then G1. Other genotypes like G3 and G4 which are among the commonest types found worldwide were not reported.

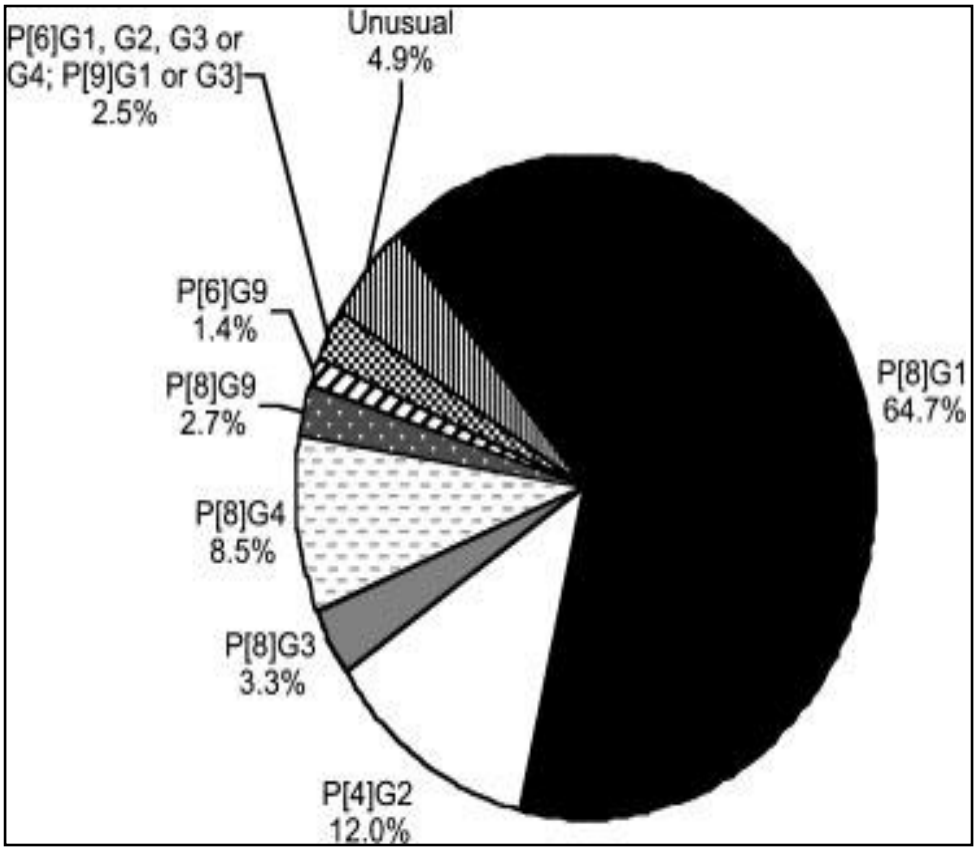


Figure 5. Global distribution of G and P types from five continents between 1989 and 2004 (Rev.Med. Virol. 2005)

5. LABORATORY DIAGNOSIS OF ROTAVIRUS

The different techniques to detect rotavirus in the laboratory are i) Virus isolation using cell culture techniques, ii) Electron microscopy, iii Serological methods to detect the antigen, including Enzyme immunoassay (EIA), Latex agglutination, Immunochromatography and iv) Nucleic acid detection.

5.1. Cell culture techniques for virus isolation

Virus isolation can be performed on rhesus monkey kidney cell lines (MA104, LLC-MK2 cell line), African green monkey kidney cell lines (BSC1, CV1) and Madin Darby bovine kidney cell line. After an incubation period of 18 hours, the cell line is examined for cytopathic effect.

5.2. Electron Microscopy

It demands well trained personnel with technical expertise and is a very expensive instrument. Moreover, the distinction between the various serogroups cannot be made based on electron microscopy. A minimum number of viral particles for detection by electron microscopy is 10^7 viral particles/ml of stool (17).

5.3. Serological tests for Antigen detection

The most common methods in serological detection employ antibodies specific for rotavirus antigens. The captured antigen is detected in a colorimetric reaction where a second rotavirus specific antibody attached to a detector enzyme is employed. The result in terms of optical density values is recorded by the standard plate reader.

Three commercial kits are recommended for surveillance in the manual on rotavirus detection and characterization by the WHO are Premier^{TM}Rotaclone (Meridian

Biosciences; Cincinnati), ProSpecT™ (Oxoid) and RIDASCREEN® (R-biopharm, Darmstadt, Germany).

Table 2. Performance of commercially available EIA kits for rotavirus detection

Study	ELISA kit	Reference test	Sensitivity	Specificity
R Gautam et al., 2013(66)	Rotaclone ProSpecT Ridascreen	Reverse transcription PCR	76.8%, 75% and 82.1% respectively	100%
Bodo RE et al., 2001(67)	1.Ridascreen 2.Pathfinder rotavirus	Electron microscopy	100%	99.73% and 79.1% respectively
Morinet F et al.,1984(68)	Rotazyme Enzygnost	-Relative sensitivity-	89% and 98% respectively	---
JL Cromien et al., 1987(69)	Rotazyme EIA Rotazyme II	EIA on MA104 cell culture supernatants using the same kits	100%	100% and 90% respectively
Dennehy PH et al.,1988(70)	1.Rotaclone 2.Pathfinder rotavirus	RNA-PAGE	100%	100% and 98% respectively

5.4. Latex agglutination methods

Latex particles are coated with the antibody specific for rotavirus antigen and if the antigen is present in the fecal specimen, there would be agglutination of the latex particles. This can be performed on a glass slide and visualised by the unaided eye or by use of a hand lens. It was thought that this test was faster and cost effective and could be performed in the paediatric wards. Initial evaluation of Rotalex, a latex agglutination kit by Orion diagnostics, Helsinki Finland, showed low sensitivity and specificity when compared to enzyme immunoassay and electron microscopy (71).

5.5. Immunochromatography

In resource poor settings, immunochromatography may help overcome the barriers in detection and studying the epidemiology of rotavirus diarrhea. Though the sensitivity and the specificity obtained using some kits was comparable to that of enzyme immunoassay, latex agglutination in some studies(72–74), others have reported discordant results (75,76).

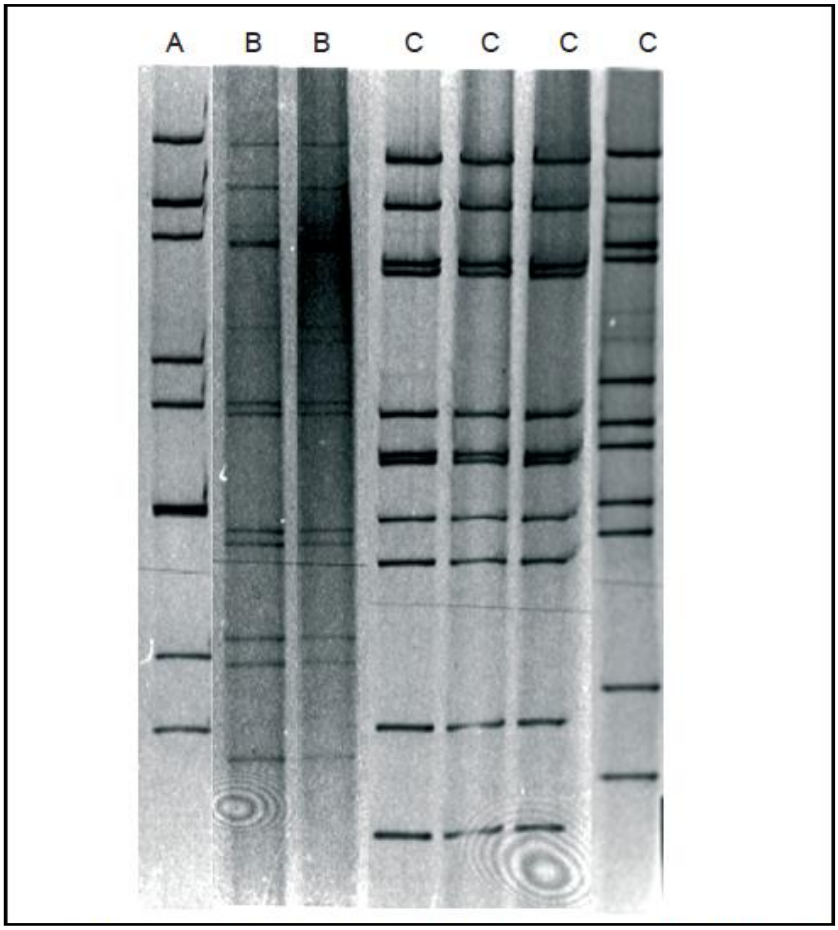
5.6. Nucleic acid detection methods

5.6.1. RNA Polyacrylamide gel electrophoresis

Large amount of viral particles are shed from stools in children with gastroenteritis and the nuclear segments can be visualised after extracting from the virus and running it electrophoretically on polyacrylamide gel after staining with either silver nitrate or ethidium bromide. Groups A, B and C are the human rotavirus groups and these have distinct bands corresponding to gene segment distribution after electrophoresis. Since this method detects both the presence of rotavirus and its serogroup, polyacrylamide gel electrophoresis (PAGE), has been used for Group A rotavirus surveillance.

The RNA which is extracted from Group A rotavirus can be categorised into four classes based on the size; large, medium, small and smallest segments. They also occur in ‘short’ and ‘long’ electropherotypes. This procedure is labour intensive and needs a trained technologist. The sample is considered positive for Group A rotavirus if the 11 segments of double stranded RNA are visible and correspond to the group A rotavirus control.

Figure 6. PAGE gel showing differences in segment migration configuration of Group A, B and C Rotavirus



5.6.2. Reverse transcription PCR

Reverse transcriptase PCR helps in improved identification of viral shedding when compared to enzyme immunoassay, latex agglutination or immunochromatography tests (77,78). Viral nucleic acid can be detected 2 to 7 days longer than when detected using the enzyme immunoassay (77). RT-PCR can detect and identify the G and P types by using primers which are specific for the VP7 and VP4 genes. The PCR products which are obtained are genotype specific and can be detected after running electrophoretically on agarose gel. Reverse transcriptase PCR takes place in three

important steps i.e., denaturation of double stranded RNA, reverse transcription of dsRNA to form the complementary DNA and amplification of cDNA. Denaturation of dsRNA is brought about by heating to separate the two strands. Complementary primers are added along with reverse transcriptase to form the cDNA. DNA polymerase enzyme extends the strand and synthesizes the new cDNA. The newly synthesized cDNA in each cycle undergoes denaturation, annealing and extension steps and gets amplified.

The genotyping methods employ a semi- nested RT-PCR where the viral RNA is reverse transcribed and amplified in the presence of consensus primers for genes encoding the VP7 and VP4 proteins (79–82). DNA obtained in the first amplification cycle functions as a template for the second PCR which takes place in presence of consensus primers and genotype specific primers. The products obtained are analysed after agarose gel electrophoresis and depending on the size of the products on the gel, the genotypes are determined.

Alternately the reverse transcription step can be carried out in presence of random hexamers instead of specific primers to obtain cDNA. The advantage here is that other enteric viruses can also be detected using specific primers for those viruses since the random hexamers prime any RNA present in the specimen (83).

5.6.3. Real time reverse transcriptase PCR

Real time PCR helps in quantitation of rotavirus particles present in the sample. It detects nucleic acids present in minute amounts.

6. REAL TIME POLYMERASE CHAIN REACTION

Post PCR analysis and quantitation following reverse transcription PCR is by gel electrophoresis and analysis of the image. Real time PCR offers a platform where the PCR product can be measured at each cycle in real time with precision using fluorescent dyes/probes which emit increasing signal with increase in amplicons. The fluorescent reporters used usually are dsDNA binding fluorescent dyes, or PCR primers/probes attached with the dye molecules. The fluorescence change is measured by an instrument that combines thermal cycling with fluorescence scanning ability. By plotting fluorescence against the cycle number, the amplification plot is generated. The entire amplification and detection occurs in a single tube eliminating the need for post PCR manipulations.

Steps involved in real time PCR

6.1. Denaturation

High temperatures (95°C) are used to separate the two strands of the dsDNA. The denaturation time can be increased based on the amount of GC content present in the template.

6.2. Annealing

This is calculated based on the melting temperature (Tm) of the primers which is usually 5°C below the Tm of the primer. Complementary sequences hybridize during this stage.

6.3. Extension

The activity of the polymerase enzyme is best at 70-72°C. When the amplicons are small, this step is combined with the annealing step using 60°C as the temperature.

6.4. Fluorescence detection systems

The most commonly used fluorescence detection systems in real time PCR are the dye based assays and 5’ nuclease assays.

6.4.1. Dye based chemistry

The commonly used DNA binding dye is SYBR Green I. It binds non specifically to dsDNA which exhibits little fluorescence in the unbound state. The fluorescence increases 1000 fold when it is bound to dsDNA. This fluorescence signal increases as the target gets amplified. A major drawback of these DNA binding dyes is their lack of specificity as they bind to any double stranded DNA.

6.4.2. Fluorescent Primer and Probe based Chemistries

The most common probe based chemistry is TaqMan chemistry. Taq polymerase is thermostable and has 5’ exonuclease activity. In addition to the exonuclease property, these assays also exhibit a phenomenon called fluorescent resonance energy transfer (FRET). In FRET, energy from a fluorescent dye can be reduced by the presence of another dye in close proximity called the quencher. It employs a sequence specific, fluorescently labelled oligonucleotide probe called the TaqMan probe. It contains a fluorescent reporter at the 5’ end and a quencher at the 3’ end. When the probe is intact, FRET phenomenon is at its optimum before the PCR starts. Once the annealing / extension takes over, the probe hybridizes to the target and the 5’ exonuclease activity of the Taq polymerase cleaves off the reporter. The fluorescence is not quenched as the reporter is separated from the quencher and it increases with each amplification cycle.

6.4.2.1. Types of Taqman probes

The two types of Taqman probes are the MGB and non-MGB probes. The non-MGB probes were used initially and where TAMRA® dye was used. The annealing temperatures of these probes had to be higher than the primers to allow cleavage to take place and hence the probes had to be longer than the primers.

The MGB probes have a minor groove- binding molecule on the 3' end. A minor groove is formed in the DNA where the probe binds and this raises the melting temperature of the probe. Taqman MGB probes are short. Hence even a single base mismatch has an impact on probe binding. Hence real time PCR employing Taqman MGB probes are highly specific.

6.5. Passive reference dye

Use of passive reference dye in real time PCR helps in normalizing the fluorescence signals that are not related to the PCR cycle and provide a stable baseline.

High specificity and high signal to noise ratio are the main advantages of using TaqMan probes. Detection of rotavirus using real time PCR is higher than that detected using reverse transcriptase PCR and the time is half that required for conventional PCR. Real Time PCR when initially performed on rotavirus, amplified a highly conserved region in the non structural protein 3 which is 87- bp long (84).

In addition to the need for understanding the biology of replication, there is a need for viral load estimation to attribute a causal relationship in patients with diarrhoea because some healthy individuals also can be positive for rotavirus when very sensitive nucleic acid detection methods are employed.

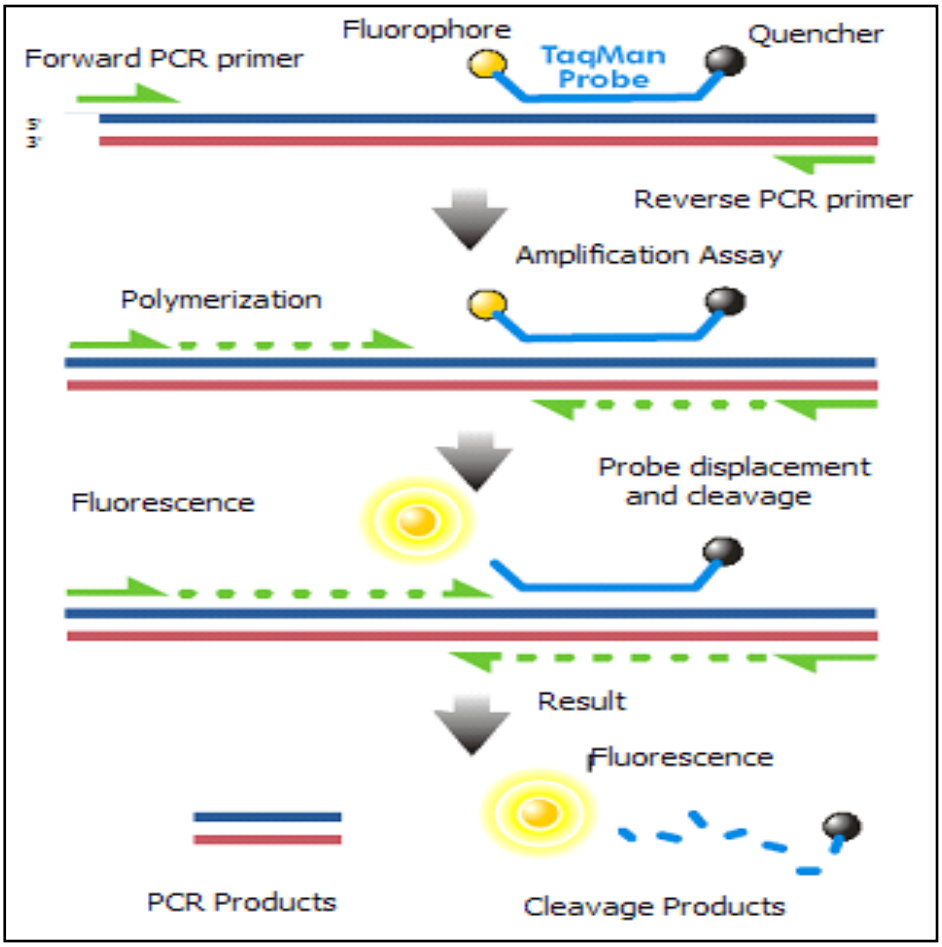


Figure 7. Diagrammatic representation of the steps in real-time PCR thermal cycling process

6.6. Amplification curve

Amplification curve in a PCR run can be divided into three important phases.

6.6.1. Exponential phase

A doubling of the reaction products happen during this phase. The reaction runs at 100% efficiency because all of the reagents are available. Hence double the amplicons accumulate at the end of the cycle.

6.6.2. Linear phase

As the reaction progresses, the reagents get consumed whenever there is doubling of the amplicons. At this point, the reaction starts to slow down and there is no more exponential increase seen in the amplicons.

6.6.3. Plateau phase

The reaction stops in this phase and no more amplicons will be made. The PCR products may even degrade if left long enough. Each sample has its own plateau phase depending on the reaction kinetics of the sample.

6.7. Real time PCR analysis

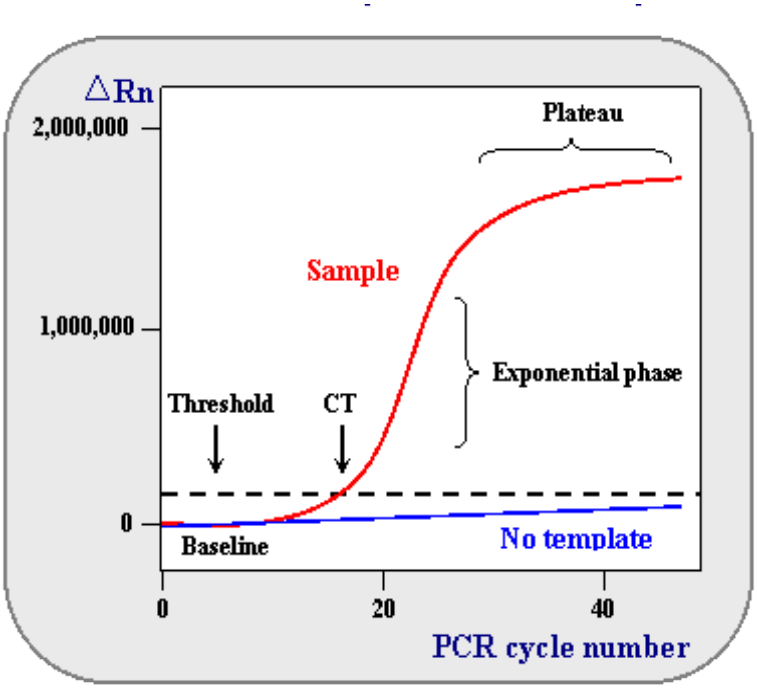


Figure 8. Real time PCR plot

6.7.1. Baseline

It is the signal level in the initial cycles of the real time PCR where there is little or no change in fluorescent signal. If low level signal can be detected at this point, it is considered as the background ‘noise’. Baseline should be determined carefully so that enough cycles contributing to background noise are eliminated and at the same time, care should be taken to see that no amplification signal is missed.

6.7.2. Threshold

Threshold is the level which separates background noise from relevant real amplification. It can be set manually or automatically. In the automatic mode, the machine sets it at 10 times the standard deviation of the fluorescence value of the baseline.

6.7.3. Threshold cycle (Ct)

It is the cycle number at which the fluorescent signal of the reaction crosses the threshold. Ct value is inversely related to the starting amount of the target.

6.7.4. Standard curve

A known template is serially diluted and a standard curve is constructed to determine the initial amount of the target template. The log of each concentration on the x-axis is plotted against the Ct value for that concentration. Using the standard curve, the performance of the reaction and the various reaction parameters can be deduced. The concentrations of the known template should be chosen in such a way that they encompass the expected concentration range of the target templates used in the real time PCR.

6.7.5. Correlation coefficient (R^2)

It determines how well the data fits the standard curve. It is a reflection of the linearity of the standard curve.

6.7.6. Slope of the standard curve

The slope of the log linear phase of amplification reaction is a measure of efficiency of the reaction. A reaction efficiency of 100% corresponds to a slope of -3.32.

6.7.7. Efficiency

An ideal efficiency of 100% means that the template doubles after each thermal cycle during exponential amplification. Length, secondary structure and GC content of the amplicon are the experimental factors that can influence efficiency. Using non optimal reagents, presence of PCR inhibitors in the reagents are a few of the reaction factors that contribute to altered efficiencies. A reaction efficiency between 95% and 105% is considered acceptable.

6.7.8. Dynamic range

This is the range over which an increase in starting material concentration results in a corresponding increase in amplification product.

7. TREATMENT

Treatment mainly focuses on rehydration and ameliorating the effects of dehydration caused by diarrhea. There is no specific treatment other than fluid replacement and zinc. Low osmolality solutions are preferred over conventional formulations of oral rehydration salts (89). Since rotaviral diarrhea is also associated with vomiting, the extent of dehydration is much more, and oral rehydration alone may not be sufficient in severe cases. The child may need hospitalization and intravenous rehydration. The health care seeking in these developing countries is also less owing to social, economic and cultural constraints. Thus vaccines hold a promising future in restraining the mortality and morbidity due to rotavirus gastroenteritis.

However, the effectiveness of these vaccines in the poorest regions, which contribute to majority of mortality, is still questionable due to the existence of other concomitant infections and the lack of information on the circulating and newly emerging strains in those geographical regions.

Some studies showed that passive immunization using hyperimmunised bovine colostrum or chicken yolk immunoglobulin were protective against rotavirus diarrhea (86–88). Racecadotril which is an encephalinase inhibitor acts on the enteric nervous system mediated secretory component of rotavirus diarrhea and can be used in the treatment of rotavirus diarrhea (89). Oral administration of certain lactobacillus species in children with rotavirus diarrhea reduced the duration of watery diarrhea (90). Laboratory studies using recombinant monovalent antibody fragments derived

from llama immunized with rotavirus showed neutralizing effect on various common genotypes of rotavirus (91).

8. ROTAVIRUS VACCINES

Studies conducted on the natural history of rotavirus infections showed that the initial infections after the neonatal period were symptomatic but conferred protection on the subsequent infections (33,92,93). These findings provided the necessary scientific rationale that this immunity could be imparted using live oral vaccines. It was also seen that vaccines of low immunogenicity showed high efficacy in field trials. Hence conducting clinical trials on a large scale may be the only secure means to estimate the effectiveness of a vaccine (94,95).

The use of vaccines should ideally be a component of a comprehensive strategy targeted to control diarrheal diseases. The other components should include improvement in general hygiene and sanitation, zinc supplementation, administration of oral rehydration solution and better management of cases (96). Efforts to manufacture a vaccine against rotavirus started in the1980s. In 1983,the first trial with a candidate oral vaccine derived from RIT4237strain, which is a bovine rotavirus strain was carried out. Its results showed four basic principles regarding live oral rotaviral vaccines. 1. They can shield children against severe diarrhea. 2. Protection was more against severe gastroenteritis as compared to milder episodes. 3. Animal strains can protect individuals from infections caused by human strains. 4. The antibody levels did not determine the effectiveness of the vaccine. The RIT vaccine developed by Vesikari did not show consistent outcome in clinical trials in developing

countries and was abandoned. After 15 years, the first vaccine Rotashield™ (Wyeth-Lederle, Pearl River, NY, USA) was licensed in the United States in August 1998. It was developed at the National Institute of Health and was a rhesus derived tetravalent reassortant vaccine (97,98).

Nine months after administering it to well over 60000 children, it was withdrawn from the market because of several cases of intussusception following its administration (99). The risk was assessed to be 1 in 10,000 vaccine recipients (100). Intussusception was seen more in the 'catch-up' group who were older than 90 days of age. Since the risk of natural intussusception is lower in the first 3 months of life and hence subsequent vaccines targeted this age group for clinical trials.

Two vaccines which are currently widely available are Rotarix® from GlaxoSmithKline Biologicals, Rixenstart, Belgium and Rotateq® from Merck & Co. Inc., West Point, PA, USA. Both are live attenuated oral vaccines. Rotarix is a monovalent vaccine from a G1P[8] strain isolated from a case of gastroenteritis in an infant which was subjected to serial passages in tissue culture for attenuation. RIX4414 was the resulting strain and it is propagated in Vero cell lines. It is administered as two doses; the first after six weeks of age and the second before 24 weeks of age. Severe combined immune deficiency syndrome is a contra indication (101,102) because of the danger of prolonged shedding.These vaccines can be administered along with the routine childhood vaccinations.

The pentavalent vaccine is a human- bovine reassortant vaccine. Its origins are from human and bovine parent strains where five reassortants express either one of the four VP7 proteins (G1, G2, G3 or G4) belonging to human strains and P7[5] from the

parent bovine strain or P1A[8] which is a VP4 protein from a human strain and and G6 protein from the parent bovine strain. All the five reassortants are finally propagated in Vero cell lines employing standard techniques. Three doses of pentavalent vaccine are given at 2,4 and 6 months of age. During the first week after administration of any of the rotavirus vaccines, the child has to be carefully watched for any episodes of severe crying, vomiting, irritability, blood in stools, which may be indicative of intussusception.

8.1. EFFICACY OF THE VACCINES

Rotarix and Rotateq vaccines showed an efficacy of 80% – 90% against severe rotaviral gastroenteritis in WHO regions with very low or low child and adult mortality rates, but 40% - 60% efficacy was seen in WHO regions with high child and very high adult mortality (103,104). In Asian and African countries, efficacy is low but there is a substantial public health impact because of the high burden of disease, since these countries account for almost 85% of rotaviral diarrheal deaths worldwide. A great diversity in existing strains, and occurrence of mixed infections and the ongoing reassortment between the human and animal strains have so far not posed a challenge to the vaccines' ability to offer cross protection.

8.2 Other vaccines

The Lanzhou strain used was isolated from a lamb with diarrhea and was grown on cell lines of primary calf kidney. It was licensed as a vaccine in China. Not much data is available on the safety, immunogenicity and efficacy of this vaccine (105).

The Rotavac vaccine is monovalent, based on the 116E strain of rotavirus which was recovered from asymptomatic infections in neonatal units in New Delhi. This strain was shown to offer protection against severe diarrhea (92). It was characterized at AIIMS Delhi as G9P[11] human – bovine reassortant, with VP4 of bovine origin and the rest of the segments of human origin (80). The vaccine completed the first two phases of clinical trials successfully and interim reports from the phase III trials from three different centres in India ie. NewDelhi, Pune and Vellore showed good results. This vaccine will be available at a low price if approved for licensure.

9. CLINICAL SCORING OF GASTROENTERITIS

Early vaccine studies revealed that the vaccines did not protect against all severities of diarrhea equally and that a vaccine should be able to protect against severe clinical outcome. There were no clear definitions or cut offs for measuring severity. Different descriptions and definitions were used in different studies to measure severity.

To measure the vaccines' ability to cause a reduction in severity of diarrhea, numerical scores can be given to most important clinical symptoms in diarrhea.

A 0-9 point scoring system was put forth by Hjelt et al which took four clinical parameters into consideration (106). They are duration of diarrhea, number of episodes of diarrhea in a 24 hour period, duration of vomiting and fever. Flores et al added degree of dehydration and treatment as additional parameters and brought forth a 0-14 point scoring system (107).

Thus different vaccine trials were evaluated by a different scoring system eg. Scoring system devised by Duffy *et al* 1986, Gotheforset *al* 1989, Clark *etal* 1988, Vesikarie*t*

al 1984 (4,108–110). Two of these scoring systems, the Clark scoring system and the Vesikari scoring system have been routinely used in vaccine trials. The Clark scoring system does not assess the degree of dehydration. There have been discrepancies between the various scoring systems in assessing the clinical severity even when they were compared in the same clinical trial (111). One of the important components to be assessed when comparing the different scoring systems is their ability to detect severe diarrhea. Vesikari scoring system is considered the best scoring system for gastroenteritis in rotaviral clinical trials (112) with relatively standardised endpoints.

9.1 Parameters and Scoring in the Vesikari scoring system

It is a 0-20 point numerical scoring system based on the clinical features with which the child presents to the health care facility and the distribution of clinical history into the the respective categories of the scoring system (4). According to theVesikari scoring system, a score below 5 is considered ‘mild’, between 6 to 10 is ‘moderate’, between 11- 15 is ‘severe’ and above 16 is considered ‘very severe’ gastroenteritis.

In children below 6 months of age, any alteration in number or stool consistency which the mother felt was diarrhea was considered diarrhea. Atleast one day of diarrhea preceeded and followed by two or more diarrhea free days was considered an episode of diarrhea (113). The episode ends on the day the child starts to pass normal stools.

Components of the scoring system

10.1.1. Diarrhea

The highest number of times the child passes stools is captured in the record and scored accordingly.

10.1.2. Vomiting

The highest number of times the child vomits in a 24-hour period is captured and scored accordingly.

10.1.3. Temperature

The highest temperature in a 24-hour period is taken. Rectal temperature is ideal. Axillary, oral or otic temperatures can also be taken.

10.1.4. Duration of diarrhea

The total number of days from the beginning of the diarrheal episode till the child turns symptom free.

10.1.5. Duration of Vomiting

The total number of days from the beginning of vomiting episodes till the child is emesis free. This is calculated in a manner similar to diarrhea duration.

10.1.6. Dehydration

The degree of dehydration is calculated using four parameters. They are, appearance of the eyes, general condition of the child (irritable, restless, lethargic, unconscious), thirst level and skin turgor. This can be done per the physician's assessment or using the WHO Integrated Management of Childhood Illnesses dehydration treatment criteria..

10.1.7. Treatment

Hospitalization is more of concern than rehydration according to the scoring system because in some of the developing countries IV rehydration may be administered even without admitting the child to the hospital. Hence hospitalization is given a score of 2 and rehydration (IV or oral rehydration) is given a score of 1.

Table 5. Vesikari scoring system

	0 points	1 point	2 points	3 points
Diarrhea duration (days)	0	1 – 4	5	≥ 6
Maximal number of diarrheal stools per 24-h period	0	1 – 3	4 – 5	≥ 6
Vomiting duration (days)	0	1	2	≥ 3
Maximal number of vomiting episodes per 24-h period	0	1	2 – 4	≥ 5
Maximal recorded temperature, (fever) Celsius scale	<37	37.1 - 38.4	38.5 - 38.9	≥ 39.0
Degree of dehydration	None		Some	Severe
Treatment	None	Rehydration	Hospitalization	

MATERIALS AND METHODS

Study design

This was an observational study conducted at the Wellcome Trust Research Laboratory, Christian Medical College, Vellore in collaboration with the different units of Child Health Department. The institution is a 2400 bedded tertiary care centre with several paediatric wards that offer both general and specialist care to children. The institution also runs a separate emergency department for paediatric age group patients. The Wellcome Trust Laboratory is involved in studies on paediatric diarrhoea. It is the World Health Organisation Rotavirus Reference Laboratory for the South East Asian Region.

Inclusion criteria

Children below 5 years of age presenting with diarrhea to the Paediatrics department.

Study duration

The study was conducted over a period of 15 months from June 2012 to August 2013 after obtaining approval from the Institutional Review Board and Ethics Committee.

Study samples

Faecal specimens from children below 5 years of age were collected. A total of 150 positive samples was aimed for with 50 samples in each of the three clinical categories, i.e., mild, moderate and severe. The sample size was calculated based on the feasibility of the study as the outcome was not predictable. Vesikari scoring system was employed to score severity of diarrhea in the study children. The majority of the samples were taken from the studies being conducted on rotavirus strain surveillance at the Wellcome Trust Research Laboratory. The rest of the samples were collected from the in-patient and out-patient wards and the emergency department of Child Health after obtaining the necessary consent from the parent/ guardian.

Collection, Transport and Storage

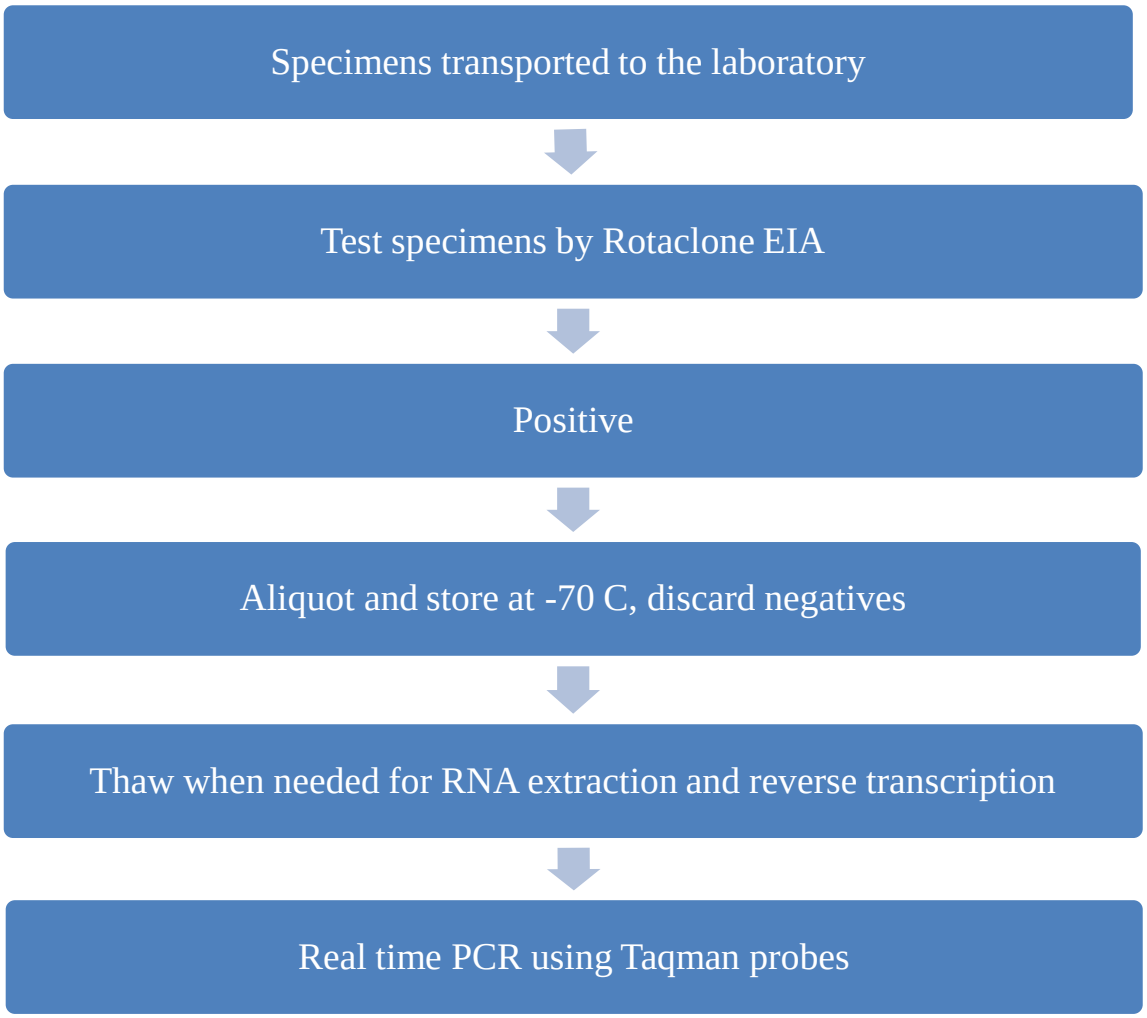
Faecal specimens which are collected as part of the Indian National Rotavirus Strain Surveillance program are screened for at the Wellcome Trust Research Laboratory routinely for rotavirus using the Rotaclone ELISA kit to detect VP6. These samples are collected by the parent or nurse, kept in the ward refrigerator and handed over to the study staff for transport to the laboratory. Parents were informed about the study, and after the consent was obtained, a clinical assessment was performed by a nurse using the components on the modified Vesikari Scoring system and the appropriate score was attributed to that particular sample. Passage of three or more loose or watery stools in a 24 hour period was considered diarrhea.

If the sample was received in the laboratory too late for the ELISA to be performed on the same day, the specimens were stored at 4°C for ELISA to be done on the

subsequent day. All the specimens were aliquoted into vials and stored at -70°C for further characterization. Rotavirus positive stool samples after ELISA were taken for the current study and were subjected to RNA extraction, cDNA conversion and real-time polymerase chain reaction.

Since most of the samples thus obtained were from the in-patient wards and children with moderate to severe diarrhea usually got admitted, children from the out-patient clinic and emergeny department who would not need admission to the wards were also recruited into the study. Consent was obtained by the field nurse/staff or the principal investigator from the parent/ guardian after providing information about the study. The process employed was similar to that of the samples collected for strain surveillance.

STUDY ALGORITHM

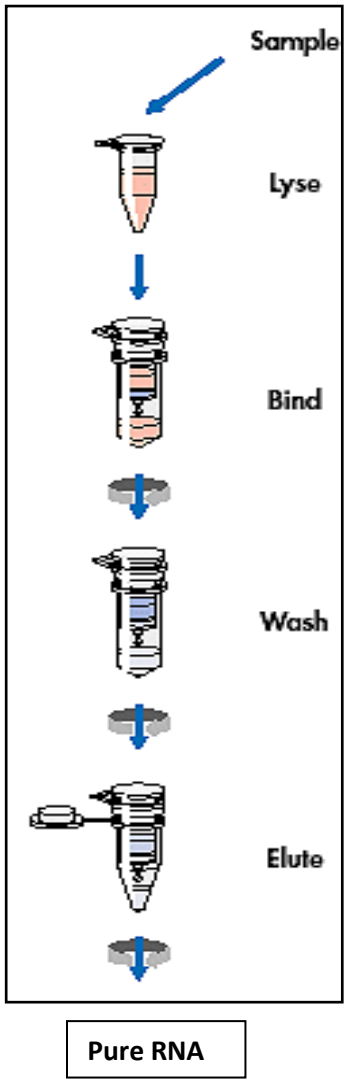


ENZYMEIMMUNOASSAY FOR ANTIGEN DETECTION FROM FECAL SPECIMENS

Figure 9. Rotaclone ELISA kit

Premier Rotaclone® kit was used for antigen detection in stool fecal specimens by ELISA. The microtitre wells that are provided in the kit are coated with murine monoclonal antibody against the protein encoded by the 6throtaviral gene (VP6). The same monoclonal antibody conjugated to horse radish peroxidise is used in the detector system. The results of the test were read using a spectrophotometer at 450nm. The samples were considered positive when the A_{450} was > 0.150. The procedure described in the Standard Operating Procedure and kit manual was strictly adhered to while performing the ELISA. Personal protective equipment and a biological safety cabinet were used for all sample handling.A 10% faecal suspension made using the diluent provided in the kit was used for EIA (see Appendix for details).

RNA EXTRACTION BY QIAGEN KIT METHOD



QIA amp Viral RNA Mini Kit is away to extract and purify viral RNA for use in amplification techniques. The kit uses silica gel based membranes which ensure recovery of pure and intact RNA without the use of any phenol or chloroform extraction or alcohol precipitation which may interfere with PCR. The silica gel based membrane has selective binding capacity and its combination with microspin helps in extraction from multiple samples at a time. The sample is first lysed under denaturing

conditions. This inactivates the RNases and ensures recovery of intact RNA. Different buffers are added to provide optimum binding of RNA to the QIAamp membrane of the spin column provided with the kit. Contaminants are washed away using two different wash buffers. RNA of high quality is eluted using RNase free buffer. This can be converted to cDNA or stored at appropriate temperatures.

REVERSE TRANSCRIPTION

The RNA that was extracted using the Qiagen kit extraction method was converted to cDNA. This was carried out in the presence of random primers and a reverse transcriptase enzyme derived from Moloney Murine leukemia virus (Invitrogen, Life Technologies, Carlsbad, CA).

The procedure for RNA extraction and reverse transcription are in the appendix.

ROTAVIRUS REALTIME PCR USING TaqMAN PROBES

In real-time PCR, DNA is amplified and detected using fluorescent chemistry. Dual probes which are short oligos labelled with a fluorescent reporting dye at the 5'end and a fluorescence quencher at the 3' end are used. Both the reporter dye and the quencher are in close proximity because of the size of the probes which are only 15 – 25 base pairs in length. Hence no or very little fluorescence is detected. Taq DNA polymerase starts to extend the DNA strand from each primer during the cycling process. The polymerase also has exonuclease activity and this results in cleavage of the probe as the polymerase extends the strand downstream. The probe is degraded, thus separating the reporter dye from the quencher.

With every additional cycle in amplification, there is an increase in fluorescence emitted due to repeated cleavage of the probe. This helps monitor DNA amplification in real time and avoids post amplification procedures like gel electrophoresis. This also reduces the chances for cross contamination. It is a highly sensitive and specific method of detection of double stranded DNA and enables quantitation of specific pathogen levels. A 379-bp region from nucleotides 747 to 1126, which codes for aa 241 to 367 of the VP6 gene is amplified using the VP-F and VP6-R primers (114).

Figure 11. Standard curve

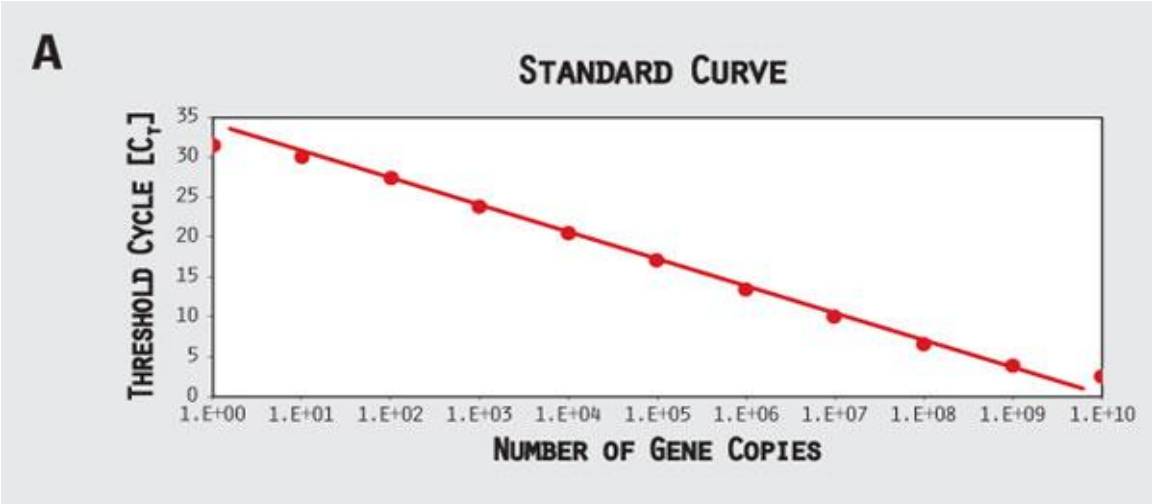
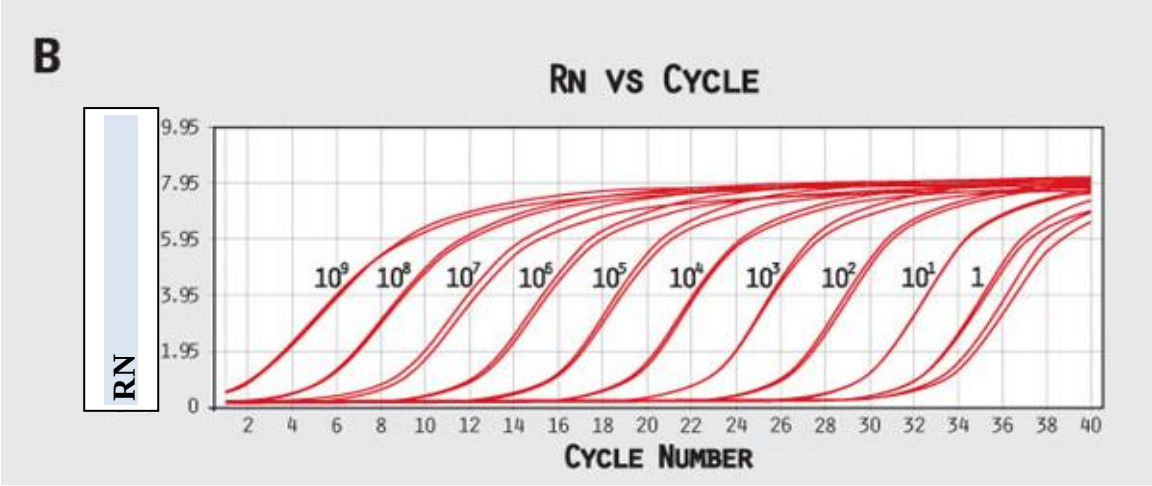


Figure 12. Amplification plot of the standard dilutions



Interpretation of results

1. The measurement of rotavirus specific cDNA was calculated using absolute quantitation by normalizing the Ct values with that of the internal plasmid control.
2. The threshold was determined automatically in the exponential phase which reflected the highest amplification rate.
3. For the crossing points resulting from the amplification curves, the threshold indicated the relation between the cycle number and the log concentration of cDNA.
4. By linear regression of these data, a standard curve was constructed which helped in the extrapolation of concentration of cDNA present in the samples as copy numbers.
5. The raw fluorescence data from the reporter dye in every well was plotted by the software. This was analysed to create a critical threshold and use this value to provide any Ct values.

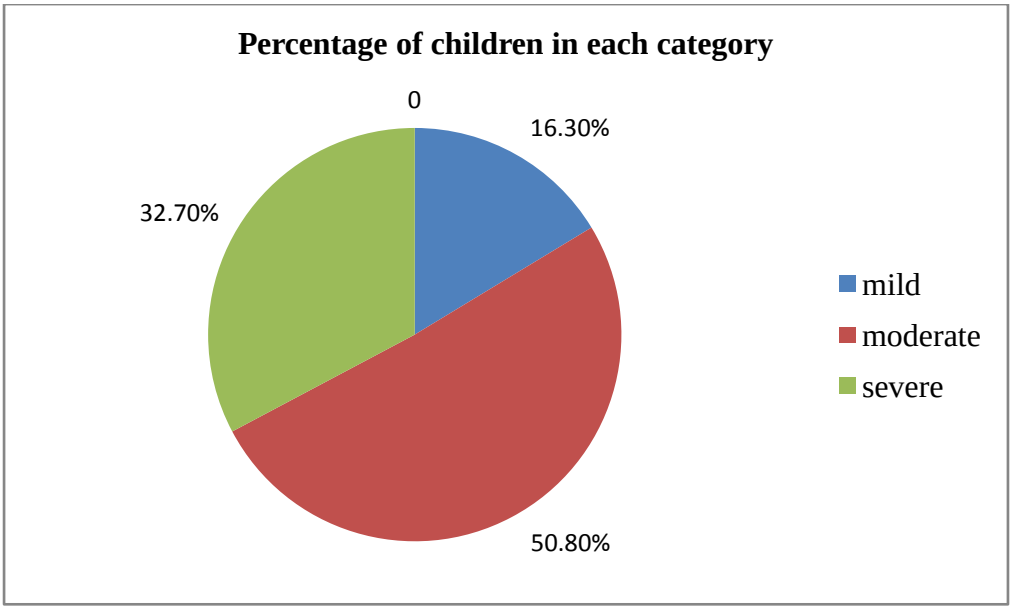
Statistical Methods

The median Ct values and the median viral load (as copy numbers) values were calculated across the mild, moderate and severe categories. They were compared against the clinical category obtained after applying the modified Vesikari scoring system using the non parametric Kruskal Wallis test.

RESULTS

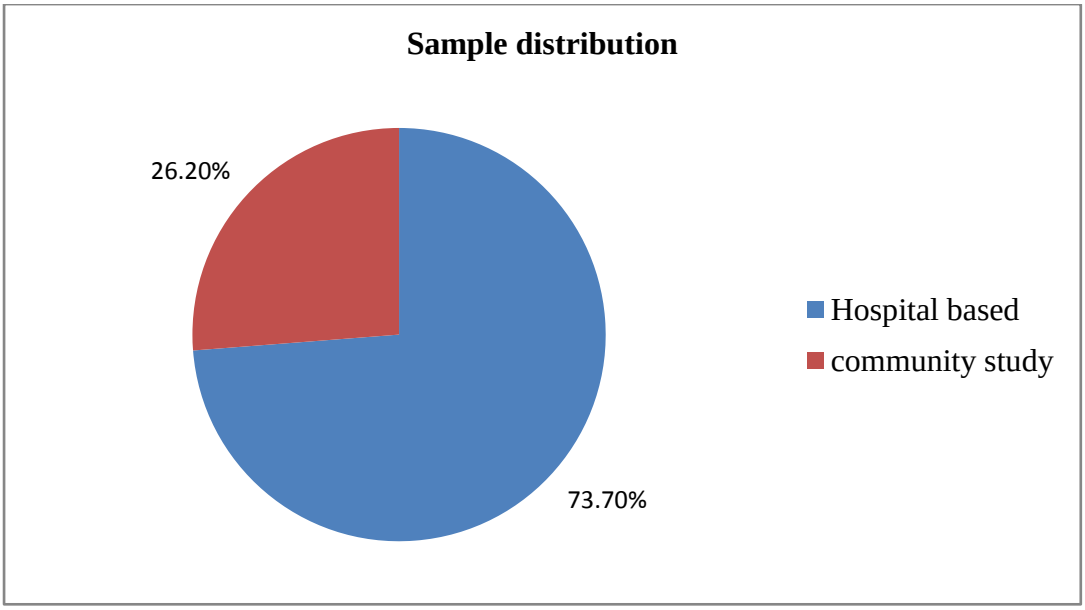
A total of 124 fecal specimens from children <5 years of age satisfying the inclusion criteria were included in the study. Information regarding the number of days of diarrhea, frequency of diarrhea in a 24 hour period, number of days of vomiting and its frequency, dehydration, temperature and treatment received by the child were captured on the case report forms of each child. In addition to this, information about other concomitant infections, the diagnoses made and the treatment given was also captured. Information on time taken for recovery from diarrhea was obtained from all the children admitted to inpatient wards.

The study algorithm was followed and only samples which were positive by enzyme immunoassay (Rotaclone) were included. RNA extraction, reverse transcription and real-time PCR were performed on these 124 samples. Though 150 samples with 50 from each category respectively were aimed for, in the stipulated period only 124 samples could be obtained and analysed. The corresponding Vesikari score categories were 22‘mild’, 62‘moderate’ and 40 ‘severe’. After real time PCR was performed, two samples from ‘mild’ category had to be left out of analysis because there was no amplification seen and hence the final number of samples analysed were 122.



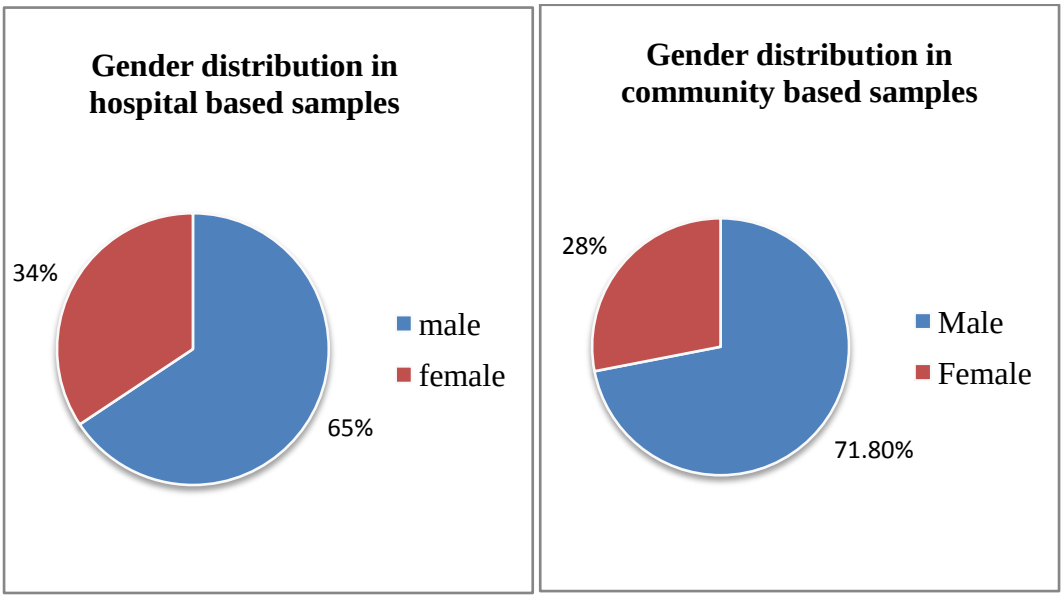
Demographic data

Twenty nine samples were obtained from children who were brought to the paediatric out-patient and emergency departments and 63 samples were from children admitted to the inpatient wards. The above two sets of samples were collected as part of the National Rotavirus Strain Surveillance Program from children < 5 years of age. Another 32 samples were taken from a community based study in children with rotavirus diarrhea. Children recruited into this study belonged to an urban slum setting. They were brought by the mothers to the study clinic established in that area by the Wellcome Trust Laboratory. The samples were all collected from symptomatic children under 5 years of age.



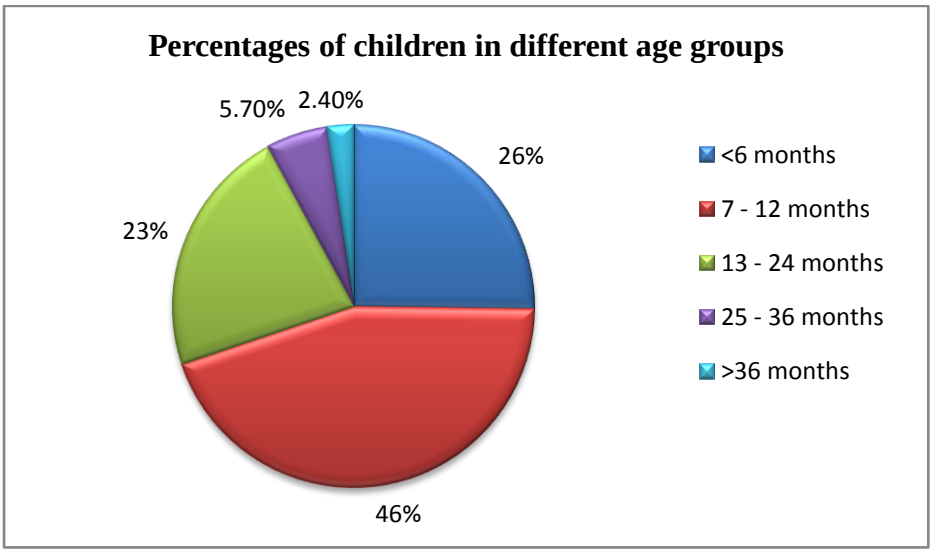
The mean age in children from the hospital based samples was 13.8 months (SD 9.23) and from the community samples was 7.75 months (SD 1.86). The overall mean age of the children was 12.08 months (SD 7.96). Children from the community are of younger age group when compared to the children attending the main hospital.

Gender distribution



Two thirds of the children enrolled into the study are males and the remaining one third are female children. This ratio is maintained in the hospital based samples whereas it is more in the community based samples.

Age distribution



The age groups ranged from 2 months to 46 months. The majority of the children were older than 6 months of age.

Other Clinical details

Of the 32 samples collected from the community, 15 children (44%) presented with upper respiratory tract infection and one child had chicken pox. All the children were managed conservatively at home with oral rehydration solution for diarrhea. None of them received intravenous fluids.

The most common medications prescribed to children attending the hospital out-

patient department was zinc sulphate suspension and oral rehydration salts. Among the children admitted to the inpatient paediatric wards, 3 children presented with sepsis of which one child was admitted to the intensive care unit. One child had urinary tract infection, one had dengue and another child presented with hypoglycaemia. Three fourths (74.6%) of the children admitted to the in- patient wards received intravenous fluids followed by supervised oral rehydration therapy. The other one fourth (24.7%) of hospitalised children received only supervised oral rehydration treatment while monitoring for signs of worsening dehydration. All the children recruited into the study recovered completely from diarrhea.

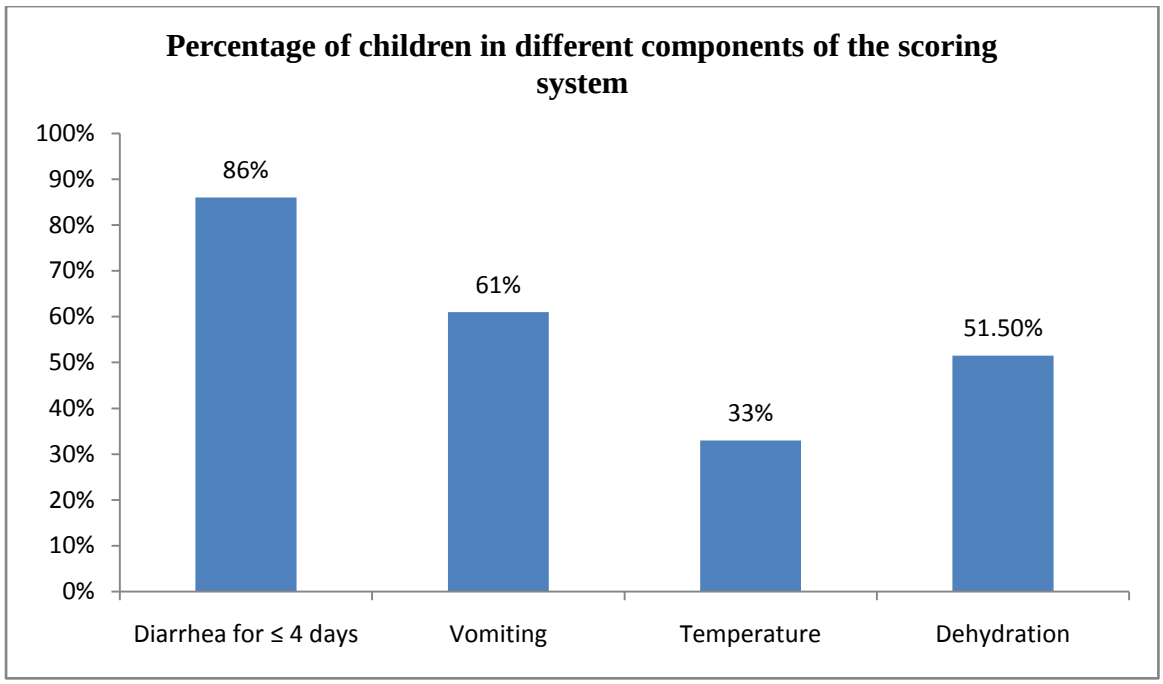
Data pertaining to the Vesikari scores

Data regarding the scores for each component of the Vesikari scoring was collected from the case report forms and entered into excel sheet. The mean and median Vesikari scores were calculated. Vesikari scores ranged from 3 to 15.

Table 6. Mean and median Vesikari scores in the different categories

Category	**Vesikari score Mean (SD)**	**Median**
Mild	4.71 (SD 0.64)	5
Moderate	7.91 (SD 1.34)	8
Severe	12.65 (SD 1.31)	13

The mean and median Vesikari scores in the samples collected from the community and hospital were 8.43 (SD 2.97), 8 and 9.13 (SD 3.1), 9 respectively.



More than half of the children presented with vomiting along with diarrhea and a third of the children presented with temperature above 37ºC.

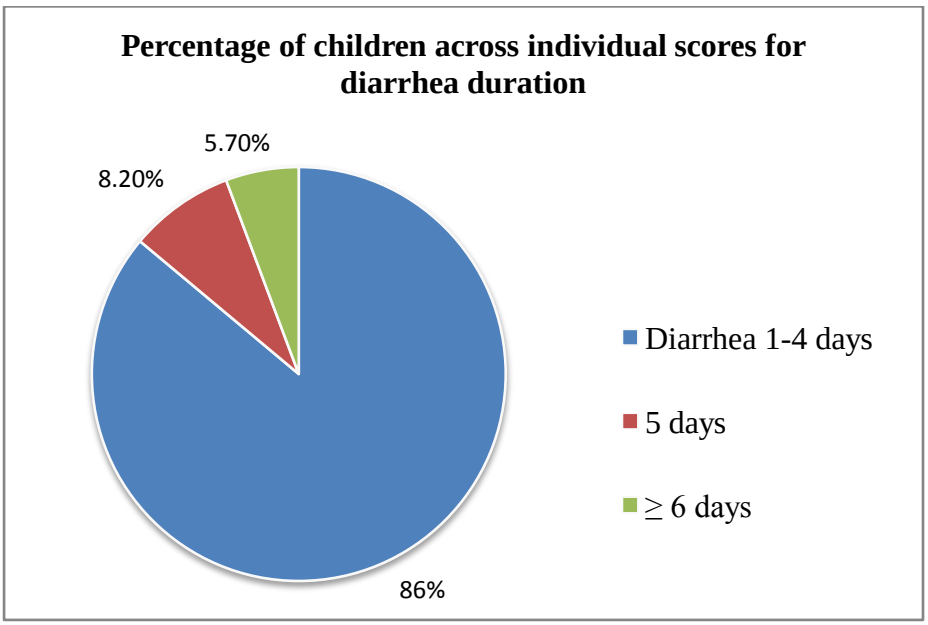
Almost all the children (except four) received treatment either in the form of oral rehydration salts or intravenous fluids, with 39.3% receiving intravenous fluids followed by supervised oral rehydration. The mean and median duration at recovery in hospitalised children are 2.1 (SD 1.01) and 3 days respectively.

Crossing points (Ct values) which were obtained in the real time PCR runs were used to calculate the viral load. Ct is a direct correlate of viral load and hence these values were plotted against the various parameters to study the possible associations.

The crossing points ranged between 13.8 and 38.9. There was no association between the crossing points and the age even after adjusting for the site of recruitment of children (hospital or community, *p* value 0.09). There was no significant difference in crossing points between the community samples and hospital samples.

Scoring of the individual components of the Vesikari scoring system

1. Duration of diarrhea



Majority of the children presented with diarrhea lasting for 1 to 4 days

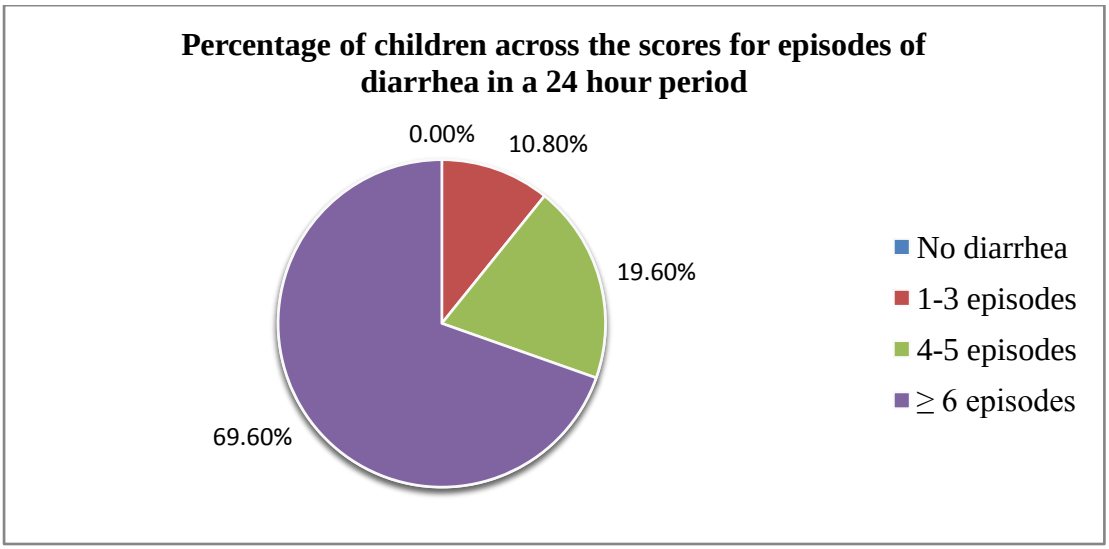
Table7. Median and mean Ct values with confidence intervals across the individual scores of 'duration of diarrhea'.

Duration score	Median Ct	Mean Ct	95% confidence Intervals
1	29	29.3	28.46 – 30.15
2	27.8	27.5	23.3 – 31.6
3	28	27.43	23.15 – 31.7

The median duration of diarrhea at the time of presentation to the hospital was 1 to 4 days and it remained the same across all the age groups. Applying the Kruskal Wallis

test, there was no significant association found between number of episodes of diarrhea and median Ct values (*p* value 0.62).

2. Number of episodes of diarrhea



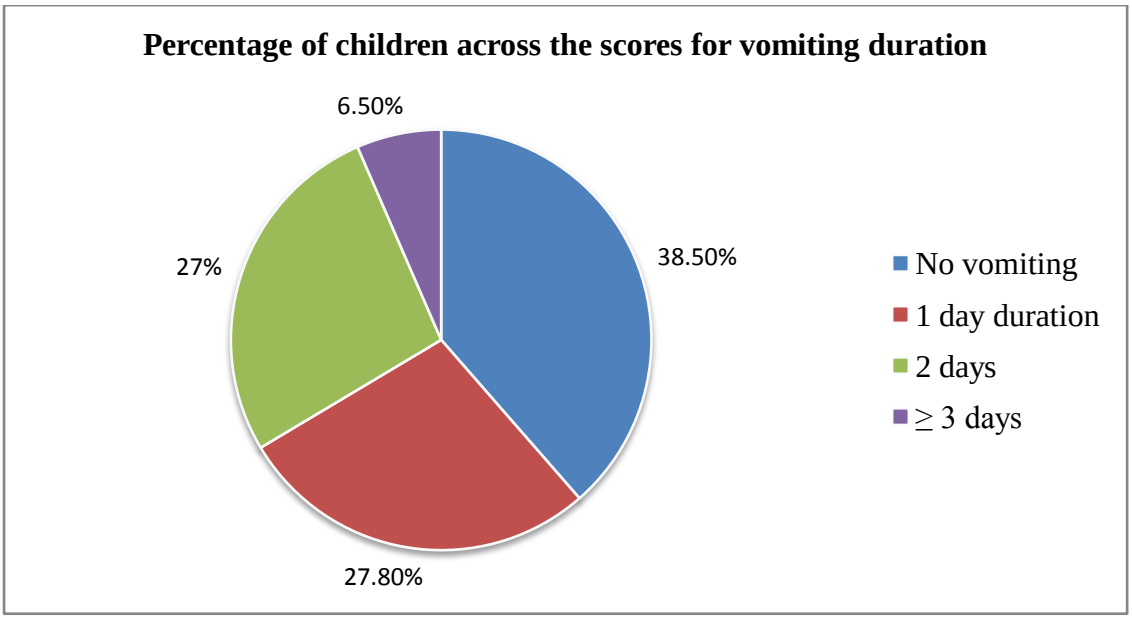
Majority of the children (70%) had more than six episodes of diarrhea in a 24 hour period.

Table 8. The median and mean Ct values along with confidence intervals across the individual scores of 'number of episodes of diarrhea'

Vesikari Score Category	Median Ct	Mean Ct	95% Confidence Intervals
1	32	32.06	29.78 – 35.13
2	28	27.02	24.61 – 29.22
3	28	29.14	28.28 – 29.99

Applying the Kruskal Wallis test, there was a significant association observed between the number of episodes of diarrhea and the median Ct values (*p* value 0.031).

3. Duration of vomiting



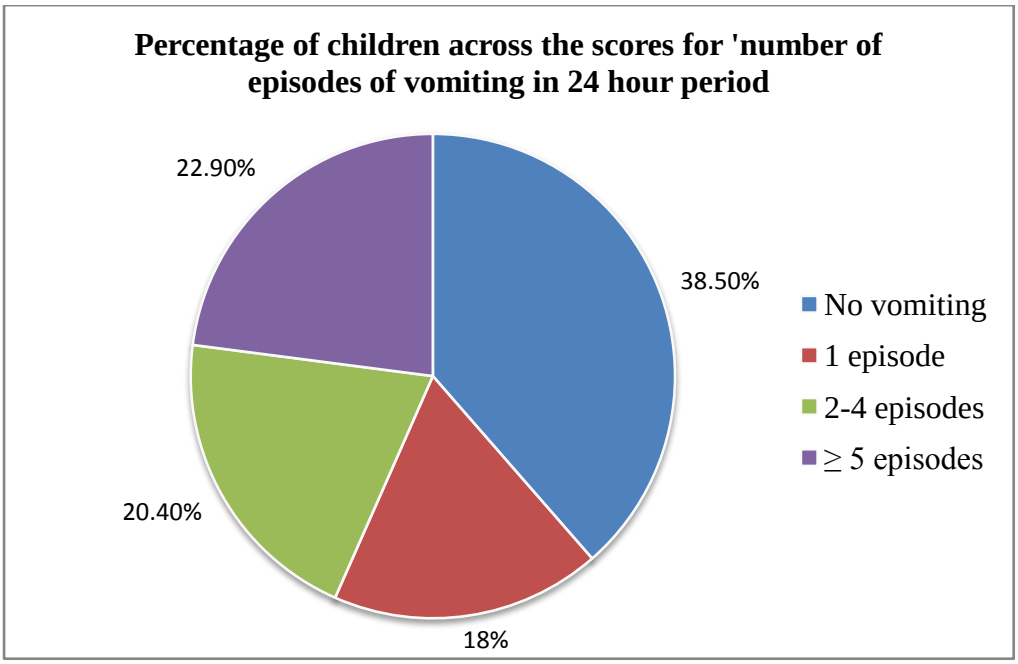
More than 60 percent of children presented with vomiting along with diarrhea and 55% had vomiting lasting for 1 to 2 days before they were brought to the health care facility.

Table 9. Median and mean Ct values with confidence intervals across the individual scores for 'duration of vomiting'

Vesikari score for duration	Median	Mean	95% Confidence Interval
0	28	28.8	27.3 – 30.4
1	29	29.3	27.6 – 31
2	28.5	28.8	27.5 – 30.1
3	30	29.6	27.5 – 31.7

Applying the Kruskal Wallis test, there was no association observed between the duration of vomiting and the median Ct values (*p* value 0.94).

4. Number of episodes of vomiting in a 24 hour period



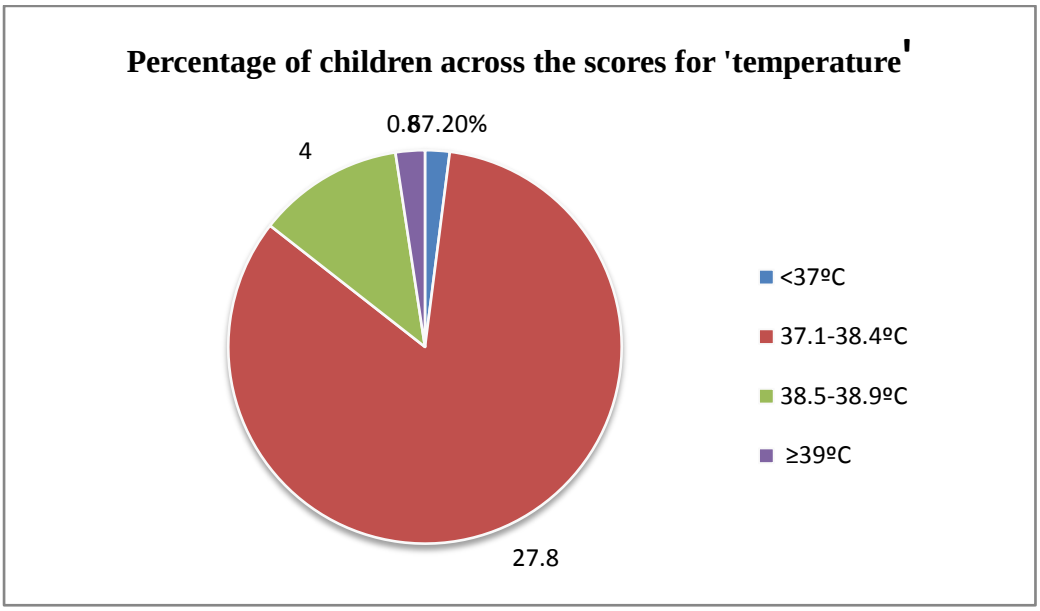
Majority of the children presented with vomiting along with diarrhea.

Table10. Median and mean Ct values with confidence intervals across the individual scores for 'number of episodes of vomiting in a 24 hour period'

Vomiting episodes score	Median Ct	Mean Ct	95% Confidence Interval
0	28	28.8	27.3 – 30.4
1	29	29.8	27.7 – 31.9
2	29	29.04	27.4 – 30.6
3	29	28.72	27.2 – 30.19

Applying the Kruskal Wallis test, there was no association observed between the number of episodes of vomiting in a 24 hour period and the median Ct values (*p* value 0.48).

5. Temperature



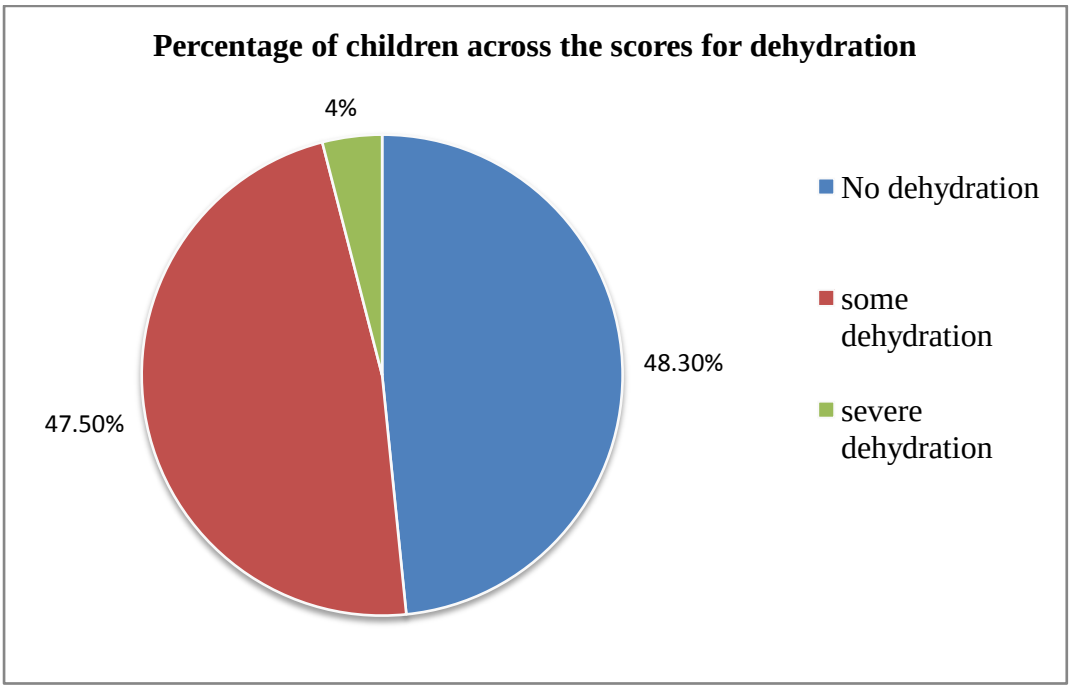
About 33 percent of the children presented with temperature above 37ºC.

Table 11. Median and mean Ct values with confidence intervals across the individual scores for 'temperature'

Temperature score	Median Ct	Mean Ct	95% Confidence Interval
0	28.5	29.1	28.1 – 30.1
1	28	28.6	27.1 – 30.2
2	26	28	23.2 - 32.7
3	24	24	

Applying the Kruskal Wallis test, there was no association observed between the temperature recorded and the median Ct values (*p* value 0.5)

6. Degree of dehydration



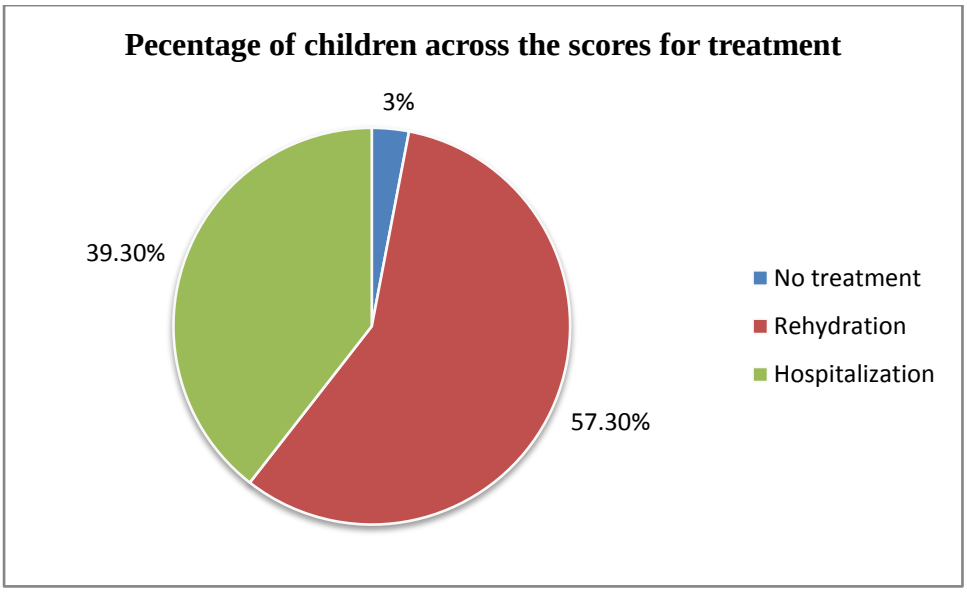
About half the number of children recruited presented with dehydration

Table 12. Median and mean Ct values with confidence intervals across the individual scores for 'degree of dehydration'

Dehydration score	Median Ct	Mean Ct	Confidence Interval
0	28	29.2	28 – 30.3
2	28	28.7	27.5 – 29.9
3	27	27.2	24.8 – 29.5

Applying the Kruskal Wallis test, there was no significant association observed between the degree of dehydration and median Ct values (*p* value 0.57)**.**

7. Treatment



3% of the children were reassured and sent back without any treatment (score 0). A majority of children (nearly 60%) received received oral rehydration therapy.

Table 13. Median and mean Ct values with confidence intervals across the individual scores for 'Treatment' component

Treatment score	Median Ct	Mean Ct	95% Confidence Interval
0	32.1	32.17	27.8 – 36.4
1	28	28.9	27.8 – 29.9
2	29	28.7	27.4 – 30.1

Applying the Kruskal Wallis test, there was no association observed between the treatment administered and the median Ct values (*p* value 0.73).

Real time PCR runs

The samples were divided into five batches and real time PCR was performed over five different runs. The viral load was assessed in terms of crossing point (Ct) which is the real time PCR cycle number where fluorescence crossed the threshold.

Statistical analysis

All the information was entered in Excel spread sheets. Statistical analysis was done using SPSS version 17. Descriptive statistics like percentages, median, interquartile range and frequencies were calculated. Kruskal Wallis test was used to study the correlation between Ct values and severity on the Vesikari scoring system and also to study the correlation between the crossing points and the different parameters of Vesikari scoring system. Two of the 124 samples had undetermined crossing points and were excluded from analysis.

Validity of the assay

The negative control and 'no template' control included with each batch had Ct values greater than 38.The positive control included with each batch had a Ct value within 2 cycles of the previous run and the Ct values ranged from 22 to 26. R^2 value obtained using the standards was > 0.9. Efficiency obtained using the standards in all the runs varied between 96% -98%.

Table 14.Validity of the Real time PCR runs

Real time PCR batch	Efficiency	Slope	R^2
1	95.92	-3.4	0.99
2	96.45	-3.4	0.95
3	97.1	-3.3	0.99
4	97.1	-3.3	0.99
5	98.04	-3.4	0.95

Reproducibility of the assay

A control VP6 plasmid was included with each PCR run and the crossing points of each dilution of the tenfold dilutions of the plasmid were similar across all the 5 runs.

The plasmid dilutions and cDNA of each sample were assessed in duplicates,

The crossing points (Ct) of the samples ranged between 13.8 and 38.9

Figure 13

Serial dilutions of standard VP6 plasmid dilutions across the five real time PCR runs

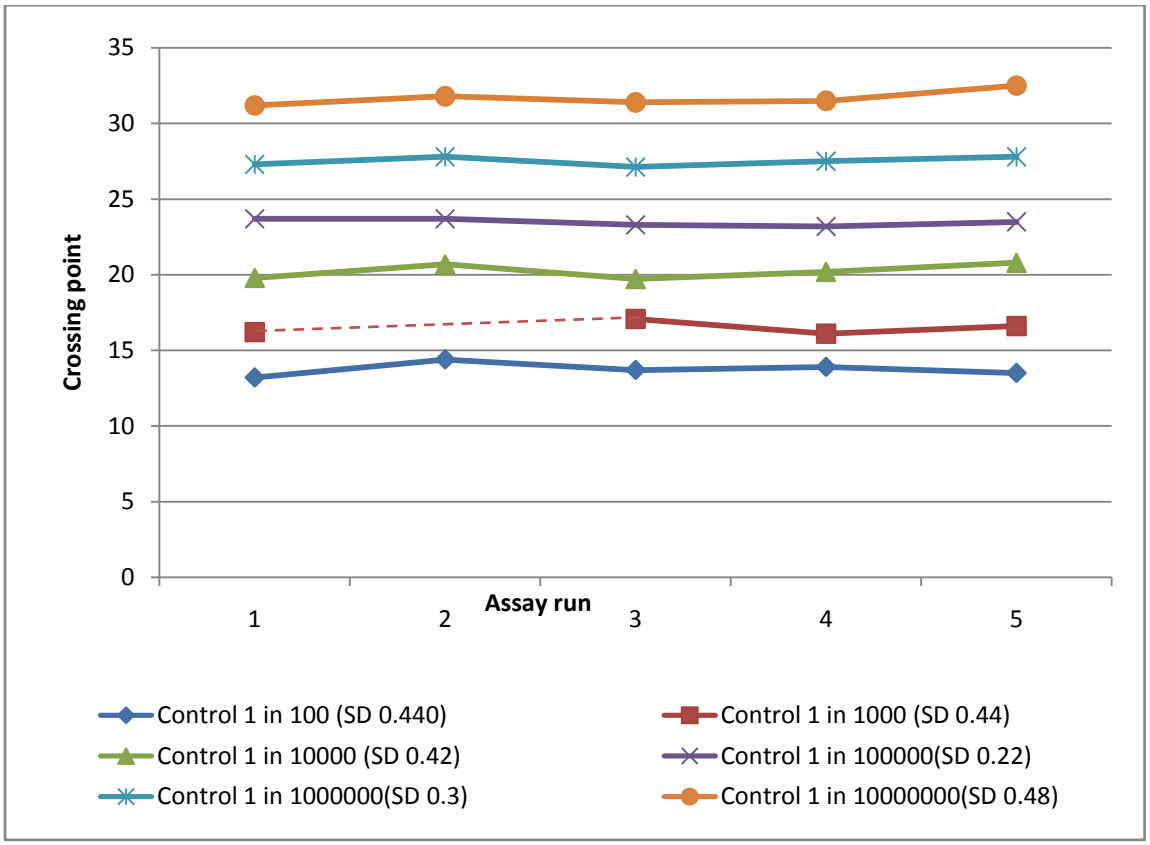
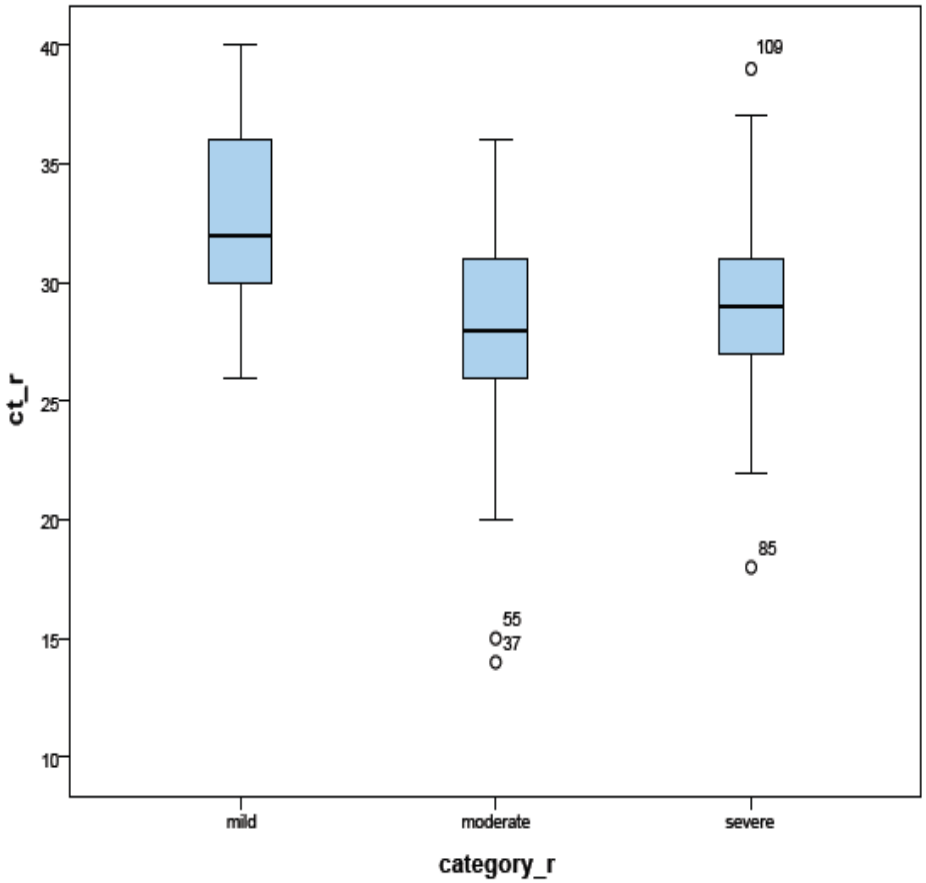


Table15. Median Ct values in the different categories

Category	Median Ct	Inter quartile range
Mild	32	29.5– 36.5
Moderate	28	26 - 31.5
Severe	29	27 – 31

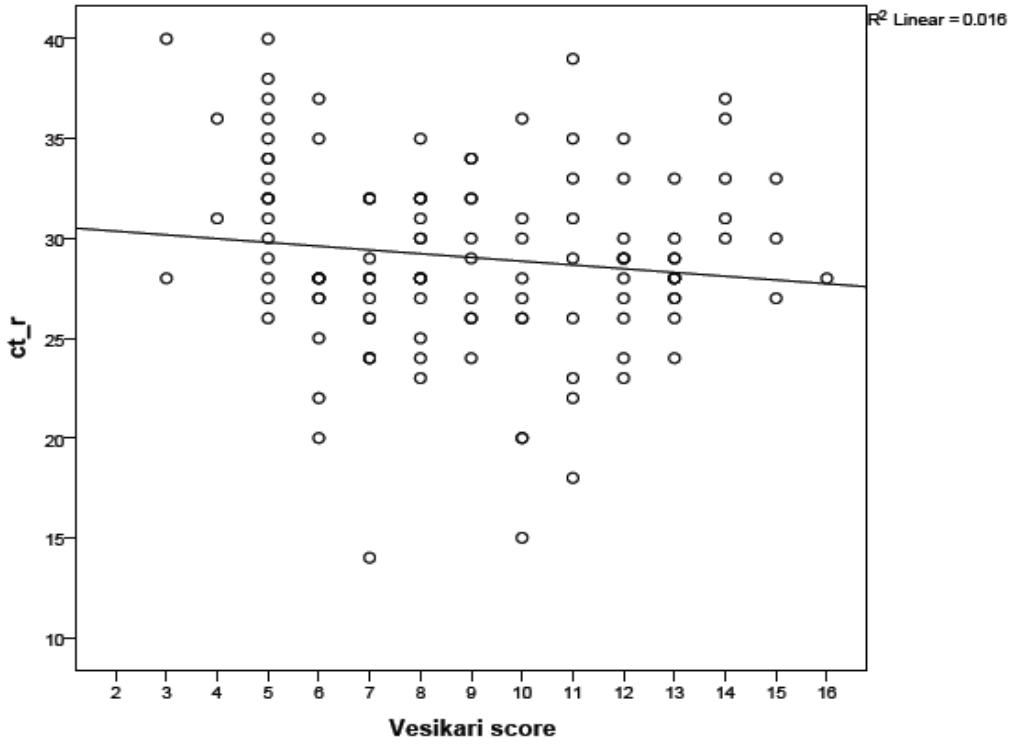
Though the median Ct values of the samples in mild category were higher than those in the moderate and severe categories, there is little gradation observed between the moderate and severe categories.

Figure 14. Box plot representing the median Ct in the different categories



The median Ct value is lowest in the ‘moderate’ category which implies a high viral load when compared to the mild and severe categories, but the linear regression shows no correlation between severity of gastroenteritis and virus excretion (p=0.16) .

Figure 15. Scatter plot of Vesikari scores with Crossing points



DISCUSSION

Diarrhea is the second leading cause of childhood mortality and rotaviruses are responsible for more than one third of these cases (2). The widespread use of molecular biology based techniques, are contributing to better understanding of the epidemiology of rotaviruses. Studies conducted so far on circulating genotypes convey that more than a single genotype exist in a particular geographical region and there are also periodic fluctuations in the circulating strains with the emergence of unusual strains intermittently. Environmental contamination with faeces and living conditions where the domestic areas are in close proximity to animals is not an uncommon feature in developing countries. The possible explanation for the constant emergence of new genotypes is the segmented nature of the genome and the reassortment taking place between human and animal strains.

The virus replicates in the gut and hence measurement of viral load in stool samples is a marker of its replication. Studies have shown a correlation between severity of diarrhea and viral load in faecal samples and also antigenemia in serum. Real time polymerase chain reaction helps in quantitation of viral load and its use in rotavirus studies enabled the study of the shedding patterns and associations with various pathophysiological aspects of rotavirus diarrhea. The current study was intended to study the correlation between severity of diarrhea and viral load. The study employed the Vesikari scoring system which is the best-known and the most frequently used scoring system in assessing severity of diarrhea measuring valid end points with minimal inter individual variation (115).

Faeces samples from children with gastroenteritis positive by Rotaclone EIA, had RNA extracted and reverse transcription followed by real time PCR was performed. The samples were broadly divided into hospital based samples which were collected from children attending the out-patient and emergency department and admitted to paediatric wards and community based samples collected from children attending the community clinic in a urban slum area of Vellore. The mean age of all the children is 12 months (SD 7.96). In this study rotavirus affected majority of the children between 7 to 14 months (IQR 7 – 14). This age group corresponds to that period when the passively acquired maternal antibody levels in the child starts to wane.

Vesikari scores ranged from 3 to 15. The difference in the mean Vesikari scores between hospital based samples (8.4 and SD 2.97) and community based samples (9.13 and SD 2.97) was not statistically significant. The majority of the samples had Vesikari scores between 6 and 12 (IQR 6 – 12) implying most of the samples had moderate diarrhea as opposed to mild and severe categories.

Crossing points in real time PCR, being a direct correlate of viral load, were analysed with the components of the Vesikari scoring system. There was no correlation observed between the severity of diarrhea and the rotavirus load. However, when the individual components of the Vesikari score were analysed for any association with the viral load, there was a significant negative correlation seen between the number of episodes of diarrhea in a 24 hour period and crossing points on the real time PCR indicating that the greater the number of diarrheal episodes the greater is the viral load (p value 0.031). This is in agreement with findings from a previous study conducted on rotavirus quantitation using real time PCR where there was a significant correlation

between the number of episodes of diarrhea and viral load (6). However, the same study showed significant association between severity of diarrhea and viral load. The current study did not reveal any significant associations between the other individual components of the scoring system and the viral load. The incubation period in rotavirus infection is 48 hours and viral excretion is expected to be high in the initial days of diarrhea. There was no association between duration of diarrhea and viral load probably because of unavoidable delays in bringing the children to health care facility. The scoring on most of the hospital based samples including the in-patient samples was mostly carried out in the busy Paediatric emergency department. Dehydration which is more commonly seen in rotavirus diarrhea is prone to be scored subjectively. The symptoms may worsen if adequate rehydration measures are not taken immediately and this increase in severity may not be directly related to increase in viral load. Modifications in the scoring system with reference to degree of dehydration have been introduced in some studies to ensure wider acceptance (115).

LIMITATIONS OF THE STUDY

Limitations of the study include lack of testing for other aetiology of gastroenteritis and lack of controls with asymptomatic infection. Though care was taken to ensure that cold chain was maintained, the time interval which elapsed between collection of stool specimen from the child till it was transported to the laboratory was not recorded. Its bearing on viral load estimation cannot be completely ignored.

Genotyping of rotavirus was beyond the scope of the study. Certain genotypes are associated with more severe diarrhea whereas others are asymptomatic in

presentation. On the other hand, the same genotype may exhibit different clinical presentations in different geographic locations (57,116).

A previous study on the protective effect of natural rotavirus infection in an Indian birth cohort showed that more than half the children were infected by the age of six months (59). Almost 90% of the children acquire antibodies against rotavirus by the age of 5 years, and prior infections confer some degree of protection from subsequent infections (59,92). The level of already existing antibodies may also contribute to mismatch between severity and viral load.

A small representative amount of 0.2gm of the stool sample was processed. Measuring the total amount of stool passed by the child each day would lead to more accurate quantitation, since both frequency and volume of stool in gastroenteritis may reflect gut damage and virus replication.

The Vesikari scoring system is widely used, but conclusions on viral load can be drawn only if strict adherence to measuring the individual parameters of the scoring system is followed while taking into consideration other factors including existing antibody levels and genotypes.

CONCLUSION

In this study to quantify rotavirus load using real time PCR and correlate viral load with clinical severity using the Vesikari scoring system, there was no significant correlation studied between the severity of rotavirus gastroenteritis and the viral load. However, with increasing episodes of diarrhea, the viral load also increased.

APPENDIX

ENZYME IMMUNOASSAY USING PREMIER ROTACLONE® KIT

Materials Required

Equipment: Microplate reader, Pipettes, Pipette tips, Refrigerator, Biological Safety Cabinet

Reagents

Premier Rotaclone®

Deionized water, pyrogen free

Bovine Rotavirus lysate – Internal Control

Procedure

All the reagents were thawed and brought to room temperature before testing and replaced at 2 – 8°C soon after use. It was ensured that the wells did not dry between the steps. All the kit components were labelled with the date they were opened before proceeding with the ELISA.

Sample Preparation

Using the diluent provided in the kit, a 10% fecal suspension was prepared. This could be used for upto 3 days when stored at 2 – 8°C without affecting the assay outcome. This fecal suspension was vortexed for 15 minutes at room temperature to emulsify in the diluent. Then it was left to stand at room temperature for the sediment to settle at the bottom.

Steps

1. The required number of wells were set in the microtitre well holder.
2. Two drops of diluted fecal sample, positive control, negative control (sample diluent) and 100 µl of bovine rotavirus (BRV) were added to the bottom of separate wells.
3. Two drops of enzyme conjugate were added to each well. All the reagents in the wells were mixed by gently swirling it on the table top.
4. After step 3, the plate was allowed to incubate at room temperature for 1 hour.
5. The liquid in the wells was poured out and the microtitre well plate is held upside down and tapped vigorously against absorbent paper to ensure complete removal of the reagents from the wells.
6. All the wells were filled to the brim with milliQ water, which was then poured out and the wells tapped vigorously against absorbent paper.
7. This washing procedure was repeated four times.
8. Two drops from substrate A solution were added to each well.
9. Two drops from substrate B solution were added to these wells.
10. The microtitre well plate with all the reagents in it was incubated at room temperature for 10 minutes.
11. Two drops of stop solution were added to each well.
12. The absorbance of each well was read at 450 nm for the Optical Density (OD) values.

Data interpretation

Validity criteria

1. The validity of the assay was evaluated by the BRV values which was used as the internal control. The range had to be 0.8 -0.9.
2. The cut off value for positive control had to be greater than 0.150.
3. The cut off value for negative control had to be less than 0.150.

Acceptance Criteria

1. The samples were considered positive for rotavirus if the absorbance units (A_{450}) was greater than 0.150.
2. If the absorbance was less than or equal to 0.150 , the samples were considered negative for rotavirus.
3. The samples had to be retested if the validity and acceptance criteria were not obtained.

RNA EXTRACTION USING QIAGEN KIT METHOD

Materials

Equipment: Pipettes**,** Pipette tips**,** Disposable gloves**,** Microcentrifuge tubes**,** Vortex shaker**,** Biological Safety Cabinet**,** Ultracold freezer**,** Centrifuge

Reagents

QIA amp Viral RNA Mini Spin Kit Qiagen

Controls:

Positive Control which is a known rotavirus positive fecal suspension

Negative Control which is a known rotavirus negative fecal suspension

Water control : DEPC treated water

Procedure

Preparation of 20% Fecal Suspension

This is made by using Minimal Essential Medium. Fecal specimens which are ELISA positive were stored at -70°C. These were taken out, thawed and brought to room temperature. About 0.2gm or 200µl of stool was measured and taken in a microcentrifuge tube.

One ml of minimal essential medium was added and vortexed for 1 minute. Centrifugation was carried out at 10000 rpm for 5 minutes. Faecal extracts were stored at -20°C to -70°C until RNA extraction

RNA EXTRACTION

RNA extraction is carried out using Qiagen kit extraction method (QIAamp® Viral RNA minikit). RNA extraction is carried out in three steps using the spin columns provided with the kit. These spin columns fit well into any standard microcentrifuge tube. These tubes are RNase free.

Adsorption to the QIAamp membrane

Viral RNA is adsorbed onto silica-gel membrane during centrifugation. Salt and pH conditions in the lysate makes sure that proteins which inhibit the downstreamreactions are not retained on the QIAamp membrane.

Removal of residual contaminants

Two wash buffers AW1 and AW2 wash the RNA bound to the membrane and make it free of contaminants.

Elution

Buffer AVE which is RNase free water that contains 0.04% sodium azide prevents microbial growth and resulting RNA contamination.

Preparation of reagents

Addition of buffer AVE to Carrier RNA

AVE buffer was checked for any precipitates and was made precipitate free.

AVE buffer (310µl)was added to the vial containing lyophilised carrier RNA. The needed volume of this was used (up to a maximum of 6 times) and the rest stored at 2

to 8°C. When needed, an appropriate volume of this AVE-carrier RNA is mixed with stipulated amount of AVL as per the protocol standards and used.

Buffer AW1

This buffer is available as a 25 ml concentrate and before using it, 19ml of absolute alcohol (100% ethanol) was added to it to make up to a final volume of 44ml.

Buffer AW2

This buffer is also available as a 13 ml concentrate and before using it, 30 ml of absolute alcohol is added to it to make upto a final volume of 43 ml.

Handling of spin columns

The following precautions were taken to overcome cross contamination in view of the sensitivity of the nucleic acid amplification techniques.

The sample was carefully pipetted and applied to the spin column without wetting the rim of the spin column.

Aerosol- barrier tips were used and pipette tips were changed between the transfer steps.

After each pulse vortexing step, the spin columns were spun briefly to remove drops accumulated towards the inside portion of the lid.

Gloves were worn throughout the procedure and were changed everytime they got into contact with the sample accidentally.

Spin Protocol

The spin columns were always set in the respective collection tubes and remained closed. Only when adding reagents, they were opened one at a time.

After adding the necessary reagents, the spin column was closed and centrifuged. The filtrate obtained in the collection tube was discarded and the column was transferred to a new collection tube. Before starting the extraction procedures the samples and reagents were thawed and brought to room temperature before starting the extraction procedure.

Steps

1. 560 µl of the already prepared AVL containing carrier RNA was pipetted into a microcentrifuge tube.
2. 140 µl of the fecal extract supernatant was added to it. They were mixed by pulse vortexing for 15 sec .
3. After step 2, incubation was carried out at room temperature (15-25C) for 10 minutes. Viral particles lyse completely in this duration. AVL also inactivates RNases and other infectious agents.
4. Brief centrifugation was carried out to remove any droplets that accumulated on the underside of the lid while carrying out the previous steps.
5. 560 µl of ethanol was taken into these centrifuge tubes and mixed by pulse-vortexing for 15 seconds. A brief centrifugation was carried out to remove droplets under the lid. Only ethanol was used in this step as other alcohols are likely to reduce the RNA yield.

6. 630 µl of sample from step 5 was pipetted carefully into a QIAamp spin column nested in a 2ml collection tube. Adequate measures were taken not to wet the rim of the spin column. This was centrifuged at 8000 rpm for 15 seconds and the spin column was carefully transferred into a second clean collection tube. The filtrate from the first collection tube was discarded.
7. With another 630µl from the microcentrifuge tube taken into the spin column, step 6 was repeated.
8. 500µl of AW1 buffer was pipetted into the spin column after carefully opening the cap and then closed. This was centrifuged at 8000 rpm for 1 minute and the spin column was transferred to another clean collection tube. The filtrate collected was discarded appropriately.
9. The spin column cap was carefully opened and 500µl of AW2 buffer was added to it. Centrifugation at full speed, i.e. 13200 rpm, was carried out for 3 minutes.
10. The spin column was placed in a microcentrifuge tube which was not provided with the kit. The old collection tube with the filtrate was discarded.
11. 60µl of AVE buffer which was at room temperature was pipetted into the spin column after carefully opening it. The spin column was closed and left to stand at room temperature for 1 minute.
12. The microcentrifuge tube along with the column was centrifuged at 8000rpm for 1 minute. This is enough to elute atleast 90% of RNA present in the spin column.
13. 40 µl of this eluted RNA was used for reverse transcription and conversion to cDNA whereas the rest was stored at -70°C.

REAL TIME PCR

Procedure

1. Preparation of plasmid standards for quantitative PCR
2. The plasmid has 10^{10} copies per sample.Ten fold dilutions were made to obtain 10^{9} to 10^{1}plasmid standards per 2µl sample for carrying out the real-time PCR.
3. Eight of these serial dilutions containing rotavirus VP6 fragment were included with each run as positive control and to derive the standard curve for quantitation of the virus from the fecal specimen.
4. One set of the diluted plasmids was stored as stock plasmid at -70°C

Preparing the Real time PCR Master Mix

1. Master mix was aliquoted before use.
2. Working concentrations were prepared and labelled accordingly.
3. Preparation of the Mastermix was carried out in the clean room.
4. All the reagents were thawed first and then briefly vortexed and centrifuged.
5. Reagents required for 1 sample of cDNA

Taqman Mix	12.5µl
VP6- F Primer	2.0 µl
VP6- R Primer	2.0 µl
VP6- Probe	0.125 µl
H_20	8.3 µl

6. 23 µl of the above reagents mix was added to 2 µl of the cDNA obtained from the fecal specimen.

Addition of samples and controls

1. 2µl of the sample cDNA was added to 23µl of master mix.
2. Gloves were changed frequently and adequate care taken to avoid cross contamination.
3. Once cDNA was added to the master mix, the wells were firmly fitted with caps.
4. Samples for standard curve were included in every run for absolute quantitation.
5. The strips after sealing with the caps underwent spinning to remove any bubbles.

Running the Reactions

1. ABI 7500 Fast Real Time PCR machine was used. The computer and the machine were switched on first.
2. The strips with the cDNA and master mix were loaded into the real time machine.
3. The ABI 7500 software was accessed to prepare the template.
4. On the real time software, the wells were labelled accordingly. The standards were labelled ‘standard’ and the samples along with a known positive and negative were labelled ‘unknown’. A no template control was included and labelled ‘negative’.
5. The number of copies in the known dilutions were entered against the sample identity in the template provided.

6. The run method belonging to “Invitrogen Rota quantitation using VP6 probes and TaqMAN chemistry” was selected from the panel displayed so that the preset temperatures would take over automatically.

7. After loading the strips and setting the run method, the ‘start run’ button was selected and the program commenced.

8. After completion of a run, the analysis tools were used to analyse the data. The standard curves were checked for a good slope and R^2 value (A perfect slope would be -3.3 and R^2 1).

9. The data was analysed in Microsoft Excel 2010.

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Office of the Addl. Vice Principal (Research)

Christian Medical College,
Vellore 632 002

Ref: Res/09/2011

February 16, 2012

Dr. P Susmitha Karunasree
PG Registrar
Department of Microbiology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL**:
Quantitation of Group A Rotavirus by real-time polymerase chain reaction in children with varying severity of gastroenteritis
Dr. P Susmitha Karunasree, PG Registrar, Microbiology, Dr. Gangandeep Kang, GI Sciences, Dr, Anna Simon, Dr. Leni Mathew, Dr. Indira Agarwal, Dr. Prabhakar D. Moses, Dr. Kala Ebenezer, Child Health Unit IV, Dr. Sudhir Babji, Wellcome Trust Research Laboratory.

Ref: IRB Min. No. 7744 dated 6.2.2012

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
Editor, Indian Journal of Psychological Counseling
Chairperson, Ethics Committee, IRB

Dr. Alfred Job Daniel, MS Ortho
Chairperson, Research Committee &
Principal

Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

February 16, 2012

Dr. P Susmitha Karunasree
PG Registrar
Department of Microbiology
Christian Medical College
Vellore 632 002

Sub: **FLUID Research grant project NEW PROPOSAL**:
Quantitation of Group A Rotavirus by real-time polymerase chain reaction in children with varying severity of gastroenteritis
Dr. P Susmitha Karunasree, PG Registrar, Microbiology, Dr. Gangandeep Kang, GI Sciences, Dr, Anna Simon, Dr. Leni Mathew, Dr. Indira Agarwal, Dr. Prabhakar D. Moses, Dr. Kala Ebenezer, Child Health Unit IV, Dr. Sudhir Babji, Wellcome Trust Research Laboratory.

Ref: IRB Min. No. 7744 dated 6.2.2012

Dear Dr. Karunasree,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Quantitation of Group A Rotavirus by real-time polymerase chain reaction in children with varying severity of gastroenteritis" on February 6, 2012.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Patient Information Sheet and Consent Form (English and Tamil)
3. Case reporting form
4. Cv of Drs. Gagandeep Kang, Indira Agarwal, Sudhir Babji, Prabhakar D Moses, Lenin Grace Mathew
5. A CD containing documents 1 – 4

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
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Dr. Nihal Thomas
MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

The following Institutional Review Board (Ethics Committee) members were present at the meeting held on February 6, 2012 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore- 632002.

Name	Qualification	Designation	Other Affiliations
Dr. B.J.Prashantham	MA (Counseling), MA (Theology), Dr Min(Clinical)	Chairperson(IRB)& Director, Christian Counselling Centre	Non-CMC
Mr. Harikrishnan	BL	Lawyer	Non-CMC
Mrs. S. Pattabiraman	BSc, DSSA	Social Worker, Vellore	Non-CMC
Mrs. Ellen Ebenezer Benjamin (on behalf of Dr. Jayarani Premkumar)	M.Sc. (Nursing), Ph.D.	Nursing Superintendent, CMC.	
Mrs. Shirley David (on behalf of Dr. Jayarani Premkumar)	M.Sc. (Nursing), Ph.D.	Nursing Superintendent, CMC.	
Dr. Nihal Thomas	MD MNAMS DNB(Endo)FRACP(Endo) FRCP(Edin)	Secretary IRB (EC)& Dy. Chairperson (IRB), Professor of Endocrinology & Addl. Vice Principal (Research), CMC.	

We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent and requires a copy of the final report.

INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002, INDIA

Dr.B.J.Prashantham, M.A.,M.A.,Dr.Min(Clinical)
Director, Christian Counseling Centre
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MD, MNAMS, DNB(Endo), FRACP(Endo), FRCP(Edin)
Secretary, Ethics Committee, IRB
Additional Vice Principal (Research)

A sum of ₹ 40,000/- (Rupees Forty thousand only) can be sanctioned for 12 months. A subsequent installment of 40,000/- will be released at the end of the first year following the receipt of the progress report (Total amount 80,000/-).

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Secretary
Institutional Review Board
(Ethics Committee)
Christian Medical College
Vellore - 632 002, Tamil Nadu, India

Information to the Participant

Rotavirus is responsible for a majority of diarrhea related deaths in children below 5 years. 1 in every 250 children die of diarrhea with this virus every year. Hence control measures are of great need. Our country is trying to make vaccinations to suit our population and at the same time are cost effective. Children presenting with diarrhea may have mild to very severe clinical symptoms. This study is being done to see the amount of virus passed out in the stools of children with diarrhea presenting with varying severity of symptoms. This can help us in the long run to evaluate the upcoming vaccines in their ability to control this rotaviral diarrhea.

You are being requested to participate in the above mentioned study as it will help us develop a good tool to measure severity.

What will you have to do?

If you wish to take part in the study, you will have to sign the consent form first and then collect stool sample of your child in the container provided.

Will you have to pay for the tests to be done in the study?

You will not need to pay any amount towards the tests.

Can you withdraw from the study?

You can withdraw from participating in the study at any point of the study.

Will you personal details be kept confidential?

Information is taken in terms of severity of diarrhea and no personal details are sought.

For further queries please contact Dr. Susmitha (Mob No. 09944642476)

CONSENT TO PARTICIPATE IN THE STUDY

Study title:

Quantitation of Group A Rotavirus in children with varying severity of gastroenteritis.

Study Number:

Date of Birth / Age (in years):

I_____

_____, parent/guardian of _____

(Please tick boxes)

Declare that I have read the information sheet provided to me regarding this study and have clarified any doubts that I had. [ ]

I also understand that participation in this study is entirely voluntary and that I am free to withdraw permission to continue to participate at any time without affecting any treatment or legal rights [ ]

I also understand that the tests done on the specimen will be done free of cost. [ ]

I understand that the child's identity will not be revealed in any information released to third parties [ ]

I voluntarily give my consent to let my child take part in this study [ ]

Name:

Signature:

Date:

Name of witness:

Date:

Case Reporting Form

Date Hospital number:

Patient serial number

Address :

Diagnosis by the attending physician:

Age of the child: Date of birth: Sex of the child: M/F

Clinical information

Temperature: ____C

Vomiting : Yes/No Duration : ____ days Max no. of episodes in 24hrs: __

Diarrhea: Yes/No Duration: _____ days Max no. of episodes in 24 hrs: ___

Signs of dehydration:

Lethargy: Yes/ No Restless/ Irritable: Yes/No

Feeding well: Yes/No Eyes sunken: Yes/ No

Skin pinch: Normal ( ) slow return ( ) Very slow return ( )

Degree of dehydration: None ( ) some( ) Severe ( )

Treatment

Supervised oral / Intravenous rehydration

Laboratory Information

Rotavirus identified in stool by ELISA : Yes/ No

Person Completing the form: Signature

Vesikari Score:

S.No	Hosp No	Age	Sex	Vesikari score	Category	EIA OD	Viral load	Ct	Sample ID	Diarrhea days	No.of episodes of diarrhea	vomiting days	no.of episodes of vomiting	Temperature	Dehydration	Treatment	AREAS
1	379430F	5	M	5	mild	2.601	20601.09	33.7	RQ 001	1	1	1	1	0	0	1	Hosp In
2	364484F	12	M	5	mild	0.579	3491.81	36.3	RQ 013	1	1	1	1	0	0	1	Hosp In
3	389329F	6	M	5	mild	1.214	67269.56	32	RQ 014	1	1	1	1	0	0	1	Hosp In
4	LBS 083	9	M	5	mild	1.306	399	36.9	LBF 435	1	1	1	1	1	0	0	Comm
5	LBS 075	9	F	3	mild	0.204	100.22	39.9	LBF 947	1	1	0	0	0	0	1	Comm
6	LBS 156	6	M	5	mild	0.518	106727.5	28.7	LBF 2077	1	2	0	0	1	0	1	Comm
7	LBS 241	7	M	5	mild	1.115	16632.39	31.3	LBF 4289	1	1	0	0	0	2	1	Comm
8	LBS 283	7	M	5	mild	0.473	344324.723	26.2	LBF 5232	1	3	0	0	0	0	1	Comm
9	LBS 003	11	M	5	mild	0.818	57108.806	32.2	LBF 6819	1	3	0	0	0	0	1	Comm
10	046246F	20	F	5	mild	0.194	3100.07	34.8	CM0221	1	3	0	0	0	0	1	Hosp In
11	294145F	3	M	5	mild	0.535	30840.5	29.8	CM0007	1	1	1	1	0	0	1	Hosp In
12	136433F	7	M	5	mild	0.328	137.22	38.4	CM0023	2	2	0	0	0	0	1	Hosp out
13	804918D	16	M	3	mild	3.262	807756	27.7	CM0023	1	1	0	0	0	0	1	Hosp in
14	852962D	14	M	5	mild	0.634	275498.5	27.9	CM0027	1	3	0	0	0	0	1	Hosp out
15	858213D	18	F	5	mild	1.723	20033.6	32.6	CM0205	1	3	0	0	0	0	1	Hosp out
16	098971F	8	M	5	mild	0.338	29494.05	31.6	CM0239	1	3	0	0	0	0	1	Hosp out
17	597502D	34	M	5	mild	0.909	976058.8	26.6	CM0289	1	3	0	0	0	0	1	Hosp out
18	046964F	3	M	4	mild	0.247	363.886	36.3	CM0423	1	3	0	0	0	0	1	Hosp out
19	032947F	10	F	4	mild	0.331	52283.2	31.2	CM0218	1	2	0	0	1	0	1	Hosp out
20	331778F	6	M	5	mild	2.071	423.947	36.8	CM0045	1	3	0	0	0	0	1	Hosp out
21	788941D	18	M	5	mild	0.164	157.694	39.7	CM0276	1	1	1	1	0	0	1	Hosp out
22	379621F	18	M	6	moderate	1.808	142779.9	27.5	RQ 007	1	1	1	1	0	0	1	Hosp out
23	384232F	16	M	6	moderate	1.323	71886	28.5	RQ 010	1	2	1	1	0	0	1	Hosp out
24	389328F	6	M	6	moderate	2.378	5572291.1	22.2	RQ 015	1	2	1	1	0	0	1	Hosp out
25	379497F	17	M	8	moderate	2.063	101727.5	28	RQ 009	1	1	1	1	1	2	1	Hosp out
26	LBS 211	6	M	6	moderate	1.469	193787.829	27.1	LBF 4473	1	3	0	0	1	0	1	Comm
27	LBS 008	11	M	6	moderate	2.095	168002.4	28	LBF 2497	1	3	0	0	1	0	1	Comm
28	LBS 308	7	M	6	moderate	0.261	2174.916	34.3	LBF 3744	1	1	0	0	0	2	1	Comm
29	LBS 054	8	F	6	moderate	2.757	134118.493	27.6	LBF 6648	1	2	1	2	0	0	0	Comm

30	LBS 187	6	F	8	moderate	3.033	28925.61	30.5	LBF 2332	1	3	1	1	1	0	1	Comm
31	LBS 059	11	F	10	moderate	3.056	406047.2	26.7	LBF 4008	1	3	2	3	0	0	1	Comm
32	LBS 217	7	M	7	moderate	0.281	279086.5	27.2	LBF 4158	1	3	0	0	0	2	1	Comm
33	LBS 257	6	M	8	moderate	1.079	87443.399	28.2	LBF 5791	2	3	0	0	0	2	1	Comm
34	LBS 329	6	F	10	moderate	0.605	389027.276	26.1	LBF 5816	1	3	1	2	0	2	1	Comm
35	LBS 024	9	M	7	moderate	1.185	1794970647	13.8	LBF 5937	2	2	0	0	0	2	1	Comm
36	LBS 094	9	F	9	moderate	0.566	20876.84	33.7	LBF 6279	1	2	1	1	1	2	1	Comm
37	LBS 301	6	M	7	moderate	0.541	56491.767	28.9	LBF 6405	1	3	0	0	0	2	1	Comm
38	LBS 269	6	M	10	moderate	2.272	137494.582	27.6	LBF 6573	1	3	1	1	1	2	1	Comm
39	LBS 084	7	M	7	moderate	2.767	104684.606	28	LBF 6810	1	3	0	0	0	2	1	Comm
40	LBS 227	7	M	8	moderate	0.528	3351629.754	22.9	LBF 7652	1	3	1	2	0	0	1	Comm
41	LBS 299	11	F	7	moderate	1.892	88727.1986	28.2	LBF 7680	1	3	0	0	0	2	1	Comm
42	LBS 216	9	M	9	moderate	0.342	55854.737	28.9	LBF 8409	1	2	1	2	0	2	1	Comm
43	389328F	6	M	7	moderate	1.514	147243.8	24.2	CM0149	2	3	0	0	1	0	1	Hosp out
44	367536F	12	M	6	moderate	1.119	30899070.8	19.7	CM0111	1	3	0	0	0	0	2	Hosp in
45	309472F	29	M	10	moderate	1.708	364302.726	26.2	CM0009	1	3	2	2	0	0	2	Hosp in
46	085396F	13	M	9	moderate	2.348	562765.25	25.5	CM0030	1	3	0	0	1	2	2	Hosp in
47	082213F	11	M	8	moderate	1.908	73435.84	31.9	CM0070	1	3	0	0	0	2	2	Hosp in
48	927039D	22	F	10	moderate	1.416	17641404.65	20.5	CM0074	1	3	1	1	0	2	2	Hosp in
49	347835F	12	F	8	moderate	1.822	59895.12	32.2	CM0076	1	3	0	0	0	2	2	Hosp in
50	170495F	8	M	8	moderate	0.498	9607.98	34.8	CM0100	1	3	2	2	0	0	0	Hosp in
51	367550F	12	M	7	moderate	2.118	298765.754	26.5	CM0114	1	2	0	0	0	2	2	Hosp in
52	374544F	28	M	8	moderate	0.78	116747.3883	24.5	CM0123	1	3	0	0	0	2	2	Hosp in
53	207201F	7	F	10	moderate	0.78	874050062.3	14.8	CM0124	3	2	0	0	1	2	2	Hosp in
54	180938F	6	M	12	moderate	0.626	1289.531	34.6	CM0130	1	3	2	2	0	2	2	Hosp out
55	189063F	8	F	8	moderate	0.701	60032.28	32.3	CM0133	1	3	0	0	0	2	2	Hosp In
56	379497F	13	M	10	moderate	0.887	18216.806	30.6	CM0134	1	3	2	2	0	0	2	Hosp In
57	001863F	18	M	10	moderate	0.244	340.18	36.5	CM0136	1	3	0	0	2	2	2	Hosp In
58	395521F	16	F	9	moderate	0.286	226662.925	26.8	CM0147	1	3	0	0	0	3	2	Hosp In
59	389329F	6	M	9	moderate	1.614	6387.42	32.1	CM0150	1	3	0	0	0	2	2	Hosp In
60	014698F	5	M	8	moderate	1.368	10074.27	31.6	CM0153	1	3	0	0	0	2	2	Hosp In
61	314159F	29	M	7	moderate	1.22	1253697.25	24.4	CM0168	1	3	0	0	1	0	2	Hosp In
62	411128F	7	M	10	moderate	1.682	179534.7	29.6	CM0180	1	2	3	2	0	0	2	Hosp In

63	054881F	15	F	9	moderate	0.465	186557.5	29.6	CM0186	1	2	2	3	0	0	1	Hosp In
64	113103F	14	F	9	moderate	1.83	3854479	23.6	CM0194	1	3	2	2	0	0	1	Hosp In
65	179904F	13	M	10	moderate	0.498	70556307.2	20.5	CM0197	1	3	2	3	0	0	1	Hosp In
66	406355F	6	F	8	moderate	0.939	28891.86	30.6	CM0204	2	3	0	0	2	0	1	Hosp In
67	259672F	8	F	8	moderate	2.585	394879.57	27.5	CM0218	1	3	0	0	0	2	2	Hosp In
68	352539F	3	F	8	moderate	3.078	174237.8	25.3	CM0231	1	3	0	0	0	2	2	Hosp In
69	244516F	9	M	7	moderate	2.647	4108985.4	23.9	CM0232	1	2	0	0	3	0	1	Hosp out
70	354842F	7	F	7	moderate	0.314	67283.73	32	CM0083	1	2	1	1	1	0	1	Hosp In
71	074615F	12	M	6	moderate	1.319	928560.6	26.9	CM0149	1	3	0	0	1	0	1	Hosp In
72	765652D	27	M	6	moderate	0.175	1058.966	34.7	CM0347	2	3	0	0	0	0	1	Hosp out
73	399797F	8	F	6	moderate	0.331	290323.52	25.00	CM0381	2	3	0	0	0	0	1	Hosp out
74	152136F	11	F	8	moderate	0.761	868091.4	27.6	CM0030	1	2	2	2	0	0	1	Hosp out
75	162180F	7	M	8	moderate	0.28	15179.92	29.60	CM0038	1	3	1	2	0	0	1	Hosp out
76	171179F	8	F	9	moderate	0.73	15093	32	CM0039	3	3	1	1	0	0	1	Hosp out
77	937094D	12	F	7	moderate	0.499	592652.4	27.6	CM0130	1	2	2	1	0	0	1	Hosp out
78	992345D	11	F	9	moderate	0.2	6807.9	34.2	CM0170	1	3	2	2	0	0	1	Hosp out
79	162155F	6	M	8	moderate	2.262	113246.50	26.6	CM0232	1	2	2	2	1	0	1	Hosp out
80	810872D	24	F	9	moderate	3.327	589665.9	26.5	CM0308	1	3	1	2	1	0	1	Hosp out
81	280282F	2	M	7	moderate	0.391	51045	31.5	CM0330	3	3	0	0	0	0	1	Hosp out
82	110480F	10	F	7	moderate	1.419	5607011.272	25.7	CM0334	1	3	1	1	0	0	1	Hosp out
83	200807F	15	M	11	severe	1.454	117567652.2	17.7	RQ 008	1	2	1	3	1	2	1	Hosp out
84	014698F	12	F	11	severe	1.031	91029421	21.5	RQ 016	1	2	1	1	0	0	1	Hosp out
85	LBS 060	12	M	13	severe	0.798	1836414.1	25.6	LBF 054	1	2	3	3	1	2	1	Comm
86	LBS 249	6	M	11	severe	0.318	66739.58	29.4	LBF 1831	3	3	1	3	1	0	0	Comm
87	LBS 321	6	M	12	severe	0.877	201699.5	28.6	LBF 4445	1	3	2	2	1	2	1	Comm
88	LBS 235	6	M	12	severe	1.166	2971795	23.2	LBF 4718	2	3	0	0	2	3	2	Comm
89	LBS 086	9	F	12	severe	1.01	57976.195	28.8	LBF 4995	1	3	2	3	0	2	1	Comm
90	LBS 195	7	M	13	severe	0.849	71936.052	28.5	LBF 5633	3	3	2	2	0	2	1	Comm
91	LBS 157	8	F	12	severe	1.386	105079.659	27.8	LBF 5979	3	3	1	1	1	2	1	Comm
92	LBS 183	6	M	14	severe	0.323	22024.809	30.2	LBF 7307	1	3	3	3	0	3	1	Comm
93	LBS 272	7	M	12	severe	0.173	316051.3844	26.4	LBF 8800	1	3	2	3	0	2	1	Comm
94	002992F	13	M	13	severe	2.432	25422.05	30	CM0002	1	3	1	3	1	2	2	Hosp in
95	835864D	21	M	15	severe	0.159	18723.6088	30.5	CM0004	2	3	3	3	0	2	2	Hosp in

96	304506F	3	M	12	severe	3.212	50715.54	29	CM0006	1	3	2	2	0	2	2	Hosp in
97	309760F	14	F	13	severe	0.883	192014.258	27.1	CM0015	1	3	2	3	0	2	2	Hosp in
98	341986F	18	F	13	severe	1.112	235272.0098	26.8	CM0059	1	3	2	1	1	3	2	Hosp in
99	347401F	19	M	12	severe	1.13	2467.023	33.4	CM0067	1	3	1	2	1	2	2	Hosp in
100	080788F	16	M	11	severe	1.767	7044.4	35.3	CM0073	1	3	1	1	1	2	2	Hosp in
101	299574F	10	M	13	severe	0.733	1453710.47	24.1	CM0081	1	1	2	3	2	2	2	Hosp in
102	119944F	10	M	13	severe	2.34	54757.63	28.9	CM0082	1	3	2	3	0	2	2	Hosp in
103	540414D	39	M	14	severe	0.697	672.8373	36.9	CM0087	1	3	2	3	1	2	2	Hosp in
104	361083F	44	F	11	severe	1.743	14029.364	30.9	CM0088	1	2	2	2	0	2	2	Hosp in
105	119098F	13	M	12	severe	1.55	1335170.527	24.3	CM0098	1	3	2	2	0	2	2	Hosp in
106	674787D	31	M	11	severe	0.457	2634721.19	23.3	CM0106	1	2	2	2	0	2	2	Hosp in
107	384257F	5	F	11	severe	0.372	102.56	38.6	CM0137	1	3	1	2	0	2	2	Hosp in
108	804789D	27	M	12	severe	2.296	22263.805	30.3	CM0146	1	3	1	3	0	2	2	Hosp in
109	252560F	6	M	14	severe	0.186	18031.3625	30.8	CM0155	1	3	2	3	1	2	2	Hosp in
110	180514F	12	M	13	severe	0.353	97576.465	28.2	CM0166	1	3	1	3	1	2	2	Hosp in
111	394177F	11	M	13	severe	0.332	47784.78	32.7	CM0167	1	2	2	3	1	2	2	Hosp in
112	394551F	11	F	12	severe	0.646	269915.2375	26.7	CM0171	1	3	3	3	0	0	2	Hosp in
113	401189F	14	F	15	severe	0.692	1733.94	33.3	CM0176	1	3	3	3	1	2	2	Hosp in
114	415231F	12	F	13	severe	0.382	28891.856	28.9	CM0199	1	3	2	2	0	3	2	Hosp in
115	417148F	17	M	13	severe	0.511	39593.162	28.5	CM0200	1	3	2	3	0	2	2	Hosp in
116	942769D	21	M	15	severe	0.712	985712	26.8	CM0207	2	3	3	3	0	2	2	Hosp in
117	170372F	9	M	13	severe	1.706	202375.2125	28.3	CM0211	1	3	2	3	0	2	2	Hosp in
118	434162D	46	M	14	severe	2.248	17235.407	32.6	CM0225	1	3	2	3	1	2	2	Hosp in
119	439091F	8	F	14	severe	0.16	637.20898	36.4	CM0227	1	3	3	3	0	2	2	Hosp in
120	992176D	7	M	11	severe	0.164	42964.98	32.6	CM0018	1	3	2	3	1	0	1	Hosp out
121	307606F	9	M	11	severe	1.115	124637.1	26	CM0287	1	3	1	3	2	0	1	Hosp out
122	367389F	11	F	15	severe	1.803	116845.68	27.8	CM0105	3	3	2	2	1	2	2	Hosp in
Hosp in - Inpatient			Hosp out - Out patient				Comm - Community										