UNIVERSITY OF LIVERPOOL

MOLECULAR EPIDEMIOLOGY OF ROTAVIRUS GASTROENTERITIS IN THE ROYAL LIVERPOOL CHILDREN'S HOSPITAL (ALDER HEY)

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DECLARATION

This thesis is the result of my own work. The material contained in this thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

The clinical data was carried out at the Royal Liverpool Children Hospital (Alder Hey). The research work took place in the Department of Medical Microbiology and genitor-Urinary Medicine, University of Liverpool.

Amer M A Shata

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This work is especially dedicated to my children, Marwa, Osama, and Ahmed,

To the memory of my dear friend, Mohamed Toobar,

To the memory of my great teachers Ibrahim El-Keiyé, and Farouq El-Weishi,

Least but not last, this thesis is about children, and is dedicated to all children, in particular those sick children all over the world.

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Abbreviations

aa	amino acid
AHCH	Alder Hey Children Hospital
bp	basepairs
°C	degrees centigrade
EDTA	ethylene diamine tetra acetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
H_2O	water
L	litre
M	molar
mM	millimolar
mg	milligram
$MgCl_2$	magnesium chloride
MW	molecular weight
nt	nucleotide
NaCl	sodium chloride
NaOH	sodium hydroxide
pH	-log[H+]
rpm	revolutions per minute
ul	microlitre
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
PAGE	Poly Acrylamide Gel Electrophoresis
VP	Viral Protein
WHO	World Health Organization

ABSTRACT

Diarrhoeal disease is a leading cause of childhood morbidity and mortality worldwide. A large part of this disease burden is attributed to enteric viruses. Rotavirus is undoubtedly the most important viral enteropathogen, in both hospital and community based surveys. It is the major cause of dehydrating acute watery diarrhoea in children.

This was a two-year prospective descriptive study of rotavirus gastroenteritis among children attending Alder Hey Children Hospital (AHCH), Liverpool. The main objective of the study was to examine the relative importance of rotavirus as a cause of diarrhoeal diseases. Enzyme immunoassays (ELISA) were used to examine faecal samples obtained from children with acute gastroenteritis for the presence of rotaviruses. The other objectives were to examine the electropherotypes and VP7 (G genotypes) of rotaviruses circulating over the two year period. Rotaviruses were therefore examined by polyacrylamide gel electrophoresis (PAGE) for rotavirus electropherotype, and by hemi-nested reverse transcription-polymerase chain reaction (RT-PCR) for VP7 genotype (G type).
This study also examined the seasonal variation in the prevalence of different genotypes and electropherotypes. The most vulnerable age group was the focus of the study.

Between January 1st 2000 and January 1st 2002, a total of 1496 faecal samples were collected from children with diarrhoea. From these, 412 (27.8%) samples were positive for rotavirus by ELISA. These 412 rotavirus positive samples were examined by PAGE for electropherotype and by RT-PCR for VP7 genotype.

The most frequently affected group was children aged 6-12 months (42.5%), followed by those aged 13-24 months (26.7%). There was no significant difference in the age groups distribution between seasons. The age of the patients had no impact on prevalence of electropherotypes or genotypes.

The peak prevalence of infection occurred in March and April, and November and December each year, showing a biphasic pattern.

An electropherotype could be assigned to 359 (87%) strains. These comprised 286 (69.5%) long profiles, 67 (16.5%) short profiles, 6 (1.5%) mixed profiles and 53 (12.5%) were non-detectable (ND). We could conclude that 4 of those 6 profiles belonged to the phenomenon of genome re-arrangement. There was a significant difference in the prevalence of L, S and ND profiles between the two years, 2000 and 2001.

A total of 354 (86%) samples were typable, by RT_PCR and 58 (14%) were not typable (GNT). Of the 58 untypable samples, 35 had been undetectable by electropherotyping. Five different genotypes were detected. These were G1, G2, G3, G4 and G9. G1 was the most frequently-detected genotype (n=158, 38.5%), followed by G9 (n=102, 24.8%), G2 (n=67, 16%), G3 (n=1, 0.2%), G4 (n=26, 6.5%). The short electropherotype profile comprised almost all of serotype G2. The long electropherotype profiles comprised most of G1, G9 and G4. The mixed profiles were of an unusual pattern which may be described under the rearrangement category. There was a significant difference in genotype distribution (G1, 2, 3, 4, 9) over the two years, In the diarrhoea season for 2000, G1 occurred in 92 (45%), G2 in 42 (21%), G4 in 8 (4%), G9 in 27 (13%) and GNT in 33 (16%). In 2001, G1 occurred in 66 (32%), G2 in 25 (12%), G4 in 18 (9%), G9 in 75 (36%) and GNT in 21 (10%).

G9 showed a significant difference in prevalence between the seasons in each year; [n=27 (13%), n=75 (36%) respectively].

The emergence of or increase of new rotavirus strains must be considered in the design and implementation of rotavirus vaccines strategy. This study outlines the distribution patterns of rotavirus genotypes in a paediatric Liverpool hospital population and contributes to understanding of the changing patterns of diarrhoeal disease in our urban UK population.

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Chapter One

Introduction and Study Design

1.1 Diarrhoeal Diseases:

Diarrhoea in the Paediatric age group may arise from many different causes. Different aetiological factors may produce diarrhoeal stools through one or more different mechanisms. As in most cases in paediatric medicine, the differential diagnosis of diarrhoea varies markedly with age. The clinician must correlate the age of the patient, the nature of the stool, the chronicity of the problem, and other relevant historical factors in order to formulate and evaluate an adequate differential diagnosis.

1.1.1 Pathophysiology:

Diarrhoea can be classified into acute, chronic and chronic persistent. The main concern here is acute diarrhoea. It can be defined as either an increase in the frequency or a decrease in stool consistency, although this definition is somewhat imprecise. Normal infants pass 5-10g/kg/day, and stool volume in excess of 10g/kg/day in infants is often considered as diarrhoea (Weaver 1988).

By the age of 3 years stool volumes reach adult levels. Stool losses of more than 200g/day are defined as diarrhoea in both children and adults.

Diarrhoea can be sub-divided based on pathophysiology. Osmotic, secretory, motility and inflammatory diarrhoea are common although overlap exists. Rotavirus gastroenteritis is due to different mechanisms but is mainly osmotic and secretory (McClung *et al.* 1976).

Osmotic diarrhoea:

Osmotic diarrhoea is a term applied when malabsorption of an absorbable solute creates an osmotic load in the distal small intestine and colon resulting in increased fluid losses (Fordtran *et al.* 1965). Acute rotavirus infection alters the intestinal permeability to some molecules. For example, in humans, the absorption of D-xylose, L-rhammose and polyethylene glycols are reduced while permeability to lactose and lactulose is increased. Studies have shown that the transport of sucrase-isomaltase from their site of synthesis to the enterocyte membrane is reduced by rotavirus infection which can lead to the accumulation of disaccharides in the gut lumen since they can only be absorbed when hydrolysed to monosaccharides. The increase in faecal lactose levels during acute rotavirus gastroenteritis is consistent with the hypothesis that the destruction of enterocytes leads to osmotic diarrhoea secondary to carbohydrate malabsorption (Jourdan *et al.* 1998).

Secretory diarrhoea:

Intestinal absorption of water and electrolytes is governed by net difference between the fluid and electrolytes secreted and fluid and electrolytes absorbed. Total net absorption may be much smaller than either of the two unidirectional processes. Therefore, a change in the balance between these two processes can markedly alter total intestinal fluid absorption and may result in secretory diarrhoea (Powell 1987)

The rotavirus non-structural protein 4 (NSP4) is the product of genome segment 10, and is an intracellular receptor. NSP4 can play a unique role in virus assembly and may also play a critical role in the pathogenesis of diarrhoea (Estes *et al.* 1999). The comparison of the biological activities of cloned gene 10 from virulent and non-virulent strains of the porcine rotavirus OSU has provided further evidence of the potential role of NSP4 in the production of diarrhoea. NSP4 from virulent OSU increased calcium levels in intestinal cells, and caused diarrhoea in greater than 50% of neonatal mice. In contrast, NSP4 from attenuated OSU caused diarrhoea in only 16% of neonatal mice (Zhang *et al.* 1998)

1.1.2 The importance and severity of diarrhoeal diseases worldwide:

All children in the world suffer from diarrhoeal disease at some time. For the majority there will be one or a few acute attacks of watery diarrhoea followed by spontaneous and complete recovery. Unfortunately, for many children there may be repeated attacks, some of which are serious and result in death. It has been estimated that up to five million children die each year of the immediate or longer-term consequences of diarrhoeal diseases (Snyder *et al.* 1982; Murray *et al.* 1997)

The immediate effects of diarrhoeal diseases are loss of essential body water and electrolytes in loose stool and/or vomit. This often results in an upset in the body's acid-base balance. There is an important association between diarrhoeal diseases and malnutrition especially in developing countries. The animal model studies have indicated that poor nutritional status increases the severity of diarrhoea due to rotavirus (Greenberg *et al.* 1994). There is some clinical evidence that the malnourished children develop more severe gastroenteritis after rotavirus infection (Dagan *et al.* 1990). This may be one of the factors that can explain the higher mortality attributed to rotavirus infection during the childhood period in developing countries where malnutrition is most common.

The toll from the diarrhoeal diseases in developing countries is staggering. It is estimated that 1 billion diarrhoeal disease episodes and 3.3 million deaths from diarrhoea occur annually in the less than 5 years age group in developing countries of Africa, Latin America, and Asia (excluding China) (Bern *et al.* 1992)

Furthermore, diarrhoeal illnesses consistently rank as one of the top six causes of all deaths, one of the top three causes of death from an infectious disease, and one of the top two causes of death when considering years of life lost (Murray *et al.* 1997; Lopez *et al.* 1998)

In a country like USA, infectious non-bacterial gastroenteritis was the second most common disease among younger children (Ho *et al.* 1988). Diarrhoeal diseases are not the prominent cause of mortality in infants and young children in developed countries to the same scale as in developing countries. However, in a country like USA, the estimated number of deaths from rotavirus disease is around 20-40 per year and most of these deaths occurred in the less than 1 year age group (Bryce *et al.* 2005; Parashar *et al.* 2006).

In order to estimate the number of deaths attributed to rotavirus infection each year, the total number of diarrhoea deaths is multiplied by the proportion of severe diarrhoeal cases, represented by hospitalisation, in which rotavirus can be detected (severe diarrhoea has in general been used as a proxy for diarrhoea death since studies addressing cause-specific mortality from diarrhoea are rare) (Perez-Schael *et al.* 2007).

Viruses also account for up to 30% of cases of infectious diarrhoea in children in developing countries. Rotaviruses, Adenoviruses, Astroviruses and Caliciviruses are of the groups that cause damage to the gastrointestinal tract, resulting in vomiting, diarrhoea or both.

Rotavirus: is undoubtedly the most important viral enteropathogen, in both hospital and community based surveys. It is the major cause of dehydrating acute watery diarrhoea in children. The World Health Organization (WHO) has recently updated the mortality figures, and it has been estimated that in 2004, 527,000 (range 475,000-580,000) global deaths among children under 5 years of age (Parashar *et al.* 2006).

Adenovirus: is the second most important cause of viral gastroenteritis. It is responsible for 5-12% of diarrhoeal cases in most surveys. It is an unenveloped virus (70-80 nm in diameter) with double stranded linear DNA genome surrounded by an icosahedral capsid. Adenovirus types 40 and 41 are the enteropathogens. The clinical picture of illness is similar to that produced by rotavirus. Preliminary evidence suggests that infection does not protect against subsequent symptomatic infection. No vaccine is available (Unicomb *et al.* 1996)

Astrovirus: is a small round un-enveloped virus with a characteristic star shape on its surface (approximately 28nm). They are a member of the Astroviridae (Carter *et al.* 1996). They have single stranded positive sense RNA genome. With the development of new detection methods, Astrovirus may compete with Adenovirus 40/41 as the second most important cause of diarrhoeal diseases (Heath *et al.* 1997). There are eight serotypes of astroviruses and type 1 usually predominates (Shi *et al.* 1994; Glass *et al.* 1996; Palombo *et al.* 1996). It has been found that by 1 year of age, 50% of London children had antibodies to type 1 and by 5 years , 90% had been infected (Kriston *et al.* 1996). However, outbreaks of astroviruses serotype 3, have been reported in adults (Belliot *et al.* 1997).

Calicivirus: is a small unenveloped RNA virus with cup shaped depression on their surface (approximately 40nm). Their genome is single stranded. The small round structured viruses are also caliciviruses but they do not show the classical cup shaped depressions. Caliciviruses include Norwalk, Southampton and Lonsdale viruses (Clarke *et al.* 1997). Infection with classic human calicivirus tends to produce diarrhoea, whereas infection with Norwalk type tends to produce vomiting. Sporadic and epidemic infection were reported in both children (Levett *et al.* 1996) and adults (Cubitt *et al.* 1996; Jiang *et al.* 1996; Kilgore *et al.* 1996).

The human caliciviruses have recently been re-classified based on genomic content (Radford *et al.* 2004)

The classical caliciviruses that have cup-shaped depression on their surface have been named sapoviruses (after the prototype Sapporo virus) and cause predominantly diarrhoeas. The agents of winter vomiting disease have been re-designated noroviruses (after the prototype Norwalk virus) and are probably as important as rotaviruses as a cause of gastroenteritis in children and the major cause of epidemics of gastroenteritis in adults worldwide. (Radford *et al.* 2004)

1.2 Rotavirus Gastroenteritis:

1.2.1 Introduction:

Rotaviruses are the single most important cause of severe diarrhoeal illness in infants and young children in both developed and developing countries worldwide (Bern *et al.* 1992; Nakata *et al.* 1999). In this age group, diarrhoeal disease is one of the most common illnesses throughout the world. However, diarrhoeal disease assumes a special significance in developing countries where it constitutes a major cause of mortality among these vulnerable groups.

Rotavirus is the single most important pathogen because of its frequency and because it is over represented in more severe dehydrating disease. It is also the most important cause of diarrhoea requiring admission to hospital during the first 6-24 months of life. Infection with rotavirus is common worldwide from birth to old age. Attacks rates of severe rotavirus diarrhoea in young children are similar in developing and developed countries. The frequency and severity of rotavirus infection have been the justification for a vigorous search for an effective vaccine.

1.2.2 History:

The report in 1973 of reovirus like particles in epithelial cells in small bowel biopsies from children with acute non-specific diarrhoea led to a cascade of clinical and laboratory studies that have established rotavirus as the leading enteric viral pathogen (Bishop *et al.* 1973). This discovery has improved the identification rate of the causes of childhood enteritis in developed countries from 10% to 20% in the early 1970s, to 40% to 70% in the 1990s.

Despite the magnitude of the problem of infantile diarrhoea, the search for important aetiological agents was unrewarding until the 1970s. During the 1950s and 1960s scores of viruses were discovered but none was found to be an important aetiological agent of infectious diarrhoea (Yow *et al.* 1970). This frustration ended with the discovery in 1972 of the 27-nm diameter Norwalk virus and its association with epidemic gastroenteritis in older children and adults (Kapikian *et al.* 1972). This was followed by the discovery of the 70-nm diameter human rotavirus and its association with severe endemic diarrhoea in infants and young children (Bishop *et al.* 1973); Kapikian *et al*, 1974; Middleton *et al*, 1974).

Although the human rotaviruses were discovered in 1973, the efforts made

before that should be noted and appreciated such as the description of virus-like particles discovered by Adams and Kraft in 1963 (Adams *et al.* 1963) Malherbe and Harwin in 1963 (Malherbe *et al.* 1963). Malherbe and Strickland-Cholmley in 1967 (Malherbe *et al.* 1967), and Mebus et al, in 1969 (Mebus *et al.* 1969).

The Norwalk and related viruses can be responsible for over 50% of community outbreaks of non-bacterial gastroenteritis in older children and adults. These viruses although they are associated with mild gastroenteritis in infants and young children; have not been established as an important cause(Yolken *et al.* 1988) of severe diarrhoea in the infant age group (Yolken *et al.* 1988; Bridger 1994)

It has been, then, established that this 70-nm viral particle subsequently designated as rotavirus is an important aetiologic agent of diarrhoea of infants and young children causing about 35% to 50% of the hospitalization for this form of gastroenteritis during the first two years of life (Kapikian 1993)

1.2.3 Classification of rotavirus:

Rotaviruses compose a genus within the family of *Reoviridae*. Eight other genera account for the remainder of the *Reoviridae* family. That includes the following: *Orthoreovirus, Orbivirus, Coltivirus, Aquareovirus, Cypovirus, Phytoreovirus, Fijivirus and Oryzavirus.* Viruses belonging to all of the genera within the Reoviridae family share several common features such as: all are non-enveloped, icosahedral viruses 60-80nm in diameter and possess a varying number of linear, double stranded RNA (dsRNA) genome segments(Estes 2001).

The Rotaviruses themselves share common morphologic and biochemical properties, which are their structure, their genome and their replication.

Structure: 65-75nm diameter icosahedral particles, triple layered protein capsid, non-enveloped (resistant to lipid solvents), and capsid contains all enzymes necessary for mRNA production (Figure 1.1).

Genome: 11 segments of dsRNA, purified RNA segments are non-infectious, each RNA segment codes for at least one protein, and RNA segments from different viruses reassort at a high frequency during dual infections of the cells.

Replication: cultivation facilitated by proteases, cytoplasmic replication, inclusion body formation, unique morphogenesis involves transient enveloped particles, and virus is released by cell lysis or by non-classic vesicular transport in polarized epithelial cells (Estes 2001).

The prominent features of rotaviruses are that (a) mature virus particles are 100 nm in diameter and possess a triple-layered icosahedral protein capsid composed of an outer layer, an intermediate layer and an inner core layer; (b) 60 spikes extend from the smooth surface of the outer shell; (c) particles contain an RNA dependent RNA polymerase and other enzymes capable of producing capped RNA transcripts; (d) the virus genome contains 11 segments of double-stranded RNA (dsRNA); (e) the viruses are capable of genetic reassortment; (f) virus replication occurs in the cytoplasm of infected cells; (g) virus cultivation in vitro is facilitated by treatment with proteolytic enzymes, which enhances infectivity by cleavage of an outer capsid spike

polypeptide; and (h) the viruses exhibit a unique morphology pathway(i.e. virus particles are formed by budding into the ER) and enveloped particles are evident transiently at this stage of morphogenesis. Mature particles are non-enveloped, and these virions are librated from infected cells by cell lysis or by nonclassic vesicular transport in polarized epithelial cells (Estes 2001).

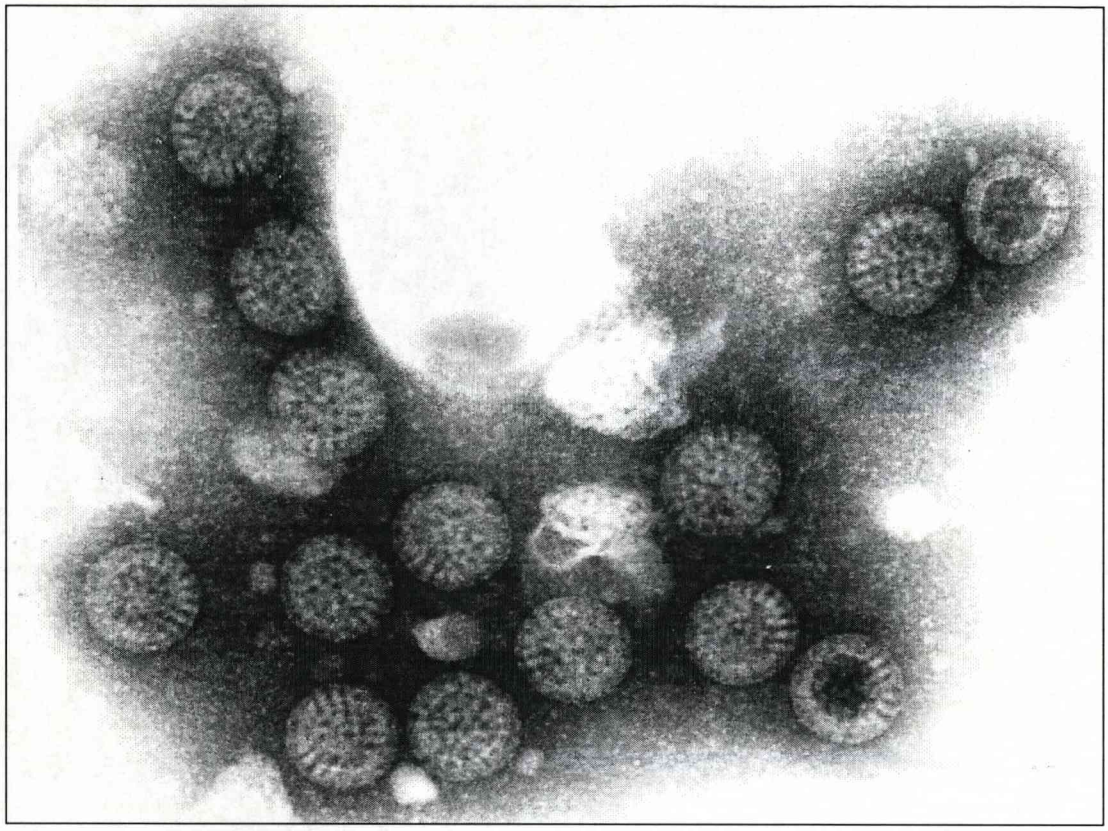


Figure 1.1 Rotavirus particles visualized immune electronic microscopy in stool filtrate from a child with acute gastroenteritis. The 70-nm diameter particles possess distinctive double-shelled outer capsid.

1.2.3.1 The serological classification of rotaviruses:

Serological classification is based on a scheme that allows for the presence of multiple groups (serogroups) and for the existence of multiple serotypes within each group. A rotavirus group (or serogroup) includes viruses that share cross-reacting antigens detectable by a number of serological tests. Rotaviruses comprise seven distinct groups (A to G). Group A, B and C rotaviruses are those currently found in both humans and animals, where as viruses in groups D, E, F and G have been found only in other animals to date. Viruses within each group are capable of genetic reassortment, but reassortment does not occur among viruses in different groups (Yolken *et al.* 1988). The common antigens in a group or the antigenic determinants of a group are found in most, if not all, of the structural proteins and probably on many of the non-structural proteins as well.

Group A viruses have clearly been established as causing significant diarrhoeal disease in young children. Group B rotaviruses have been associated with annual epidemics of severe diarrhoea primarily in adults (Midthun *et al.* 1996). Group C rotaviruses also have been sporadically detected in faecal specimens from children with diarrhoea and in several outbreaks. However, the clinical significance of group C remains unclear.

The diagnosis of non-group A viruses has been established using ELISA and Mabs and this will facilitate the study of the clinical importance of these viruses (Perez-Schael *et al.* 1990; Kapikian 1993). The main focus in this study is in group A rotaviruses. However, reviews of non-group A rotaviruses and comparisons between the proteins of group A and non-group A have been published (Bishop *et al.* 1973; Mata *et al.* 1983; Hoshino *et al.* 1987).

In summary: a classification scheme for rotaviruses has been derived from the immunological reactivities of its various components and also from genomic comparisons and has been established as follows:

1. According to VP6 reactivities, there are at least seven distinct groups A-G. VP6, the product of genome segment 6, constitutes 51% of the virion mass. Using MAb experiments, this protein was demonstrated to exhibit the major group antigen. ***(See section 1.2.6 and 1.2.8)***
2. Within group A, subgroups I, II, I+II and non-I, non-II are distinguished according to reactivities of VP6 with two monoclonal antibodies.
3. Because there are two neutralizable outer capsid proteins (VP4 and VP7), a dual classification system has emerged.

4. There are at least 15 different VP7-specific types, termed G-types (derived from glycoprotein).
5. There are at least 25 different VP4-specific types, termed P-types (derived from protease-sensitive protein).
6. All G- and P- types can be unambiguously distinguished by sequencing of the relevant genes (genotypes).
7. All G-genotypes have been characterized as serotypes.
8. However, this is not the case for all P-genotypes, i.e. the P-genotype may not be consistent with the P-serotype.
9. Therefore the following nomenclature has been agreed upon: each virus has a P-type, indicated by an order number for the serotype and by an order number in square brackets for the genotype, and a G-serotype, indicated by an order number (coinciding with genotype number). Thus, the human Wa strain is defined as P1A [8] G1 (P-serotype 1A, P-genotype 8; G-sero/genotype 1).
10. Because VP4 and VP7 are coded for by different RNA segments (RNA4, and RNA7-9, respectively) various combination of G- and P-types can be observed in vivo and in vitro, both in man and in animals (Desselberger 2000; Santos *et al.* 2005; Nakagomi *et al.* 2006; Uchida *et al.* 2006).

1.2.4 Morphology and Morphogenesis:

Rotaviruses have a distinctive morphologic appearance by Negative-stain EM. The complete particles measure about 70nm in diameter and have a distinctive double-layered icosahedral protein capsid that consists of an outer and an inner layer when viewed by transmission EM (Figure1.1).

Within the inner layer is a third layer, which is the core that contains the virus genome consisting of the 11 segments of dsRNA. The complete particle is designated smooth particles because the outermost margin of the outer capsid layer has a well-defined, smooth, circular appearance. The incomplete particles are designated rough particles because of the lack of the smooth outer layer, and the capsomers of the inner capsid project to the periphery, giving the bristly circular appearance. The core measures about 37nm in diameter and appears to be hexagonal. The term rotavirus is derived from the Latin word rota, which means a wheel (Flewett *et al.* 1974).

Studies of the three-dimensional structure of the unstained, unfixed rotavirus particles using cryoelectron microscopy and image processing techniques have yielded important new information on the ultrastructure of rotaviruses (Estes 2001). (Fig 1.2)

The three-dimensional structure of rotaviruses has now been accurately determined by electron cryomicroscopy. A distinctive feature of the virus structure is the presence of 132 large channels that link the outer surface with the inner core. These channels are involved in importing the metabolites required for RNA transcription and exporting the nascent RNA transcripts for subsequent viral replication processes (Prasad *et al.* 1996; Lawton *et al.* 1997; Lawton *et al.* 1999). Another characteristic feature is the sixty spikes that extend from the smooth surface of the outer shell. It is now confirmed that the spikes are composed of VP4 (Estes 2001).

1.2.5 Genome structure:

The rotavirus genome contains 11 segments of dsRNA that are contained within the virus core capsid. Rotaviruses are the only known agents of mammals or birds that contain 11 segments of dsRNA. They are in the size range of 0.6 to 3.3 kilobase pairs (Mattion 1994). The RNA segments fall into four size classes. This is based on the measurement by EM, nucleotide sequence analysis of each of the 11 genes and the migration of the segments during the PolyAcrylamide Gel Electrophoresis (PAGE). Characteristically, these RNA segments are numbered in order of migration during PAGE, with the slowest (heaviest) RNA segment designated gene 1, and so on (Estes 2001; Kapikian 2001) (Fig 1.2).

For group A rotavirus, there are four large segments, two medium sized segments, three smaller segments and the two smallest segments. So, it makes the pattern of 4, 2, 3, 2 (Holmes 1983). This pattern of migration of rotavirus genes can distinguish them from, for example, Reoviruses. The Reovirus genome contains only 10 segments that are distributed into three size classes, large, medium and small (Holmes 1983)

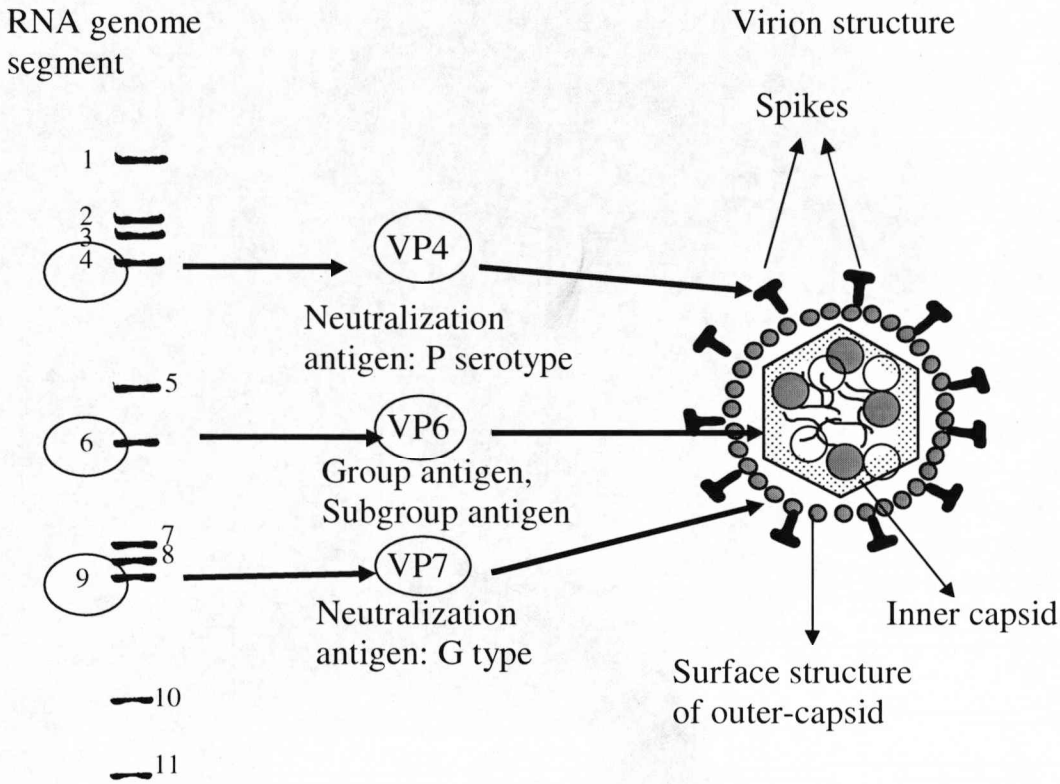


Figure 1.2 Schematic diagram showing rotavirus RNA genome segments and key structural proteins.

The neutralising protein VP4 and VP7 are coded for respectively by genome segment 4 and genome segment 9; depending on the strain, this can be genome segment 7 or 8.

(Reproduced with permission from Prof. Nakagomi & Dr. Cunliffe)

Some group A rotavirus strains do not display the characteristic RNA migration pattern described above (Bridger 1994). For example, murine rotavirus segment 11migrates close to segment 10, whereas in avian rotavirus segment 5 migrates close to segment 4, and segments 10m and 11 are difficult to resolve. A lapine rotavirus has the distribution pattern of 4,2,4,1 instead of the usual pattern of 4,2,3,2.

In some human rotavirus strains, the 11^{th} RNA segment migrates more slowly than usual and is thus located between the 9^{th} and the 10^{th} segments, yielding a "short" pattern characteristic of almost all antigenic subgroup I human rotaviruses [which are almost always viral protein 7 (VP7) serotype G2 strains] (Holmes 1996; Hoshino *et al.* 1996). Similarly, a "long" 10^{th} and 11^{th} RNA segment pattern of human rotaviruses is characteristic of almost all subgroup II human rotaviruses. There are also "super short" patterns of RNA migration in which the 11^{th} segment migrates even more slowly than the "short" pattern (Mattion *et al.* 1990). Certain strains, which belong to non-group A rotaviruses, such as groups B, C, D, F or G, have a genome with 11 dsRNA segments but do not share the common group antigen of conventional group A rotaviruses. Such strains display quite distinct migration patterns (Bridger 1994).

Individual gene segments reassort at relatively high frequencies following co-infection both in vivo and in vitro because of the segmented nature of the rotavirus RNA genome. However, reassortment between members of different groups (e.g. between group A and B) has never been documented. This suggests that each group may represent a separate gene pool (Raming 2000).

Comparison of the migration patterns of RNA during PAGE became an important laboratory and epidemiologic technique for characterization of strains, because human rotaviruses could not initially be propagated in tissue culture. The term *electropherotyping* was then applied to this method of differentiating strains (Estes *et al.* 1984; Holmes 1996). Subsequent studies indicated that there is no correlation between the electropherotyping patterns and the serotypes. This means that viruses of the same serotype can exhibit different electropherotypes, and viruses of the same electropherotype can belong to different serotypes (Kapikian 2001).

Gene segments with similar migration patterns by PAGE do not necessarily exhibit homology by hybridization; on the other hand, some gene segments that exhibit homology by hybridization vary in their electrophoretic

migration (Flores *et al.* 1982).

Rearranged genome segment(s) have been observed in strains recovered from both immunocompetent and immunodefficient hosts. Genome rearrangements occur exclusively in genes encoding non-structural proteins, except for a rare example of VP6 gene rearrangement. Rearrangement of genome segment encoding VP1, VP2, VP3, VP4 or VP7 has never been detected. Therefore, genome rearrangements have been proposed to be a third mechanism of rotavirus evolution in nature, besides genomic point mutations and accumulations, as well as genetic reassortments (Hundley *et al.* 1985; Hundley *et al.* 1987; Tian *et al.* 1993; Shen *et al.* 1994; Desselberger 1996; Desselberger 1996)

Deproteinized rotavirus dsRNAs are not infectious. This reflects the fact that viral particles contain their own RNA-dependent polymerase to translate the individual RNA segments into active mRNAs.

The nucleotide sequences of each of the 11 rotavirus RNA segments for several rotavirus strains are now known. These include SA11, human KU and bovine RF rotaviruses. The prototype simian SA11 strain was the first genome completely sequenced (Estes 2001).

1.2.6 Gene Coding Assignment and Rotavirus Proteins:

Analysis of the gene coding assignments for each of the 11 genes established that there are six structural and six non-structural proteins (ICTV 1998). The proteins of SA11 strains have been thoroughly and frequently studied, in part because this virus was among the first of this group of agents to be propagated efficiently in cell culture (Malherbe *et al.* 1963). RNA segments 1, 2, or 3 of SA11 encode a core or sub core structural VPs, designated VP1, VP2, and VP3, respectively, whereas segment 6 encodes the major inner capsid protein VP6. Segments 4 and 9 encode the outer capsid structural proteins VP4 and VP7, respectively, (Mattion 1994). The six non-structural proteins (NS), which are found in infected cells but not in virions, are encoded by segments 5, 7, 8, 10 and 11 and are designated NSP1, NSP2, NSP3, NSP4, NSP5 and NSP6. VP4 is cleaved specifically by the proteolytic enzyme trypsin to form cleavage products designated VP5 and VP8 (P-type). Proteolytic cleavage of VP4 potentates infectivity by enhancing the penetration of the virus into cells. VP4 is a multifunctional protein. The 6^{th} gene encodes VP6, which contains the subgroup antigen as well as the antigen that acts as the main determinant of group reactivity. The 9^{th} gene (or the 7^{th} or 8^{th} gene in certain rotavirus strains) encodes the neutralization protein VP7 (G-type), which is largely responsible for

serotype specificity defined by neutralization with hyperimmune antiserum (Matsui *et al.* 1989) (See Table 1.1).

Table 1.1 Rotavirus proteins and gene coding assignment

Genome segment	Protein product	Molecular Weight (kda)	Molecular Size (bp)	Location in virion	Function
1	VP1	125	3302	Core	RNA polymerase, complex with VP3
2	VP2	94	2690	Core	RNA binding, replicate activity of VP1
3	VP3	88	2591	Core	Guanylyltransferse, methyltransferase, complex with VP1
4	VP4 (VP5 +VP8)	88	2362	Outer capsid	Hemagglutinin, neutralization antigen, protease enhanced infectivity, virulence, cell attachment and protection
5	NSP1	53	1581	Non-structural	Basic, zinc finger, RNA binding
6	VP6	41	1356	Inner capsid	Subgroup antigen, protection
7	NSP3	34	1104	Non-structural	RNA binding, RNA replication
8	NSP2	35	1059	Non-structural	Basic, RNA binding
9	VP7	38	1062	Outer capsid	Neutralization antigen, Ca binding protection
10	NSP4	28	751	Non-structural	Morphogenesis, entrotoxin, protection
11	NSP5 NSP6	26 12	667	Non-structural Non-structural	RNA binding, interact with NSP2, NSP6 Interact with NSP5

1.2.7 Propagation and Assay in Cell Culture:

Most animal rotaviruses, such as SA11 and the O agent, grow readily in cell culture. However, human rotaviruses were not grown efficiently in tissue culture until relatively recently (Wyatt *et al.* 1980; Sato *et al.* 1981; Urasawa *et al.* 1981). Early attempts to propagate the non-cultivable human rotavirus DS-1 (VP7 serotype 2) in piglets were unsuccessful. Another strategy to cultivate this virus was adopted. This strategy is based on the well-known property of the Reoviridae to undergo genetic reassortment with high efficiency during co-infection in cell culture (Greenberg *et al.* 1981). With this technique, rescue of non-cultivable human rotavirus was achieved. Using this technique, 33 of 52 human rotaviruses were successfully rescued, and the serotype diversity of these viruses was demonstrated by conventional naturalization assays (Greenberg *et al.* 1982). Subsequently, efficient techniques have been developed for the direct cultivation of most human rotaviruses in cell culture (Sato *et al.* 1981; Urasawa *et al.* 1981).

1.2.8 Antigenic Composition:

1.2.8.1 Designation of serotypes and subgroups:

There are three important antigenic specificities in rotaviruses, which are the group, subgroup and serotype. The group antigen is shared among all rotaviruses of a particular group. Groups A, B, C, D, E, F and G are recognized at present (Bridger 1994; Saif 1994). The cross-reactive (group) epitopes are present on all structural proteins and most non-structural proteins. However, in diagnostic assays, VP6 is the predominant group antigen, probably because it constitutes 51% of the virion (Mattion 1994). VP6 also mediates subgroup specificity, which serves as an important epidemiological marker (Kapikian *et al.* 1981; Mattion 1994).

The serotypes of group A rotaviruses have been identified by neutralization assay using hyperimmune antisera rose in animals. In designating the antigenic composition of a rotavirus, four antigenic specificities must be considered: group, subgroup, serotype mediated by VP7 (designated a G serotype because VP7 is a glycoprotein) and serotype mediated by VP4 (designated a P serotype because VP4 is protease sensitive) (Mattion 1994). For example, the human Wa virus is group A, subgroup II, G1, and P1A, where as DS-1 virus is group A, subgroup I, G2, and P1B. The VP4

classification was referred to earlier and is discussed in theVP4 section.

The tidy distinction between subgroup I and II strains by PAGE is not as clear-cut as presumed from earlier studies because of the increasing number of ***"exceptions to the rule"***. It used to believed that human rotaviruses with "short" electropherotypic pattern belongs to a homogenous group, VP7 serotype 2 by neutralization, whereas human viruses with the "long" pattern are belonging to VP7, serotypes 1, 3, or 4. Several human or animal rotaviruses have been detected and cannot be classified into either subgroup I or subgroup II, or that have both subgroup I and subgroup II specificities (Hoshino *et al.* 1996).

So far, al least 15 VP7 (G) serotypes have been identified, 10 in humans and 13 in other animals (Hoshino *et al.* 1996; Uchida *et al.* 2006). The sharing of subgroup specificity by certain animal and human rotaviruses is well established. Also the sharing of VP7 neutralization specificity is well known (Hoshino *et al.* 1984). Extensive reciprocal cross-neutralization tests among human and animal rotaviruses revealed that: 1) only one of the 10 human rotavirus serotypes, which is serotype 12, is restricted to humans. 2) Serotype 3 viruses have the broadest host range, which includes humans,

monkeys, cats, dogs, horses, pigs, mice, rabbits and sheep. 3) Serotypes 11, 13, 14 or 16 are restricted to horses or pigs, whereas serotype 7 appears to be restricted almost exclusively to birds. 4) Although there is extensive sharing, certain serotypes occur almost exclusively in either humans or animals, for example, serotype 1 and 2 are often found in humans but rarely in animals. 5) It is of interest that the porcine rotavirus strains Gottfried and SB-2, which share serotype specificity, belong to different subgroup and this was the first example of shared neutralization but not subgroup specificity (Hoshino *et al.* 1996).

Currently, up to 25 VP4 (P-genotypes) have been described (Hoshino *et al.* 1996; Rao *et al.* 2000; Uchida *et al.* 2006).

VP6:

This is a viral protein that is located on the inner capsid of the virus, and has a molecular weight of 41,000 and constitutes about 51% of the virion protein. VP6 specifies the major group reactivity measured by various immunoassays (Greenberg *et al.* 1983). It is now believed that a separate domain of VP6 specifies subgroup reactivity, while separate domains of VP6 contain the major determinants of group A antigenic reactivity (Mattion

1994). The site of attachment of VP6 subgroup MAbs on rhesus rotavirus (RRV) has been visualized clearly by IEM. These antibodies attach to the inner but not the outer capsid of the RRV (Kapikian 1996).

The amino acids of the VP6 protein of several groups A, B and C rotavirus strains have been determined. Immunodominant sites of VP6 which contain group-specific epitopes for group A rotaviruses have been identified in four regions (amino acids residues 32-64, 155-167, 208-294 and 380-397) (Kohli *et al.* 1993). It is also reported that amino acid residues 172, 305 and 315, and region 296-299 each contributes to subgroup epitopes (Lopez *et al.* 1994; Tang *et al.* 1997). Four subgroup specificities have been recognized: subgroup I (detected in human and animal strains), subgroup II (human strains and a few animal strains), subgroup I and II (rarely detected in humans and animals), and neither subgroup I nor II (most avian strains, rarely detected in humans and animals).

Rotaviruses that share the group antigen are now classified in group A, whereas those that lack this antigen are classified as non-group A viruses. The latter also lack the sharing of subgroup antigens.

Group B rotaviruses have been associated with several large outbreaks of severe gastroenteritis in China, predominantly involved adults. It has also been associated with a mild outbreak of diarrhoea in neonates in China and an outbreak of adult diarrhoea in India (Tao 1988; Bernstein *et al.* 1995; Mackow 1995; Angel *et al.* 1998).

There is little epidemiological importance in group C rotaviruses that have been detected in various locations in humans. The non-group A rotaviruses appear to be of minor importance other than in China and Asia (Tao 1988; Mackow 1995).

VP7:

This glycoprotein is the major neutralization antigen of rotaviruses. It has served as the basis for identification of serotypes. It has a molecular weight of 38,000, and constitutes 30% of the virion protein, making it the second most abundant rotavirus protein (after VP6) as well as the major constituent of the outer capsid (Mattion 1994) [also see figure 1.2]. It is located on the outer capsid, forming the smooth external surface of the outer shell (Mattion 1994; Hoshino *et al.* 1996).

Most neutralizing antibodies in hyperimmune antiserum are directed against

the VP7 protein, which is encoded by the 7th, 8th or 9th gene, depending on the rotavirus strain (Mattion 1994; Gueguen *et al.* 1996). The site of attachment of the VP7 MAbs has been identified by IEM as the outer capsid of the virus (Kapikian 1996). Thus, so far group A rotaviruses have been classified into 14 distinct G- serotypes. Serotype identification is also facilitated by the isolation of VP7 MAbs that are specific for that particular serotype (Hoshino *et al.* 1994; Hoshino *et al.* 1996). Absolute agreement is observed between serotypes determined by neutralization, and by sequence analysis. As a glycoprotein, it contains one to three potential sites for glycosylation, which is important for antigenicity but not necessarily for infectivity.

VP4:

VP4 is present in the outer capsid as a series of 60 short spikes of approximately 10-12 nm in length. The distal ends of the spikes comprise the region of the knoblike structures that contain its proteolytic cleavage product, VP5 (Prasad *et al.* 1988; Prasad *et al.* 1990) [also see figure 1.2]. VP4 is a non-glycosylated protein and has a MW of 88,000 and constitutes 1.5% of virion protein (Mattion 1994). In spite of being a minor component of the outer capsid, it performs a variety of functions defined by

immunologic and genetic analyses. VP4 has been identified as the viral haemagglutinin (Greenberg *et al.* 1983; Kalica *et al.* 1983). Trypsin cleavage of VP4 is essential for the virus to become infectious and to initiate penetration through the host cell membrane (Konno *et al.* 1993; Mattion 1994). VP4 is the major rotavirus cell attachment protein both in vivo and in vitro (Ludert *et al.* 1996)). Also both VP5 and VP8 have been shown to mediate rotavirus attachment to cells (Zarate *et al.* 2000). It has been determined that VP4 can neutralize infectivity, a function that was thought to be mediated exclusively by VP7 (Greenberg *et al.* 1983; Greenberg *et al.* 1983). The ability of the two outer capsid proteins (VP4 and VP7) to induce neutralizing antibodies separately has necessitated the re-evaluation of serotype classification because it had been based on neutralization mediated primarily by VP7. The need for re-evaluation also became apparent when various strains were discovered that shared neutralization specificities with two distinct serotypes (Hoshino *et al.* 1985).

VP4 has a significant role in protective immunity and its function as protective antigen in vivo is independent from that of VP7 (Hoshino *et al.* 1988). In addition to its other functions, VP4 appears to play a major role in virulence of rotaviruses (i.e., diarrhoea, loose, and more frequent stools) (Flores *et al.* 1986; Gorziglia *et al.* 1988; Tian *et al.* 1996).

NSP4:

This is a non-structural, endoplasmic reticulum-specific glycoprotein. It is encoded by genome segment 10 of group A rotaviruses and has a MW of 28,000 (Mattion 1994; Tian *et al.* 1996). NSP4 has a unique role in rotavirus morphogenesis in infected cells. It functions as an intracellular receptor by mediating the conversion of double layered particles in the cytoplasm to triple layered particles in the endoplasmic reticulum (ER). It has been reported that NSP4 can act as an entrotoxin and is capable of inducing diarrhoea by itself (Ball *et al.* 1996; Estes *et al.* 1999). Tyrosine at residue 131 of the protein was reported to be a key amino acid that is associated with the toxigenic activity of the NSP4. It is also reported that the antibodies to the NSP4 protein can protect against virus-induced diarrhoea (Horie *et al.* 1999).

Sequence analysis of NSP4 genes has indicated that there are four genetic NSP4 groups or alleles. However, the relevance of such genetic diversity has not been determined (Horie *et al.* 1997; Kirkwood *et al.* 1997; Horie *et al.* 1999; Kirkwood *et al.* 1999). The role of the NSP4 enterotoxins in the natural history and pathogenesis of rotavirus infection in humans needs further studies and clarification to assess its relevance to human disease.

1.2.9 Pathology and Pathogenesis:

Rotaviruses infect the mature absorptive villous epithelium of the upper two thirds of the small intestine. After replication in the upper small intestine, infectious particles are released into the intestinal lumen and undergo further replication in the distal areas of the small intestine. Infection is generally confined to the intestinal mucosa. Although rotaviruses can be found in the lamina propria and regional lymphatics, replication at these sites and systemic spread usually do not occur in immunocompetent persons. Despite the superficial nature of mucosal infection, rotaviruses induce both local intestinal and systemic immune response (Greenberg *et al.* 1994).

Rotavirus infection is thought to be confined to the intestine. Reports of rotavirus RNA in the cerebral spinal fluids and serum of children infected with rotavirus suggest the possibility that rotaviruses escape the intestine to the circulatory system. Thus, rotavirus can escape the gastrointestinal tract in children, resulting in antigenaemia and viraemia (Blutt *et al.* 2003; Blutt *et al.* 2007; Blutt *et al.* 2007)

(See section 1.2.18, clinical features)

Infection with rotaviruses can cause lesions of the jejunal mucosa. These

lesions can be identified as: shortening and atrophy of the villi, mononuclear cell infiltration into the lamina propria, distended cisternae of the ER, mitochondrial swelling and sparse and irregular microvilli (Davidson *et al.* 1975; Suzuki *et al.* 1975; Moon 1994). Virus particles have been visualized and detected in various parts such as the dilated cisternae of ER in Enterocytes and in phagocytes in the lamina propria. Abnormal gastric motor function and impaired d-xylose absorption have been observed as well as depressed level of disaccharidases (Bishop *et al.* 1973; Mavromichalis *et al.* 1977; Bardhan *et al.* 1992).

Malabsorption is established as the mechanism of rotavirus-induced diarrhoea. This is characterized by viral replication in the small intestinal villi with subsequent cell lysis, depressed level of mucosal disaccharidases, watery diarrhoea and dehydration. However, the mucosal damage is not necessary to precede and produce the diarrhoea. Secretory diarrhoea then can be induced by rotavirus infection in a fashion similar to bacterial entero-toxin induced diarrhoea in which the intestinal inflammation can evoke fluid secretion by activation of the enteric nervous system (Kohler *et al.* 1990; Shaw *et al.* 1995). Rotavirus NSP4 may also participate in inducing intestinal inflammation and thus can have a toxin-like function. This leads

to the activation of the Entric Nervous System (ENS) and thereby a secretory type diarrhoea (Kapikian 2001).

The rotavirus non-structural protein 4 (NSP4) is the product of genome segment 10, and is an intracellular receptor. NSP4 can play a unique role in virus assembly and may also play a critical role in the pathogenesis of diarrhoea (Estes *et al.* 1999). The comparison of the biological activities of cloned gene 10 from virulent and non-virulent strains of the porcine rotavirus OSU has provided further evidence of the potential role of NSP4 in the production of diarrhoea. NSP4 from virulent OSU increased calcium levels in intestinal cells, and caused diarrhoea in greater than 50% of neonatal mice. In contrast, NSP4 from attenuated OSU caused diarrhoea in only 16% of neonatal mice (Zhang *et al.* 1998).

1.2.10 Epidemiology:

1.2.10.1 Morbidity and Mortality:

It is clear now that rotaviruses are the major aetiological agents of serious diarrhoeal illness in infants and young children throughout the world (Bishop 1994). The mortality is low in developed countries though rotavirus diarrhoea occurs in high frequency. It is estimated that 4 of 5 children under the age of 5 years will develop rotavirus diarrhoea in a country like the USA (Tucker *et al.* 1998).

The low mortality but high morbidity can be explained by the development of replacement therapy of the lost fluids and electrolytes, which are used routinely in developed countries and improved health and nutrition (Edelman *et al.* 1980).

In developing countries rotaviruses are the leading cause of severe and life-threatening diarrhoea in infants and young children. It is estimated that 130 million cases of rotavirus diarrhoea occur every year in the developing countries; 18 million of them are severe.

In order to estimate the number of deaths attributed to rotavirus infection

each year, the total number of diarrhoea deaths is multiplied by the proportion of severe diarrhoeal cases, represented by hospitalisation, in which rotavirus can be detected (severe diarrhoea has in general been used as a proxy for diarrhoea death since studies addressing cause-specific mortality from diarrhoea are rare) (Perez-Schael *et al.* 2007)

Using data from studies published between 2000 and 2004, it was estimated that 39% (range 29-45%) of childhood hospitalisations for diarrhoeal disease could be attributed to rotavirus infection, which translated into an estimated 611,000 (range 454,000-705,000) rotavirus related deaths a year (Parashar *et al.* 2006).

The World Health Organization (WHO) has recently updated these figures, and it has been estimated that in 2004, 527,000 (range 475,000-580,000) global deaths among children <5 years could be attributed to rotavirus infection. Almost 85% of these deaths occurred in Africa and Asia and 23% in India. Globally, these 527,000 rotavirus deaths accounted for approximately 29% of all deaths owing to diarrhoea and 5% of all child deaths (Bryce *et al.* 2005; Parashar *et al.* 2006)

The widespread distribution of rotaviruses in the community has led to the universal acquisition of serum antibodies to these viruses at an early age

(Kim *et al.* 1973; Kapikian *et al.* 1978). This high prevalence of rotavirus antibodies is maintained into adult life. This suggests that sub-clinical rotavirus infections can occur throughout life.

Dehydration is the most serious complication of rotavirus diarrhoea. In one study, dehydration was 14 times more frequent among children with rotavirus diarrhoea than with diarrhoea of bacterial, acute parasitic or unknown aetiology (Wyatt *et al.* 1979).

1.2.10.2 Rotavirus infection in adults:

Adults can undergo rotavirus re-infections quite commonly but with minimal or no clinical manifestation. For example, 22 (55%) of 40 adult household contacts of children hospitalized with rotavirus gastroenteritis developed serologic evidence of rotavirus infection, predominantly sub clinical, at or about the time of their children admission to the hospital (Kim *et al.* 1977). Although sub clinical rotavirus infection is the most common outcome in adults, rotavirus gastroenteritis in adults has been described (Hrdy 1987; Bishop 1994).

Several outbreaks with high attack rate and fatalities were reported in elderly patients. However, these attacks are unusual because of the high level of

rotavirus immunity that most adults have acquired from previous infections (Bishop 1994).

Severe outbreaks in adults were reported, especially with those in close contact with children such as through play groups (Rodriguez *et al.* 1979; Foster *et al.* 1980). An extensive outbreak of adult rotavirus gastroenteritis was reported from the northern Brazil, in an isolated South American Indian community. The attack rate in adults over 41 years of age was 80% (Linhares *et al.* 1981). Rotavirus is not the major aetiologic factor in traveller's diarrhoea. However it has been it has been associated with traveller's diarrhoea (Black 1990).

Group B rotaviruses have been implicated in several large outbreaks of severe gastroenteritis in adults in various parts of China. Affected individuals developed cholera-like severe watery diarrhoea, and a few elderly patients died (Tao 1988).

1.2.10.3 Nosocomial infections:

Nosocomial rotavirus infections occur frequently in children hospitalized for non-diarrhoeal diseases. Nosocomial rotavirus infections have been

described in various neonatal nurseries around the world (Ryder *et al.* 1977; Bishop 1994).

1.2.10.4 Epidemiology of rotavirus serotypes:

MAb-based ELISA has been used to determine the distribution of VP7 serotypes of rotavirus isolates from children with acute gastroenteritis over a 6 year period in various countries of Europe, North and South America, Africa and Asia. The analysis indicated that:

a) Most of the rotavirus isolates belonged to G serotypes 1, 2, 3 or 4.

b) Rotavirus serotype 1 was detected most often, followed by 2, 3 and 4 respectively.

c) Each of the four serotypes had a worldwide distribution, with no single serotype being found exclusively in one location.

d) Differences in distribution of serotypes were observed year by year and in different countries during the same years.

e) Differences were not observed in the distribution of serotypes detected in developed and developing countries. (Beards *et al.* 1989)

Almost 2 decades ago, studies demonstrated that 4 globally common rotavirus serotypes (G1-G4) represent >90% of the rotavirus strains in circulation. Subsequently, these 4 serotypes were used in the development of

reassortant vaccines predicated on serotype-specific immunity. More recently, the application of reverse-transcription polymerase chain reaction genotyping, nucleotide sequencing, and antigenic characterization methods has confirmed the importance of the 4 globally common types, but a much greater strain diversity has also been identified (we now recognize strains with at least 42 P-G combinations). These studies also identified globally (G9) or regionally (G5, G8, and P2A[6]) common serotype antigens not covered by the reassortant vaccines that have undergone efficacy trials. The enormous diversity and capacity of human rotaviruses for change suggest that rotavirus vaccines must provide good heterotypic protection to be optimally effective (Gentsch *et al.* 2005)

The temporal and geographical distribution of human rotavirus G and P types was reviewed by analysing a total of 45571 strains collected globally from 124 studies reported from 52 countries on five continents published between 1989 and 2004. Four common G types (G1, G2, G3 and G4) in conjunction with P[8] or P[4] represented over 88% of the strains analysed worldwide. In addition, serotype G9 viruses associated with P[8] or P[6] were shown to have emerged as the fourth globally important G type with the relative frequency of 4.1%. When the global G and/or P type

distributions were divided into five continents/subcontinents, several characteristic features emerged. For example, the P[8]G1 represented over 70% of rotavirus infections in North America, Europe and Australia, but only about 30% of the infections in South America and Asia, and 23% in Africa. In addition, in Africa (i) the relative frequency of G8 was as high as that of the globally common G3 or G4, (ii) P[6] represented almost one-third of all P types identified and (iii) 27% of the infections were associated with rotavirus strains bearing unusual combinations such as P[6]G8 or P[4]G8. Furthermore, in South America, uncommon G5 virus appeared to increase its epidemiological importance among children with diarrhoea. Such findings have (i) confirmed the importance of continued active rotavirus strain surveillance in a variety of geographical settings and (ii) provided important considerations for the development and implementation of an effective rotavirus vaccine (e.g. a geographical P-G type adjustment in the formulation of next generation multivalent vaccines) (Santos *et al.* 2005)

1.2.11 Transmission:

Rotaviruses are transmitted by the faecal- oral route. Nevertheless, speculation continues as to whether rotaviruses are transmitted also by the respiratory route (Gordon 1982; Cook *et al.* 1990). The source of infection for the young infant who is normally not in contact with other infants and young children with gastroenteritis is not well documented or explained. Most likely, infection is acquired from an older sibling or parent with sub-clinical infection. Resistance to physical inactivation, along with the large number of viral particles shed in faeces, may contribute to the efficient transmission of the human rotaviruses. The ability of rotaviruses to survive on various surfaces under different conditions may contribute to the rapid spread of these agents. High relative humidity (~80%) results in a rapid loss in human rotavirus infectivity (Ansari *et al.* 1991).

Although rotaviruses have been detected in raw or treated sewage, it is unlikely that contaminated water plays an important role of transmission of group A rotaviruses (Smith *et al.* 1982). Effective disinfection of contaminated material and careful hand washing constitute important measures to contain rotavirus infection, especially in a hospital or institutional setting (Black *et al.* 1981).

There has been speculation on the role of animals as a source of rotavirus infection of humans. This speculation has been intensified by some observations: a) certain animal rotaviruses share a neutralization antigen with human rotaviruses. b) certain naturally occurring animal rotaviruses strains may infect humans or form reassortants with human rotaviruses (Nakagomi *et al.* 1993). This type of interspecies transmission appears to be a rare event in most parts of the world. However, the human-bovine reassortant strain 1321 or a human-porcine reassortant strain appears to be endemic in selected areas in India and Brazil, respectively (Das *et al.* 1993; Gouvea *et al.* 1999).

1.2.12 Incubation period:

The incubation period of rotavirus diarrhoeal illness has been estimated to be 48 hours (Davidson *et al.* 1975). The incubation period of the illness under experimental conditions was 1 to 4 days (Kapikian *et al.* 1983; Kapikian *et al.* 1983).

1.2.13 Geographic Distribution:

Rotaviruses have been detected throughout the world wherever they have been sought. Furthermore, these viruses consistently constitute major etiologic agents of severe infantile diarrhoea in every country (Bishop 1994; Parashar *et al.* 1998).

1.2.14 Seasonality Distribution:

Rotavirus infection shows a strong seasonal distribution. In the UK as in other temperate northern countries, peaks of infection occur in the winter months. The precise timings, however, appear to vary by country; for example annual peaks in Spain are in December, in France in February, in the UK in February and March and in Holland and Finland in March. In contrast the bacterial diarrhoeal diseases are more prevalent in summer (Koopmans *et al.* 1999).

In tropical climates, infection rates with rotaviruses show a year-round transmission, which is more common towards the equator. However, the peak activity still occurs during the cool and dry season (Cunliffe *et al.* 1998).

The cause of this striking seasonal pattern is not known. However, the influence of low relative humidity in the home has been suggested as a factor helping the survival of rotaviruses on surfaces (Brandt *et al.* 1982).

1.2.15 Age and socioeconomic distribution:

Rotavirus gastroenteritis occurs most frequently in infants and young children from approximately 6 months to 2 years of age (Bern 1994; Bishop 1996). Infants under 6 months of age experience the next highest frequency of these infections. In certain studies, those under 6 years old are the most frequently affected and this may reflect racial and socio-economic differences (Brandt *et al.* 1981).

The highest rates of illnesses due to rotaviruses are known to occur in the first year of life in children in developing countries such as in Africa (Cunliffe *et al.* 1998), but in the second year of life in developed countries

such as the USA (Parashar *et al.* 1998). The reason for the difference in age exposure between developed and developing countries is unclear, but may be explained by the year-round transmission of rotaviruses that occurs in many tropical settings (see above).

There is a low frequency of clinical illness in most (but not all) neonates who shed rotaviruses (Bishop 1994). There may be a role for VP4 in this phenomenon (Flores *et al.* 1986; Gentsch *et al.* 1996).

Routine rotavirus and adenovirus screening in hospitalized neonates seems to be unnecessary. Viral diagnostic examinations should be considered in patients with necrotizing enterocolitis (Hallstrom *et al.* 2001).

In five separate faecal collections spanning three years, group A rotaviruses were detected by enzyme-linked immunosorbent assay in 35 (25%) of 142 specimens obtained from nondiarrhoeic, hospitalized neonates in Blantyre, Malawi. Molecular characterization of each strain identified, for the first time in neonates, a short electropherotype, genotype P[6], G8 strain type, similar to the dominant, co circulating community strain detected in symptomatic infants in Blantyre. Partial sequence analysis of the VP4 and NSP4 genes of neonatal and community strains failed to identify changes which could explain the differences in clinical outcome. Neonatal serotype

G8 rotaviruses should be considered as potential rotavirus vaccine candidates for use in Malawi (Cunliffe *et al.* 2002).

A human rotavirus strain (NB-150) was detected in stool samples from a neonate hospitalized for mild/moderate community-acquired diarrhoea. This is believed to be the first report in Brazil of a rotavirus infection involving a strain with G1 and G4 alleles, with VP8* and NSP4 genes of porcine origin. These findings strongly suggest the occurrence of interspecies transmission (Mascarenhas *et al.* 2007; Mascarenhas *et al.* 2007).

Malnutrition is thought to play an important role in the severity of clinical manifestations of human rotavirus infections. Repeated diarrhoeal infections may lead to the development of malnutrition by damaging the intestinal mucosa and compromising the mechanism of absorption (Mata 1981).

1.2.16 Rotavirus replication:

There are general features of rotavirus replication, which are based on culture in monkey kidney cells. These can be summarised as follows:

1) Cultivation of most virus strains requires the addition of exogenous proteases to the culture medium. This is to activate the viral infectivity by cleaving the outer capsid protein VP4.
2) Replication is exclusively a cytoplasmic process.
3) The virus must supply enzymes to replicate dsRNA as the cells do not contain those enzymes.
4) Transcripts have two functions, to produce proteins and to act as a template to produce negative strands. Once the complementary negative strand is synthesized, it remains associated with the positive strand.
5) The dsRNA segment is formed within nascent sub viral particles, as free dsRNA or free negative stranded ssRNA has never been found in infected cells.
6) Sub viral particles are formed within and in association with viroplasm.
7) Cell lysis releases particles from infected cells.

1.2.16.1 Stages of the Replication Cycle:

1) Adsorption, Penetration, and uncoating.

2) Transcription and replication.

3) RNA Encapsidation and Virion Assembly.

4) Virus Release.

1.2.17 Immunity and Rotavirus Infection:

The mechanism of immunity to rotavirus infections and illness are not completely understood, especially in humans. Most of the information comes from the study of animal models, where the relative importance of systemic and local immunity has been studied. In two animal model studies, it was observed that newborn calves frequently developed rotavirus diarrhoea despite the moderately high level of circulating rotavirus antibodies derived from the colostrums (Woode *et al.* 1975; Ward 1996). Subsequently, the relative roles of local and systemic rotavirus antibodies were further studied. It was then observed that antibodies in the lumen of the small intestine were the primary determinant of resistance to rotavirus infections, whereas circulating serum antibodies failed to protect (Offit *et al.* 1985). It was also observed with interest that systemic rotavirus antibodies are present in the lumen of the gastrointestinal tract of neonatal calves when the level of circulating antibodies is sufficiently high. These serum-derived mucosal antibodies can provide protection against induced infections and diarrhoea (Besser *et al.* 1988).

Thus, in addition to this immunologic mechanisms operating locally in the small intestine, non-immunologic mechanisms probably play a role in the

resolution of acute rotavirus gastroenteritis. These include shedding of infected enterocytes in the small intestine and their replacement with immature cells, which are less permissive to viral growth, and also the increase in peristalsis movement of the small intestine during the acute phase of infection (Offit 1996).

Additional evidence for the role of rotavirus antibodies was obtained from some studies of immunity in experimental animals, but the antigenic determinants of resistance have not been clarified completely. In some animal model studies, serotype-specific immunity has been demonstrated, but this could not be confirmed in other studies (Bridger *et al.* 1987; Offit 1994; Saif LJ 1994).

Information regarding correlates of rotavirus immunity was obtained from a volunteer study in humans. In one study, human rotavirus VP7 serotype 1(D strain) was administered to 18 individuals by the oral route. Five of the 18 volunteers shed rotavirus, and four of those five developed diarrhoeal illness. A critical pre-existing level of serum neutralizing antibodies to the homotypic virus or to a heterotypic VP7 serotype 2 human rotavirus correlated with resistance to diarrhoeal illness. The relationship of

neutralizing activity in intestinal fluid to resistance was less clear (Kapikian *et al.* 1983; Kapikian *et al.* 1983). This study not only confirmed the association of serum antibodies with resistance to infection but also identified a specific protective epitope on VP7. In a study of 1-24 months old infants and young children, a firm correlation between serum neutralizing antibodies and resistance to disease was observed (Chiba *et al.* 1986). Rotavirus serum IgA levels were shown to be correlated with resistance to severe rotavirus infection (Hjelt *et al.* 1987). However, serum antibodies may correlate only with, and hence reflect, local intestinal antibodies that are produced locally and /or enter the intestinal lumen by transudation (Tristram 1994).

The association between Faecal IgA and serum IgG anti-rotavirus Antibody titres and protection against infection and illness was investigated in 100 USA children under 18 months of age. These studies concluded that:
(a) a rotavirus specific faecal IgA titre of at least 1:80 or at least 1:20 correlated with protection against infection or illness, respectively.
(b) pre-existing serum anti-rotavirus IgA titres of greater than 1:200 or IgG titres of greater than 1:800 were associated with protection against infection.
(c) a high level of pre-existing G-type specific blocking antibody was

associated with protection against infection (Matson *et al.* 1993; O'Ryan *et al.* 1994). A similar study with similar outcomes was carried out in Australia (Coulson *et al.* 1992). Also, similar studies to support these findings have been carried out in animals (To *et al.* 1998; Yuan *et al.* 1998).

The protective effect of prior rotavirus infection against severe subsequent rotavirus diarrhoea was demonstrated in an Australian study, in which, 81 neonates were studied, 44 were rotavirus positive and 37 were rotavirus negative during the first 14 days of life. They were all followed up and kept under clinical and serological surveillance for 3 years. It was found that the frequencies of subsequent postnatal rotavirus infection were similar for both groups. However, the severity of diarrhoeal illness was significantly different between the two groups with fewer illnesses among those of rotavirus positive group (Bishop *et al.* 1983). Thus, neonatal rotavirus infection did not protect against re-infection but induced partial resistance against any rotavirus diarrhoeal illness and complete protection against severe rotavirus diarrhoea during re-infection. In this particular study, the rotavirus strains responsible for neonatal infection were of particular interest because these viruses exhibited the same electropherotype.

The unusual electropherotype of the neonatal strains was not encountered

among the strains that induced illness in the study group during their follow-up in the post-neonatal period. Thus, infection with neonatal strains conferred partial protection against diseases during re-infection with strains other than neonatal ones, and likely included a variety of different serotypes. This finding has been applied to the development of a candidate vaccine. In a recent study, neonatal infection with a unique rotavirus strain 116E, which shares VP7 specificity with the human W161 (G9) strain induced statistically significant partial protection against subsequent rotavirus diarrhoea during the first year of life (Bhan *et al.* 1993). It was also found that in infants and experimental animals, the predominantly serotype-specific response is expanded to include other serotypes following subsequent natural or vaccine-induced re-infection with the same or another serotype (Brussow *et al.* 1987; Brussow *et al.* 1988; Brussow *et al.* 1988).

In a Mexican study, it was concluded that in infants, natural rotavirus infection confers protection against subsequent infection. This protection increases with each new infection and reduces the severity of the diarrhoea (Velazquez *et al.* 1996)

It has been noted that VP7 and VP4 proteins independently induce neutralizing antibodies, and as a consequence each outer capsid protein plays a role in resistance to disease (Hoshino *et al.* 1988). In a recent study, it was observed that the majority of infants and young children who received oral RRV vaccine developed antibodies to the major serotype-specific neutralization epitope on RRV VP7. Almost half of these vaccinated children responded to a major neutralization epitope on VP4 (Shaw *et al.* 1987). This indicates that infection with an attenuated vaccine strain can induce antibodies to neutralization epitopes on each of these proteins. In one study, comparing serum antibody response to VP4 and VP7 following oral administration of bovine rotavirus WC3 vaccine to infants not previously infected with rotavirus, VP7 appeared to be more efficient than VP4 for the induction of neutralizing antibodies (Ward *et al.* 1990). The reverse was found in another study in adults (Ward *et al.* 1988). Interestingly, the two studies were carried out by the same authors in an overlapping period of time. Currently, it is not clear which surface protein and which region of that surface protein is most efficient for the induction of protective immunity. Sero-type specific antibodies have been demonstrated in breast milk. However, the protective effect of breastfeeding on infantile rotavirus gastroenteritis still requires additional studies. If breastfeeding does transfer

immunity to newborns and infants, it appears that its lasting protective effect is modest (Clemens *et al.* 1993; Golding *et al.* 1997; Newburg *et al.* 1998).

Lactadherin, a human-milk glycoconjugate, has been found to protect babies from the symptoms of rotavirus infection. David Newburg and colleagues studied 200 infants in Mexico City from birth by monitoring their stools for the presence of rotavirus. Milk samples from breast-feeding mothers were analysed weekly until 4 weeks post partum, then monthly. Those samples taken just before the infant had rotavirus infection were assayed for a collection of substances, including lactadherin, which was found to have the highest anti-rotavirus activity. Importantly, the protective effect of lactadherin was independent of products of the secretory immune system. The investigators suggested that milk glycoproteins such as lactadherin could form the basis of therapeutic agents suitable for oral supplementation. The full characterisation of such molecules could make possible a novel class of therapeutic agents suitable for oral supplementation (Newburg *et al.* 1998).

The effect of Lactobacillus GG and breast-feeding in the prevention of rotavirus nosocomial infection has been studied. The study concluded that

Lactobacillus GG was ineffective in preventing nosocomial rotavirus infections, whereas breast-feeding was effective (Mastretta *et al.* 2002).

While the overall health benefits of breastfeeding are significant, ingestion of maternal breast milk at the same time as administration of oral rotavirus vaccines may inactivate the vaccine. (Glass *et al.* 2006)

External factors such as vitamin A have been investigated for their effect on the immune response. It appeared that Vitamin A deficiency impaired the serum antibody and the cell mediated immunity response in infant mice (Ahmed *et al.* 1991).

The role of cell-mediated immunity in the immune response to rotavirus infection is under investigation both in humans and animals. Studies in an adult mouse model have shown: a) rotavirus specific antibodies are of primary importance in protection from re-infection. b) homologous rotavirus strains are far more potent than heterologous in inducing protective local humoral immune response. c) MHC class 1-restricted CD8+αβT cells play a major role in resolution of primary rotavirus infection, but also rotavirus specific antibodies and CD4+T cells are also involved. d) after primary

infection, CD8+T cells mediate almost complete protection (up to 2 weeks), or partial protection (3 months or less) from re-infection. e) perforin or interferon-γ is not essential in an anti-rotavirus activity mediated by CD8+T cells. f) CD8+T cells expressing α4β7 marker are more efficient in mediating rotavirus clearance than the negative α4β7 cells (Dharakul *et al.* 1990).

1.2.18 Clinical Features:

The principal presentation of rotavirus infection is acute voluminous watery diarrhoea with or without vomiting. Rotavirus infection produces a spectrum of responses that vary from sub-clinical infection, to mild diarrhoea, to severe diarrhoea and fatal dehydration. The major cause of morbidity and mortality is the dehydration and electrolyte imbalance (12org). Over a 5-year period in a Toronto/Canadian hospital, rotavirus gastroenteritis was responsible for the deaths of 21 children. Death occurred most frequently because of dehydration and electrolyte imbalance, and then next in frequency from aspiration of vomitus and seizures (Carlson *et al.* 1978).

The main symptoms and signs are non-specific and they range from vomiting, diarrhoea, fever, dehydration with or without electrolyte imbalance, irritability, and lethargy. It is interesting that some symptoms and signs of respiratory nature often happen with rotavirus infection such as, pharyngeal erythema, tonsillar exudation, rhinitis, red tympanic membranes, rhonchi or wheezing and palpable cervical lymph nodes (Rodriguez *et al.* 1977). These clinical findings may raise or support the speculation that the route of infection with rotaviruses can occur through the respiratory tract. It

also makes it difficult to design a diagnostic clinical score to distinguish rotavirus from other causes of gastroenteritis. More details about scoring systems are discussed in chapter 4. In a study, that compared gastroenteritis with rotavirus and non-rotavirus infection, it was found that:

a) there was no difference in acquiring pyrexia,

b) vomiting and dehydration in the rotavirus group were significantly more often and of longer duration and,

c) in the rotavirus group, the diarrhoea started later than vomiting but lasted longer (Rodriguez *et al.* 1977).

In the same study (referred to above), the laboratory findings reflect the high frequency of vomiting and dehydration associated with rotavirus illnesses. A BUN value (blood urea nitrogen concentration) or azotemia of greater than 18 mg/dL was observed in the rotavirus positive group, and also there was more frequently a urine specific gravity of 1.025 or greater. These rates were significantly higher than in the rotavirus negative group. However, the frequency of acidosis and electrolyte imbalance did not differ in the two groups.

Rotavirus can produce a chronic symptomatic infection or serious illness in

immunodeficient children (Jarvis *et al.* 1983; Oishi *et al.* 1991) For example, chronic diarrhoea associated with prolonged shedding of rotavirus has been described in children with primary immunodeficiency, T-cell deficiency or SCID. An unusual aspect of the illness in the former group was the rotavirus antigenaemia. Some of these children developed hepatitis, and rotavirus was found in the livers and kidneys after the death of some of them (Oishi *et al.* 1991).

Rotaviruses can escape the gastrointestinal tract in children, resulting in antigenaemia and viraemia (Blutt *et al.* 2007)

One of the interesting findings was that, during the chronic infection in immunocompromised children, rotavirus can undergo marked changes in its genome, as indicated by abnormal electrophoretic migration pattern of rotavirus RNAs (Desselberger 1996).

Rotaviruses pose a special threat to individuals who are immunosuppressed for bone marrow transplantation (Kapikian 2001). Rotavirus has also been reported as a threat to adults renal transplant recipients (Peigue-Lafeuille *et al.* 1991).

Rotaviruses do not appear to play an important role in diarrhoea occurring in adults infected with HIV (Grohmann *et al.* 1993) and the same was found in Malawian children infected with HIV (Cunliffe 2001)

Although rare, some other disease associations have been described. Recent rare associations include acute myositis, haemophagocytic lymphohistiocytosis, polio-like paralysis, encephalitis and seizures. In addition, it has recently been found that there can be prolonged (up to 57 days) excretion of rotavirus after severe diarrhoea. In some of these children there were further episodes of mild diarrhoea (Hart *et al.* 1999)

A temporal association of rotavirus infection with a variety of other diseases has been described. While rotavirus infection can cause severe manifestation in certain conditions, like immunodeficiency, necrotizing enterocolitis and haemorrhagic gastroenteritis in neonates, and pneumatosis intestinalis in infancy, its association with the reported conditions is temporal and not aetiological (Dearlove *et al.* 1983; Capitanio *et al.* 1991) .

Rotavirus was detected in cerebrospinal fluid in a case of rotavirus gastroenteritis with convulsion and may even cause encephalitis,

haemorrhagic shock and encephalopathy (Nishimura *et al.* 1993). The same findings were also reported recently (Iturriza-Gomara *et al.* 2002). Transient MR Signals Changes in the splenium of the corpus callosum in rotavirus encephalopathy have been reported (Kobata *et al.* 2002). Another study from Finland showed the same association of CNS infection and rotavirus (Koskiniemi *et al.* 2001)

Recently, group rotavirus genome has been detected in the liver or bile duct of infants with extra hepatic biliary atresia. It is noteworthy that some group A rotaviruses can replicate in murine liver and cause a similar syndrome (Hart *et al.* 1997)

There were reports of the association between intussusceptions and the administration of the quadrivalent RR vaccine. Further studies of this association are of contradictory outcomes. In a Japanese study, rotavirus was detected in the stools of 11 (37%) of 30 infants and young children with intussusceptions, who also developed a serological response to rotavirus (Katsushima 1981). However, three subsequent studies in Australia, Germany and France failed to find significant associations of intussusceptions with rotavirus infection (Mulcahy *et al.* 1982; Nicolas *et al.*

1982; Staatz *et al.* 1998).

Studies and reports have shown some rare but an interesting association of rotavirus with severe diseases. Rotavirus was reported to cause an unusual case of hepatitis. The author also referred to Reye syndrome association in this case (McMaster *et al.* 2001).

There is an interesting study that has made an alleged link between environmental triggers, including viral and rotavirus infections, and type 1 Diabetes Mellitus (Couper 2001). Another study showed association of rotavirus with poliomyelitis (Chou *et al.* 1998). Rotavirus encephalopathy has been well described (Goldwater *et al.* 2001).

The presence of portal vein gas associated with rotavirus infection in a five-month old infant has been reported. This would increase the suspicion of a rotaviral origin of the necrotizing enterocolitis (Morrison *et al.* 2001). Epileptic seizures are also associated with viral infections (Yamamoto *et al.* 2004).

In a recent study by Nakagomi, et al, they concluded the lack of significant

association between the electropherotype or G-serotype of the infecting strain and disease severity of rotavirus gastroenteritis (Nakagomi *et al.* 2006).

1.2.19 Diagnosis:

The clinical manifestations of rotavirus illnesses are not specific, and not sufficient to make a diagnosis on this basis alone. The epidemiological and seasonal pattern of rotavirus diseases at any one time may suggest the diagnosis, but laboratory confirmation is required. Vesikari has developed an used numerical scores (20 points) for the clinical severity of diarrhoeal episodes including those of rotavirus diseases in Finnish children (Ruuska *et al.* 1990) [table: 1.2]. With increasing severity, the etiological share and detection rate of rotavirus increases (Pang *et al.* 2000).

Virus detection is best diagnosed by examination of faecal samples collected within the first 4 days of the onset of diarrhoea. However, prolonged, intermittent shedding of rotavirus may be detected by RT-PCR for up to 8 weeks (Richardson *et al.* 1998). Diagnosis can be made by the detection of rotavirus particles, the antigen/ serological response and the genome.

Table 1.2 Numerical scoring system for the severity of rotavirus diarrhoea

Symptom or sign	20 points
Duration of diarrhoeal disease	
1-4	**1**
5	**2**
>6	**3**
Max No of diarrhoeal stools/24h	
1-3	**1**
4-5	**2**
>6	**3**
Duration of vomiting (days)	
1	**1**
2-3	**2**
>3	**3**
Max No of vomiting episodes /24	
1	**1**
2-4	**2**
≥ 5	**3**
Fever	
37-38.5 °C	**1**
38.5-38.9 °C	**2**
≥ 39 °C	**3**
Dehydration	
1-5%	**2**
≥ 6%	**3**
Treatment	
Hydration	**1**
Hospitalization	**2**

Initially, direct visualisation of stool samples by Electron Microscopy (EM) was the classic method for rotavirus detection. EM is a highly specific method of rotavirus detection since rotavirus has a distinctive morphological appearance. By using EM, 80 - 90% of rotavirus positive samples will be detected. EM has the advantage of detecting rotaviruses that do not carry group A specific antigens. IEM is not necessary for the detection of rotaviruses because the particle has such a distinctive morphological appearance that can be easily detected.

The method of choice in most laboratories is the confirmatory ELISA that employs polyclonal or Mabs to the group specific rotavirus antigen. ELISAs are highly sensitive, do not require specialized equipment, require minimal training by investigators, and have a built-in control for non-specific reactions.

Polyacrylamide gel electrophoresis (PAGE) can be used for the demonstration of the whole genome and carries similar sensitivity to EM. It is also highly specific because the eleven dsRNA segments are diagnostic of rotavirus. It is mainly useful in epidemiological assays. Molecular methods such as RT-PCR have improved sensitivity. However, RT-PCR and PAGE

are more technically demanding and labour intensive, and they are not suitable for routine use in diagnostic purposes in most laboratories (Cunliffe 2001)

1.2.20 Treatment:

The major aspect of treatment of rotavirus gastroenteritis is to replace the fluids and electrolyte lost by diarrhoea and vomiting, which can be achieved by oral or intravenous fluids. Oral rehydration therapy is playing a big role in developing countries where facilities for parenteral fluids are not always available. The oral rehydration salts (ORS) of various formulations, have shown to be effective in treating diarrhoea and the dehydration of rotavirus gastroenteritis, with some minor variations in their efficacy. It is said that ORS containing glucose are more rapidly effective in treating dehydration compared to those containing sucrose (Nalin *et al.* 1978).

The standard WHO ORS solution formula is composed of the following: sodium, 90 mmol/L, chloride, 80 mmol/L, potassium, 20 mmol/L, citrate, 10mmol/L, and glucose, 111 mmol/L. The overall osmolality of this combination is 310 mOsm/L, and bicarbonate, 30 mmol/L can be a substitute for citrate (Pediatrics 1996).

Recently, an ORS solution containing a reduced sodium concentration (reduced osmolality) has been found to be more effective compared to the standard WHO solution. This solution contains sodium, 75 mmol/L, chloride, 65 mmol/L, potassium, 20 mmol/L, citrate, 10 mmol/L, and glucose, 75 mmol/L, with an overall osmolality of 245 mOsm/L (mbc 71). Rice-based solutions have been compared with glucose-based ORS in infants and young children with diarrhoea and mild to moderate dehydration. Both were found to be effective, but the rice-based solution was more effective in decreasing total stool output and increasing absorption and retention of fluids and electrolytes. However, this difference was found to be of minor clinical importance (Carpenter *et al.* 1988).

The intravenous fluids must be given immediately, if the ORS does not correct the dehydration and electrolyte loss, or there is severe dehydration and shock. ORS should not be given to children with depressed levels of consciousness because of the possibility of fluid aspiration.

In most countries, certain drugs are contra-indicated in the treatment of acute diarrhoea in children 1 month to 5 years of age. For example, the American Academy of Pediatrics does not recommend the use of loperamide, anticholinergic agents, bismuth subsalicylate, adsorbents, and Lactobacillus

containing compound, opiate, and opiate atropine combination drugs (Pediatrics 1996) .

Human milk, containing rotavirus antibodies, has been used successfully for the treatment of immunodefficient children with chronic rotavirus infection and disease (Davidson 1996). Colostrums or milk concentrates from cows immunized with human rotavirus were not effective in the treatment of children with acute rotavirus gastroenteritis, though it decreased the duration of virus shedding (Ebina *et al.* 1983). The daily oral administration of rotavirus antibody-containing bovine colostrums appeared to have a protective effect during an outbreak of rotavirus diarrhoea (Ebina *et al.* 1983).

The effect of a single oral dose (300mk/kg) of gamma globulin was evaluated in the treatment of rotavirus gastroenteritis in a double-blind placebo-controlled trial. The treatment group had a significantly shorter duration of diarrhoea, shorter viral excretion and hospital stay (Guarino *et al.* 1994). The same effect was observed when oral human serum immunoglobulin, and bovine colostrums prepared by immunization of pregnant cows with human rotavirus were used (Guarino *et al.* 1991;

Hammarstrom 1999).

The efficacies of several broad-spectrum antiviral agents have been examined as they inhibit rotavirus replication in vitro. Various adenosine analogs were found to have anti-virus activity, which may be the result of inhibition of S-adenosylhomocysteine hydrolase, an enzyme essential for methylation and maturation of viral mRNA (Kitaoka *et al.* 1986).

A 3-day course of nitazoxanide significantly reduced the duration of rotavirus disease in hospitalised paediatric patients. These results are encouraging, and might lead us to think about new approaches to managing rotavirus disease in children. Nitazoxanide, a broad-spectrum anti-infective drug, which is licensed in the USA for treating diarrhoea caused by Cryptosporidium parvum and Giardia lamblia in children and adults (Rossignol *et al.* 2006)

Bismuth subsalicylate (BBS) is perhaps contraindicated because of the association between salicylate and Reye's syndrome. In a double-blind placebo-control study, it was effective and the author could fine no cases of Reye's syndrome. The author also found that the blood level of salicylate and bismuth in the treatment group were well below the toxic level (Soriano-

Brucher *et al.* 1991).

Zinc Supplementation for the Treatment of Diarrhoea:

In May 2004, WHO/UNICEF issued a joint statement on the Clinical Management of Acute Diarrhoea. This statement recommended zinc supplementation together with a new ORS formula for the clinical management of diarrhoea (Fontaine 2006; Long *et al.* 2006).

Two recent advances in managing diarrhoeal disease – newly formulated oral rehydration salts (ORS) containing lower concentrations of glucose and salt, and the use of zinc supplementation can drastically reduce the number of child deaths. The new methods, used in addition to prevention and treatment of dehydration with appropriate fluids, breastfeeding, continued feeding and selective use of antibiotics, will reduce the duration and severity of diarrhoeal episodes and lower their incidence (Arora *et al.* 2006; Aggarwal *et al.* 2007).

WHO and UNICEF currently recommend daily 20 mg zinc supplements for 10–14 days for children with acute diarrhoea, and 10 mg per day for infants under six months old, to curtail the severity of the episode and prevent

further occurrences in the ensuing 2-3 months (Fischer Walker *et al.* 2007; Luabeya *et al.* 2007).

The importance and effects of micronutrients, zinc and vitamin A have been extensively studied in relation to rotavirus diarrhoea, in the past decade (Alarcon *et al.* 2004; Valery *et al.* 2005).

1.2.21 Prevention:

1.2.21.1 General Rules and Observation:

Prevention of rotavirus diseases is needed world-wide, and the need for this is based on epidemiological and hospital based studies. Prevention is most needed in developing countries, where nearly 500,000 children die every year from rotavirus gastroenteritis. Natural infection does not provide complete protection against re-infection or mild diseases (Bishop *et al.* 1983; Velazquez *et al.* 1996) There is an urgent need for the development of a rotavirus vaccine that prevents severe rotavirus gastro-enteritis during the first 2 years of life, where in this period rotavirus disease is most serious.

Evidence from animal studies indicates that the most important role in resistance to rotavirus disease is played by the local intestinal immunity. From this observation it is probable that the effectiveness of any rotavirus vaccine will depend mainly on its ability to stimulate the local intestinal immunity and the local IgA antibody production. Therefore, the most effective way of stimulating local immunity is through the infection at the local site that can be achieved by the development of a live attenuated oral vaccine.

In the past few years, several live virus vaccines have been developed. However, the heterotypic and homotypic immunity responses have raised a number of important issues concerning the efficacy and the most effective strategies in applying and using these vaccines.

1.2.21.2 Passive immunity:

Passive immunization can be effective in preventing or modifying rotavirus diseases both in animals and humans (Barnes *et al.* 1982; Brussow *et al.* 1990; Ebina 1996). Passive rotavirus antibodies can be administered from various sources. For example, eggs from immunized hens, or milk formulas containing bovine milk immunoglobulin from cows hyper-immunized with human rotavirus (Yolken *et al.* 1988; Davidson 1996). However, providing passive immunity is not practical in humans because it requires repeated doses of antibodies. It may be of benefit in immunocompromised children.

1.2.21.3 Vaccine Interventions:

As mentioned earlier, longitudinal studies have demonstrated that naturally acquired rotavirus infections provide certain degree of protection against symptomatic disease upon re-infection (Bishop *et al.* 1983; Bhan *et al.* 1993).

In infancy, it has been shown that rotavirus infection can protect against symptomatic rotavirus re-infection (Bernstein *et al.* 1991; Ward *et al.* 1994). It has been observed and demonstrated, in a recent study in Mexico, that successive rotavirus infections can increase the degree of protection (Velazquez *et al.* 1996). Thus, children with one, two, and three rotavirus infections had progressively lower risks of subsequent rotavirus diarrhoea [relative risk 0.62, 0.40, 0.34 respectively]. Moreover, no child with two previous infections subsequently developed moderate or severe rotavirus diarrhoea (Cunliffe 2001). Some studies have helped to clarify the serotype-specific nature of the immune response, both in terms of the magnitude of the serotype-specific antibody rise following natural infection (Matson *et al.* 1992), and in the tendency for repeat infections to be with a different serotype to the primary infection (Chiba *et al.* 1986; O'Ryan *et al.* 1994; Velazquez *et al.* 1996).

As described above, the protective nature of neonatally acquired rotavirus infection is also important especially when these infections are asymptomatic. Thus, researchers believe that naturally-occurring attenuated rotavirus infection could/can induce significant protection from rotavirus diarrhoea and disease. In the light of the evidence from multiple studies of natural rotavirus infections, vaccination of infants with several doses of oral

attenuated vaccine virus has been expected to provide a high level of serotype-specific protection.

1.2.21.4 History of Rotavirus Vaccine Development:

In 1998, prior to the development and licensure of Rotarix® and RotaTeq®, the world's first rotavirus vaccine, Rotashield™, was licensed for use in the United States. Clinical trials in the United States, Finland, and Venezuela had found it to be 80 to 100% effective at preventing severe rotavirus diarrhea, and researchers had detected no statistically significant serious adverse effects (Cunliffe *et al.* 2002).

The manufacturer of Rotashield™, however, withdrew it from the market in 1999, after it was discovered that the vaccine may have contributed to an increased risk for intussusceptions, or bowel obstruction, in one of every 12,000 vaccinated infants (Cunliffe *et al.* 2002).

The experience provoked intense debate among public health officials, scientists, vaccine manufacturers, and others about the relative risks and benefits of a rotavirus vaccine. As the debate continued, so did deaths, disease, and hospitalizations caused by rotavirus.

Today's vaccines against rotavirus were rigorously studied to determine any

association with an increased risk of intussusceptions. In the studies conducted to date, both vaccines were found to have no association with the disorder (Ruiz-Palacios *et al.* 2006; Vesikari *et al.* 2006; Vesikari *et al.* 2006)

1.2.21.5 New Rotavirus Vaccines:

Two live, oral, attenuated vaccines against rotavirus infection (Rotarix®, manufactured by GlaxoSmithKline; and RotaTeq®, manufactured by Merck & Co., Inc.) were licensed by the European Medicines Agency and the US Food and Drug Administration, respectively, in 2006. Clinical trials in Europe, Latin America, and the US have demonstrated that these vaccines are safe and highly efficacious at preventing rotavirus-associated severe gastroenteritis. A number of countries, including some developing countries, have licensed these vaccines, and they are beginning to be introduced in routine immunization programs in some settings. To date, both vaccines have been primarily studied in middle- and high-income countries. But historically, oral vaccines have been shown to perform differently in different regions of the world. The global health community recognizes the need to carry out additional studies of the safety and efficacy of these vaccines in developing countries of Africa and Asia, where the

burden of disease is very high (Cunliffe *et al.* 2005; WHO 2006; WHO 2006)

Neither breastfeeding nor concurrent administration of other childhood vaccines appears to diminish the efficacy of a 3-dose series of RotaTeq®. Among 1,566 infants exclusively breastfed, the efficacy of RotaTeq® against rotavirus gastroenteritis of any severity (68%; CI = 54%--78%) was comparable to that among 1,632 infants who were never breastfed (68%; CI = 46%--82%). Among 204 vaccinated infants born prematurely (<37 weeks' gestation), the estimate of vaccine efficacy against rotavirus gastroenteritis of any severity was comparable to that among non-premature infants (70%; CI = -15%--95%), but the confidence limits included zero because of the small sample size (Parashar *et al.* 2006).

The simultaneous administration of oral vaccine and breast milk may not be appropriate. Breastfeeding may play a role in reducing the vaccine's efficacy. While the overall health benefits of breastfeeding are significant, ingestion of maternal breast milk at the same time as administration may inactivate the vaccine. (Glass *et al.* 2006)

The two new rotavirus vaccines:

Simultaneous administration:

Rotavirus vaccine can be administered together with DTaP, Hib vaccine, IPV, hepatitis B vaccine, and pneumococcal conjugate vaccine. Available evidence suggests that the rotavirus vaccine does not interfere with the immune response to the Hib vaccine, IPV, hepatitis B vaccine, and pneumococcal conjugate vaccine, and the diphtheria and tetanus antigens in DTaP.

Because validation of the pertussis assays is still under review, insufficient immunogenicity data are available to confirm lack of interference of immune responses when rotavirus vaccine is concomitantly administered with childhood vaccines to prevent pertussis.

Contraindications:

Rotavirus vaccine should not be administered to infants who have severe hypersensitivity to any component of the vaccine or who have experienced a serious allergic reaction to a previous dose of rotavirus vaccine.

Precautions:

Altered immunocompetence:

Practitioners should consider the potential risks and benefits of administering rotavirus vaccine to infants with known or suspected altered immunocompetence. Children and adults who are immunocompromised because of congenital immunodeficiency, hematopoetic transplantation, or solid organ transplantation sometimes experience severe, prolonged, and even fatal rotavirus gastroenteritis. However, no safety or efficacy data are available for the administration of rotavirus vaccine to infants who are potentially immunocompromised, including

- infants with blood dyscrasias, leukemia, lymphomas of any type, or other malignant neoplasms affecting the bone marrow or lymphatic system;
- infants on immunosuppressive therapy (including high-dose systemic corticosteroids);
- infants with primary and acquired immunodeficiency states, including human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) or other clinical manifestations of infection with HIV; cellular immune deficiencies; and hypogammaglobulinemic and dysgammaglobu-linemic states. Data are insufficient from the clinical trials to support administration of

rotavirus vaccine to infants with indeterminant HIV status who are born to mothers with HIV/AIDS; and infants who have received a blood transfusion or blood products, including immunoglobulins, within 42 days. In general, rotavirus vaccine should be deferred for 42 days following receipt of an antibody-containing product if possible. However,

- if the 42-day deferral would cause the first dose of rotavirus vaccine to be scheduled for age ≥13 weeks, a shorter deferral interval should be used to ensure the first dose is administered before age 13 weeks.

Acute gastroenteritis:

In usual circumstances, rotavirus vaccine should not be administered to infants with acute, moderate-to-severe gastroenteritis until the condition improves. However, infants with mild acute gastroenteritis can be vaccinated, particularly if the delay in vaccination might be substantial and might make the child ineligible to receive vaccine (e.g., aged ≥13 weeks before vaccination is initiated).

Rotavirus vaccine has not been studied among infants with concurrent acute gastroenteritis. In these infants, the immunogenicity and efficacy of rotavirus vaccine can theoretically be compromised. For example, infants who receive

oral poliovirus vaccine (OPV) during an episode of acute gastroenteritis in some instances have diminished poliovirus antibody responses to OPV.

Moderate to severe illness:

Infants with moderate-to-severe illness should be vaccinated as soon as they have recovered from the acute phase of the illness. This precaution avoids superimposing adverse effects of the vaccine on the underlying illness or mistakenly attributing a manifestation of the underlying illness to the vaccine.

Pre-existing chronic gastrointestinal disease:

Practitioners should consider the potential risks for and benefits of administering rotavirus vaccine to infants with preexisting chronic gastrointestinal disease. Infants with preexisting chronic gastrointestinal conditions who are not undergoing immunosuppressive therapy should benefit from rotavirus vaccine vaccination, and the benefits outweigh the theoretical risks. However, the safety and efficacy of rotavirus vaccine have not been established for infants with these preexisting conditions (e.g., congenital malabsorption syndromes, Hirschsprung's disease, short-gut syndrome, or persistent vomiting of unknown cause).

Intussusceptions:

Following administration of a previously licensed rotavirus vaccine, RRV-TV, an increased risk for intussusceptions was observed. Available prelicensure data from a trial of 70,000 infants indicated no evidence of an association between intussusceptions and the current vaccine. However, additional postlicensure surveillance data are required to confirm that the vaccine is not associated with intussusceptions at a lower rate than would have been detected in prelicensure trials. In addition, data suggest that infants with a history of intussusceptions might be at higher risk for a repeat episode than other infants. Therefore, until postlicensure data on safety of rotavirus vaccine are available, the risks for and the benefits of vaccination should be considered when vaccinating infants with a previous episode of intussusceptions.

Special Situations:

Premature infants (<37 weeks' gestation):

Practitioners should consider the potential risks for and benefits of vaccinating premature infants against rotavirus. Limited data suggest that premature infants are at increased risk for hospitalization from viral gastroenteritis during their first year of life. In clinical trials, the safety and

efficacy of rotavirus vaccine appears to be similar for premature and term infants, although a relatively small number of preterm infants have been evaluated. The lower level of maternal antibody to rotaviruses in very low birth weight, premature infants theoretically could increase the risk for adverse reactions from rotavirus vaccine. In USA, they support vaccination of prematurely born infants if they are at least aged 6 weeks, are being or have been discharged from the hospital nursery, and are clinically stable. Until further data are available, physicians should consider that the benefits of rotavirus vaccine vaccination of premature infants outweigh the theoretical risks.

Exposure of immunocompromised persons to vaccinated infants:

Infants living in households with persons who have or are suspected of having an immunodeficiency disorder or impaired immune status can be vaccinated. The majority of experts believe the protection of the immunocompromised household member afforded by vaccination of young children in the household outweighs the small risk for transmitting vaccine virus to the immunocompromised household member and any subsequent theoretical risk for vaccine virus-associated disease. To minimize potential virus transmission, all members of the household should employ measures

such as good hand washing after contact with the feces of the vaccinated infant (e.g., after changing a diaper).

Exposure of pregnant women to vaccinated infants:

Infants living in households with pregnant women can be vaccinated. The majority of women of childbearing age would have pre-existing immunity to rotavirus and so the risk for infection and disease from potential exposure to the attenuated vaccine virus strain is low. In addition, no evidence exist that rotavirus infection or disease during pregnancy poses any risk to the fetus. Furthermore, vaccination of young children would avoid potential exposure of the pregnant women to wild virus if the unvaccinated infant suffers from rotavirus gastroenteritis.

Regurgitation of vaccine:

The practitioner should not re-administer a dose of rotavirus vaccine to an infant who regurgitates, spits out, or vomits during or after administration of vaccine. The infant can receive the remaining recommended doses of rotavirus vaccine at appropriate intervals. Data are limited regarding the safety of administering a dose of rotavirus vaccine higher than the recommended dose and on the efficacy of administering a partial dose.

Additional data on safety and efficacy are needed to evaluate the benefits of and risks for re-administration.

Hospitalization after vaccination:

If a recently vaccinated child is hospitalized for any reason, no precautions other than routine universal precautions need be taken to prevent the spread of vaccine virus in the hospital setting. From: (Glass *et al.* 2006; Parashar *et al.* 2006)

1.2.21.6 The Promise of a Vaccine

The world health community believes that vaccines offer the best hope for protection against rotavirus. Why?

- Naturally acquired rotavirus infections provide protection against disease; a vaccine could also stimulate immunity (Cunliffe *et al.* 2002)
- Rotavirus transmission rates seem unaffected by improvements in sanitation and hygiene, one of the principal public health approaches to controlling other types of diarrhoeal disease.

- Life-saving intravenous treatment to rehydrate children with severe rotavirus diarrhoea is unavailable to many children in the developing world.

For many countries of both the developed and developing world, a vaccine will be the most cost-effective way to stop rotavirus.

1.3 Study Design:

1.3.1 Place of the study:

This study has been carried out at The Royal Liverpool Children Hospital, Alder Hey, and the Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool.

All children with diarrhoeal diseases, who attended Alder Hey, were included in the study. Most of those children were admitted to the inpatient wards where stool samples were collected (the main starting point). However, stool samples were also collected from other sites, e.g. Accident and Emergency Department and outpatient clinics whenever the children presented with diarrhoea.

Case definition: acute watery diarrhoea with or without vomiting or fever. Any child who presented with ≥ 3 episodes/24 h of watery stool was included. There were no age limitation or exclusion criteria. However, there was no stool collection from the Neonatal Unit at the Liverpool Women Hospital.

Consent: No parental consent was requested.

Seasonality

The season is defined as the peak period of rotavirus gastroenteritis.

1.3.2 Aims:

1. To describe the relative importance of rotavirus as a cause of diarrhoeal diseases in children attending Alder Hey Children Hospital.
2. To determine the most vulnerable age group infected and affected by rotaviruses.
3. To assess the optimal child age for the administration of rotavirus vaccines.

1.3.3 Specific objectives:

1. To determine the different electropherotypes.
2. To determine the G genotypes of rotaviruses circulated over a two-year period in children attending AHCH.
3. To determine whether there are seasonal differences in the prevalence of circulating G genotypes.

1.3.4 Rationale and significance

This is the first complete systematic study of rotavirus G genotypes in one city in UK. It will provide much-needed information of rotavirus genotypes

to aid rational design of vaccines.

1.3.5 Research Design:

This is a prospective descriptive study of rotavirus diarrhoeal diseases with laboratory studies on rotavirus electropherotypes and genotypes.

1.3.6 Subject:

All children attended AHCH with diagnosis of rotavirus diarrhoea in the two-year study period are included.

1.3.7 Methods:

chapter II (Materials and Methods).

Chapter Two

Materials and Methods

2.1 Study Site:

This study was based at the Royal Liverpool Children Hospital, Alder Hey and the Department of Medical Microbiology and Genito-Urinary Medicine, the University of Liverpool.

2.2 Subject Enrolment:

This study was conducted between December 4th, 2000 and August 30^{th,} 2002. Samples were regularly collected at Alder Hey from January 1st 2000 to January 1st 2002. A two-year study was undertaken so that the rotavirus seasonality of infection and strain types could be assessed over 24 months.

Faecal samples were collected regularly from all children presenting to Alder Hey with acute diarrhoea whether or not they had any other symptoms and signs of gastroenteritis. There were no limitation or selection criteria for the collection of stool samples. The collection was undertaken by the nursing staff. The nursing staff collected stool samples in sterile containers from all children who presented with acute diarrhoea, in inpatient wards, Accident & Emergency department and outpatient. No parental consent was necessary in collecting the stool samples.

Stool samples were passed first to the Medical Microbiology Department at Alder Hey to test for rotavirus using an antigen detection ELISA.
There was no limitation to the age groups studied. There was no other clinical data collection.

2.3 Specimen collection, labelling and storage:

Faecal samples were collected in sterile containers by trained nursing staff. The samples were labelled with the name of the child, hospital number, date of birth and the date of collection before being sent to the Medical Microbiology at Alder Hey. All samples, tested positive to rotavirus with ELISA, were separated, labelled and stored there for a short period. The rotavirus positive samples were then collected from Alder Hey and brought to the Department of Medical Microbiology and Genito-Urinary Medicine where they were immediately stored and frozen at –80°C until further testing, later.

2.4 Testing with ELISA:

The ELISA test was the IDEIA Rotavirus (DAKO, Cambridgeshire, UK) and was performed at Alder Hey prior to the transfer of the samples.

The IDEIA rotavirus test is an enzyme immunoassay for the detection of Group A rotavirus in faecal specimens.

The test utilises a polyclonal antibody to detect group specific proteins, including the major inner capsid protein (VP6), present in Group A rotaviruses.

2.5 Preparation of Faecal Supernates:

Approximately 10-20% faecal suspensions were prepared in 0.01M phosphate-buffered saline (PBS) of pH 7.4. The PBS solution was prepared by dissolving a single PBS tablet (Sigma, Poole, UK) in 50ml of distilled water. Approximately, 100-200ul of liquid stool (or a pea-sized portion of solid stool) was mixed thoroughly in a 1.5ml Eppendorf tube with 900ul of PBS of pH 7.4 by vortexing in a class 1 Biosafety cabinet. The mixture was then spun at 1300rpm for 10 minutes in a bench-top microcentrifuge. The supernate was pipetted off and then placed in a clean 1.5ml Eppendorf tube and then stored at –80°C for further rotavirus testing.

2.6 Extraction of Rotavirus dsRNA from Faecal Samples:

Extraction of rotavirus dsRNA from faecal samples was performed by using a guanidine isothiocyanate/silica glass powder extraction method [modified

by Gentsch et al., (1992), from the method of Boom et al., (1990)]. This method removes inhibitors of RT-PCR present in stool by partially purifying Rotavirus dsRNA by binding to glass powder in the presence of guanidine thiocyanate. The dsRNA is then eluted from the glass powder and can be used directly for RT-PCR. All spins were performed in a bench-top microcentrifuge.

2.6.1 Reagents:

Guanidine isothiocyanate, molecular biology grade (Sigma)

Tris-HCl (Sigma)

Ethanol, 100%

Silica powder (RNAID, distributed by Stratech Scientific, Luton, UK)

Kit wash buffer (RNAID, distributed by Stratech Scientific, Luton, UK)

High Pressure Liquid Chromatography (HPLC)-grade water (BDH, Poole, UK)

2.6.2 Procedure:

- 250ul of faecal supernate (10-20% supernatant in PBS) was prepared in a 1.5ml Eppendorf tube. This was done in a class 1 Biosafety cabinet.

- 500ul of 6M guanidine isothiocyanate (made in 50mM Tris-HCl, pH 7.5) was added to each tube and was vortexed well. This was done in a class 1 Biosafety cabinet.
- Following incubation at 65°C for 10 minutes, 8.5ul of silica powder was added to each sample. The tubes were vortexed well and rocked on a rotator for 30 minutes at room temperature. The mixture was centrifuged on a bench top centrifuge for 60 seconds at 4000 rpm (the lowest speed that pelleted the particles completely was used).
- The supernate was removed using a separate pipette for each sample, and washed once with 700ul of guanidine wash solution (ratio of 2:1 6M guanidine: 50mM Tris-HCl). Fresh guanidine wash solution was used for each RNA extraction procedure.
- The tubes were centrifuged on a bench top centrifuge for one minute at 4000 rpm. The supernate was removed and discarded.
- The tubes were washed three times with an ethanol wash solution and after the first two washes were centrifuged for one minute at 4000 rpm; after the third wash the tubes were centrifuged for two minutes at 1300 rpm.

- All residual liquid from the samples was removed by pipetting, and the samples were dried for 10 minutes in the centrifuge drier or in room temperature for 30-60 minutes.
- RNA was extracted by adding 35ul of autoclaved HPLC-grade H_2O and incubating for 10 minutes at 65°C, followed by centrifugation on an Eppendorf microfuge for two minutes at 13000 rpm.
- The supernate was aspirated with separate pipette tips and transferred to 0.5 ml microcentrifuge tubes.
- The H_2O extraction step (step 8) was repeated. The supernates were combined to give a final RNA volume of 70ul.
- The tubes were centrifuged at 13000 rpm for two minutes to pellet any remaining silica powder. The RNA was stored at –80°C until used for PAGE and RT-PCR.

2.7 PolyAcrylamide Gel Electrophoresis (PAGE) of Rotavirus dsRNA:

2.7.1 Preparations:

Stock solutions:

Laemmli sample buffer (Biorad, Hemel Hempstead, UK)

Sample buffer was diluted in an equal volume of water

Acrylamide: Bisacrylamide (Biorad)

A 30 g bottle of 29:1 bis-acrylamide was used. The acrylamide powder was dissolved in 73ml H_2O using a magnetic stirrer. The solution was refrigerated in the dark. Gloves and goggles were used all times when working with acrylamide.

Lower gel (separating gel) stock solution:

1.5M Tris solution was prepared by dissolving 18.17 g of Tris base (Sigma) in 100 ml of H_2O; 37% HCl was added to give a final pH of 8.8.

Upper gel (stacking gel) stock solution:

0.5M Tris solution was prepared by dissolving 6.06g of Tris base (Sigma) in 100 ml of H_2O; 37% HCl was added to give a final pH of 6.8.

Ammonium persulfate (Biorad):

A 10% solution weight/volume was prepared (typically 0.1g of ammonium persulfate per 1ml water)

Temed (Biorad):

This was used as supplied by the manufacturer.

Running buffer:

Running buffer was made by dissolving the following in one litre of distilled water:

Tris base (Sigma) 3.03g

Glycine (Sigma) 14.4g

2.7.2 Procedure:

(A) 10% acrylamide separating (lower) gel was made as follows:

Lower gel stock (pH 8.8)	6.0ml
Acrylamide: Bis	8.0ml
H_2O	10ml
105 ammonium persulfate	0.12ml
TEMED	12ul (added when ready to pour)

(B) 3% acrylamide stacking (upper) gel was made as follows:

Upper gel stock (pH 6.8)	2.5 ml
Acrylamide: Bis	1.5ml
H_2O	6ml
10% ammonium persulfate	0.03ml
TEMED	10ul (added when ready to pour)

2.7.3 Assembly of plates:

The Biorad Mini-Protean vertical electrophoresis system was used. Each of the pieces of apparatus was washed thoroughly using soapy water followed by alcohol. Dividing strips were placed along the lateral edges of the glass plates and secured firmly into the apparatus using bulldog clamps. Heated 1.5% agarose was poured to a line on the surface of a plastic plate and the apparatus was placed on the bench to cool.

2.7.4 Pouring of gels:

The separating gel was poured, and layered with a small volume of 21-fold diluted lower gel stock. The gel was allowed to polymerise. After polymerisation, the 21-fold diluted lower gel stock was poured off, and the plates were rinsed with distilled water. The apparatus was drained well, and blotting papers were used to dry surfaces between the glass plates. The stacking gel was poured and the comb inserted, avoiding air bubble formation. After polymerisation in the stacking gel, the comb was removed. The gel plates were assembled on the electrophoresis chamber by means of bulldog clamps. To avoid leakage of reservoir buffer, the plates were sealed to the chamber by pipetting melted agarose along the edges. The centre of

the electrophoresis chamber was filled with the running buffer until the buffer overflowed into the gel.

2.7.5 Samples preparation and loading:

30ul of RNA was added to a 1.5 Eppendorf tube and dried in the vacuum centrifuge. 10ul of 1x sample buffer was added per sample of dried RNA. The buffer/ RNA were heated at 65°C for 10 minutes, vortexed briefly and centrifuged for a few seconds prior to use. The samples were loaded with a finely drawn Pasteur pipette (Bioquote, York, UK). The outer chamber was filled with running buffer until the lower end of the glass plate was covered. The leads were connected to a power pack and the gels were run at 150 volts for 120 minutes.

2.7.6 Removal of the gels:

The apparatus was removed and excess buffer was poured off. The gels were removed from the apparatus and were transferred to a fixing solution (solution A, below)

2.7.7 Silver Staining of Gells to visualize the Rotavirus Genome:

The Gels were stained with silver nitrate using the method of Herring et al., (1982).

Solutions

10% ethanol, 0.5% acetic acid (fixing solution)

100% ethanol	20ml
Glacial acetic acid	1ml
Distilled water to	200ml

0.01 1M silver nitrate (Sigma)

Silver nitrate (Sigma)	0.4g
Distilled water to	200ml

0.75m NaOH (Sigma); 0.1M formaldehyde (Sigma) [developing solution]

* The developing solution was made at the same time as solution B, since NaOH takes at least 30 minutes to dissolve.

NaOH	6.0g
37% formaldehyde	1.5ml
Distilled water to	200ml

5% Acetic acid (stop solution)

Glacial acetic acid	10ml
Distilled water to	200ml

2.7.7.1 **Procedure:**

Staining was carried out in glass dishes. Step 3 was done in a safety cabinet.

The gels were detached from the plates and placed in solution A for 30 minutes.

The gels were transferred to solution B for 90 minutes and then rinsed briefly with distilled water.

The gels were transferred to solution C and development allowed to proceed until all bands were visible in the control lanes (which contained rotavirus strains Wa and DS1 as controls)

The gels were transferred to solution D for 30 minutes.

The gels were rinsed and stored in 20% ethanol/ 1% glycine until photographed.

2.8 RT-PCR Genotyping of Rotavirus:

2.8.1 Principles:

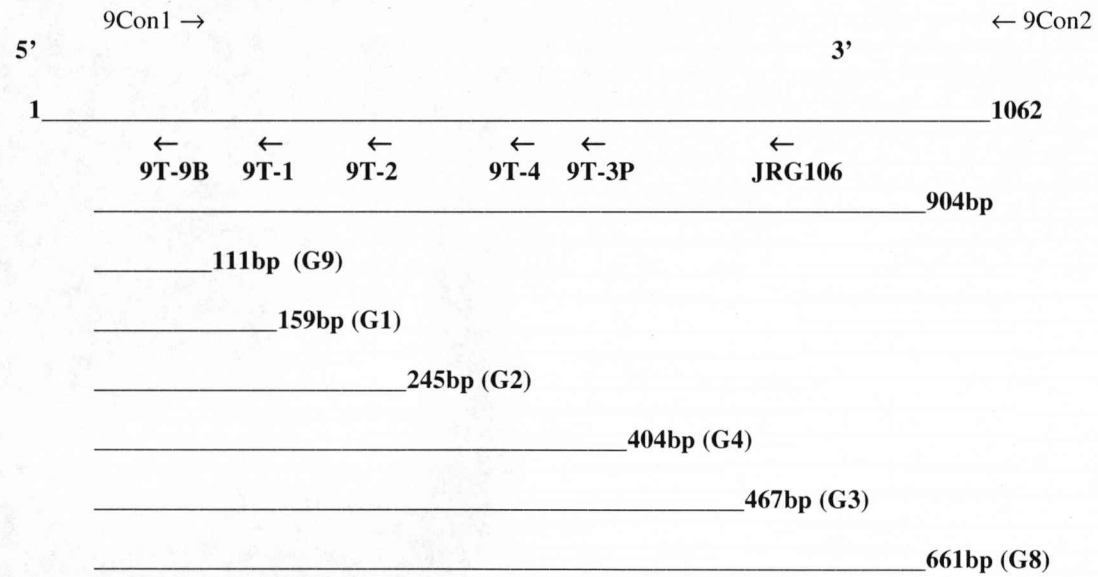
Rotavirus VP7 (G) genotypes were determined by using hemi-nested, multiplex, RT-PCR. The methods were originally described for G typing by Gouvea et el., (1990) and Das et al, (1994). The dsRNA template was first denatured (to separate the strands) and each strand was then reverse transcribed to complementary DNA (cDNA). A pair of consensus primers was used in the RT-PCR reaction to amplify a fragment of the VP7 gene. Type-specific primers representing each of the common G types gave products of different (type-specific) lengths when used in a second amplification reaction. The products were then resolved by electrophoresis in an agarose gel and visualised by ethidium bromide staining. A positive control (strain Wa or Ds-1) and a negative control (H_2O) were included in each RT-PCR experiment.

2.8.2 The VP7 Typing:

Detection of rotaviruses in clinical specimens and determination of the G-types was accomplished by extraction of the viral dsRNA from faecal specimens and analysis by RT-PCR with primers specific for the VP7 genes of G serotypes 1,2,3,4,8 and 9 (Figure 2.1)

Figure 2.1 Primers used for VP7 (G) typing

Primer	Strain (ST)	NT	Sense	Sequence	Primer Type
9Con1	Wa (G1)	37-56	+	TAGCTCCTTTTAATGTATGG	Consensus
9Con2	Wa (G1)	922-941	-	GTATAAAATACTTGCCACCA	Consensus
9T-1	Wa (G1)	176-195	-	TCTTGTCAAAGCAAATAATG	Type Specific
9T-2	S2 (G2)	262-281	-	GTTAGAAATGATTCTCCACT	Type specific
9T-3P	107E (G3)	484-503	-	GTCCAGTTGCAGTGTTAGC	Type Specific
9T-4	ST3 (G4)	423-440	-	GGGTCGATGGAAAATTCT	Type Specific
9T-9B	116E (G9)	131-147	-	TATAAAGTCCATTGCAC	Type Specific
JRG106	HMG89 (G8)	681-698	-	TCTTCAAAAGTCGTAGTG	Type Specific



2.8.3 Reagents:

Autoclaved HPLC grade H_2O (BDH)

Super Reverse transcriptase (Molecular Genetic Resources, Tampa, Florida, USA)

10x PCR buffer (PE Applied Biosystems, Warrington, UK)

25mM $MgCl_2$ (PE Applied Biosystems)

Amplitaq DNA Polymerase (PE Applied Biosystems)

2.5mM dNTP (PE Applied Biosynthesis)

2.8.4 Procedure:

All PCR mixes (containing enzymes, dNTPs and $MgCl_2$) were prepared in a clean area.

These were separated from the RNA processing area, which was separated from the DNA processing area. Filter pipette tips were used throughout. All amplification reactions were conducted with a Techgene thermal cycler, (Techgene, Princeton, NJ, USA)

2.8.4.1 First amplification reaction:

2ul of consensus primer mixture (20uM each of 9con1 and 9con2) was added to each tube.

The RT and Amplitaq mixes were prepared on ice and vortexed prior to use.

1x RT (ul components)		1x Amplitaq (ul components)	
H_2O	17.9	H_2O	36.5
2.5mM dNTP	8	2.5mM dNTP	8
10x PCR buffer	5	10x PCR buffer	5
25mM $MgCl_2$	9	TAQ	0.5
RT (6-9U)	0.1		
	40ul		50ul

The tubes with 9con1/9con2 primer mix were transferred to the RNA area. 8ul of RNA were combined with the 2ul of the 9con1/9con2 primer mix and heated at 97°C for five minutes.

The tubes were cooled in ice for one minute, spun for 10 seconds at 13000 rpm and returned to the ice bath.

40ul of the RT mix was added to each tube, which was incubated for 60 minutes at 42°C in the thermal cycler.

The samples were returned to the ice bath, and 50ul of the Amplitaq mix was added to each tube.

The tubes were centrifuged for a few seconds, returned to the ice bath and then added to the thermal cycler for 10 cycles of PCR.

2.8.4.2 PCR cycling conditions:

1 cycle	94°C, 1 minute
10 cycles	94°C, 0.5 minutes
	42°C, 0.5 minutes
	72°C, 1 minute
1 cycle	72°C, 7 minutes
1 cycle	soak at 4°C

2.8.4.3 Second amplification:

1x reaction mix (ul components):

H_2O	27.5	
2.5mM dNTP	8	
10x PCR buffer	5	
25mM $MgCl_2$	4	
10uM G9 pool*	3	(1ul G9 pool + 1ul 9con1 + 1ul JRG 106)
Amplitaq	0.5	
	48	

Mixture at a concentration of 20uM each of primers 9T-1, 9T-2, 9T-3P, 9T-4, and 9T-9B

2.8.4.4 Procedure:

The first amplification product was centrifuged for 5 seconds at 13000 rpm and 2ul of product was transferred to 48ul of 1x reaction mixture. The samples were run for 30 cycles of PCR

2.8.4.5 PCR cycling conditions:

1 cycle	94°C, 1 minute
30 cycles	94°C, 0.5 minutes
	42°C, 0.5 minutes
	72°C, 0.75 minutes
1 cycle	72°C, 7 minutes
1 cycle	soak at 4°C forever

10ul of PCR product was electrophoresed at 150 volts for 90 minutes on an agarose gel {volume = 100ml of 0.5 TBE running buffer (Gibco BRL, Paisley, Scotland) + 2g agarose}. 2ul of 123bp DNA ladder (Gibco BRL, Paisley, Scotland) was run on each gel. The products were stained by adding 6ul of Ethidium Bromide solution/100ml.

2.8.4.6 Interpretation of results:

The sample was positive for G-types 1,2,3,4,8, or 9 if the sample gave PCR products of the appropriate size.

2.8.4.7 Limitations of the test:

This test is not a serological proof of the serotypes of the tested strains, although a strong correlation exists between the results of PCR and the monoclonal-based serotyping methods (Gouvea et al., 1990)

2.9 Nucleotide Sequencing:

The first amplification products were used for sequencing in which 9Con1 and 9Con2 were the primers. The products were run for 35 thermal cycles instead of 10 cycles as in step 8 above.

2.9.1 PCR product purification:

PCR products were purified prior to sequencing by the dideoxy chain termination method. Purification (including the removal of oligonucleotide primers and dNTPs) was performed by one of two methods. For single-banded PCR products, spin columns were used according to the manufacturer instructions (Qiagen, Crawley, UK). The spin columns contain a silica-gel membrane, which adsorbs DNA but allows contaminants to pass through. For PCR products containing multiple bands, purification was undertaken by using gel extraction. A low melting point agarose gel was used (typically a 1.2% seaplaque gel made in TAE buffer) and the PCR products were cut out by using a scalpel with the aid of ultraviolet illumination. The gel fragment was weighed and purified with a gel extraction kit according to the manufacturer's instructions (Qiagen). Using this kit, DNA fragments adsorb to silica particles and non-nucleic acid impurities (e.g. agarose, proteins, salts, and ethidium bromide) are removed

by washing. Purified DNA products were typically resuspended in 30-50ul of autoclaved, HPLC-grade water. In order to estimate the concentration of DNA in the sample prior to sequencing, 5ul of product was electrophoresed on a 2% agarose gel together with a standard mass ladder (Gibco BRL)

2.9.2 Cycle sequencing:

PCR products were sequenced completely in both directions by using the service provided by the Liverpool School of Tropical Medicine. This service utilises the PRISM Ready Big Dye Terminator Cycle Sequencing Kit (PE Applied Biosystems) with an automated sequencer (Applied Biosystems model 377). Sequences were received electronically and as hard copies.

2.9.3 Sequence analysis:

Sequences were edited manually and overlapping fragments were aligned by using DNASTAR (Madison, Wis., USA). Consensus sequences were generated with the aid of the seqman and editseq DNASTAR programmes. Using consensus nucleotide sequences, the Wisconsin Genetics Computer Group (GCG) computer programs (Devereux et al., 1984; Felsenstein, 1989) generated amino acid sequences (translate programme); nucleotide and amino acid identities (gap programme); and multiple sequence files (pileup

programme). Phylogenetic trees were drawn by using PHYLIP version 3.5c (copyright J. Felsenstein and the University of Washington) and CLUSTALX (EMBL, Heidelberg, Germany). The trees were visualised using the Treeview programme (http:// taxonomy.zoology.gla.ac.uk/rod/treeview.html).

2.10 Data analysis and statistical methods:

Statistical analysis was performed on a Pentium 4 personal computer using statistical package for social sciences (SPSS) for windows software, version 14.0.

The following significant tests were used:

Chi square, Fisher exact, Student's t and ANOVA (analysis of variance, using ANOVA, allowed us to extend the test to more than two populations or measurements).

P value

A p value of less than 0.05 was considered significant

Seasonality

The season is defined as the peak period of rotavirus gastroenteritis.

2.11 Ethical Approval:

The Research Committee at the Royal Liverpool Children Hospital, Alder Hey gave ethical approval for the study.

Chapter Three

Results

3.1 Total number of samples collected from the cases of diarrhoeal illnesses over the study period:

1496 samples were collected from children presented with diarrhoea. From these, 412 (27.8%) samples were positive for Rotavirus by ELISA.

3.2 Age groups:

The 412 ELISA positive stool samples were collected from children of different age groups.

A total of 93 (22.6%) samples out of 412 were from children less than 6 months of age. It is of interest that no positive case of rotavirus infection was detected under the age of 4 (0-4=0). The positive cases at the age of 4-5 were only 10 and 83 cases were 5-6 years.

A total of 175 (42.5%) samples were from the group of children aged 6-12 months. There were 110 (26.7%) samples from the children group aged 12-24 months and 34 (8.3%) samples in the children older than 24 months. [Figure 3.1, 3.2, table 3.1]

The most frequently-affected group was children aged 6-12 months followed by those aged 13-24 months. There was no significant difference in the age groups in the two seasons (Student's T. test, $p < 0.24$).

The age of the patients itself showed no significant difference between the two years (Anova Statistical test $p < 0.24$). The age was not a significant factor between the peak seasons of each year.

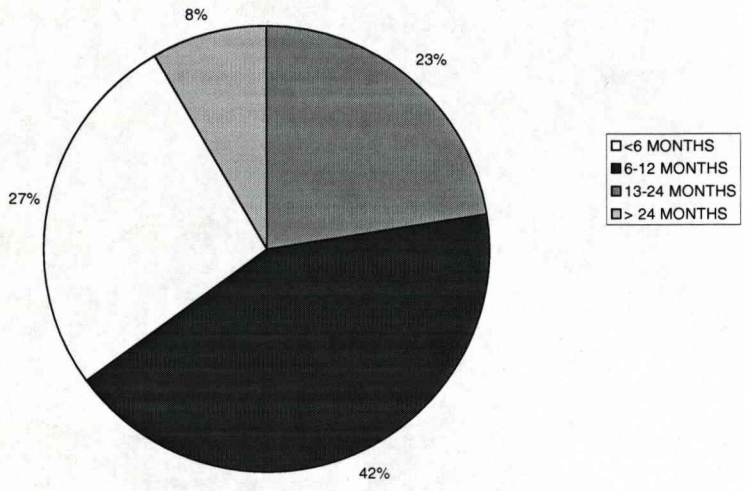


Figure 3.1 Age groups of rotavirus infected children two years (total)

NB: 06 (0-2=0, 2-4=0, 4-5=10, 5-6=83)

Age (months)	No positive	% positive of total positives
0-6 (total) 0-2 2-4 4-5 5-6	93 00 00 10 83	22.6% 0% 0% 2.6% 20%
6-12	175	42.5%
13-24	110	26.7%
>24	34	8.3%

Table 3.1 Age group v frequency

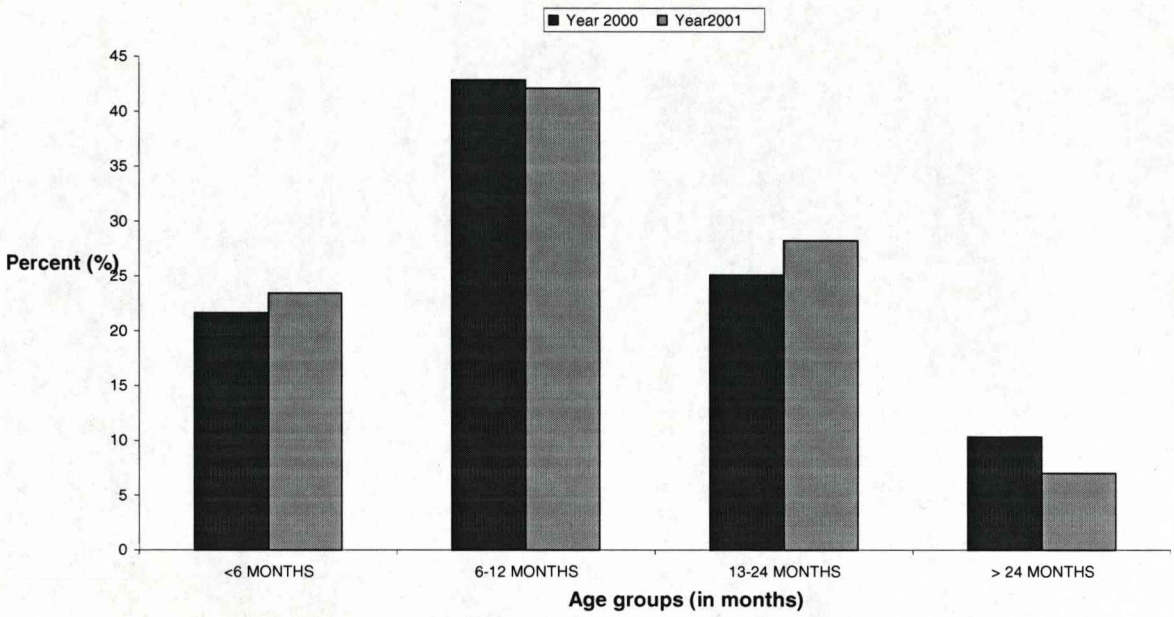


Figure 3.2 Age groups comparison of rotavirus infected children in the 2000 and 2001

3.3 Seasons:

The positive samples were obtained over two consecutive years from 1st January 2000 to 1st January 2002. A total of 203 (49.3%) samples were obtained in the 2000, and a total of 209 (50.7%) samples in 2001.

From figures 3.3 and 3.4, the highest prevalence of infection can be seen in February, March and April, followed by November and December each year, peaking in March and April. While the predominant peak of rotavirus diarrhoea occurred in Feb, March and April, there appeared a second smaller peak in November-December. Apparently, the second smaller peak was prominent in year 2000 compared to year 2001. This gives a picture of biphasic peaks.

There was no significant difference between the total numbers of ELISA positive samples that were collected in each season (203 and 209 respectively). Although the peak time (high prevalence) remained the same in both years (Feb, March, April), there was an increase in the prevalence at the peak time on 2001 compared to that of 2000. Also a decrease in prevalence was noticed on November and December 2001 compared to the same time in year 2000 (Fig 3.3, 3.4). The statistical analysis showed no

correlation between the age of the patients and the peak seasons, both the main and the smaller peaks (Anova test, $p < 0.24$).

There was significant difference between the distribution of the genotypes and electropherotypes in the two peak seasons (details under Electropherotypes and Genotypes).

Table 3.2 shows a record of temperature and humidity during the peak seasons of year 2000 and 2001.

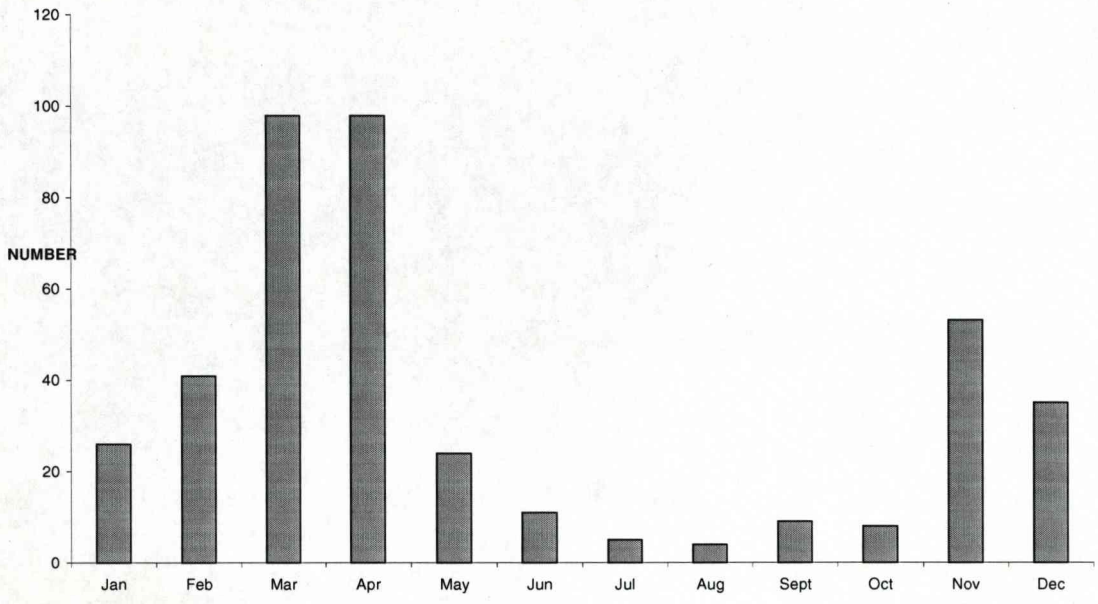


Figure 3.3: Total number of samples over two years combined from January to December

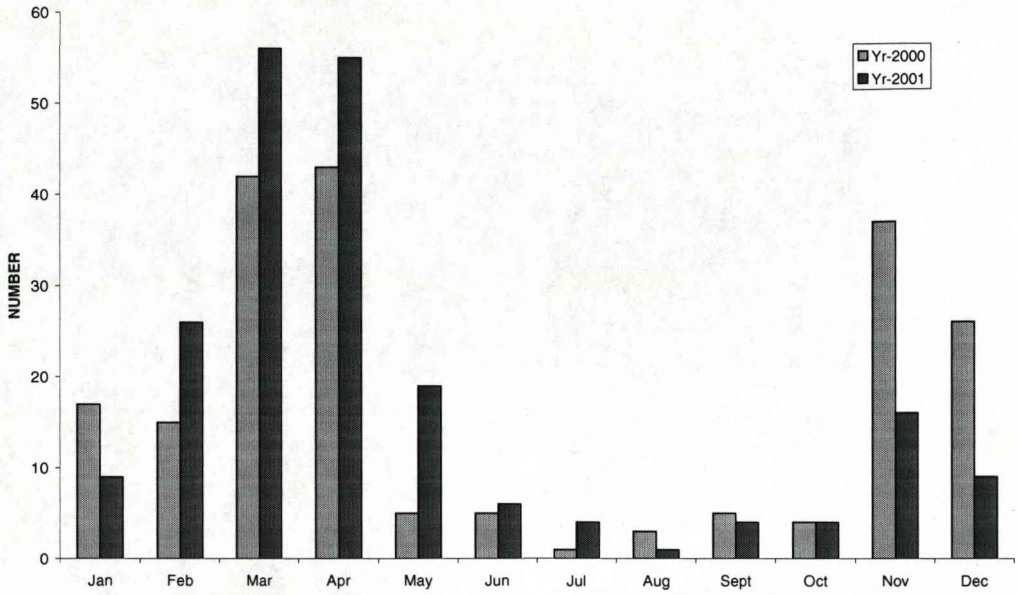


Figure 3.4: Number of rotavirus positive cases in each of the year 2000 and 2001

Month/year	Week 1		Week 2		Week 3		Week 4	
	Temp.	Humid %	Temp.	Humid %	Temp.	Humid %	Temp.	Humid %
Jan/2000	37 °F / 3 °C	85%	40 °F / 4 °C	67%	37 °F / 3 °C	76%	38 °F / 4 °C	72%
Feb/2000	47 °F / 8 °C	77%	47 °F / 8 °C	66%	37 °F / 3 °C	77%	-	-
Mar/2000	-	-	-	-	-	-	-	-
Apr/2000	-	-	-	-	-	-	-	-
Nov/2000	42 °F / 6 °C	89%	44 °F / 6 °C	84%	42 °F / 6 °C	83%	44 °F / 7 °C	80%
Dec/2000	46 °F / 8 °C	75%	47 °F / 8 °C	81%	34 °F / 2 °C	94%	32 °F / 0 °C	82%
Jan/2001	44 °F / 7 °C	90%	34 °F / 2 °C	96%	28 °F /-2 °C	92%	37 °F / 3 °C	87%
Feb/2001	42 °F / 6 °C	94%	42 °F / 6 °C	80%	34 °F / 2 °C	94%	36 °F / 2 °C	67%
Mar/2001	31 °F / -0 °C	71%	50 °F / 10 °C	85%	36 °F / 2 °C	68%	38 °F / 4 °C	76%
Apr/2001	52 °F / 11 °C	79%	50 °F / 10 °C	76%	46 °F / 8 °C	59%	46 °F / 8 °C	81%
Nov/2001	50 °F / 10 °C	74%	42 °F / 6 °C	60%	45 °F / 8 °C	85%	47 °F / 8 °C	77%
Dec/2001	37 °F / 3 °C	93%	34 °F / 1 °C	91%	36 °F / 2 °C	88%	34 °F / 2 °C	85%

Table 3.2 A record of temperature and humidity during the peak seasons of 2000 and 2001

(From http://www.wunderground.com/history/)

3.4 ELISA:

Total of 412 stool samples were positive by the Rotavirus ELISA. The sensitivity and specificity data of IDEIA kit (DAKO, Cambridgeshire, UK) is not published. However, the kit has been used worldwide and is recommended by World Health Organization.

The IDEIA rotavirus test is an enzyme immunoassay for the detection of Group A rotavirus in faecal specimens. The test utilises a polyclonal antibody to detect group specific proteins, including the major inner capsid protein (VP6), present in Group A rotaviruses.

3.5 Electropherotypes (PAGE Results):

An electropherotype could be assigned to 359 (87%) of the strains (Figure 3.5).

These comprised 286 (69.5%) long profiles, 67 (16.5%) short profiles, 6 (1.5%) mixed profiles and 53 (12.5%) were non-detectable (ND). (Figure 3.5, 3.6) [Table 3.3, 3.4, 3.5].

The electropherotypes of a variety of strains, representing long, short and mixed electropherotype profiles are demonstrated in figures 3.7 and 3.8.

In the 6 mixed profiles we could identify 2 of them as typical-mixed infection (Fig 3.8, Lane 5) and 4 re-arranged profiles. This is a well described but rare phenomenon (Fig 3.7, Lane 3, 6, 7) [table 3.6]. There is an aberrant migration of the genome segment that emerged between segment 4 and 5.

A total of 35 samples were both unclassifiable by electropherotyping, and non-typeable (GNT) by RT-PCR genotypes. Out of the 53 ND samples by electropherotryping, 18 samples were typeable by RT_PCR.
Conversely, the total number of GNT strains was 58. Out of these 58 GNT samples, 23 samples had demonstrable PAGE patterns (Table 3.3)

Apart from the mixed profiles which were present equally in both seasons, there was a significant difference between the prevalence of L, S and ND profiles in 2000 and 2001 (Fisher's Exact Test, $p < 0.01$). There was also a significant difference between the prevalence of L, S and ND profiles in the peak seasons of 2000 and 2001 (Fisher's Exact Test, $p < 0.002$).

Although there was a significant difference of the Electropherotypes in the two years and the peaking seasons, it was found that the age of the patient

had no association with that difference (Anova test analysis of Age, year, PAGE, $p > 0.89$). Also, it was found that the age of the patient had no significant association with the presence of any particular Electropherotype (L, S, Mix, ND) {Anova test $p < 0.42$}.

		Genotype						Total
		G1	G2	G3	G4	G9	GNT	
PAGE	L	148	0	1	25	92	20	286
	S	0	65	0	0	0	2	67
	MIX	2	1	0	0	2	1	6
	ND	8	1	0	1	8	35	53
Total		158	67	1	26	102	58	412

Table 3.3 Rotavirus electropherotypes and G-types

Total samples n=412
total detected by PAGE n= 359
L n= 286
S n=67
MIX n=6
ND n=53
Total typeable by RT-PCR n=354
total GNT n=58
G1 n=158
G2 n=67
G3 n=1
G4 n=26
G9 n=102
total ND and GNT n=35
Total ND but G-typeable n=18
total detected by PAGE but GNT n=23

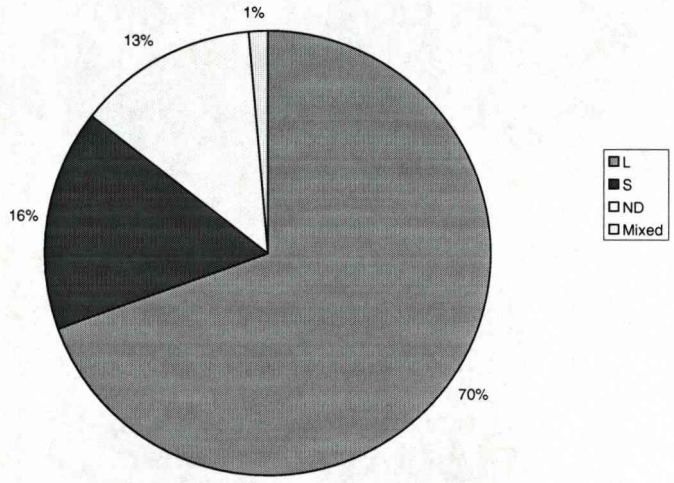


Figure 3.5 Distribution of Electropherotypes (PAGE). L=long, S=short, Mix=mixed infection, ND=non detectable

Electropherotype	No positive	% positive of total positives
L	286	69.4%
S	67	16.3%
Mixed	6	1.5%
ND	53	12.9%

Table 3.4 Electropherotype profiles Distribution (percent.)

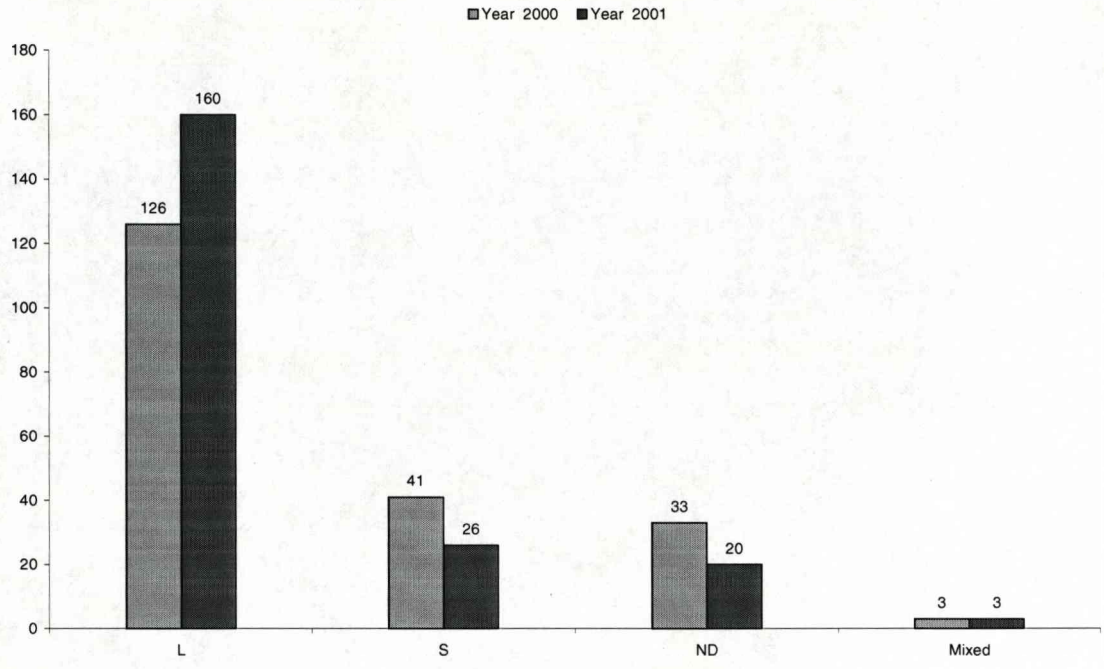
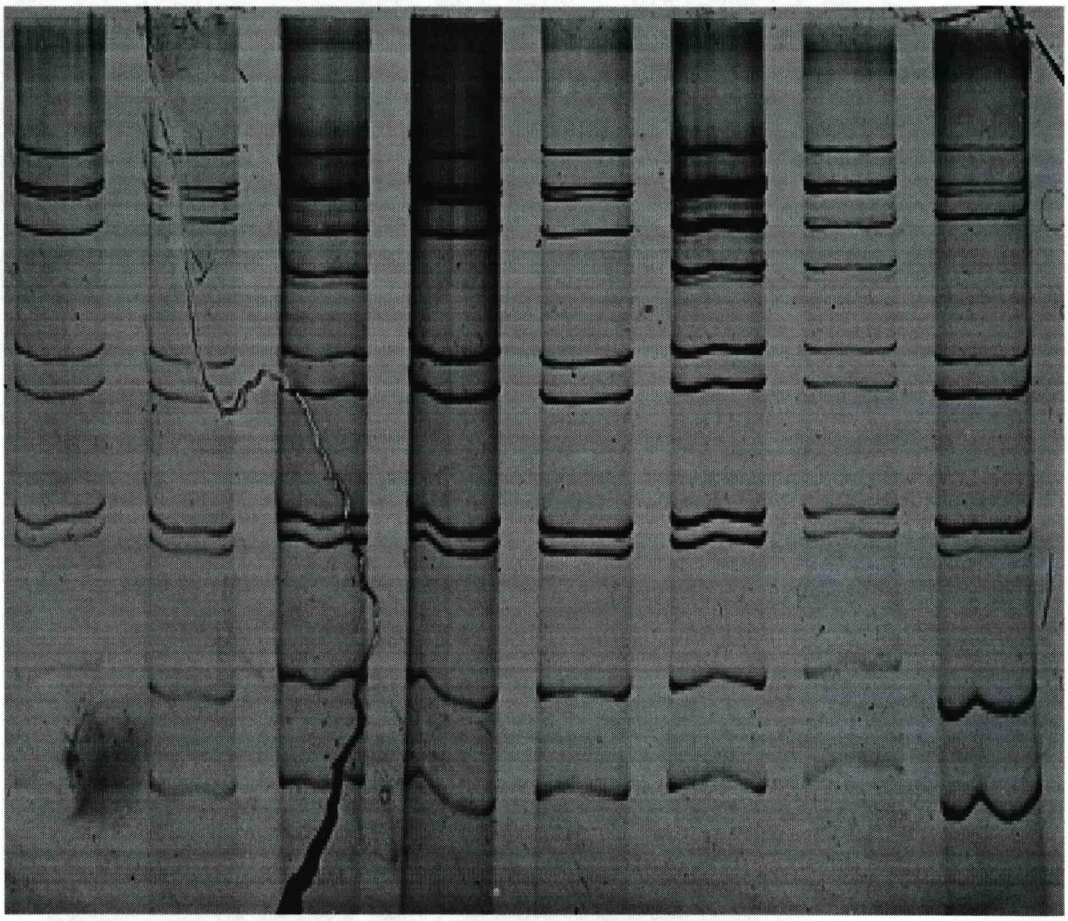


Figure 3.6: Distribution of Electropherotypes in two years, 2000 and 2001.

Season/Electropherotypes		No positive	% positive of total positives'
Year 2000	L	126	62.1%
	S	41	20.2%
	Mix	3	1.5%
	ND	33	16.3%
Year 2001	L	160	76.6%
	S	26	12.4%
	Mix	3	1.4%
	ND	20	9.6%

Table 3.5 Distribution of Electropherotypes profiles in two years.



1 2 3 4 5 6 7 8

Figure 3.7 Silver-stained rotavirus genome following polyacrylamide gel electrophoresis

Lane 1: Standard strain showing electropherotype profile (strain Wa)

Lane 2, 4, 5, 8: Long electropherotype profiles

Lane 3, 6, 7: Re-arranged of electropherotype profiles

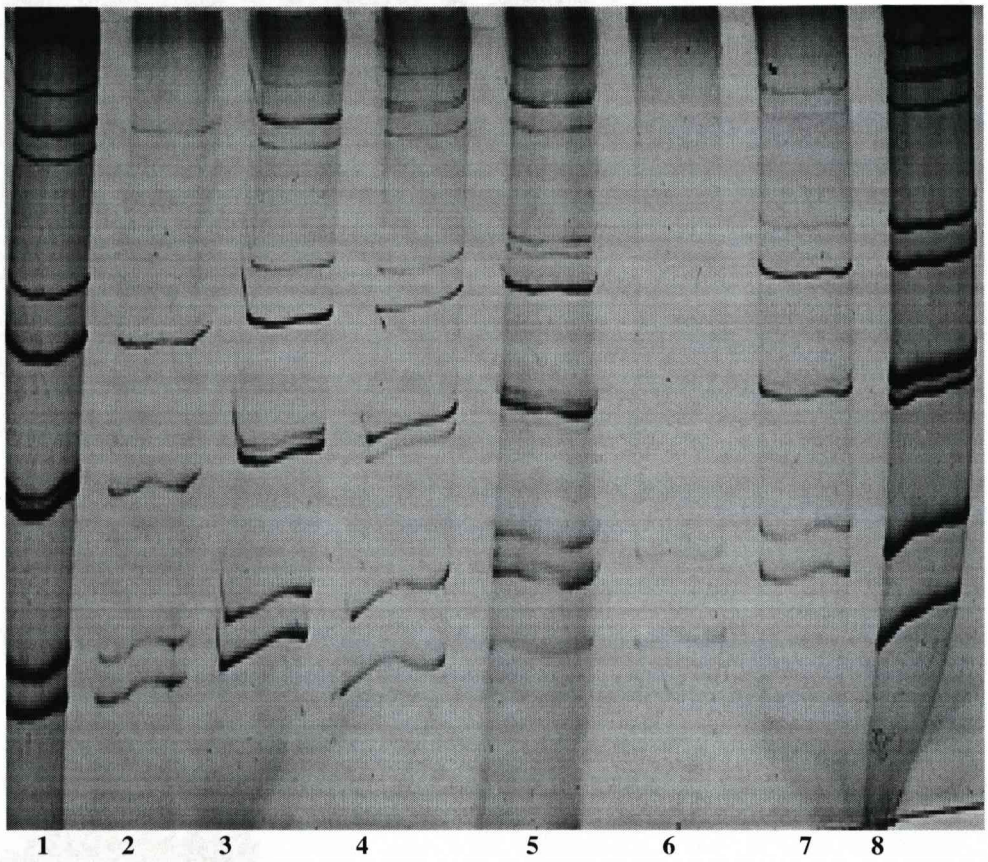


Figure 3.8 Silver-stained rotavirus genome following polyacrylamide gel electrophoresis

Lane 1: Standard strain showing short electropherotype profile (strain DS-1)

Lane 2, 3, 7: Short electropherotype profiles

Lane 4, 6, 8: Long electropherotype profiles

Lane 5: Long mixed electropherotype profile

Probable Rearranged genome	Age	Gender	PAGE	Genotype
1	23	F	L	G1
2	10	M	L	G1
3	10	F	L	G9
4	12	F	L	G1

Table 3.6 profiles of rearranged genomes

3.6 G types:

A total of 412 rotaviruses were detected in faecal samples from children with diarrhoea. The rotavirus samples were collected over two years and were characterized by RT-PCR Genotyping (Table 3.2, 3.5 & Figure 3.9)

A total of 354 (86%) samples were typable.
58 (14%) samples were not typable (GNT). Of the 58 nontypable samples, 35 samples also had been undetectable by electropherotypes (table 3.3).

Five different genotypes were detected. These were G1, G2, G3, G4 and G9. G1 was the most frequently-detected genotype strain (n=158, 38.5%), followed by G9 (n=102, 24.8%), G2 (n=67, 16%), G3 (n=1, 0.2%), G4 (n=26, 6.5%). {Table 3.3, 3.7, 3.8 & Figure 3.9, 3.11, 3.12}

The short electropherotype profile comprised almost all of serotype G2.
The long electropherotype profiles comprised the majority of serotype G1, G9, G4 and G3 (table 3.3).

On comparing the genotype distribution (G1, 2, 3, 4, 9) over the two years, there was a significant difference (Chi-Square Test p <0.0001) [table 3.7, table 3.8 & Figure 3.9, figure 3.11]. In the year 2000, G1 was 92 (45%), G2 was 42 (21%), G4 was 8 (4%), G9 was 27 (13%) and GNT was 33 (16%). In the year 2001, G1 was 66 (32%), G2 was 25 (12%), G4 was 18 (9%), G9 was 75 (36%) and GNT was 21 (10%).

Also, on the comparison of the genotype distribution (G1, 2, 3, 4, 9) over the peak seasons, there was a significant difference (Chi-Square Test p <0.002)

The age of the children had no impact or effect on the distribution of different genotypes (Anova test p <0.25). Although there was a significant difference between the two years in the genotype outcome as mentioned above, the age proved to have had no influence or reflection on that difference (Anova test, comparing age, year, genotypes, p <0.41).

G9 was clearly of significant difference between the two years [n=27 (13%), n=75 (36%) respectively] {table 3.3 and table 3.7, 3.8 & Figure 3.9, 3.10, 3.11). Thus, 13 % of the serotypes strains in 2000 were G9 compared with 36% in 2001 (p <0.001).

The emergence of G9 in the second year (2001) was one of the most important findings in this study. It was also found that the age of subjects had no effect correlation with the prevalence of G9 over the two years and seasons (t-Test $p < 0.93$).

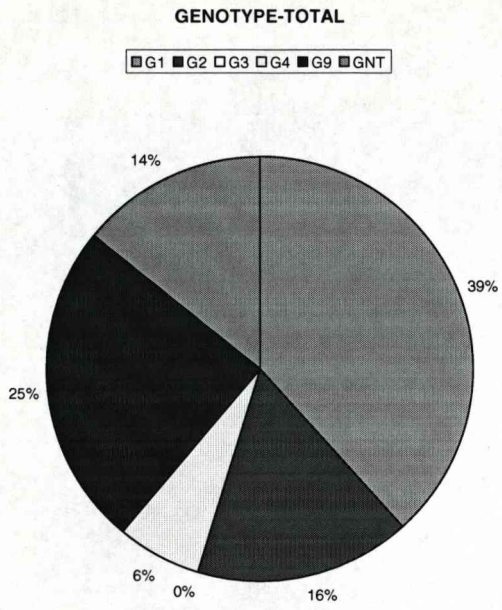


Figure 3.9 Rotavirus G types in two years, 2000 and 2001 (combined)

Serotype	No. positive	% positive of total positive
G1	158	38.3%
G2	67	16.3%
G3	1	0.2%
G4	26	6.3%
G9	102	24.8%
GNT	58	14.1%

Table 3.7 Rotavirus G-types total distribution

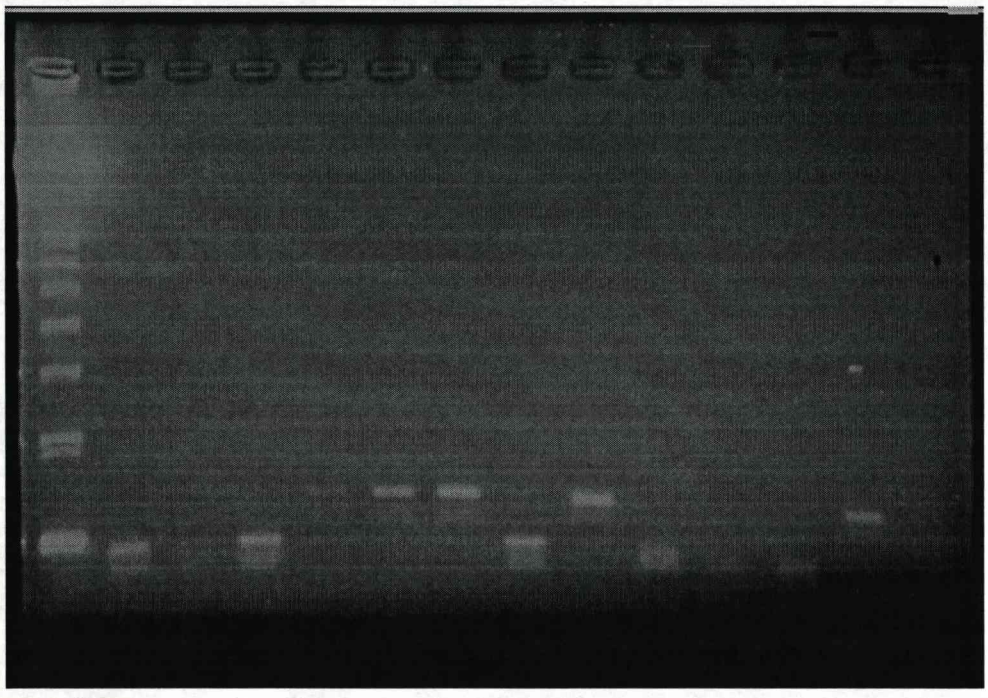


Figure 3.10 Ethidium bromide-stained agarose gel showing G type-specific PCR products.

Lane 1: 123bp ladder

Lane 2, 4, 8, 10, 13 G –type G9

Lane 5, 6, 7, 9 G-type G1

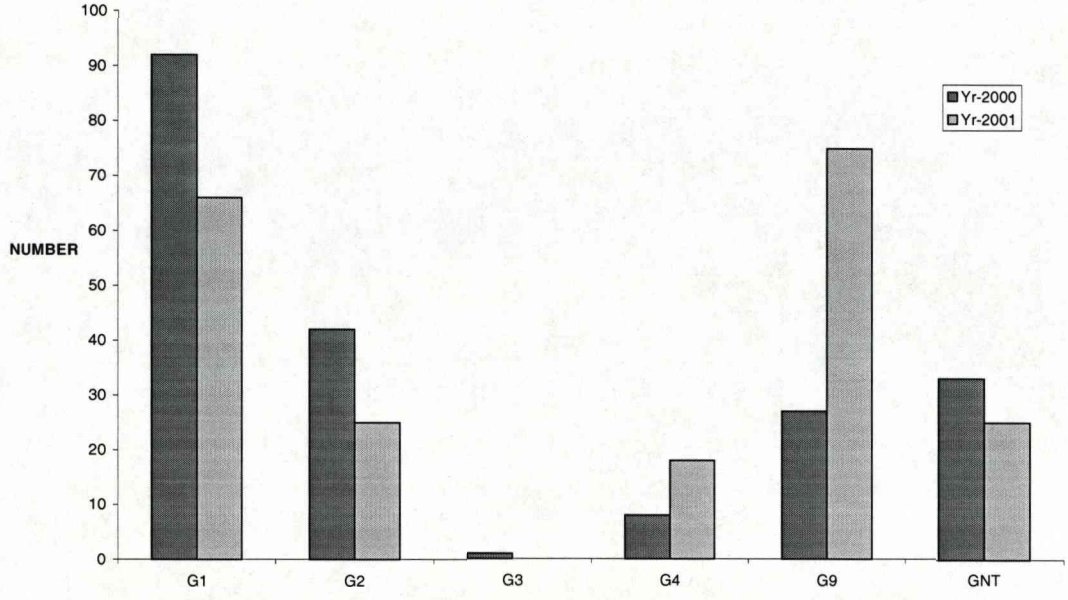
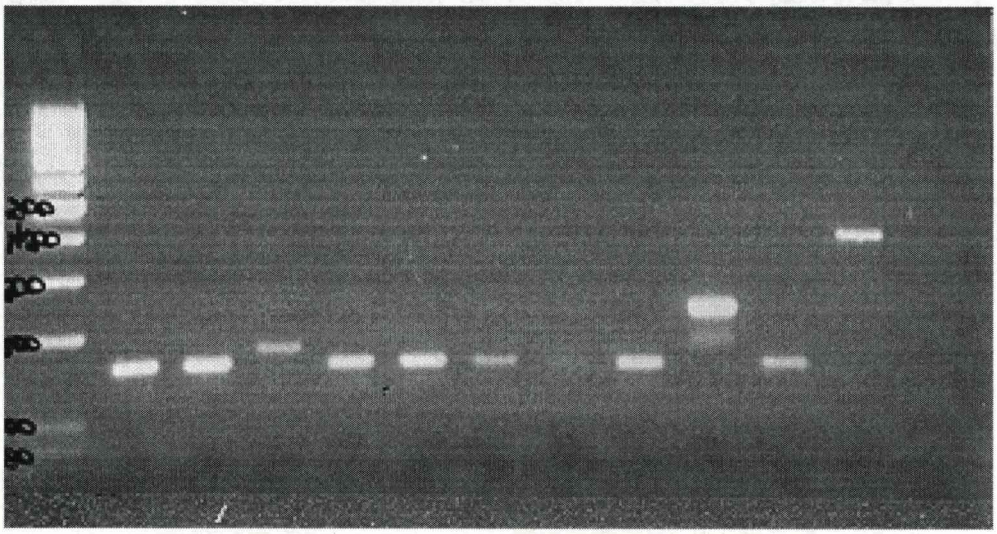


Figure 3.11 Rotavirus G-types in each year (2000-2001)

Year	G1 No (%)	G2 No (%)	G3 No (%)	G4 No (%)	G9 No (%)	GNT No (%)	Total
2000	92 (45%)	42 (21%)	1)	8 (4%)	27 (13%)	33 (16%)	203
2001	66 (32%)	25 (12%)	0 0	18 (9%)	75 36%)	25 10%)	209
p value	<0.02	<0.01	-	<0.02	<0.001	<0.03	-

Table 3.8 Year genotype cross tabulations



1 2 3 4 5 6 7 8 9 10 11 12 13

Figure 3.12 An Ethidium bromide-stained agarose gel showing G type-specific PCR products.

Lane 1: 100 bp ladder

Lane 2, 3, 4, 5, 6, 7, 8, 9, 11 G –type G1

Lane 10 G-type G2

Lane 12, G-type G4

3.7 Nucleotide Sequencing:

A partial VP7 sequence was performed on G9 strains. The sequence was 99% identical to lineage 3 of G9 genotypes. (Figure 3.10, 3.13)

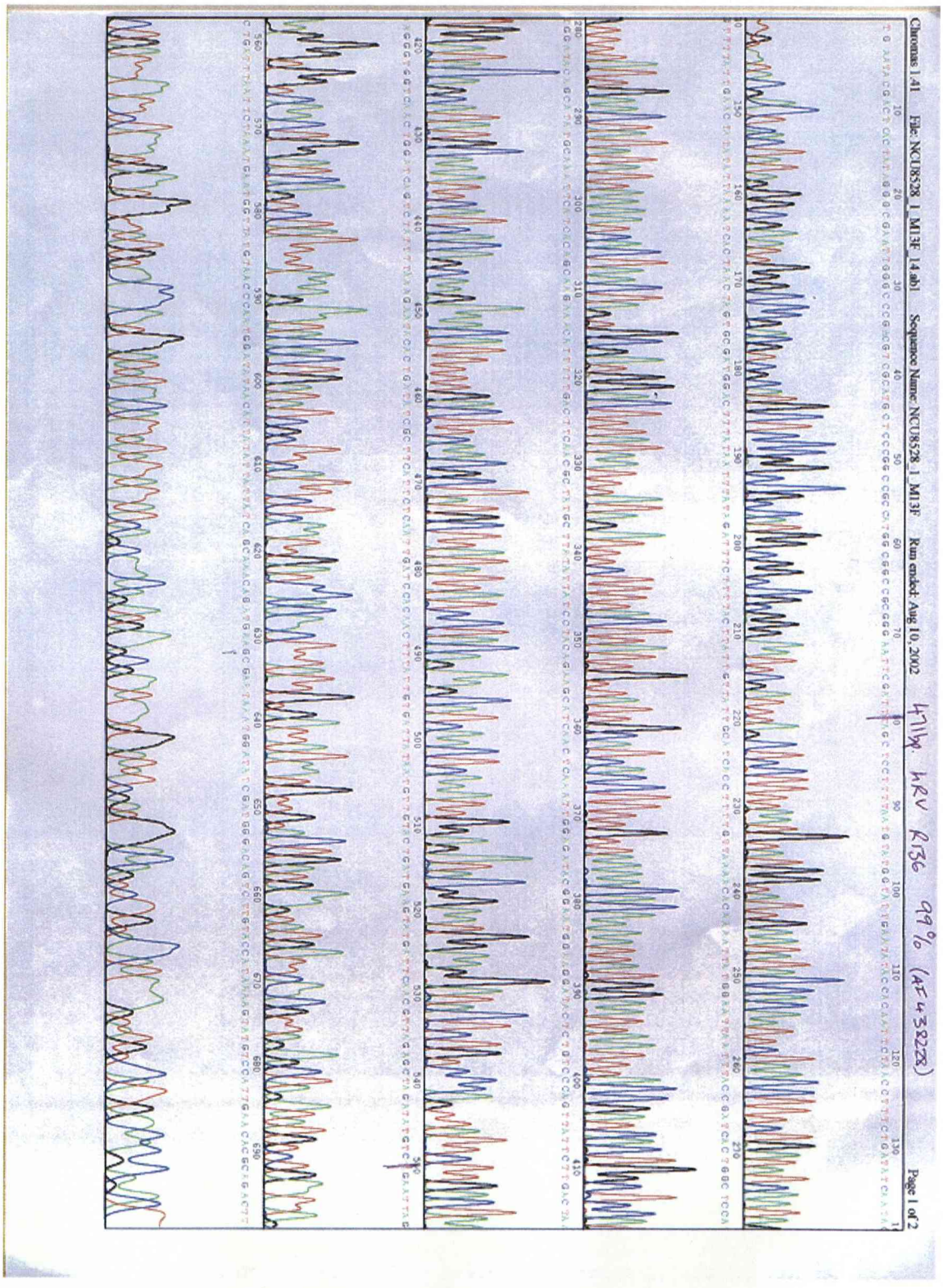


Figure 3.13 Chromatogram of a partial VP7 sequence showing Genotype G9

Chapter Four

Discussion and Conclusion

4.1 Summary of the study:

This is a prospective descriptive study of rotavirus diarrhoeal diseases at our tertiary Paediatric hospital.

This study is the first to investigate rotaviruses as the cause of diarrhoea and diarrhoeal diseases in children attending Alder Hey Children Hospital (AHCH). Thus, the study has provided the opportunity to study the epidemiological features of infection with rotavirus. It also provided the opportunity to confirm what has become well known that rotaviruses are the main and important cause of diarrhoeal diseases in young infants and children. The total number of children with diarrhoea and diarrhoeal diseases, who attended AHCH was 1496. Out of those, 412 children tested positive for rotavirus (27.8%).

This study identified a remarkable diversity among rotavirus strains circulating in the Paediatric population at Alder Hey. It also identified the variety in seasonal frequency of each genotype which was noted even within a relatively short period of surveillance (two years).

Genotype G1 was the most predominant strain. It comprised 38.5% of the total strains. This was followed by G9 (25%) and then by G2, G4, and G3 respectively. There was a significant annual variation in the genotypes of different strains. This seasonal difference was most evident for G9 strains [n=27 (13%) in year 2000 compared to n=75 (36%) in 2001].

The most vulnerable and affected group of children were those under 2 years. In this study, the most frequently infected group of children were those aged between 6-12 months, followed by those of 13-24 months and then those under 6 months of age. There were no children with rotavirus diarrhoea below the age of 4 months.

The peak time (season) of the year was the month of February, March and April. There was a significant difference in the prevalence of the circulating G genotypes and electropherotypes in the two years period. The age of the children had neither association with the peak season of each year nor with the prevalence of electropherotypes and genotypes outcome of each year.

This study has made important observations regarding the role of rotavirus as a major factor in diarrhoeal diseases, and observation of the annual variation of different geno-types.

4.2 Aims and specific objectives of this study:

Aim 1:

Since rotavirus generally results in more severe gastroenteritis than that caused by other enteric pathogens, its detection rate rises with increasing severity of the disease. According to the published data of the detection rate and its relation to severity of disease, our study showed detection rate of 27.8% which correlated with 8-12 of the 20 points severity score (Pang *et al.* 2000).

Although the detection rate of 27.8% is not in the highest level, it is still showing rotavirus as the most important aetiological factor in diarrhoeal diseases.

Aim 2 and 3:

A large number of infected children were 6-12 months. In developed countries, the usual pattern of infection occurs in those ≥12 months. However, our results showed that 144 children aged ≥ 12, were infected

(110 aged 12-24, 34 aged ≥24, respectively). In one similar study in Japan, it was reported that only 32% of rotavirus cases were from those less than one year age group (Nakagomi *et al.* 2005).

Although both UK and Japan are developed countries, they are different in geographical location and weather. In the Japanese study, all children were hospitalized with severe diarrhoeal gastroenteritis. Their severity score was 16.5 compared to the estimated severity score in our study which was between 8 and 12. So, the detection rate in the Japanese study was higher compared to our detection rate (27.8%). In our study, all children with diarrhoeal disease of any severity score were included. Also all children who attended Alder Hey were included. Although most of children were from inpatient department, stool samples were collected from other places; hence the total number of diarrhoeal stool samples was 1496. In the Japanese study the stool samples were exclusively taken from children who were admitted (inpatient) with severe gastroenteritis; hence the severity score was higher (16.5).

Our study showed that not one child was infected in the age range of 0-4 months, and only 10 cases were detected from those 4-5 months. This is consistent with the currently recommended age for the administration of the new vaccines. The first three trial phases of the new vaccines have been very

successful. The new rotavirus vaccines are now in their last phase of field trial. The result of these field trials will determine the child's age at which the maximum efficacy of the vaccine can be achieved.

Specific objective 1:

This study demonstrated the correlation between the short electropherotypes and the VP7of G2 serotype. All G2 genotypes were of short electropherotype profiles.

It can be noted that 4 of those 6 mixed profiles (by PAGE) belong to the rare gene rearrangement phenomenon.

Rearranged genome segment(s) have been observed in strains recovered from both immunocompetent and immunodefficient hosts. Genome rearrangements occur exclusively in genes encoding non-structural proteins, except for a rare example of VP6 gene rearrangement. Rearrangement of genome segment encoding VP1, VP2, VP3, VP4 or VP7 has never been detected. Therefore, genome rearrangements have been proposed to be a third mechanism of rotavirus evolution in nature, besides genomic point mutations and accumulations, as well as genetic reassortments

(Hundley *et al.* 1985; Hundley *et al.* 1987; Tian *et al.* 1993; Shen *et al.* 1994; Desselberger 1996).

In our study, there was an aberrant migration of the genome segment emerged between segment 4 and 5 (Fig 3.7, Lane 3, 6, 7). This is most probably due to the rearrangement phenomenon. Those rearranged genomes (Fig 3.7, Lane 3, 6, 7) are easily distinguished from the typical mixed infection genome (Fig 3.8, Lane 5).

Specific objective 2 and 3:

Seasonality:

The vast majority of children were infected in cold weather in the two-year period. While the predominant peak of rotavirus diarrhoea occurred in the months of February to April, there appeared a second smaller peak in November to December. Apparently, the second smaller peak was prominent in 2000 compared to 2001. Although the second smaller peak was atypical observation, it was still within the cold weather period, which is typical of rotavirus infection.

The cause of this striking seasonal pattern is not known. However, the influence of low relative humidity in the home has been suggested as a factor helping the survival of rotaviruses on surfaces (Brandt *et al.* 1982)

The emergence of G9 in this study:

Beside G1 serotype, the emergence of G9 in the second year (2001) was one of the most important findings in this study. It was also found that the age had no effect or correlation with the prevalence of G9 over the two-year study.

Emergence of G9, in UK and abroad:

G9P[6] and G9P[8] rotavirus strains were identified during 1995/96 through the molecular epidemiological surveillance of rotavirus strains circulating in the UK between 1995 and 1998. An increase in the incidence and spread of sporadic infections with rotavirus genotype G9P[8] across the UK was detected in the two following seasons. Partial sequencing of the VP7 gene showed that all the UK strains shared a high degree of homology and were related very closely to G9 strains from the US and from symptomatic infections in India (> or =96% homology). The UK strains were related more distantly to the pathogenic Indian strain 116E (85-87.8% homology).

Phylogenetic analysis revealed clustering of the UK strains into 3 different lineages (I to III) and into two sub-lineages within lineage I. There were correlations between VP7 sequence clustering, the P type and the geographical origin of the G9 strains. Partial sequencing of the VP4 gene showed high degree of homology (>98%) among all the P[6] strains, and the sequences obtained from the P[8] strains clustered into 2 of the 3 global lineages described for P[8] strains associated with other G types. These data suggest that G9 strains may be a recent importation into the UK, and that G9P[8] strains may have emerged through reassortment in humans between G9P[6] strains introduced recently and the more prevalent co-circulating G1, G3 and G4 strains that normally carry VP4 genes of P[8] (Iturriza-Gomara *et al.* 2000).

In a recent study in Brazil, G9 was the predominant genotype (Santos *et al.* 2005).

Thus, G9 is globally emerging as one of the common genotypes infecting children. The next generation of rotavirus vaccines will need to provide adequate protection against disease caused by G9 viruses.

Would the emergence of G9 serotypes have an impact on the vaccines which are currently licensed for use worldwide including the children population in UK? Further rotavirus epidemiological studies and data analysis of vaccine efficacy, will probably provide the answer in the future.

In a recent study in Belgium, G9 rotavirus strains were found to have increased (24%) as well as the other common serotypes such as G1, G2 and G4. In this Belgian study, the authors raised the same point which is "The emergence of G9 as an important pathogen in both developing and developed countries which necessitates the urgent consideration of incorporation of the G9 epitopes in rotavirus vaccines (Rahman *et al.* 2005).

G9 was also found recently to be in increased prevalence in Thailand. In this Thai study, G9 comprised 91.6% of the total rotavirus serotypes. (Khamrin *et al.* 2006).

G9 has been found to be circulating in the United Kingdom, and there was evidence of reassortment between co-circulating strains in the population in the United Kingdom in 1995-1998. Totals of 19, 18, and 51 G9 rotavirus strains were detected during the 1995–1996, 1996–1997, and 1997–1998

periods, respectively. In 1995–1996, G9 strains were detected in only three of eight regions of the United Kingdom (south, north, and east of England), increasing to five regions (Wales and south, middle, north, and west of England) in 1996–1997 and to six regions (Scotland and south, middle, north, west, and east England) in 1997–1998 (Fig. 1). No G9 strains were found in Northern Ireland throughout the study. A concomitant year-by-year increase in the number of G9 isolates was detected in two locations (Leeds and Reading) over 3 years and in four different locations (Dundee, Peterborough, Birmingham, and Bristol) over 2 years. Statistically significant increases in the percentage of G9 strains isolated in Leeds and Birmingham were also detected, rising in Leeds from 3.2% of all strains in 1995–1996 to 5% of all strains in 1996–1997 and 28% of all strains in 1997–1998 and rising in Birmingham from 4% of all strains in 1996–1997 to 13% of all strains in 1997–1998. G9 rotaviruses were the second most frequently found type in Leeds and Birmingham in 1997–1998

(Iturriza-Gomara *et al.* 2000; Iturriza-Gomara *et al.* 2000).

4.3 Rotavirus detection rate and estimation of number of diarrhoea deaths:

In order to estimate the number of deaths attributed to rotavirus infection

each year, the total number of diarrhoea deaths is multiplied by the proportion of severe diarrhoeal cases, represented by hospitalisation, in which rotavirus can be detected (severe diarrhoea has in general been used as a proxy for diarrhoea death since studies addressing cause-specific mortality from diarrhoea are rare) (Perez-Schael *et al.* 2007)

With increasing severity, the etiological share and detection rate of rotavirus increases. Using the severity score of modified 20 points, the detection rate is 68% (score ≥14), 50% (score 11-13), 27% (score 8-10) and 24% (all episodes) (Pang *et al.* 2000).

Recent studies suggest that as global deaths from childhood diarrhoea decreased during the past 2 decades, the proportion of diarrhoea hospitalizations attributable to rotavirus may have increased. For example, prospective, sentinel hospital–based surveillance of rotavirus disease in 9 Asian countries demonstrated a median rotavirus detection of 45% among children hospitalized with diarrhoea, a figure that was considerably greater than the detection rates in previous studies from the same countries. Similarly, a more extensive study of 5,768 children hospitalized from 1998 through 2000 in 6 canters in Vietnam identified rotavirus in 56% of patients,

a proportion that was more than twice the 21% detection rate reported among children hospitalized with diarrhoea in a hospital in Hanoi, Vietnam, from 1981 to 1984 (Parashar *et al.* 2006)

4.4 General discussion:

While important observations have been made in this thesis, a number of questions still remain that could not be answered by this study. First, rotaviruses from the areas outside those served by AHCH were not examined. It is quite possible that rotavirus populations elsewhere differ from that within the populations served by AHCH.

Second, the emergence of new rotavirus genotypes elsewhere in the world could make it possible to find those new ones in the future studies in AHCH and surrounding areas. Examples of new Genotypes are G10 and G12 (Pietruchinski *et al.* 2006). G11 has been found recently together with G12 (Uchida *et al.* 2006). In India G10 together with G16 were found [equine rotaviruses] (Gulati *et al.* 2006)

In the long term, the best hope in reducing the burden of disease due to rotavirus infection in Alder Hey, in UK and in all developed and developing countries, is the use of rotavirus vaccine. Studies, so far, have shown that the

vaccines are safe and immunogenic. Also the follow-up surveillance with continuous strain collection and characterization will help to detect the emergence of new strains and to determine the persistence of dominant genotypes. This would lead to either modification of current vaccines or formulation of new ones. However, the data available so far about the new vaccines are encouraging, regarding safety, tolerance and reasonable effectiveness (Clark *et al.* 2006; Keating 2006;Vesikari *et al.* 2006).

There are some clinical data or numerical scores systems which were developed to describe the rotavirus diseases. Vesikari et al score is one example (Ruuska *et al.* 1990). From my observation of cases of rotavirus gastroenteritis in both the developed and developing world, those scores are not practical in the developed countries. From the medical and scientific point of view one has to exclude other important causes of vomiting, fever, and dehydration, in children with severe gastroenteritis, even with positive rotavirus in their stool. Infection with rotavirus can co-exist with other infections such as Respiratory Syncythial Virus simultaneously. The child can be admitted to the hospital with fever and dehydration for any other reason and then later can develop rotavirus gastroenteritis, in the same hospital bed. However, the scores can be used, carefully and cautiously, in

the developing world where the resources and facilities are slim. If we have to add to the numerical score, one should consider scoring for the time of the season of the diarrhoeal episodes, and whether it is consistent with typical season of the rotavirus infections in that particular geographical area.

4.5 Epidemiology, morbidity, mortality and economic burden of rotavirus infection:

From the total number of diarrhoeal diseases (1496 samples in 2 years) one can recognize the effect and pressure on the children units, as most of these children are usually admitted to the acute wards. Those who are admitted normally go to the isolation wards, which add pressure to the health system.

Prevention of rotaviruses infection by mass vaccination could have a positive impact on the incidence of rotaviruses by reducing the number of children hospitalized for gastroenteritis, therefore reducing the number of hospital cross-infections and associated costs. There are a number of studies that have estimated the economic burden of rotavirus gastroenteritis and also the positive economic effect of introducing the Rotavirus vaccines (Fischer *et al.* 2005; Constenla *et al.* 2006; Gleizes *et al.* 2006; Rheingans *et al.* 2006; Isakbaeva *et al.* 2007)

The seasonal pattern of rotavirus infections coincides and more or less overlaps with that of respiratory syncytial virus (RSV). This again adds to the pressure and demand on the health system at that time of the year, giving the fact that some of those children infected with viruses may need Intensive Care beds at some time.

Although, we do not have mortality data on any of the children whose specimens were examined, it is a well-established fact that rotaviruses rarely cause mortality in the developed world. For example, a recent study gave estimates of 3.2 to 3.8 rotavirus deaths per year in England and Wales (Jit *et al.* 2007).

Unfortunately, a large number of children die every year in the developing world from the diarrhoeal diseases in general and in particular from rotavirus gastroenteritis (Bern *et al.* 1992) {also see chapter I}. However, morbidity is a reality in association with rotavirus gastroenteritis both in the developed and developing world. It can cause anxiety and distress to the sick children and their parents. Further, it has implications on the economy and on health care systems.

4.6 Current and prospective vaccines:

Accelerated development and introduction of rotavirus vaccines into global immunization programs has been a high priority for many international agencies, including the WHO and the Global Alliance for Vaccines and Immunizations. Vaccines have been developed that could diminish or even prevent the enormous morbidity and mortality from rotavirus and their effect should be measurable within 2-3 years. Two live oral rotavirus vaccines have been licensed in many countries; one is derived from an attenuated human strain of rotavirus and the other combines five bovine-human reassortant strains. Each vaccine has proven to be highly effective in preventing severe rotavirus diarrhoea in children and safe from the possible complication of intussusceptions. In developed countries, these vaccines could substantially reduce the number and associated costs of child hospitalizations and clinical visits for acute diarrhoea. In developing countries, they could reduce deaths from diarrhoea and improve child survival through programs for childhood immunizations and diarrhoeal disease control. Although many scientific, programmatic, and financial challenges face the global use of rotavirus vaccines, these vaccines - and new candidates in the pipeline - hold promise to make an immediate and

measurable effect to improve child health and survival from this common burden affecting all children.

4.7 Recommendation and future needs:

Surveillance of rotavirus gastroenteritis

Establishing rotavirus disease surveillance systems that are adequately sensitive and specific to document the effectiveness of vaccination programs will be necessary. National surveillance systems for rotavirus infections include review of national hospital discharge databases for rotavirus-specific or rotavirus-compatible diagnoses and reports of rotavirus isolation from laboratories. Special studies (e.g., case-control studies and retrospective cohort studies) will be needed to confirm the effectiveness of rotavirus vaccine in routine programmatic use.

Detection of unusual strains of rotavirus

A national strain surveillance system should be (or should have been) established to monitor the prevalence of rotavirus strains before and after the introduction of rotavirus vaccines. This system should be designed to detect new or unusual strains that might not be effectively prevented by vaccination and might affect the success of the vaccination program.

Rotavirus vaccine research

Future research should include studies to determine the safety and efficacy of rotavirus vaccine administered to infants born prematurely, infants with immune deficiencies, infants who live in households with immunocompromised persons, and infants with chronic gastrointestinal disease. Studies also should be conducted to determine the protective efficacy of <3 doses of vaccine and to address the cost effectiveness of vaccination programs in various settings.

Education of health-care providers and parents

The success of a rotavirus vaccination program depends on the acceptance and enthusiasm of physicians and other health-care providers who care for children and caretakers of infants. In light of the experience with the withdrawal of RRV-TV vaccine because of its association with intussusceptions, some health-care providers and parents might have concerns about vaccination with current rotavirus vaccine. Vaccination program personnel will benefit from education about rotavirus disease and rotavirus vaccine. Parental education on rotavirus gastroenteritis and on the vaccine will be essential to establish and maintain public confidence in this vaccine and to avoid confusion caused by cases of gastroenteritis in early

childhood resulting from non-rotavirus etiology and not preventable by rotavirus vaccine.

4.8 Conclusion:

Rotaviruses are the most common cause of severe diarrhoea in children worldwide and diarrhoeal deaths in children in developing countries.

This study has provided the essential descriptive data about rotavirus infections and gastroenteritis in the children served by AHCH. In the future, a combination of clinical, epidemiological and laboratory-based research should be undertaken, to generate data that will facilitate the evaluation of current and future new vaccines. Also the cumulative data regarding current and future vaccines will determine whether or not alternative approaches to the management of rotavirus gastroenteritis should be considered, such as the use of oral immunoglobulins.

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