Molecular Epidemiology of Rotaviruses Isolated from Hospitalised Children in Melbourne, Australia

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Doctor of Philosophy

by

Kiran Shah M.Sc.

Environment and Biotechnology Centre
Faculty of Life and Social Sciences
Swinburne University of Technology

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Abstract

Rotaviruses are the leading cause of severe gastroenteritis in children worldwide. The morbidity due to rotavirus related illness in developed countries is significant, and results in an estimated 610,000 childhood deaths annually. Until the mid 1990s, four major G serotypes (G1-G4) of rotavirus had been determined to circulate in Australian communities and around the world. Since then, the emergence of G9 rotaviruses has been reported worldwide, and with its unique ability to spread rapidly, many countries have reported the presence of this serotype. In Australia, serotype G9 strains were first isolated in 1997. A recent outbreak (in 2001) caused by serotype G9 rotavirus in Central Australia highlights the epidemiological importance of this serotype.

The main aim of this thesis was to analyse the evolution of serotype G9 rotaviruses in Melbourne and characterise their antigenic and genetic properties. An epidemiological study conducted in this study in 2002 revealed a predominance of serotype G9 rotaviruses (58%) in rotavirus positive isolates obtained from hospitalised children. This thesis further explored the extent of genetic variation among serotype G9 rotaviruses collected over a longer period of six years (1997-2002) from children admitted to the Royal Children's Hospital in Melbourne. Six different antigenic groups of the VP7 protein among serotype G9 isolates were defined in this study. Genetic analysis of the VP7 gene showed that the Melbourne strains were closely related to each other, however, no conserved amino acid difference were found to correlate with the antigenic group.

Genetic variation was observed in two of the non-structural proteins, NSP1 and NSP4, which were analysed in this study. Phylogenetic analysis indicated the existence of two clusters of NSP1 gene present in Melbourne serotype G9 strains. Strain R1 had an NSP1 gene that was closely related (96% sequence identity) to the serotype G1 strain, Wa, suggesting a possible gene reassortment between rotaviruses of different serotypes. Strain R1 also shared close identity with the NSP4 gene of the serotype G1 strain, Wa (95% sequence identity). Most interestingly, strain R24 exhibited significant variation to the rest of Melbourne isolates studied (73-78% sequence identity) and only 66%-78% sequence identity with other prototype NSP4 genes. These findings suggest that R24 may belong to a new NSP4 genotype. These findings contribute new information regarding the extent of genetic variation among non-structural proteins of serotype G9 rotaviruses.

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Declaration

The experimental work and writing of this thesis have been performed entirely by me, except in instances where due acknowledge is made. This thesis is less than 100,000 words in length, exclusive of tables, figures, bibliography, appendices and footnotes. The material contained in this thesis has not been presented previously for the award of any other degree or diploma in any university or institution**.**

Kiran Shah

Journal Publication

Genetic Variation of NSP1 and NSP4 Genes among Serotype G9 Rotaviruses Causing Hospitalization of Children in Melbourne, Australia, 1997–2002.
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Publication arising from parts of this thesis is contained in Appendix III.

List of Conference Presentations

Parts of the results of this thesis have been presented at the following scientific meetings:

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4. K. Shah, M. Bhave, C.D. Kirkwood and E.A. Palombo (2005). Molecular Diversity of Serotype G9 Rotaviruses in Melbourne, Australia. Australian Society for Microbiology, Annual Meeting, 25-29 September 2005, Canberra, Australia. Oral presentation.

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List of Abbreviations

SI units are used throughout. Other abbreviations used are as follows

aa	amino acid
AMV-RT	avian myeloblastosis virus reverse transcriptase
bp	base pair
^{o}C	degree centigrade
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
dGTP	2'-deoxy-guanosine-5'-triphosphate
dNTP	2'-deoxy-nucleotide-5'-triphosphate
dTTP	2'-deoxy-thymidine-5'-triphosphate
DNA	deoxyribonucleic acid
DIG	digoxigenin
dsRNA	double stranded ribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EIA	enzyme immunoassay
ER	endoplasmic reticulum
Gr	group
HA	hydroxyapatite
hr	hour
IgA	immunoglobulin class A
IgG	immunoglobulin class G
IgM	immunoglobulin class M
kDa	kilodalton
min	minute
MMC	Monash Medical Centre
Nd	not determined
nt	nucleotide
N-MAb	neutralising monoclonal antibody
NSP	non-structural protein
OD_{450}	optical density at 450 nm
PBS	phosphate buffered saline
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
RT-PCR	reverse transcriptase-polymerase chain reaction
RCH	Royal Children's Hospital
SDS	sodium dodecyl sulphate
SG	subgroup
TAE	tris acetate buffer
TBE	tris borate buffer
TEMED	N',N',N',N'-tetramethylethlenediamine
UV	ultra violet
U	units
VP	viral protein
[v/v]	volume per volume
[w/v]	weight pre volume
xg	times gravity

Chapter One

Introduction and Literature Review

1. Introduction

Rotaviruses are the most common cause of severe diarrhoeal illness in infants and young children in both developing and developed countries. Rotavirus infection is of special significance in developing countries where they constitute a major cause of mortality among the young children. Although diarrhoeal diseases are not a prominent cause of mortality in infants and young children in developed countries, the toll from diarrhoeal disease in developing countries is staggering (Fig 1.1). Due to the lack of proper disease management and/or the unavailability of health care facilities in the developing world, rotavirus kills over half a million infants a year, about one child each minute (Newsrx, 2006). The death toll has been estimated at 610,000 per year, 82% of which occurs in poor countries (Parashar *et al.*, 2006). Rotavirus causes about 10,000 hospital admissions in Australia (Carlin *et al*., 1998) and 70,000 hospital admissions in the United States annually, or about 5% of all paediatric admissions. It also results in 250,000 visits each year to emergency rooms in the U.S. (Newsrx, 2006). A recent study in Australia has reported thirteen rotavirus related deaths from 1990-2002 due to the severity of infection (Newall *et al.*, 2006).

Rotavirus is ubiquitous and endemic in nature and infections occur in almost all children. It was discovered in Australia in 1973 in a duodenal biopsy from an infected person (Bishop *et al.*, 1973) The rotavirus virion has a round structure with a double-shelled capsid and measures 75nm in diameter. Soon after its discovery, it became apparent that rotavirus was an important etiological agent of diarrhoea in infants and young children causing about 35%-50% of all hospitalisations for this form of gastroenteritis during the first two years of life. The severity of rotavirus infection ranges from no symptoms to dehydrating gastroenteritis that can be fatal. Rotaviruses are also associated with diarrhoea in various animals and birds.

In a thirteen-year survey published in Australia, out of 4,637 children from 0 to 14 years of age admitted to a paediatric hospital due to acute diarrhoea, 39.6% of admissions were caused by Group A rotavirus. Fifty five of these children were aged from12 to 23 months. Rotavirus was a frequent cause of acute gastroenteritis in children under

6 months (18.7%) and in those aged 5 to 13 years (16%) (Carlin *et al.*, 1998). Rotaviruses were solely responsible for the peak of admissions during winter (Barnes *et al.*, 1998; Carlin *et al.*, 1998). The incidence of hospitalisation due to rotavirus infection in New York has been calculated at 8.7% of all diarrhoea-associated hospitalisations (Chang *et al.*, 2003). A total of 136 patients with diarrhoea died during their hospitalisation (hospital fatality rate, 1.6 per 1000), and the 12 deaths among patients with rotavirus had a distinct winter pattern. During 1997, 46% of the hospitals reported diarrhoea in children that was due to rotavirus, and 12% of hospitals reported rotavirus in >30% of all diarrhoea-associated hospitalisations. Infants >6 months of age were more likely to get infected than older children. A higher proportion of nosocomial infection with rotavirus were seen in children with congenital malformations (Chang *et al.*, 2003).

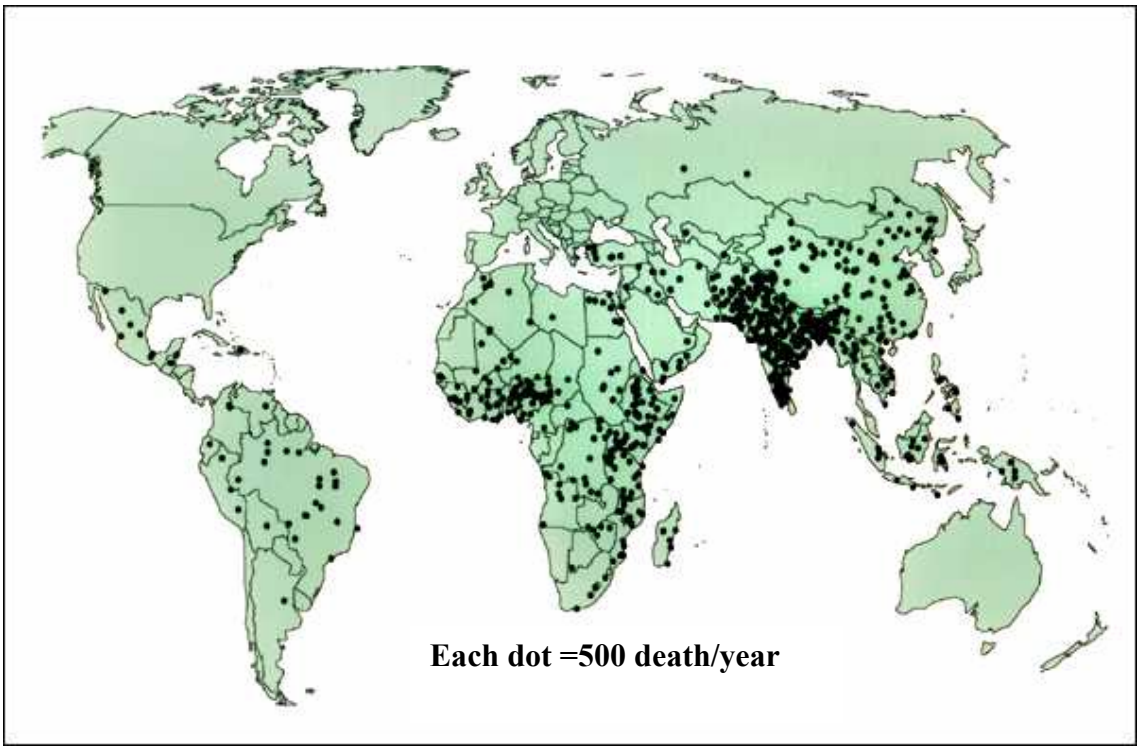

Figure 1.1: Distribution of rotavirus mortality worldwide. Source: Harder, Ben, Science News, 9/27/2003, Vol. 164 Issue 13, p204.

Rotaviruses are classified as a genus in the family *Reoviridae*, and have distinctive triple-layered icosahedral protein capsids that consist of an outer and an inner layer. Within the inner layer is third layer, the core, which contains the virus genome consisting of 11 segments of double stranded RNA. Rotavirus RNA segments range in

size from 0.6 to 3.3 kilobase pairs (Matton *et al.*, 1989). A detailed description of rotavirus structure follows in section 1.1. Characteristically, these RNA segments are numbered in order of migration during polyacrylamide gel electrophoresis (PAGE); with the slowest migrating RNA segment designated gene 1 and so on. In the 1980s and early 1990s, electropherotyping of rotavirus became the most common technique for the characterisation of different strains of the virus. The resultant RNA migration pattern in polyacrylamide gels is called an electropherotype (e-type). It soon became apparent that human rotaviruses exhibited a wide variety of electropherotypes and that these patterns differed from those observed for animal rotavirus, indicating the existence of huge diversity among rotavirus strains (Holmes, 1996). However, PAGE does not provide accurate or detailed genetic information about the various strains and subsequent studies showed that viruses of the same serotype can exhibit different electropherotypes, and that viruses of the same electropherotype can belong to different serotypes, when molecular typing methods such as reverse transcriptase-polymerase chain reaction (RT-PCR) are used to classify the virus (Holmes, 1996). The extent of genetic relatedness and variation among rotavirus strains has been studied by using labelled (+) single stranded RNAs transcribed from viral genome as hybridisation probes (Nakagomi and Nakagomi, 1996). These studies have indicated the existence of three major 'genogroups' of human rotaviruses and numerous other genogroups of animal strains.

Molecular epidemiology has become an important tool in studying the various rotavirus strains circulating in different parts of the world at a given time and will aid significantly in determining the appropriate strategies for use in the development of rotavirus vaccines. Such vaccines are urgently needed to prevent the huge number of fatalities caused by this virus. The first licensed vaccine, a tetravalent vaccines based on the rhesus strain, RRV (RRV-TV) incorporated serotype G1, G2, G3 and G4 specificities, the most common serotypes worldwide at the time. The vaccine was subsequently withdrawn in 1999 due to an association with of intussusception in vaccine recipients (Murphy *et al*., 2001; CDC., 1999a). Extensive research followed to develop a new and effective vaccine and recently, two new rotavirus vaccines, Rotarix and RotaTeq, have been approved in the US and several countries in Latin America (Arvin and Greenberg, 2006). Rotarix, which is a monovalent, attenuated vaccine developed by GlaxoSmithKline has recently been licensed in many European countries

(Linhares and Villa, 2006). Both vaccines have been licensed in Australia and have recently been included on childhood routine immunisation schedule. The second generation of vaccines needs to focus on targeting the common types and any of the new and emerging types such as serotype G9, which is now the fifth common global type. Thus, continued surveillance and the genetic and antigenic analysis of the various strains of rotavirus circulating worldwide will aid significantly in assessing the effectiveness of these vaccines and monitor emergence of new strains.

1.1 Virus structure

When examined by electron microscopy, the morphology of the rotavirus particle resembles that of a wheel, as it consists of three distinct layers. There are about 60 spikes of viral protein VP4 protruding from the outer surface, which has well defined surface rim, giving a wheel-like appearance (Latin 'rota' wheel) hence the name (Fig 1.2).

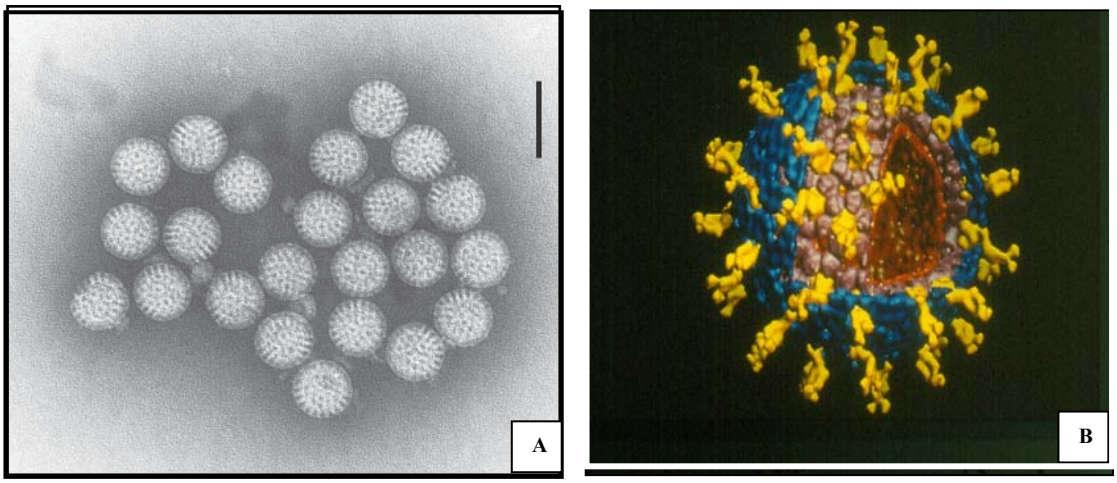

Figure 1.2 Structure of rotavirus (A) Negative-Stain Electron Micrograph (B) Cryoelectron Microscopy of rotavirus. Bar represents 100 nm in length (Figure: Glass & Parashar, 2006)

The virus appears rough when its outer surface is removed showing its intermediate layer which consists of projecting trimers of viral protein VP6. Methods developed by Prasad *et al.* (1988) using cryoelectron microscopy show the reconstruction of three-dimensional structure and have helped the study of the three distinct layers in more

detail (Fig 1.2B). The outer layer, made up of molecules of VP7 proteins encoded by one of gene segment 7, 8 or 9 (depending on the strain), forms a triangular shape, surrounding the many aqueous channels present on the outer surface. The thickness of the VP7 layer is ~35 Å. Spikes made up of protein VP4, encoded by gene segment 4, surround the outer capsid. The length of the spikes extending from the surface is 120 Å. Each spike has a distinct bilobed structure at the distal arc of diameter ~25 Å. Particles containing only the intermediate VP6 layer are 705 Å in diameter (Fig 1.3). The VP6 and the VP7 molecules interact with one another through their upper domains. The inner layer, or core, is comprised of the protein VP2 and has a radius of 270 Å. The entire genome and the VP1 and the VP3 proteins are contained within the core (Estes and Cohen, 1989; Prasad *et al.*, 1988).

1.2 Viral Proteins

The 11 segments of viral RNA genome code for different structural and non-structural proteins (Fig 1.3). There are six structural viral proteins (VP1-4, 6 and 7) and 6 non-structural proteins (NSP1-6) with various functions as listed in Table 1.1.

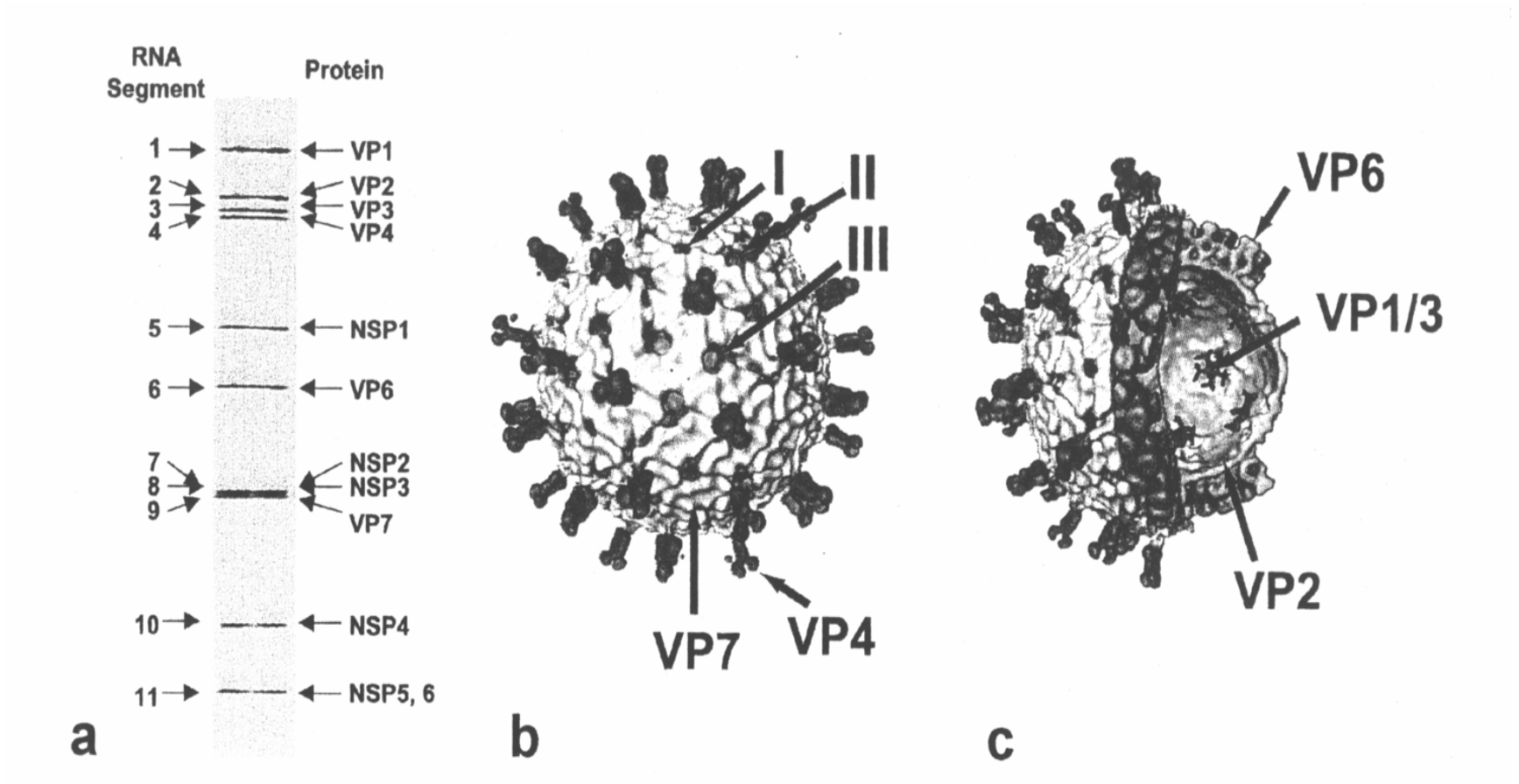

Figure 1.3: Genome and coding assignment of rotavirus (a) Genome Segments*:* PAGE separation of the 11 segments of the rotavirus genome. Encoded proteins*:* the PAGE separation of the intracellular proteins synthesized by SA11 and the genome segment in which they are encoded. (b) Outer capsid proteins I, II and III represents various channels on outer capsid. (c) Arrangement of the proteins into 3 concentric capsid layers, and the location of the VP1/VP3 (Fig. source Pesavento *et al*., 2006).

1.2.1 The VP6 protein

The VP6 protein is located on the inner capsid of the virus and is encoded by gene segment 6. This protein constitutes 51% of the total viral protein. Virus classification is based on VP6, which confers group and subgroup specificities and differentiates rotavirus into seven groups (A-G). Within group A, four different subgroups are defined based on the reactivity of subgroup specific monoclonal antibodies: subgroups I, II, I & II and non-I/non-II (Estes *et al.*, 2001). Group A is the major type causing infection in humans. Monoclonal antibodies (MAbs) developed against the human rotavirus strain, Wa (subgroup II), or the rhesus rotavirus, RRV (subgroup I), react with almost all mammalian group A rotavirus (Greenberg *et al.*, 1983). The majority of subgroup II and subgroup I & II rotaviruses exhibit 'long' electropherotypes, based on the mobility of gene segment 11 whereas almost all subgroup I rotaviruses exhibits 'short' electropherotypes (Kapikian *et al.*, 2001).

Table 1.1: Characteristics and functions of rotavirus proteins*:

Genome segment	Protein product	Polypeptide (Dalton)	Location in the virus	No. of molecules /virion	Function
1	VP1	125,005	Core	12	RNA Polymerase, SS RNA binding.
2	VP2	94,000	Core	120	RNA binding, required for replicase activity
3	VP3	88,000	Core	12	guyanylyltransferase, methyltransferase.
4	VP4	86,782	Outer Capsid	120	hemagglutinin, neutralisation antigen, protease enhanced infectivity
5	NSP1	58,654	NS	-	basic, RNA binding, virus spread.
6	VP6	44,816	Inner capsid	780	hydrophobic, trimer, subgroup antigen, protection
7	NSP3	34,600	NS	-	acidic dimers, binds 3'end viral mRNA, inhibits host translation.
8	NSP2	36,700	NS	-	basic, RNA binding, NTPase
9	VP7	37,368	Outer capsid	780	integral membrane glycoprotein, neutralising antigen,
10	NSP4	20,290	NS	-	RER transmembrane glycoprotein, role in morphogenesis, enterotoxin
11	NSP5/NSP	21,725/12,00	NS	-	basic, phosphoprotein, RNA binding,

	6	0			

*adapted from Estes *et al.* (2001)

A separate domain of VP6 contains the group specific epitopes for group A rotaviruses, corresponding to four different regions on VP6 deduced amino acid sequence: residues 32 to 64, 155 to 167, 208 to 294 and 380 to 397 (Estes, 1996). Rotaviruses that share the group specific antigenic determinants are classified in group A and those that do not share this feature are classified into non group A (Tao, 1988). Presently, non group A rotavirus are classified into groups B, C, D, E, F or G. Group B has been found in children, cattle, sheep, pigs, goats and rats (Gueguen *et al.*, 1996) and also have been reported to cause diarrhoea outbreaks in adults in China (Kuzuya *et al.,* 2005*;* Su *et al.*, 1986; Tao, 1988; Ward *et al.*, 1992). Group C has been found in pig, cattle and dogs (Otto *et al.*, 1999) and is also been found to cause sporadic infection in humans (Kuzuya *et al.*, 2005; Phan *et al.*, 2004). Group D rotaviruses are found in chickens, pheasants and turkeys (Devitt and Reynolds, 1993). Group E rotavirus is detected in pigs and Group F and G are isolated from chickens and turkeys (Saif and Jiang, 1994).

1.2.2 The VP7 Protein

The VP7 protein, which constitutes 30% of the viral protein, is the major outer capsid protein and is the major neutralising antigen of the virus. Based on antigenic determinants found on this glycoprotein, it serves as the basis for viral classification into G-serotypes. VP7 is encoded by one of gene segments 7, 8 or 9, depending on the strain (Greenberg *et al.*, 1983a; Mattion *et al.*, 1990). So far group A rotaviruses have been classified into 15 G-serotypes based on the reactivity of these antigenic sites in enzyme immunoassay (EIA) (Hoshino *et al.*, 2005). G-serotype identification of rotaviruses has been facilitated by the isolation of VP7 MAbs that are specific for the epidemiologically important human G-serotypes (Hoshino and Kapikian, 1994; 1996).

The amino acid sequences of VP7 of rotavirus strains belonging to each G-serotype have been deduced from VP7 nucleotide sequences. Comparisons among different group A rotaviruses have shown that there are nine regions of the amino acid sequence of VP7 that are highly divergent between strains belonging to different G-serotypes (Nishikawa *et al.*, 1989). Each of these regions is highly conserved among rotavirus strains within the same G-serotypes, allowing prediction of the G-serotype of strains by

direct sequence analysis of any two of these variable regions (Green *et al.*, 1988). A common technique used to identify the G-serotype of group A rotaviruses involves multiplex semi-nested PCR using a pool of G-serotype specific primers (Table 2.5). This technique is carried out in two steps; the first involves full length amplification of the VP7 gene using gene-specific end primers by RT-PCR which is followed by a second round multiplex semi-nested PCR using one conserved end primer and a pool of G-serotype specific primers (Gouvea *et al.*, 1990; 1991).

1.2.3 The VP4 Protein

The VP4 protein is situated on the outer capsid layer in the form of spikes that protrude from the surface of the virus. There are 60 such spikes and their length is up to 10 –12 nm in a mature virion. The VP4 protein constitutes 1.5% of total viral protein. This protein is susceptible to trypsin proteolytic cleavage yielding its subunits VP5* and VP8*, the asterisk is conventionally used to denote the cleaved subunits. VP4 has a very important function in terms of viral attachment to the host cell and it also induces the production of neutralising antibodies. The protein is coded by the fourth segment of the viral genome (Mattion *et al.*, 1994). VP4 helps the virus to penetrate the host cell by trypsin-associated cleavage and also causes infectivity (Isa *et al.*, 1997). The VP4 protein has neutralising antigenic sites different from those of the VP7 protein. Thus a dual classification system exists based on the VP7 and VP4 neutralising antigens, designating as G-serotypes for the VP7 glycoprotein and P-serotypes for the protease sensitive VP4.

While no correlation between rotavirus virulence and the G-type of the infecting strain is apparent, i.e. each of four major G-serotypes (G1, 2, 3 and 4) are associated with both symptomatic and asymptomatic infections (Hoshino *et al.*, 1985a), it was observed that the VP4 gene of rotavirus strains causing asymptomatic infections was highly conserved (95.5% to 97.5% sequence identity) and was distinct from the VP4 gene of strains from symptomatic infections (25.4% to 27.1% sequence divergence) (Gorziglia *et al.*, 1988). Although VP4 antigenic specificities had not been assigned for all rotavirus strains, RNA-RNA hybridisation, reactivities with neutralising monoclonal antibodies and/or sequencing analysis of various rotaviruses have defined at least 26 P-types (Hoshino *et al.*, 2005; Rahman *et al.*, 2005a).

The VP8* subunit of VP4 is the location of the major antigenic sites determining P-serotype specificity, whereas the VP5* subunit contains sites responsible for cross-reactivity among various VP4 types (Larrade *et al.*, 1991).

1.2.4 The Core Proteins

The proteins VP1, VP2 and VP3 comprise the rotaviral core proteins and have important roles in viral replication and RNA transcription. Studies have shown that VP1 is the viral RNA-dependant RNA polymerase and functions as both the viral RNA transcriptase and replicase (Estes *et al.*, 1984; Patton *et al.*, 2004). All sequenced rotavirus VP1 proteins have four common motifs conserved in RNA-dependant RNA polymerases and depend on VP2 for replicase activity (Burns *et al.*, 1989; Patton *et al.*, 1993). The VP2 protein binds with RNA non-specifically and interacts with dsRNA (Boyle and Holmes, 1986; Labbe *et al.*, 1994; Prasad *et al.*, 1996). VP2 plays an important role in replication, as it has the ability to bind to mRNA (Liu *et al.*, 1992; Patton *et al.*; 1996; 1997; 2004). VP3 can bind to the N-terminus of VP2, with labelling experiments using α^{32}P –guanosine triphosphate (GTP), indicating it is a guanylyltransferase and also a methyltransferase (Boyle and Holmes, 1986; Chen, 1999 Pizzaro, 1991).

1.2.5 The Non-structural proteins (NSPs)

There are six NSPs coded by the rotavirus genome, namely NSP1-6. With the exception of NSP4, all of these NSPs bind to the RNA. They have various functions in viral replication, assembly of various proteins and in viral packaging during replication (Mattion *et al.*, 1990; Poncet *et al.*, 1993).

1.2.5.1 The NSP1 protein

NSP1 is encoded by gene segment 5 and its gene size ranges from 1564 bp to 1611 bp in length which encodes a polypeptide of 486-495 amino acids in length (Barro and Patton, 2005). NSP1 appears to be the least important in the replication of virus. However, it has an important role in cell-to-cell spread during infection (Barro and Patton, 2005; Hua *et al.*, 1994) and contains cysteine-rich zinc fingers, involved in

binding of RNA. Recently, it has been demonstrated that this protein has an important anti-interferon activity which destroys innate immunity of host cells (Barro and Patton, 2005) and thus has a pathogenic function. This protein is also the least conserved showing more sequence variation among various strains than VP4 and VP7 discussed earlier. NSP1 contains one cysteine-rich region near its amino terminus. The cysteine-rich domain of group A and C rotaviruses is the motif C-X_2-C-X_5-C-X_2-C-X_3-H-X-C-X_2-C-X_5-C located at amino acids 42-69 (Hua *et al.*, 1993). This motif forms one or two zinc fingers which are highly conserved among many rotaviruses and are involved in RNA binding suggesting that it plays a critical role in the function of the virus (Hua *et al.*, 1993; Kojima *et al.*, 1996a). The carboxyl half of the protein is not involved in virus replication but has a pathogenic role, such as the anti-interferon activity as mentioned above and viral cell-to-cell spread (Barro and Patton, 2005). Although the rotavirus gene segment 5 is believed to be highly variable in sequence, NSP1 is conserved to some extent among rotaviruses infecting the same host (Kojima *et al.*, 1996a; Mattion *et al.*, 1994). Analysis of 22 NSP1 sequences from various host species showed that this protein exhibits a higher degree of host specificity compared to both VP7 and VP4. The gene 5 nucleotide sequence homology was found to be more specific within a single species than among different species (Dunn *et al.*, 1994) suggesting a role in host range restriction. However, when interspecies relatedness was examined, a high degree of amino acid identity between the human strain, Wa, and the porcine strain, OSU (86%), and between the human strain, AU-1 and the bovine strain UK, (87%), have been observed (Dunn *et al.*, 1994; Nakagomi and Kaga, 1995).

1.2.5.2 The NSP4 Protein

The NSP4 protein is 175 amino acids in length and is encoded by gene segment 10 (751 bp). It is an endoplasmic reticulum (ER) specific glycoprotein (Ball *et al.*, 2005; Mattion *et al.*, 1994; Tian *et al.*, 1996) and is one of the most important non-structural proteins in relation to virus virulence and pathogenesis and is the first multifunctional viral enterotoxin reported (Ball *et al.*, 1996). This protein acts as an enterotoxin and is responsible for diarrhoea in infants and age-dependant diarrhoea in mice (Ball *et al.*, 1996; Estes *et al.*, 2001). The protein plays a unique role by functioning as an intracellular receptor and converting double-layered viral particles in the cytoplasm into the triple-layered form in the ER (Estes *et al.*, 2001; Horie *et al.*, 1999; Maass and

Atkinson, 1990; Taylor *et al.*, 1996). It is proposed that NSP4 interacts with epithelial cells of the intestine and stimulates a calcium-dependant signal transduction pathway that increases plasma membrane and stimulates chloride secretion resulting in secretive diarrhoea (Ball *et al.*, 1996). The development of antibodies against NSP4 in mice pups immunised with a peptide derived from SA11 NSP4 (residues 114 to 135) resulted in diarrhoea of less severity and shorter duration compared to controls (Horie *et al.*, 1999; Mori *et al.*, 2002a) suggesting that NSP4 induces a protective antibody response in the host.

A recent computational analysis of the 176 NSP4 genes sequences available in the GenBank database derived from all the different groups of rotavirus, revealed the presence of four different genotypes of NSP4 and many amino acids which were found to be well conserved regardless of genotype (Lin and Tian, 2003). However, considerable variation among the NSP4 from mammalian and avian groups has been observed such that a fifth genotype has been reported. A major difference observed in avian NSP4 was the absence of highly probable coiled–coil region responsible for the quaternary structure seen in mammalian NSP4 (Lin and Tian, 2003; Taraporewala *et al.*, 1999).

1.2.5.3 Other NSP proteins

Rotavirus non-structural proteins are actively involved in viral replication. NSP2 is an oligomeric nucleotide tri-phosphatase (NTPase), is localised in the viroplasm and possesses helix destabilising activity (Poncet *et al.*, 1993; Taraporewala *et al.*, 1999). NSP3 binds with the 3' end of the viral mRNA, needing only a four nucleotide sequence to recognise the binding site. NSP3 inhibits cellular protein synthesis by binding with the poly A mRNA of the host cell (Piron *et al.*, 1998; 1999, Poncet *et al.*, 1993; Vende *et al.*, 2000). NSP3 also enhances viral translation (Gonza'lez and Burrone, 1991; Vende *et al.*, 2000). NSP5 is a self-assembled dimer (Arias *et al.*, 1996; Gonza'lez and Burrone, 1991) with an autokinase property that exists abundantly in infected cells in various isomeric forms (Arias *et al.*, 1996; Blackhall *et al.*, 1997; Poncet *et al.*, 1997) and interacts with NSP2 during replication (Afrikanova *et al.*, 1998; Gonza'lez *et al.*, 1998; Mattion 1991; Poncet 1997) NSP6 is encoded by gene segment 11 and is known to interact with NSP5 (also encoded by gene 11) and reside in the

viroplasm (Mattion *et al.*, 1991; Torres-Vega *et al.*, 2000). NSP2, NSP5 and NSP6 are involved in RNA encapsidation and movement of viral particles from the viroplasm to the membrane of endoplasmic reticulum (ER) (Estes *et al.*, 2001; Fabbretti *et al.*, 1999).

1.3 Replication of rotavirus

Rotavirus replication takes place in the cytoplasm of the host cell. The natural host cell of rotavirus is the differentiated enterocyte in the small intestine (Clark *et al.*, 1979; Estes *et al.*, 2001). The various steps of the rotavirus replication cycle have been studied by infecting monkey kidney cells in cell monolayers (Fig 1.4). In these cells, the replication cycle is fairly rapid with high yields of virus being found after 10 to 12 hours at 37°C when cells are infected with high multiplicities (10 to 20 PFU/cell) (Clark *et al.*, 1979; McCrae and Faulkner-Valle, 1981; Ramig, 1982).

While rotavirus replication takes place entirely inside the cytoplasm, the addition of exogenous protease is required in order to cleave VP4, the outer capsid spike protein. The cell does not have the enzyme to replicate dsRNA, so the virus has to provide the necessary enzymes. Transcripts have two functions: acting as mRNA for the production of proteins, and also acting as a template to form negative strand RNA, which remains associated with the positive strand (Estes, 1996; Silvestri *et al.*, 2004). As shown below (Fig 1.4) the nascent sub-viral particles mature by budding through the membrane of the ER, during which they acquire the outer capsid (Estes, 1996; Petrie *et al.*, 1981).

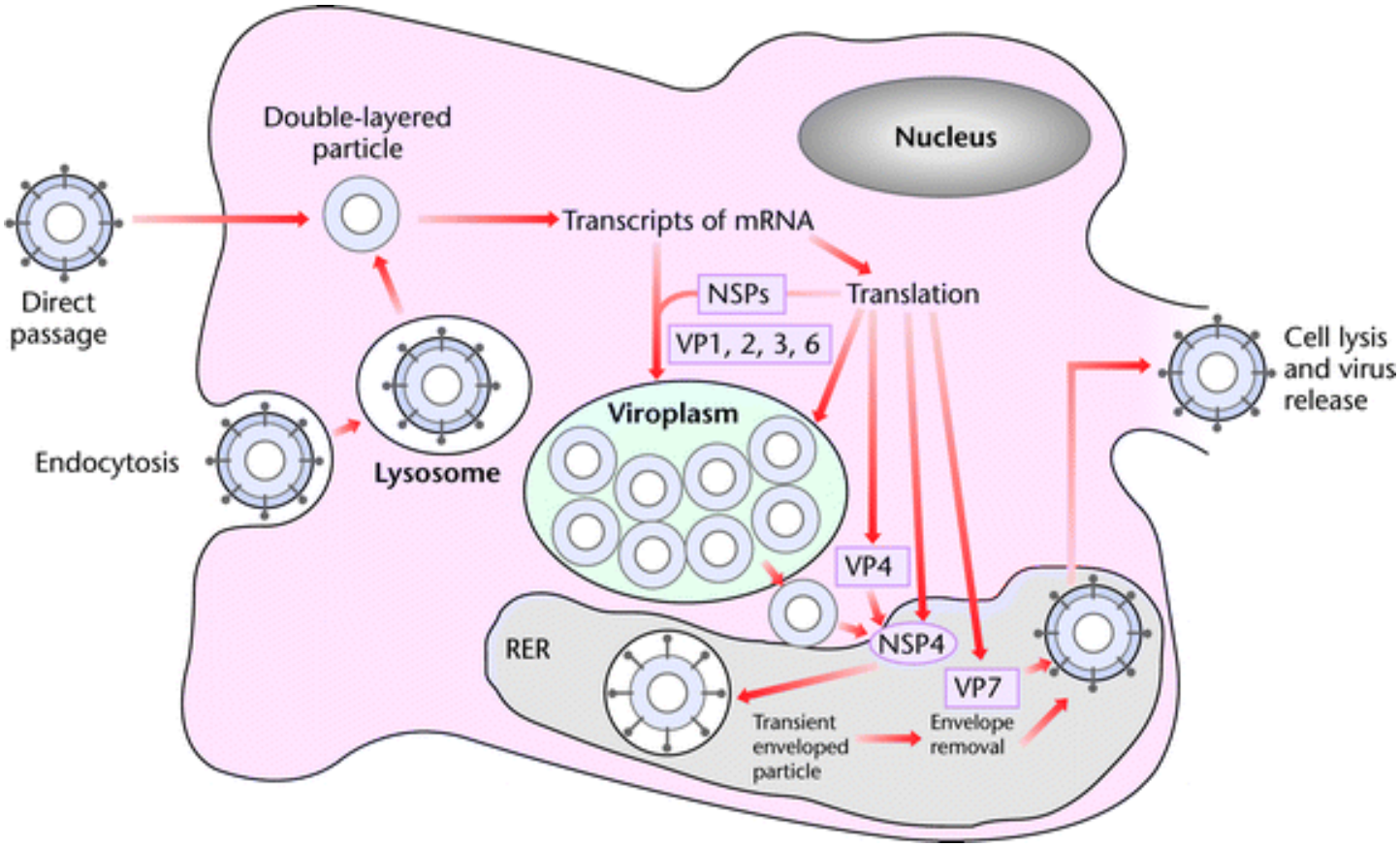

Figure 1.4 Schematic representation of replication of rotavirus: various steps of viral protein synthesis. (Fig source Dr Enzo A Palombo)

1.3.1 Steps in viral replication

1. Adsorption to the host cell

The first step in viral replication is attachment to host cells with the help of VP4 (Fukudome *et al.*, 1989; Jourdan *et al.*, 1998; Petrie *et al.*, 1981). The type of glycoconjugates that actually function as the *in vivo* receptor for a given rotavirus depends on the strain and host cell type (Franco *et al.*, 1994; Fukudome *et al.*, 1989). Two different methods of cell attachment are known: sialic acid-dependant and sialic acid-independent viral attachment to the host cells. Rotaviruses that are sialic acid-dependent infect only polarised epithelial cells and attack only the apical membrane, whereas sialic acid-independent strains attack non-polarised cells on either the apical or basolateral surfaces (Ciarlet *et al.*, 2000; Ciarlet and Estes, 1999; Franco *et al.*, 1994; Mendez *et al.*, 1999). These results indicate that sialic acid-dependant and independent viruses use different cell receptors and they may share a second receptor (Bastardo and Homes, 1980; Mendez *et al.*, 1999).

2. ***Penetration***

After binding occurs, the virus is internalised, probably by direct penetration. Virus entry into the cell is a multi-step processes (Mendez *et al.*, 1999). Radiolabelling studies in the monkey kidney cell line (MA104) using the simian virus, SA11, have found that there are 13,000 receptors units per cell; initial binding is sodium dependant, pH sensitive (5.5-8.0) and dependant on sialic acid residues in the membrane (Bastardo and Homes, 1980; Imai *et al.*, 1983).

3. Transcription and Replication

Once the virus enters the cells, the synthesis of viral transcripts is initiated by an endogenous viral RNA-dependant RNA polymerase (transcriptase). The enzyme has a number of different enzymatic activities e.g. transcriptase, nucleotide phosphohydrolase, guanyltransferase and methylase (Imai *et al.*, 1983). The transcriptase is a component of VP1 and VP3 localised together in a flower shaped structure attached to the top of VP2 at the axes of VP2 protein unit (Cohen, 1977;

Fiore *et al.*, 1991; McCrae and McCorquodale, 1983; Sandino *et al.*, 1986; Spencer and Ariar, 1981). The exact site of transcription within the cytoplasm has not been precisely localised, although it is thought to occur in the viroplasm. Transcription requires ATP for initiation or elongation of RNA molecules and also for polymerisation (Lawton *et al.*, 1997; Spencer and Ariar, 1981). RNA synthesis occurs inside the core of the virion. Newly transcribed RNA exits the core through the type I channels in the core VP2 layer and multiple mRNA transcripts can be released simultaneously from an actively transcribing particle (Lawton *et al.*, 1997; Stacy-Phipps and Patton, 1987). Each genome segment is thought to be transcribed by a specific polymerase complex. The synthesis of positive and negative stranded RNA has been studied in SA11 infected cells (Patton, 1986; Stacy-Phipps and Patton, 1987) in a cell-free system using extracts of infected cells and an electrophoretic system that allows separation of the positive and negative strands in acid urea agarose gels (Patton and Stacy-Phipps, 1986a). RNA synthesis in infected cells results in positive and negative RNA strands that are detectable within three hours of infection (Silvestri *et al.*, 2004; Stacy-Phipps and Patton, 1987). Both newly synthesised and pre-existing positive-stranded RNA can act as templates for negative strand synthesis throughout infection (Estes *et al.*, 1996; Silvestri *et al.*, 2004). During replication, dsRNA remains within partially uncoated particles, afterwards dsRNA remains associated with sub-viral particles, and thus free dsRNA is not found within the cell at anytime (Estes, 1996; Petrie, 1983).

4. RNA encapsidation and virion assembly

Sub-viral particles assemble in cytoplasmic viroplasms as shown in Fig 1.4 and bud through the membrane of the ER, acquiring a transient envelope. This is one of the characteristics features of rotavirus that differentiates it from other members of *Reoviridae* family. The temporary envelope is then lost as the particle moves from the membrane to the inner part of the ER and is then coated with outer capsid proteins as the virus matures. Most of the rotavirus proteins, structural or non-structural, are synthesised on free ribosomes that are associated with the membrane of the ER (Richardson *et al.*, 1986). Viroplasms are probably the sites of synthesis of double layer particle that contain RNA. This conclusion is based on the localisation of several viral proteins (VP2, NSP2, NSP5 and NSP6) to the viroplasm

and of VP4 and VP6 to the space between the periphery of the viroplasm and the outside of the ER (Gonza'lez *et al.*, 2000; Silvestri *et al.*, 2004). Recently, it has been demonstrated by immunofluorescence analysis of bromouridine (BrU) labelled RNA that plus strand RNAs are made in viroplasms (Silvestri *et al.*, 2004).

5. Virus release

The infectious cycle ends when progeny virus is released by host cell lysis in nonpolarised cells as shown by electron microscopy (Altenburg *et al.*, 1980; Chasey, 1977; Musalem and Espejo, 1985). Extensive cytolysis occurs late during infection and drastic alteration in the permeability of the plasma membrane of infected cell results in the release of cellular and viral proteins (Estes *et al.*, 1996; Musalem and Espejo, 1985).

1.4 Virus classification

Rotavirus belongs to family *Reoviridae*. It shares the following common general features of the family (Estes, 1996)

- possess segmented double stranded RNA as a genome
- non-enveloped
- mature virus measures 65-75nm
- icosahedral particles
- purified viral RNA is not infective
- cultivation is facilitated by protease

Rotaviruses are classified serologically into several groups based on the VP6 protein (serogroup). Rotavirus groups share the cross-reacting antigens detected by a number of serological tests like Enzyme Linked Immunosorbent Assay (ELISA), immunofluorescence, and immunoelectron microscopy (Estes *et al.*, 2001). Rotaviruses comprise seven distinct groups (A-G) based on the group determinants present in the VP6 protein. Groups A, B and C are found to cause infection in both humans and animals, whereas groups D, E, F and G have been found only in animals.

Viruses within each group are capable of genetic reassortment. Group A rotaviruses have been found as a cause of diarrhoea in significant numbers of children. This group remains the most common type circulating worldwide. Group A is further divided into the following subgroups based the presence or absence of specific antigens: subgroup I; subgroup II; subgroup I & II; and subgroup non I & non II.

Within group A, viruses are classified into serotypes defined by reactivity of virus neutralisation assays using hyperimmune serum prepared in antibody negative animals. Using this assay, 15 VP7 serotypes have been identified (Hoshino *et al.*, 2005). Strains from both human and animal origin may fall within the same serotypes (Tables 1.2 and 1.3). Neutralisation assays can measure reactivity against the two outer capsid antigens, VP4 (P-type) and VP7 (G-type). However, in many cases, the predominant reactivity is determined against, VP7, simply because VP7 covers a greater percentage of outer capsid and induces highly specific antibodies (Birch *et al.*, 1988; Coulson *et al.*, 1987; Hoshino *et al.*, 1987a; Taniguchi and Urasawa, 1985). The minor neutralising antigen, VP4, does not always allow rotavirus strains to react clearly in reciprocal neutralising assays with hyperimmune antiserum (Hoshino *et al.*, 1985). These two different neutralising antigens thus were made basis for a dual classification system for rotavirus. As mentioned in sections 1.2.2 and 1.2.3, rotavirus classification based on neutralising VP7 antigens is termed G-type (since VP7 is a glycoprotein), and classification based on VP4 neutralising antigens is termed P-type (since VP4 is a protease sensitive protein). Genes that encode VP7 and VP4 can segregate separately, resulting in a large amount of antigenic diversity (Hoshino *et al.*, 1985; Su *et al.*, 1986). There are 42 different combination of G and P types that have been recognised so far (Gentsch *et al.*, 2005).

The P-typing system of classification has been hampered by lack of readily available serological typing reagents to the different VP4 serotypes. Instead, the antigenic properties of VP4 have been studied by gene sequence analysis and have indicated that there are 26 different P- genotypes (Santos, 2005). Genotypes of VP4 and VP7 are determined by sequence analysis or hybridisation, whereas serotypes have been determined by reactivity with polyclonal or monoclonal antisera (Estes, 1996; Imai *et al.*, 1983). For VP7, a correlation between genotypes and serotypes has been established; however such a correlation is not yet available for VP4.

Table 1.2: Group A rotavirus VP7 (G) serotypes

	Strains followed by species of origin	
Serotypes	***Human***	***Animal***
1.	Wa, KU, RV-4, K8, D, M37, Mont, WI79, AU32, HN-1, GR475/87.	Bo/T449, Po/C60, Po/C91, Po/CN117, Po/C86, Po/S7, Po/S8.
2.	DS-1, S2, RV-5, RV-6, KUN, HN-126, 1076, E201, HU5, TA3, TA20	Si/Sa11CI3, Si/SA114fm, Si/SA11-SEM, Si/SA11-FEM, Si/RRV
3.	Ito, YO, P,M, Nemoto, AU-1, RV-3, WI78, AK-35, ST8, MO, McN13, McN14, HCR3, CHW927A, Ro1845, O264, AU228, PA710, 02/92, Ch-32, CHW2, CHW17, CH-55, AI-75, J-12, TK08, TK28	Ca/K9, Ca/CU-1, CA/A79-10, Ca/LSU79C-36, Ca/RS15, La/ALA, La/C-11, La/R-2, La/BAP-2, Eq/311, Fe/Cat97, Fe/Cat2, Fe/Cat22, Fe/Taka, Fe/FRV64, Fe/FRV-1, Mu/EW, Mu/EB, Mu/EL,, Mu/EC, Mu/ EHP, Mu/YR-1, Po/ CRW-8, Po/A131, Po/A138, Po/LCA 843, Po/A411, Po/BEN-307, Po/PRV 4F, Po/AT/76, Ov/LRV1, Po/MDR-13.
4.	Hochi, Hosokawa, ST3, 57M, ST4, VA70.	Po/ Gottfried, Po/ SB-1A, Po/SB-2A, Po/BEN-144, Po/BMI-1,
5.	Ial28, BR1054	Po/OSU, PO/EE, Po/TFR-41, PO/A34,Po/A46, Po/C134, Po/CC117, Po/S8, Eq/H-1
6.	PA151, PA169, MG6	Bo/NCDV-Lincoln, Bo/UK, Bo/B641, Bo/RF, Bo/C486, Bo/WC3, Bo/KN-4, Bo/IND, Bo/BRV033, Bo/B-60, Bo/CR231/39, Ov/LRV2, Cap/Cap455.
7.		Ch/Ch-2, Ty/Ty-1, Ty/Ty-3, Pi/PO-13, Bo/993/83.
8.	69M, B37, HAL1166, HAL1271, HMG89.	Bo/678, Bo/A5, Bo/NCDV-Cody, Bo/J2538, Eq/26/94.

Table 1.2 contd..

9.	WI61, F45, Mc523, AU32, US1205, Mc345, 116E	Po/ISU-64, Po/S8, Ov/LRV
10.	I321, Mc35, A28, A64	Bo/B223, Bo/61A, Bo/V1005, Bo/KK3, Bo/B-11, Bo/A44, Bo/CR129, Bo/BR65/255, Eq/R-22, Ov/lp14, Ov/K923.
11		Po/YM, PO/A253
12	L26, L27 ISO-1	
13		Eq/L338.
14		Eq/F123, Eq/FR4, Eq/FR5, Eq/FR8, Eq/EQ431, Eq/CH3.
15		PoRV

Species of origin of animal strains indicated by abbreviation: Si: simian, La: Lapine, Po: Porcine, Ca:canine, Mu: murine, Ty: turkey,Ch: Chicken, Pi: pigeon, Eq: equine, Fe: feline, Bo: bovine, Ov: ovine, Cap:caprine. Data obtained from ref: Estes (1997).

Table 1.3 Group A rotavirus VP4 (P) serotypes

		Strains followed by species of origin	
Genotype:	**Serotypes:**	**Human**	**Animal**
1	6		Bo/C486, Bo/NCDV, Bo/BRV033, Bo/A5, Bo/Cr231/39, Bo/J2538, Si/SA11 4fm, Si/SA11-FEM, Eq/26/94, Ov/LRV1
2	5B		Si/Sa11, CI3, Si/SA11-SEM.
3	5B		Si/RRV, Fe/FRV64
	5A	Ro/1845, HCR3	Ca/K9, Ca/CU-1, Ca/RS15, Fe/Cat97
4	1B	RV-5, DS-1, S2, L26, KUN, E210, CHW17	
5	7		Bo/UK, Bo/WC3, Bo/B641, Bo/61A, Bo/678, Bo/V1005, Bo/IND, Po/4S
6	2A	M37, 1076, Rv-3, ST3, McN13, US1205	
	2B		Po/Gottfried, Po/BEN-144, Po/S5, Po/S7, Po/SB-2A
7	9		Po/OSU, PO/TFR-41, PO/C60, Po/A138, Po/YM, PO/A253, Po/BMI-1, Po/AT/76, Po/C95, Po/C134, Po/CC117, PO/SB-1A, PO/CRW-8, Po/BEN-307, Po/A131, Po/EE, Po/ISU-64, Eq/H-1
8	1A	Wa, KU, P, YO, VA70, D, AU32, CH-32 CH-55, CHW2, CH927A, WI61, F45, Ai-75, Hochi, Hosokawa, BR1054	Po/S8, Ov/LRV
9	3	K8, AU-1, PA151, M318, AU2 28, 02/92	Fe/FRV-1, Fe/Cat2
10	4	69M, 57M	
11	8	116E, I321	Bo/B223, Bo/A44, Bo/KK3, Bo/B-11, Bo/KN-4, Bo/CR129, Bo/BR65/255, Eq/R-22, Ov/LRV2
12	4		Eq/H-2, Eq/FI14, Eq/FI23, Eq/FR4, Eq/FR8, Eq/69, Eq/124, Eq/EQ 431, Eq/K311, Eq/K1673
13	13		Po/MDR-13, Po/A46, Po/Clon8
14	11	PA169, HAL1166, Mc35, MG6, GR475/87	La/ALA, LA/C-11, La/BAP-2, Cap/Cap455
	11?		La/R-2
15			Ov/lp14, Ov/lp16
16	10		Mu/Eb, Mu/EW, Mu/EC
17			Pi/PO-13, Bo/993/83, Ch/Ch-1, Ty/Ty-1, Ty/Ty-3
18	12		Eq/L338

Table 1.3 contd.

19			Po/4F
20			Mu/EHP
21			
22			lapine LRV
23	14		Por A34
24	11		Mc323
25	?	Dhaka6	
26	?		PoRV strain 134/04-15

Species of origin of animal strains indicated by abbreviation: Si: simian, La: Lapine, Po: Porcine, Ca:canine, Mu: murine, Ty: turkey,Ch: Chicken, Pi: pigeon, Eq: equine, Fe: feline, Bo: bovine, Ov: ovine, Cap:caprine. Data obtained from (Estes, 1997).

1.5 Molecular Biology of Rotavirus

1.5.1 Genome of Rotavirus

Rotavirus is the only mammalian virus that has a genome of 11 segments of double stranded RNA (dsRNA). The genome is found inside the core (Fig 1.5). The virus contains its own RNA-dependant RNA polymerase required for replication, thus, deprotenised RNA is not infective. The rotavirus gene sequences are rich in A+U (58%-67%), are base paired end to end and the positive strand contains the 5'- cap sequence, $m^7GpppG^mGP_y$ (Estes *et al.*, 1996; Imai *et al.*, 1983; Mccrae and Mccorquodale, 1983; Patton *et al.*, 2004). One of the most important features of rotaviruses is that it co-ordinately replicates and packages the 11 segments with great efficiency. The 11 segments share common cis-acting signals because all the segments of RNA are replicated by the same polymerase (Estes *et al.*, 1996; Mattion *et al.*, 1991).

The nucleotide sequence of all 11 RNA segments is now known for several rotavirus strains. The simian rotavirus strain, SA11, was the first one to be completely sequenced and is the prototype group A rotavirus. Each positive strand starts with a 5'- guanidine followed by a set of conserved sequences that are part of the non-coding sequence. An open reading frame (ORF) then follows, which encodes a structural or non-structural protein. Almost all mRNAs end with the consensus sequence 5'-UGUACC-3', an important signal for RNA replication and gene expression. All the gene segments possess at least one ORF after the first initiation codon. Gene segment 1, 9 and 10, however, has an additional ORF. The entire gene segments are monocistronic, with the exception of gene 11 (Estes and Cohen, 1989; Mattion *et al.*, 1991).

The 11 genome segments can be well segregated by polyacrylamide gel electrophoresis (PAGE). In most cases, the electrophoretic pattern for group A rotaviruses shows a typical pattern of four high molecular weight bands (segments 1-4; 3302, 2690, 2591 and 2362 bp respectively for strain SA11), two middle sized bands (segments 5 and 6; 1611 and 1356 bp respectively for strain SA11), followed

by a triplet (gene segments 7-9 ;1105, 1059, 1062 bp respectively for strain SA11) and two low molecular weight segments (10 and11; 751 and 667 bp respectively for strain SA11). However, the electrophoretic patterns may not always be the same for particular groups, due to various reasons such as gene rearrangements, insertions or deletions of nucleotides during replication. Electrophoretic analysis is however relatively easy, rapid and thus a popular method for virus detection and molecular epidemiology (Bishop, 1996).

Northern blot assays are commonly used to classify rotaviruses into genogroups, based on genome similarities, for identifying the origin of a viral gene in reassorted strains and to identify viruses involved in cross-species transmission (Ballard *et al.*, 1992; Estes *et al.*, 2001; Nakagomi and Nakagomi, 1991; 1993).

The rotavirus genome undergoes genetic changes by different means. Gene rearrangement is responsible for different electrophoretic patterns within a given group. Around 1,800 nucleotides can be inserted into a particular gene segment without causing any significant changes in the gene expression or particle diameter, but the density of the virion may increase proportionately. Deletions of nucleotides have also been reported. In most cases the rearranged gene does not cause any noticeable changes, which indicates that the rearrangement has left the ORF and its expression unaltered. Gene sequence analysis of rearranged genes has confirmed this (Ballard *et al.*, 1992; Desselberger, 1996; Palombo, 2002).

1.5.2 Coding assignment of the genes of rotavirus:

Based on *in vitro* translation using mRNA and denatured dsRNA, and by analyses of reassortant viruses, the protein assignments of rotavirus genes and the properties of the encoded proteins have been determined. Much of this information has been obtained from investigations of the prototype rotavirus, SA11. The ability to obtain sequence information directly from dsRNA or ssRNA and the accumulated information on nucleic acid databases has made it possible to identify similarities among strains based on sequence homology. The structural proteins are known as VP

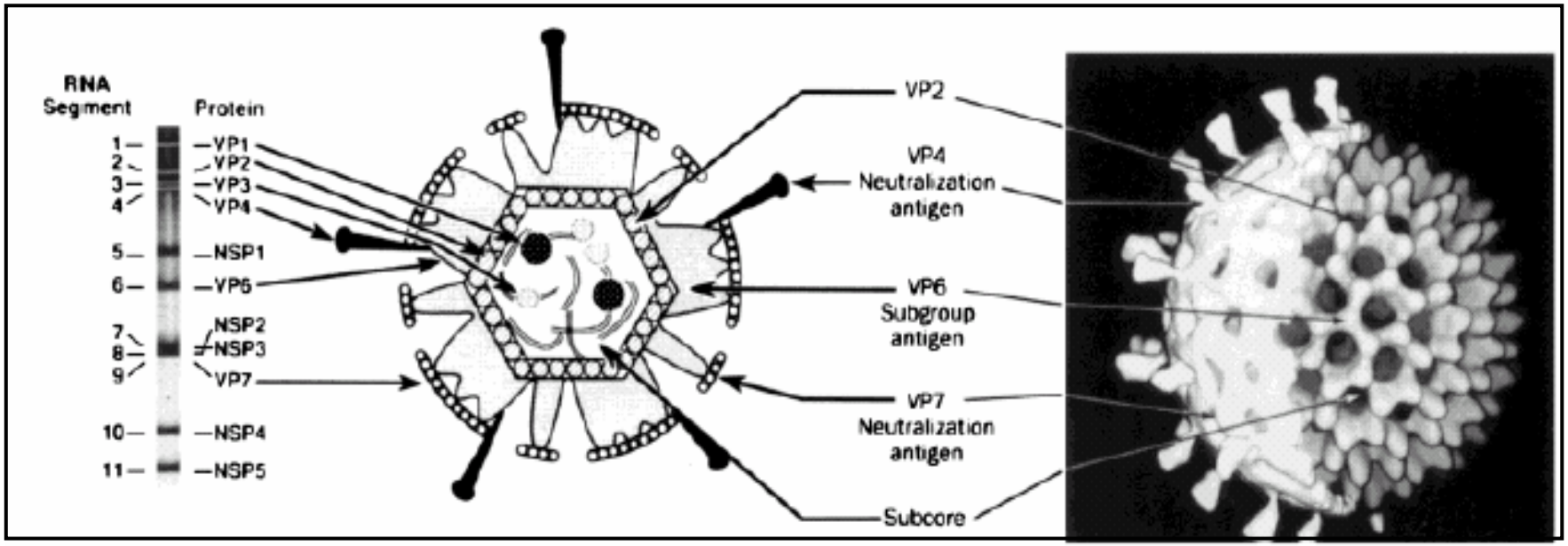

Figure1.5 Profile of rotavirus genes (10% SDS polyacrylamide gel, silver stained), protein products (gene protein assignment) and particle structure (diagram and reconstruction from cryoelectron micrograph) of rotaviruses (Fig. Mattion *et al.*, 1991)

followed by a number and non-structural proteins are name as NSP followed by a number. VP1 has the highest molecular weight. See Table 1.1 for details.

1.5.3 Genetics of rotavirus

The genetics of rotavirus has been studied to better understand the various and unusual combinations of VP4 and VP7, to investigate the natural phenomenon of reassortment occurring between strains *in vivo* and to assign functions to particular genes. Several temperature sensitive (ts) mutants of SA11, UK and RRV have been collected in order to study the genetics of these strains (Estes *et al.*, 2001). The phenotypes of these mutants were consistent with functions assigned for that particular gene segment, based on biochemical or sequence information (Estes *et al.*, 2001). Reassortment, which occurs maximally at the initial phase of infection, can take place at non-suitable temperatures and mutants from all reassortment groups can interfere with the growth of wild-type virus (Estes *et al.*, 2001). *In vitro* reassortment of ts viruses has been carried out successfully to derive reassortant progeny with desired subsets of genome segment derived from each ts parent. These reassortant strains have been used to assign the ts mutation to a specific gene segment and correlate the biological properties of the specific gene segment (Desselberger, 2000; Gonza'lez and Burrone, 1991).

1.5.4 Genetic variability of rotavirus

The rotavirus genome has the potential for rapid genetic change. Different mechanisms create variation among rotavirus populations and result in the evolution of the virus and the development of atypical strains:

- Point mutation
- Genetic rearrangement
- Gene reassortment

The replication of the rotavirus genome occurs via a viral encoded RNA-dependant RNA polymerase (Desselberger, 2000; Ramig, 1997) and the main source of the genetic variation is considered to be mutations due to the error-prone nature of this enzyme. A mutation rate of $< 5X10^{-5}$ per nucleotide per replication round has been calculated for rotaviruses (Beards *et al.*, 1989; Ramig, 1997). The contribution of mutations in generating genetic variants of rotavirus isolates collected from various geographical locations worldwide has been well documented (Beards *et al.*, 1989; Desselberger, 2000; Estes, 1996; Palombo, 2002). Genetic rearrangement also contributes to the formation of diverse strains of rotavirus. Although there is no direct evidence for homologous recombination during rotavirus replication, polymerase jumping during plus and minus strand synthesis can result in intermolecular recombination. This leads to the rearrangement of segments (Fig 1.6), usually their partial duplication (Desselberger, 1996; Ramig, 1997). The rearranged genome may leave the ORF conserved and thus the function of gene remains unaltered (Ballard *et al.*, 1992) (Fig 1.6), the rearrangement could lead to an extended ORF (Tian *et al.*, 1993) or in some cases, the duplication of the gene along with a point mutation in the ORF leads to the premature truncation of the protein (Fig 1.6)(Hua and Patton, 1994a).

The unique segmented nature of the rotavirus genome provides a novel way of genetic variation via the process of genetic reassortment (Fig 1.7). This occurs during mixed infections where packaging of viral segments into sub-particles can lead to the mixing of genes from two different parental viruses (Palombo, 2002; Ramig, 1997). Reassortment has the capacity to rapidly generate new genetic and antigenic variants and is a distinguishing feature of viruses with segmented genomes (Palombo, 2002; Prasad *et al.*, 1990). The generation of possible reassortant progeny genomes can be detected using electrophoresis (Fig 1.7).

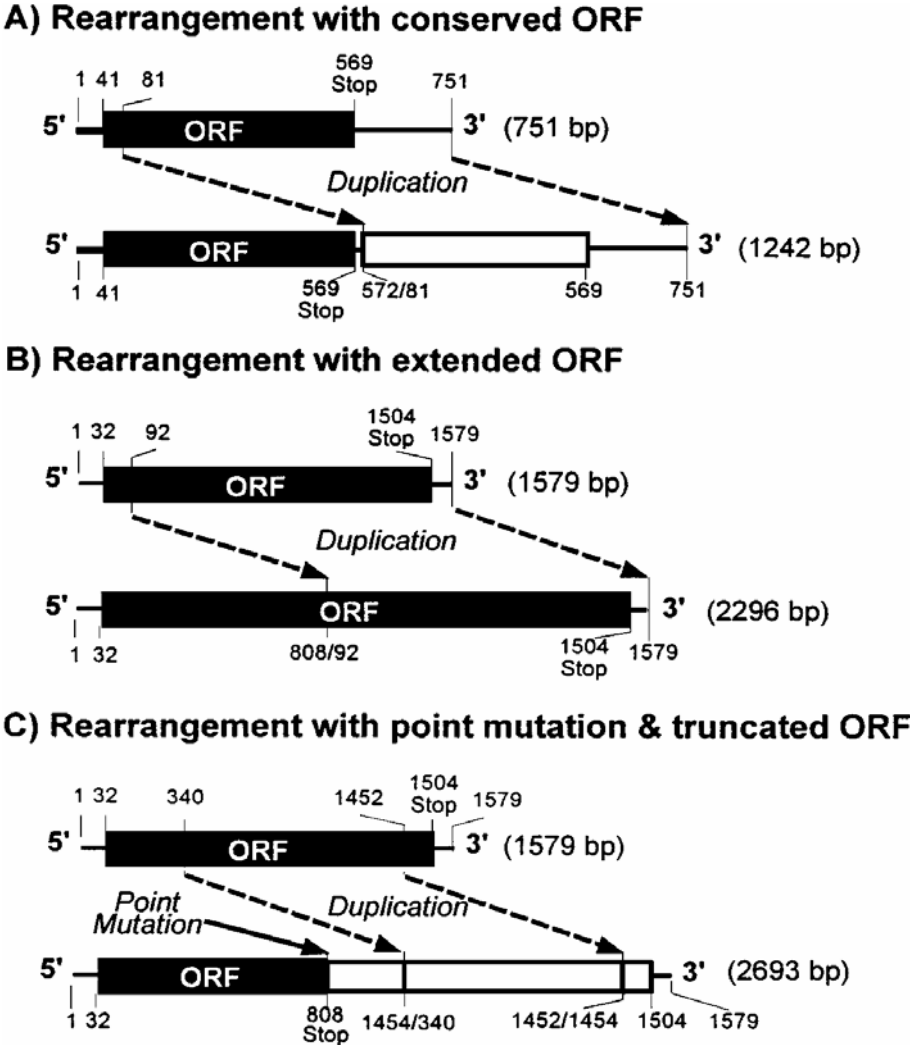

Figure 1.6 Structures of rearranged rotavirus genome segments. *Lines* indicate noncoding regions (NCR), *solid bars* show open reading frames (ORF), and *open bars* show untranslated regions derived from ORF sequences that were duplicated. *Position numbers* indicate nucleotide numbers in normal genes. The lengths are of the parental and rearranged segments are indicated in base pairs. (*A*) Rearrangement of genome segment 10 from a human rotavirus, showing conservation of the ORF with generation of an extended 30-NCR (Ballard *et al.,* 1992). (*B*) Rearrangement of segment 5 from a bovine rotavirus with an in-frame duplication generating an extended ORF (Tian *et al.,* 1993). (*C*) Rearrangement of segment 5 from a bovine rotavirus with a duplication and a point mutation generating a truncated ORF (Hua and Patton, 1994)) (Source: published by Ramig , 1997*)*.

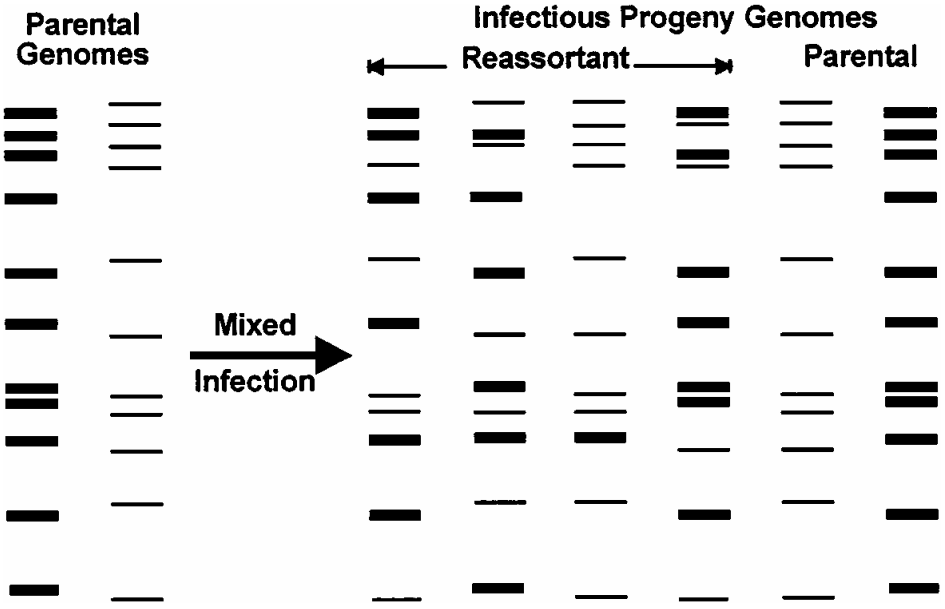

Figure 1.7 Schematic representation of reassortment in rotavirus during mixed infection with two different types of genome segments. On the left, two parental genomes, on the right, possible combinations of genome exchange between two separate genomes in progeny viruses. Electropherotype can be used to identify the parental and progeny genome pattern.(Kapikian *et al.*, 2001)

1.6 Antigenic properties of rotavirus

Rotavirus consists of two major proteins, VP6 and VP7, which comprise the inner and outer capsid of the virus, respectively. The VP6 protein has contains subgroup specific antigens whereas VP7 has serotype specific antigens. Furthermore, there is third protein known to possess antigenic properties, VP4, which is a spike protein, also found on the outer capsid. VP4 is composed of dimers and has hemagglutinin activity (Greenberg *et al.*, 1983a; Prasad *et al.*, 1990). VP7 and VP4 are known to stimulate neutralising antibodies found in the sera and stools of infected patients, are believed to confer the protection against rotavirus infection (Dyall - Smith *et al.*, 1986; Greenberg *et al.*, 1983; Hoshino *et al.*, 1988) and are therefore the major targets for candidate vaccines. Six major antigenic sites have been located on VP7 and these are designated as regions A, B, C, D, E and F (Dyall-Smith and Holmes, 1984; Estes and Cohen, 1989; Huang, 2000; Lazdins *et al.*, 1995; Kirkwood *et al.*, 1993). These regions contain epitopes that are found to induce protective antibodies (Huang, 2000; Kapikian and Chanock, 1996).

1.6.1 Molecular analysis of antigenic sites on rotavirus VP4

VP7 and VP4 are both located on the outer capsid proteins and play important roles in viral attachment and penetration during infection, and both proteins independently induce neutralising antibodies (Jolly *et al.*, 2001; Kapikian and Chanock, 1996). Detailed knowledge of the antigenic properties of these proteins is critical to develop an effective rotavirus vaccine. The VP4 protein modulates important functions such as viral attachment to cellular receptors and helps in consequent host cell penetration (Fiore *et al.*, 1991; Jolly *et al.*, 2001). Prior to cell penetration, VP4 must be cleaved to its subunits, VP5* and VP8*, by treatment with trypsin (Fiore *et al.*, 1991; Fuentes-Panana *et al.*, 1995). Various studies have demonstrated that VP4 is the viral attachment protein and have provided evidence that amino acid residues 150,180,183, 187 and 194 may be involved in binding to the sialylated cell receptors (reviewed by Jolly *et al.*, 2001). Neutralisation epitopes on VP4 have been identified using serotype specific and serotype cross reactive N-MAbs.

1.6.2 Molecular analysis of antigenic sites on rotavirus VP7

The VP7 glycoprotein is the second most abundant protein of rotavirus and, as stated earlier, six antigenic regions have been mapped (Fig 1.8). The locations of these are as follows: region A (aa 87-101), B (aa 143-152), region C (208-221), region D (aa 65-76), region E (aa 189-190) and region F (aa 235-242) *(*Dyall - Smith *et al.,* 1986; Kirkwood *et al.*, 1993). Reactivity patterns of neutralising antibody escape variants has indicated that region C shows cross reactivity between serotypes G4 and G9 (Kirkwood *et al.*, 1993). Moreover, while all the antigenic regions are widely separated on the linear sequence, some of them are located in close proximity to one another when considering the three dimensional structure of VP7. Regions A and C are juxtaposed in serotypes G1, G2, and G3 and may influence each other (Dunn *et al.*, 1993; Dyall-Smith *et al.*, 1986; Lazdins *et al.*, 1995; Taniguchi and Urasawa, 1995). Also, it has been observed that regions A, C and E are in close proximity with each other in serotype G2 (Das *et al.*, 1993; Kirkwood, 1996). The antigenic region F is reactive to serotype G3, G4 and G9 specific monoclonal antibodies and contains serotype-specific and serotype cross-reactive epitopes detectable in the absence of glycosylation at position 238 (Dunn *et al.*,

1993; Kirkwood *et al.*, 1996a; Kirkwood *et al.*, 1993). The antigenic region E may only be identified in some rotaviruses (Dunn *et al.*, 1993; Lazdins *et al.*, 1995; Caust *et al.*, 1987).

Glycosylation is an important property of VP7 that influences its antigenicity. In one study, SA11 escape mutants selected with specific N-MAbs were found to have a new glycosylation site in region C that led to marked antigenic alterations (Caust *et al.*, 1987; Lazdins *et al.*, 1995). The presence or absence of glycosylation can determine whether a MAb will select substitutions in the antigenic regions A or C, with glycosylation at aa190-192 blocking access of N-MAb to region A (Lazdins *et al.*, 1995; Kirkwood *et al.*, 1993). Antigenicity was also altered by glycosylation in region F (Kirkwood *et al.*, 1993; Mendez *et al.*, 1996).

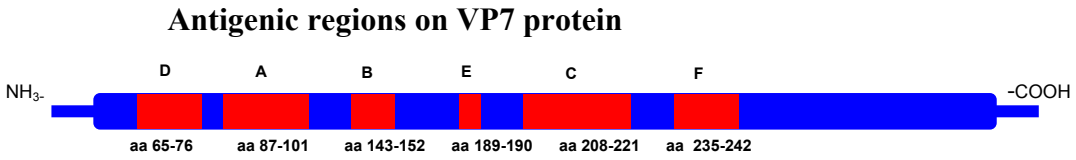

Figure 1.8 Location of antigenic regions on VP7 protein of rotavirus. Each red box indicates one antigenic domain.

Furthermore, the two outer capsid proteins of rotavirus are known to interact with each other (Fig 1.9) and thus mutation in one protein have been proposed to affect the antibody binding activity of the other (Mendez *et al.*, 1999; Zhou *et al.*, 1994). An epitope on the VP8* subunit of VP4, at residues 180-183, has been suggested as a site which interacts with VP7 (Zhou *et al.*, 1994).

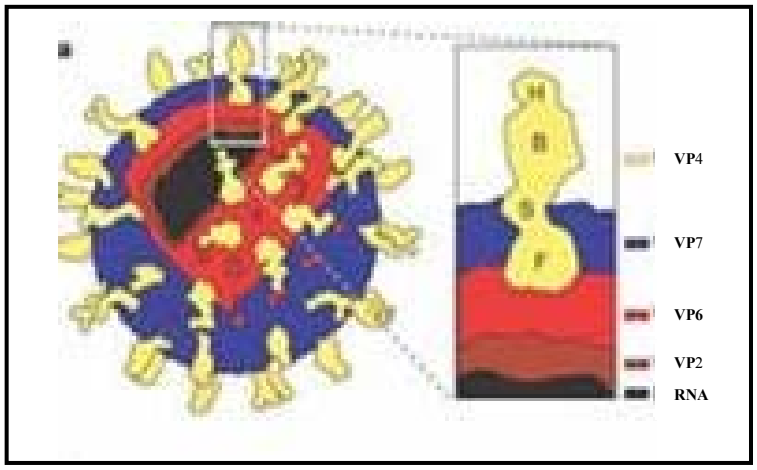

Figure 1.9 Structural proteins of rotavirus and their interaction with each other. Note the spike protein, VP4, interacts with both VP7 and VP6 proteins. Source: www.medspace.com

1.7 Pathogenesis of rotavirus

Rotavirus infection causes disease by three different pathogenic mechanisms. First, rotavirus infects mature villus tip enterocytes, thus affecting absorption of nutrients and liquid in the gut (see below). Second, rotavirus is able to secrete the virus-encoded non-structural protein, NSP4, which acts as an enterotoxin and mobilises fluid secretion (section 1.2.5.2). Finally, effects on the enteric nervous and vascular systems stimulate indirect secretion (Arvin and Greenberg, 2006; Estes *et al.*, 2001; Glass *et al.*, 1996).

1.7.1 Manifestation of rotavirus infection

Rotavirus is a major cause of viral gastroenteritis in infants, children and the young of various animals. Rotavirus represents 80% of recognised viral etiologies of gastroenteritis and 140 millions cases of diarrhoea per year worldwide. In developing countries, rotavirus disease accounts for one-third of all hospitalisations for diarrhoea (Glass *et al.*, 1996; Jiang *et al.*, 2002; Parashar *et al.*, 2006). In developed countries, where general sanitation and hygienic conditions are better, rotavirus still poses a serious health problem and results in substantial medical costs (Jiang *et al.*, 2002; Parashar *et al.*, 2003). The virus strikes with similar frequency throughout the world

but mortality rates are higher in developing countries. The followings are important features of rotavirus disease (Ball *et al.,* 2005; Hsu *et al.*, 2005; Parashar *et al.*, 2006):

- Rotavirus causes up to 610,000 deaths annually.
- Rotavirus infection leads to 2.3 million hospitalisations each year worldwide
- 82% of total rotavirus mortality occurs in developing countries
- In Australia there are10,000 hospital admissions annually as a result of rotavirus infection (Barnes, 2002)
- Rotavirus causes 20-40 deaths and 55-80,000 hospitalisations each year in the US (Newsrx, 2006)
- In Australia, thirteen rotavirus related deaths have been reported (Newall *et al*., 2006).

1.7.2 Pathology of rotavirus-infected cells

One of the rotavirus non-structural proteins, NSP4, acts as a viral enterotoxin, contributing to its pathogenesis (Ball *et al.*, 2005; Estes and Morris, 1999). The enterotoxin can trigger a signal transduction pathway that leads to mobilization of intracellular calcium and chloride ion secretion (Ball *et al.*, 1996; Estes *et al.*, 2001; Estes and Morris, 1999). Rotavirus infects the villi of the small intestine, causing the shortening of the villus and thus malabsorption of fluid (Fig 1.10A and 1.10B). The infection causes disruption of epithelial cell structure and functions within 18 –24 hours after infection and may lead to cell death. The acute infection usually resolves in 7 days in immunocompetent hosts, however, in severe cases, death can occur due to severe loss of fluid, poor absorption of fluid and nutrients, and shock (Estes *et al.*, 2001; Holmes *et al.*, 1975; Parashar *et al.*, 2003a).

The pathological changes observed in the jejunal mucosa in young children hospitalised with rotavirus disease including shortening and atrophy of the villi, cell infiltration in the lamina propria and irregular microvilli (Holmes, 1975; Mebus *et al.,* 1977; Moon, 1994). Detailed information on pathogenic changes has been obtained by directly infecting animals. Within half an hour of infection, morphologic changes such as

swelling of the villi and flattening of epithelial cells were observed in the upper intestine, but the lower intestine was intact. Seven hours after the infection, upon onset of diarrhoea, the lower intestine showed morphological changes similar to that of large intestine and forty hours later, the intestine appeared normal (Davidson *et al.*, 1977 Mebus *et al.*, 1977). In another experiment, involving administration of a human rotavirus to a baby pig, physiological alterations in small intestine were observed; glucose transport was affected, sucrase activity was decreased and thymidine kinase activity was increased (Davidson *et al.*, 1977). In permissive cells *in vitro*, cytopathic effect was observed and cell death occurred due to shutting off of host cell DNA, RNA and protein synthesis (Carpio *et al.*, 1981; Ericson *et al.*, 1983; Kim *et al.*, 1977; McCrae and Faulkner-Valle, 1981).

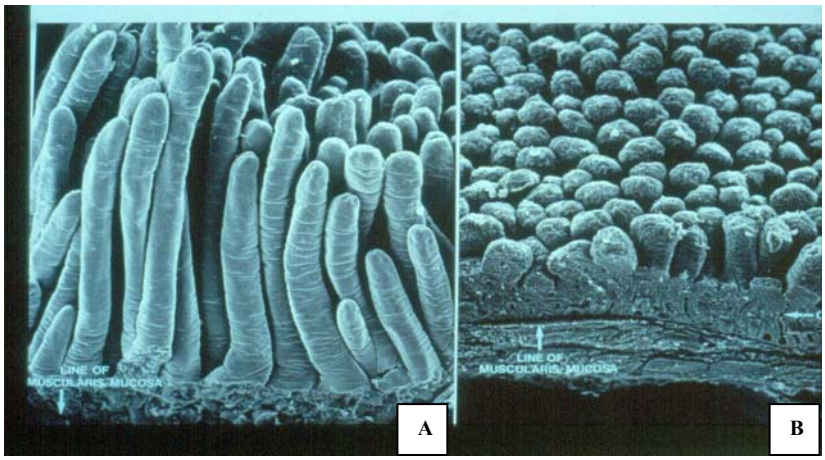

Figure 1.10 Electron Microscopy (EM) of (A) normal villi. (B) EM of villi after rotavirus infection. (Photo courtesy of D. Snodgrass)

1.7.3 Age distribution of rotavirus infection

Rotavirus is a major cause of severe infection in infants and young children under five years of age. Though it is found to cause infection in adults, the number remains comparatively low. Children under six months of age are believed to be protected by maternal antibodies, however, neonatal strains are commonly identified in hospital nurseries in many countries, including Australia, and cause asymptomatic to mild symptomatic infections in neonates (Bishop, 1996).

The major age group burdened by rotavirus related illness falls between children aged 6-23 months (Bishop, 1996). For example, in a longitudinal epidemiological study of 2,897 patients in the US conducted from 2000-2004 approximately 50% of total population infected with rotavirus ranged between 9-12 months of age and the incidence decreased as the age of patients increased (Fig 1.11).

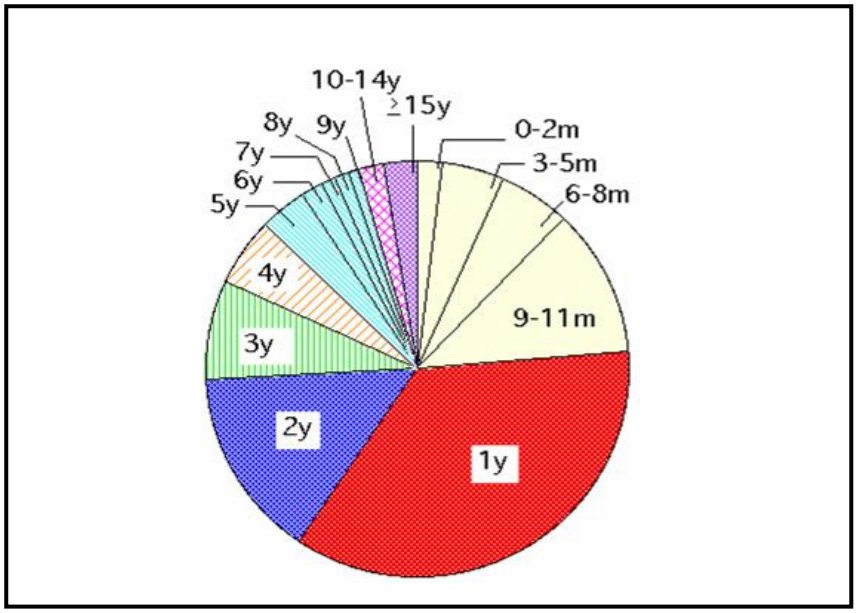

Figure 1.11 Age distribution of rotavirus detected cases from 2000-2004, total cases 2,897. Source: IASR (Infectious Agents' Surveillance Report, 2004, CDC).

1.7.4 Rotavirus infection in adults

Adults are infected by rotavirus quite frequently but immunity from previous exposure protects against symptomatic illness or results in only minimal clinical manifestations. Fifty five percent of adult showed serologic evidence of rotavirus infection at the time their children were admitted to hospital due to rotavirus infection (Anderson and Weber, 2004; Hoshino *et al.*, 1985; Kapikian *et al.*, 1983). A recent report from India demonstrated the spread of rotavirus infection from children to grandparents, causing diarrhoea. This highlighted the potential risk of transfer of rotavirus from children to the elderly (Awachat and Kelkar, 2006). There have been a few outbreaks of rotavirus infection in adults in army recruits in Finland as well as in geriatric wards in other countries like Japan, Thailand and India (Anderson and Weber, 2004; Bishop and

Barnes, 1997). Due to high level of the rotavirus immunity acquired from earlier infections, the symptoms are not as severe as in children. However, in immunocompromised adults, rotavirus infection can have a variable course from symptomless to severe infection (Anderson and Weber, 2004; Tao, 1988).

Group B rotaviruses have been reported to cause severe gastroenteritis in adults in various parts of China involving 12,000 to 20,000 individuals showing cholera-like watery diarrhoea and the deaths of a few elderly patients (Cox and Medley, 2003; Tao, 1988). A recent serological survey in the UK measured IgM antibodies as a marker of recent infection due to rotavirus. The serum of healthy adult patients was collected every month for a year. The results showed high and variable levels of IgM antibodies titres which increased during some seasons. The study suggests the need for further exploration of the area of adult rotavirus infection and especially the role of IgM antibodies, their persistence in the serum and their cross reactivity (Cox and Medley, 2003).

1.7.5 Nosocomial infections

Hospital-acquired infections by rotaviruses occur frequently, as reported in several studies (Berner *et al.*, 1999; Bishop *et al.*, 2001; Gelber and Ratner, 2002). Nosocomial infections have been reported in several parts of the world in neonatal nurseries (Bishop *et al.*, 2001). In Germany, over a 10 year survey, the nosocomial infection rate in premature neonates was 26% of total rotavirus-associated infections (Berner *et al.*, 1999; Piednoir *et al.*, 2003). A study conducted in a paediatric ward in a hospital in France, indicated that the attack rate and the incidence of healthcare-associated acquired rotavirus infection were 6.6% and 15.8 per 1000 hospital days, respectively, during a winter outbreak (Kapikian *et al.*, 1983a; Piednoir *et al.*, 2003). These findings clearly demonstrate the high risk and substantial expense incurred as a result of health-associated infections caused by rotavirus in children.

1.7.6 Transmission of the virus

Rotaviruses are predominantly transmitted by faecal-oral route, although contaminated food, water or fomites are involved in spread of the virus. In an early study, rotavirus contaminated stool particles, when administered orally, induced the diarrheal illness in volunteers (Bernstein *et al.*, 1986; Kapikian *et al.*, 1983; Morris *et al.*, 1975). There has been speculation about the respiratory route of transmission with scattered reports of the detection of group A rotavirus in upper respiratory tract (Jian *et al.*, 1991).

Resistance to physical conditions of the environment and their stability at ambient temperature has contributed to efficient transmission of the human rotaviruses (Morris *et al.*, 1975). A report from Guinea-Bissau, West Africa, observed that rotavirus-positive faecal samples, unintentionally left at ambient temperature of above 30^{o}C for two and half months due to an armed conflict, retained infectivity and contained functional RNA. After the conflict ceased and the samples were tested, VP4 and VP7 genotypes were able to determined and a few samples were also successfully cultivated (Fischer *et al.*, 2002a). While this study showed that rotavirus can survive elevated temperature, high humidity (80%) inactivates rotavirus (Ansari *et al.*, 1991; Koopman, 1978). Effective disinfection of contaminated materials and adherence to good hygienic practices help to control the spread of rotavirus infection in hospitals and institutional settings (Black *et al.*, 1981; Nakagomi and Nakagomi, 1993).

It has been speculated that animals have contributed to the spread of rotavirus infections to humans, speculation supported by the observations that certain rotavirus share neutralising antigens with human rotaviruses and that certain naturally-occurring animal strains may infect humans or form reassortants with human strains (Das *et al.*, 1993; Nakagomi and Nakagomi, 1991; 1993). Although such reassortment is a rare event, certain human-bovine or human-porcine reassortant strains appear to be endemic in parts of India and Brazil (Das *et al.*, 1993; Davidson *et al.*, 1975; Gouvea *et al.*, 1999).

1.7.7 Incubation Period

The incubation period of rotavirus infection has been estimated to be around 48 hours (Kapikian *et al.*, 1983; Ramig, 2004). The disease is usually self-limiting in immunocompetent hosts. In adults, the incubation period may vary from 1 to 4 days (Kapikian *et al.*, 1983a; Yolken and Wilde, 1994).

1.8 Clinical Features

Rotavirus causes infection in all age groups, but children are more susceptible to the disease. The infection produces subclinical to mild to severe diarrhoea and occasionally fatal dehydrating illness. Rotavirus infection is most commonly characterised by the onset of acute diarrhoea with a temperature of 37.9°C or above and vomiting in most cases, causing severe dehydration (Fig 1.12). Other clinical symptoms such as irritability, lethargy, pharyngeal erythema, rhinitis and palpable cervical lymph nodes are also associated with the diarrhoea (Estes *et al.*, 2001).

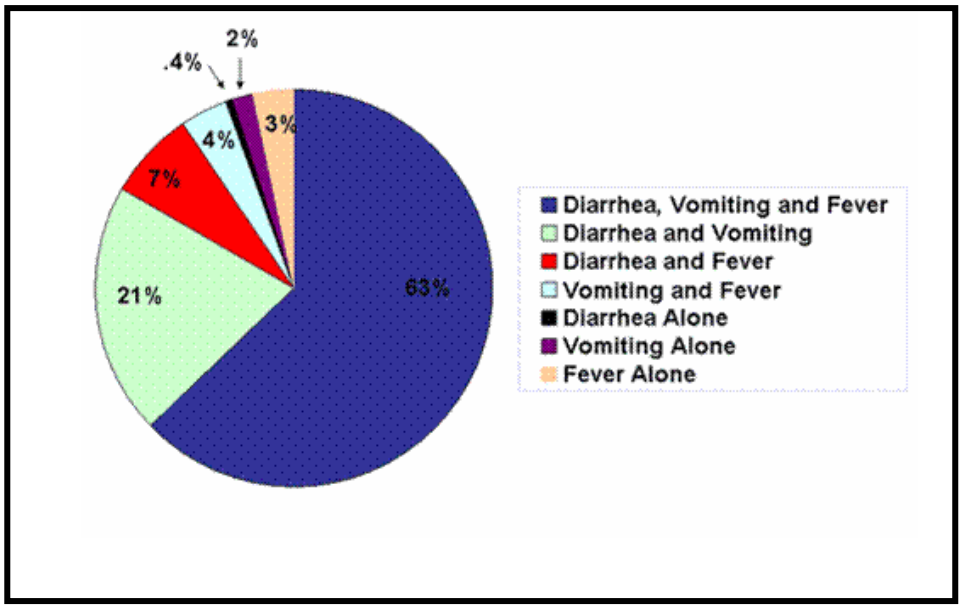

Figure 1.12 Symptoms caused by rotavirus infections. Source: www.medscape.com

Rotavirus can produce chronic illness in some individuals, especially the immuno-suppressed, bone marrow transplant recipients, and person suffering from hepatitis (Thea *et al.*, 1993; Yolken and Wilde, 1994). Rotaviruses, however, have not been found to cause any severe illness in AIDS patients (Konno *et al.*, 1977; Konno *et al.*, 1978; Thea *et al.*, 1993). Rotavirus was detected in stools of 11 out of 30 children in Japan with intussusception; 5 of the 7 rotavirus positive children tested developed a serologic response, suggesting that wild type rotavirus might have been the cause of intussusception. Another investigation showed that 33% of intussusception cases (61 patients studied) had rotavirus infection (Konno *et al.*, 1977; Konno *et al.*, 1978; Mulcahy *et al.*, 1982). Three other studies conducted in Australia, France and Germany failed to find a significant association between rotavirus and intussusception (Mulcahy *et al.*, 1982; Staatz *et al.*, 1998; Ward, 1996). The first rotavirus vaccine, RotaShield, licensed in the USA in 1998, was linked to the cases of intussusception in vaccinated children. Simonsen *et al*. (2005) found strong correlation between the Rotashield and cases of intussusception in 80% vaccinated children of age >three months. Similarly, rotavirus was identified in 7 of 8 tissue samples of children who received Rotashield vaccine and developed intussusception (Lynch *et al*., 2006), due to this link, RotaShield was withdrawn from the market a year later (CDC, 1999).

1.9 Immunity

Rotavirus infection induces both local intestinal and systemic immune responses (Kapikian *et al.*, 2001; Ward, 1996). A rapid humoral antibody response is induced by rotavirus infection that is correlated with intestinal immunoglobulin A (IgA) (Ebina *et al.*, 1983; Kapikian *et al.*, 2001). It is understood that locally produced antibodies in the intestinal mucosa play a key role in protective immunity against rotavirus-induced diarrhoea (Coulson *et al.*, 1990). Rotavirus specific IgA has been detected in duodenal fluid, serum, faecal material and breast milk (Bernstein *et al.*, 1986; Coulson *et al.*, 1990; Schwartz-Cornil *et al.*, 2002). An interesting observation is that systemic rotavirus antibodies are present in the lumen of the gastrointestinal tract of neonatal calves, if the level of circulating antibodies is sufficiently high. Such serum derived

mucosal antibodies can provide protection against experimentally induced infection and diarrhoea (Armstrong and Dimmock, 1992; Besser *et al.*, 1988).

IgA is found to prevent epithelial cell attachment by virus by cross-linking and aggregation of virus particles (Armstrong and Dimmock, 1992; Gilbert *et al.*, 2001). Studies in mouse models have also shown that IgA inhibits the assembly of rotavirus by preventing association with the outer capsid VP7 protein (Blutt *et al.*, 2004; Estes *et al.*, 2001; Gilbert *et al.*, 2001). VP7 induces polyclonal B-cell activation independent of T-cell lymphocytes (Blutt *et al.*, 2004; Franco and Greenberg, 1995). The specific cellular mechanism of the B-cell response is yet to be clearly determined. Although the B-cell arm of the immune response plays a major role in controlling rotavirus infection, experiments involving the infection of B-cell-deficient mice indicate that the actual mechanism itself is not clear (Burns *et al.*, 1996; Franco and Greenberg, 1995; Kapikian *et al.*, 2001; McNeal *et al.*, 1995). It has found that both structural and non-structural proteins of rotavirus induce the production of antibodies, although antibodies to VP6 are non-neutralising but stimulate protective immunity in some animals (Burns *et al.*, 1996; Hjelt *et al.*, 1987). Rotavirus IgA antibody levels were also shown to correlate with resistance to severe diarrhoea (Hjelt *et al.*, 1987; Matson *et al.*, 1993). The association between faecal (IgA) and serum (IgG) anti-rotavirus antibody titres and protection against disease was investigated in 100 children in the US (O'ryan *et al.*, 1994; Matson *et al.*, 1993). During two seasons, seven outbreaks of G1 and G3 rotavirus were observed. These studies concluded that: a) anti-rotavirus IgA titres of 1:80 and 1:20 correlated with protection against infection or illness, respectively, b) pre- existing serum anti-rotavirus IgA titre of greater than 1:200 or IgG titre of greater than 1:800 was associated with protection against infection and c) a high level of pre-existing G type-specific blocking antibody was associated with protection against infection. Similarly, in a study, a direct correlation between a high level of anti-rotavirus intestinal IgA antibody and a significant degree of protection of children against natural rotavirus infection and illness was observed (González, *et al*., 2005).

VP4 and VP7 proteins independently induce neutralising antibodies and each antibody plays a role in resistance to disease (Clemens *et al.*, 1993; Hoshino *et al.*, 1988; Ward *et al.*, 1998). Although serotype-specific antibodies have been demonstrated in breast

milk, the effect of these on protection is yet to be established (Clemens *et al.*, 1993; Golding *et al.*, 1997; Newburg *et al.*, 1998; Ward, 1996).

Mouse models have been used to study the immune mechanisms involved in rotavirus-induced diarrhoea (Feng *et al.*, 1997; Franco and Greenberg, 1999). The following results have been concluded from those studies: i) rotavirus specific antibodies are of primary importance in protection against rotavirus reinfection, ii) in general, homologous rotavirus strains are far more potent than heterologous rotavirus strains in inducing protective local humoral immune responses, iii) MHC class I-restricted CD8+ αβT cells play a major role in resolution of primary rotavirus infection, iv) after primary infection, CD8+ T cells mediate almost complete protection (up to 2 weeks) or partial protection (3 months or less) from reinfection, however this protection diminishes after 8 months after primary infection, v) perforin and interferon-γ are not essential in anti-rotavirus activity mediated by CD8+T cells and vi) CD8+T cells expressing the $\alpha_4\beta_7$ marker (an integrin receptor) are more efficient in mediating rotavirus clearance than $\alpha_4\beta_7$ negative cells (Feng *et al.*, 1994; Franco and Greenberg, 1995; McNeal *et al.*, 1995; Moser *et al.*, 1998; Rose *et al.*, 1998; Sack *et al.*, 1994). These observations indicate that the success of any rotavirus vaccine will depend greatly on its ability to produce mucosal immune responses, since intestinal immunity is found to be protective against rotavirus diarrhoea.

1.10 Treatment

Replacement of lost fluid and electrolytes is the main treatment for rotavirus diarrhoea. Intravenous fluid administration has been used normally with best results. However, in many parts of world, intravenous fluid replacement is not always possible, thus, oral rehydration method using various formulations of oral rehydration salts (ORS) has been used. ORS mainly contains sodium, chloride, potassium, citrate and glucose. The various concentrations of these ingredients have been described by the World Health Organisation (WHO). The standard formula of oral glucose electrolytes recommended by WHO is composed of the following: sodium, 90mmol/L; chloride 80mmol/L; potassium, 20mmol/L; citrate, 10mmol/L; and glucose, 111mmol/L resulting in an

overall osmolarity of 310 mmol/L (Alam *et al.*, 1999; Prasad *et al.*, 1988; Sack *et al.*, 1994).

1.11 Methods of detection of Rotavirus

Rotavirus is detected in stool by various laboratory techniques:

1. Electron Microscopy (EM)
2. Enzyme Linked immunosorbent assays (ELISA)Latex agglutination
4. Polyacrylamide Gel Electrophoresis (PAGE)
5. Reverse Transcription Polymerase Chain Reaction (RT-PCR)
6. Nucleic acid hybridisation (Northern Blots)
7. Cell Culture

1. Electron Microscopy (EM)

Heavy shedding of virus takes place in infected patients from the first to the fourth day of illness. Direct visualisation of rotavirus in stools after negative staining using electron microscopy gives the confirmatory diagnosis, as rotavirus has a distinct morphology (Fig 1.13). Complete rotavirus particles measure around 75 nm in diameter with icosahedral symmetry. The capsid consists of an outer and inner shell and can be also be clearly visualised using cryomicroscopy (Prasad *et al.*, 1988; Yolken and Wilde, 1994).

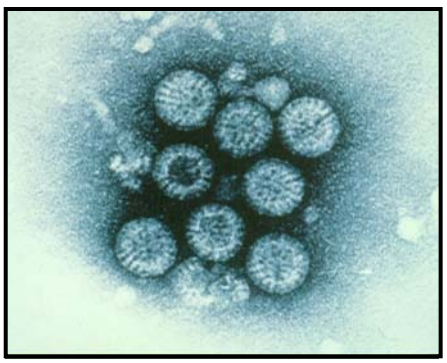

Figure 1.13 Electron microscopy (negative stain) of rotavirus

2. Enzyme Linked immunosorbent assays (ELISA)

This is a highly sensitive method of rotavirus diagnosis used by many laboratories worldwide to detect the virus directly in stool samples. It does not require any special equipment and is a comparatively less expensive technique to detect rotavirus in stool samples (Fujii *et al.*, 1992; Yolken and Wilde, 1994). Rotavirus can be detected by reactive antibodies against epitopes of VP6 shared among group A rotaviruses. Monoclonal antibodies (MAbs) are also used for the determination of subgroups within group A rotavirus. An enzyme immunoassay has also been developed for the detection of groups B and C (Coulson *et al.*, 1987). This method can also be used to determine the G- and P-type of rotaviruses (Coulson, 1996).

3. Latex agglutination (LA)

Latex particles coated with anti-rotavirus antibodies agglutinate in the presence of rotavirus antigens to produce macroscopically visible agglutinates. This method is used in various hospital diagnostic laboratories to quickly determine the cause of diarrhoeal infection. Although this method is comparatively rapid and easy, it may give false positive reactions due to non-specific reactivity against other enteric viruses. This method is commonly used in hospital for screening of rotavirus in stool samples only. Positive tests need to be confirmed by ELISA and/or RT-PCR.

4. Polyacrylamide Gel Electrophoresis (PAGE)

The 11 segments of the double stranded RNA genome, extracted from rotavirus-positive stool samples, are electrophoresed in polyacrylamide gels under specified electrophoretic conditions. The characteristic pattern of 11 segments of RNA is then observed by staining the gel with silver nitrate or ethidium bromide (Coulson *et al.,* 1987; Dunn *et al.*, 1993). This method is highly specific and extensively used to study the epidemiology of rotavirus strains circulating in a community. Similarities and differences in migration patterns can provide information about the extent of genetic variability between strains of the same serotype (Dunn *et al.,* 1993; Gouvea *et al.*, 1991).

5. *Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and semi-nested PCR*

This sensisitivty of PCR-based methods is 100,000 times more than that of PAGE (Coulson *et al.*, 1999). Commonly used methods are based on the genes encoding VP7 and VP4 as these can be used to detect the presence of rotavirus and determine the virus genotype. First round RT PCR, using gene-specific consensus primers, is followed by semi-nested second round PCR incorporating a pool of primers specific for each G-type or P-type (Eiden *et al.*, 1987; Gentsch *et al.*, 1992; Gouvea *et al.*, 1990; 1991). The size of the resultant amplicon in the second round PCR is used to determine G- or P-genotype.

6. Nucleic acid hybridisation (Northern Hybridisation)

Dot blot hybridisation assays can be used to detect rotavirus *in situ* by immobilising denatured dsRNA onto a nitrocellulose membrane. This method is very specific and sensitivity is similar to direct EM, PAGE or ELISA (Eiden *et al.*, 1987; Flores *et al.*, 1983). In a comparative study, dot blot hybridisation was 10 to 100 fold more sensitive than ELISA (Greenberg *et al.*, 1981; Flores *et al.*, 1983). By using this technique, a virus RNA concentration as low as 8 picograms can be detected. Northern hybridisation, which involves separation of dsRNA segments by PAGE prior to transfer to a membrane, is carried out to determine the genogroup of a rotavirus strain (Nakagomi and Nakagomi, 1996). Liquid hybridisation techniques are also used to determine genogroups (Browning *et al*., 1992).

7. Cell Culture Adaptation of Rotavirus

Rotaviruses can be recovered from stool specimens by direct cell culture with a reasonable success rate. Growth of rotavirus in tissue culture makes it possible to determine serotypes by neutralisation assays. Growing human rotaviruses *in vitro* can be difficult and usually requires initial adaptation in primary monkey kidney cells before passage in continuous monkey kidney cell lines. Various animal rotavirus strains, e.g. SA11, can be readily cultivated in cell culture (Greenberg *et*

al., 1982). The first human rotavirus to be grown successfully in cell culture was a mutant of the Wa strain. Efficient propagation and cultivation of rotaviruses *in vitro*, especially human strains, requires trypsin treatment (Benureau, *et al*., 2005; Gilbert and Greenberg, 1997).

1.12 Epidemiology of Rotavirus

Rotavirus is ubiquitous and extremely diverse in terms of the variety of circulating serotypes and genotypes, as discussed in section 1.4. Information about the antigenic and genomic variability is important for virus epidemiology and surveillance studies. Such studies are of great importance for monitoring the effectiveness of new rotavirus vaccines.

Most common rotavirus strains causing diarrhoea in children belong to group A, however group B rotaviruses have caused outbreaks in adults in China (Caul *et al.*, 1990) and group C viruses have caused occasional small outbreaks in some families but occur less frequently in children (Caul *et al.*, 1990; James *et al.*, 1998; Jiang *et al.*, 1995). However, a study in the UK showed that 43% of the population were seropositive to human group C rotavirus, when diagnosed with an antigen-detection ELISA developed with hyperimmune antibodies raised to recombinant human group C rotavirus VP6 (Bristol strain) expressed in insect cells. This unexpected result suggests that the extent of group C rotavirus infection may be greater that previously thought, probably due to the fact that a routine diagnostic test for this virus is not commonly available (Blackhall *et al.*, 1996; James *et al.*, 1998).

1.12.1 Molecular epidemiology of rotavirus

Like most RNA viruses, the rotavirus genome is very flexible and it is observed that the genome can undergo point mutation at the rate of $5X10^{-5}$ per nucleotide, 1000 times higher than for most of DNA viruses (Blackhall *et al.*, 1996; Rahman *et al.*, 2005a). Since this study is based on human rotaviruses, about the following discussion will focus on group A rotaviruses, which is a leading group causing infection in children.

As described previously (section 1.4), a dual classification system based on the outer capsid proteins, VP7 (Glycoprotein, G-type) and the VP4 spike protein (Protease sensitive, P-type) has been developed. So far, 15 G-types and 26 P-types of rotavirus have been reported (Desselberger *et al.*, 2001; Martella *et al.*, 2005; Rahman *et al.*, 2005). Various combinations of G-and P-type exist in nature giving a huge variety of rotavirus types. Based on this dual classification, a particular strain is known by describing its G and P types together, eg. G1P2A[8], meaning this rotavirus strain belongs to G-type 1, P-serotype 2A and P-genotype 8. There has been complete agreement between the genotype and serotype of G types, but such agreement is not available for P types, due to difficulty in developing serological typing reagents, such as monoclonal antibodies, against VP4.

Different G- types and P-types co-circulate in the community at any time. The most common types globally are G1P1A[8], G2P1B[4], G3P1A[8] and G4P1A[8]. The relative frequencies of strains in circulation change over time. The possibility of reassortment taking place during mixed infections leads to the generation of various G and P type combinations, which contributes to the wide genetic diversity and the evolution of unusual strains. Unusual G/P combinations such as G8P[8], G1P[6] or G9P[6] have been reported worldwide. G1P1B[4], G2P1A[8] are reassorted strains that could have arisen from animals or exotic human sources (Desselberger *et al.*, 2001; Maunula and Bonsdorff, 2002). Apart from reassortment, two other genetic phenomena are involved in creating new strains: point mutation and genetic rearrangement.

Point mutations occur spontaneously, but can accumulate over time and result in the development of different lineages or sub lineages within types (Desselberger, 1996; Iturriza-Gomara *et al.*, 2001; Iturriza Gomara *et al.*, 2004). Genetic rearrangement has been shown to occur both *in vitro* and *in vivo* (Desselberger, 1996; Ramig, 1997). However, reassortment is considered to be most important and significant factor in the generation of strain diversity. This process occurs *in vitro* in mixed infections with different strains of Group A rotavirus and has also been used to study gene-protein assignment (Browning *et al.*, 1992; Ramig, 1997). Comparison of various unusual strains and comparing their gene homology has provided ample evidence that

reassortment also occurs *in vivo* (Browning *et al.*, 1992; Cunliffe *et al.*, 1999; Das *et al.*, 2002; Palombo, 2002; Santos *et al.*, 1999; Unicomb *et al.*, 1999; Zao *et al.*, 1999).
Epidemiological studies conducted in various parts of the world have shown a wide variety of rotavirus strains occurring in nature. In a study by Iturriza-Gomara *et al.* (2001), a collection of over 3000 rotavirus isolates collected during 1995-1998 indicated the presence of four common types, G1P[8], G2P[4], G3P[8] and G4P[8], that constituted over 94% of typed isolates. However, uncommon strains like G1P[4], G2P[8], G4P[4], G9P[6] and G9P[8] were found in smaller frequencies. In contrast, an epidemiological survey conducted in India from 1986-88, showed that 70% of strains belonged to the unusual combination of G9P[11] (Das *et al.*, 2002; Ramachandran *et al.*, 1996). A survey conducted in 1993 showed equal distribution of the common strain G2P[4] and the unusual strain G9P[6], which were found in 21% and 24% of isolates, respectively (Arista *et al.*, 1997; Ramachandran *et al.*, 1996). In two European studies, 38% of isolates belonged to G1P[8], 52% to G4P[8] and 3% to the unusual combination of G9P[8] (Arista *et al.*, 1997; Santos *et al.*, 1994). In a North and central American study carried out in 1990-92, 71% of isolates were of type G1P[8], with 20% of isolates belonging to either G3P[8] or the unusual type G1P[6] (Ramachandran *et al.*, 1998; Santos *et al.*, 1994). In a similar study carried out a few years later (1996-97) the emergence of G9P[6] was observed, increasing from 0% (in 1990) to 6% (Ramachandran *et al.*, 1998). While the four major global rotavirus serotypes continue to be detected, a striking feature of most studies carried out over the last decade is the worldwide emergence of serotype G9.

1.13 Emergence of serotype G9 rotaviruses worldwide

Serotype G9 is an emerging serotype reported globally in high numbers. This serotype is not new and was first detected in the USA in 1983, but was not isolated again until more than a decade later in 1996. To date, serotype G9 rotavirus strains have been reported in more than ten countries, including Thailand, India, Brazil, Bangladesh, Malawi, Italy, France, United States, United Kingdom and Australia (Ramachandran *et al.*, 2000), and is reported as endemic in the US, Bangladesh and Thailand (Jiraphongsa *et al.*, 2005; Ramachandran *et al.*, 1998; Unicomb *et al.*, 1999). In Australia, serotype

G9 was first reported in Melbourne and Perth in 1997 (Kirkwood *et al.*, 2003; Palombo *et al.*, 2000). Soon after their isolation, there was gradual increase in its prevalence, from 0.6% of strains in 1997 to 29% in 2000. In Melbourne, serotype G9 strains made up 11% to 26% of all strains from 1999-2001 (Fig 1.14) (Kirkwood *et al.*, 2004a). In 2001, an outbreak of G9P[8] rotavirus occurred in Central and Northern Northern. In Alice Springs, 69% of rotavirus infection were due to serotype G9P[8] rotaviruses. In Darwin the incidence rate was 81%, while 100% of rotavirus infections in Mount Isa and Gove were due to this serotype, emphasising the huge impact on public health.

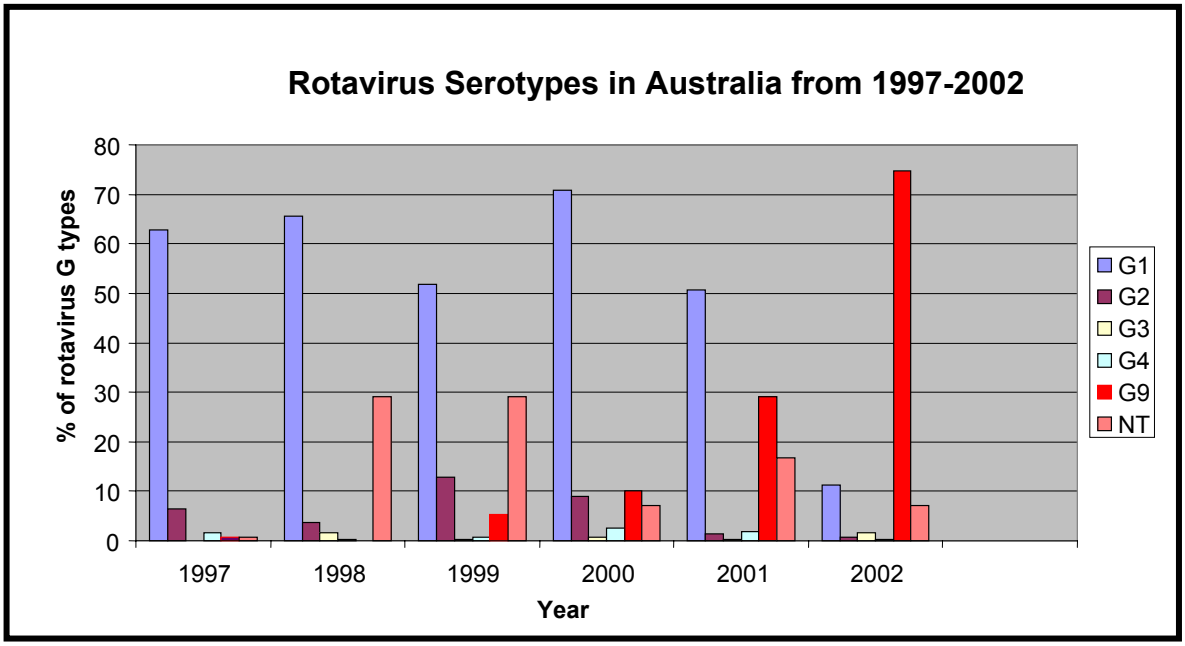

Figure 1.14 Serotype distribution of rotavirus isolates in Australia from 1997-2002. Note the emergence of serotype G9 rotavirus and its gradual increase and sharp rise in incidence in 2002 (data adapted from Kirkwood *et al*., 2004a).

These isolates had similar electropherotypes, the same subgroup specificity, and identical VP7 nucleotide sequences (Kirkwood *et al.*, 2004).

In the UK, serotype G9 was first detected in combination with P[4] in 1996, but later seemed to be replaced by G9P[8] strains towards 1997/98 (Iturriza-Gomara *et al.*, 2001; Unicomb *et al.*, 1999). It is likely that the P[8] gene was acquired by reasortment with other strains, such as G1P[8], G3P[8], and G4P[8], co-circulating in the community (Iturriza-Gomara *et al.*, 2001; Ramachandran *et al.*, 2000; Unicomb *et al.*, 1999).

Serotype G9 rotavirus has an interesting history considering that it first appeared in 1983 in the US (Clark *et al.*, 1987) and in Japan in 1985 (Hoshino *et al.*, 2005; Nakagomi, 1990b) but it did not again reappear in either countries or anywhere else until about a decade later. While serotype G9 has re-emerged as one of the major strains causing infection in children worldwide since the mid 1990s (Clark *et al.*, 2004a; Hoshino *et al.*, 2005), the recent G9 strains have distinct genetic and molecular properties compared to the earlier serotype G9 strains (Clark, 2004; Santos, 2005). Unlike other four common G-types (G1-G4) which occur mostly in conjunction with P[8] and P[4], G9 rotaviruses have been detected in association with a wide variety of P types including P[4], P[6], P[8], P[9], P[11] and P[9] (Hoshino *et al.*, 2005; Santos, 2005). The diverse nature of this serotype may have important implications for the development and success of new rotavirus vaccines. Hoshino *et al.* (2005) and Zhou *et al.* (2003)) found that the VP7 genes of a global collection serotype G9 rotaviruses belonged to three different lineages. Lineage 1 includes the strains isolated in 1983 and 1985, which had not been detected anywhere else in the world for about 20 years until recently reported isolates from Japan (Laird *et al.*, 2003; Zhou, 2003). Lineage 2 strains have only been detected in Indian strains causing asymptomatic infection of neonates. Lineage 3 contains the most recent G9 strains identified around the world. These studies suggested that the recent G9 isolates are not the direct descendant of the first G9 isolates or prototype G9 strains that had been reported in 1980s. However, a recent study by Laird *et al.* (2003) found two G9 rotavirus strains from the US (Om46 and Om67), isolated in 1996, that had some similarity with lineage 1 strains, suggesting these two strains may be distant ancestors of the 1980s G9 strains. These interesting findings about this newly emerged rotavirus serotype makes it important to study their origin and evolution, and also suggests the inclusion of this serotype in human rotavirus vaccine formulations needs to be considered.

1.14 Unusual rotavirus strains circulating in human populations

There have been many reports of human rotaviruses with unusual combinations of subgroup, G-type and P-type, suggesting considerable diversity among rotavirus strains worldwide (Coluchi *et al.*, 2002; Das *et al.*, 2002; Fang *et al.*, 2002; Rahman *et al.*, 2003; Santos *et al.*, 2003). Moreover, there are also reports of unusual strains such G5, G8 and G12 occurring locally in certain parts of world (Coluchi *et al.*, 2002; Das *et al.*, 2002; 2003; Fang *et al.*, 2002). For example, in a survey carried out in Brazil, common serotypes G1 and G3 accounted for only 27% and 12% of isolates, respectively, while 60% of strains belonged to uncommon types such as G5 (25%), G10 (16%), and G8 (4%) or were of mixed G types (16%) (Santos *et al.*, 1998). In Thailand, serotype G12 was isolated from 11 neonatal patients with diarrhoea (Pongsuwanna *et al.*, 2002). G12 was first isolated in the Philippines in 1990 and is not reported frequently, however a recent report from Nepal suggested high number of this serotype present in this country (Uchida *et al*., 2006). Serotype G6, an uncommon human serotype but a common bovine type, was isolated in Hungary from a child and upon characterisation, it was found to possess a VP4 gene of type P[9], an unusual combination of G- and P types (Banyai *et al.*, 2003). Two unusual strains, G8P[4] and G10P[9], have recently been reported in Brazil (Volotao *et al.*, 2006). In Australia, strain G6P[13], a strain normally found in cattle, was isolated from a hospitalised child suffering from acute gastroenteritis suggesting a possible reassortment between human and animal rotaviruses (Palombo and Bishop, 1995). These findings indicate that there is a huge diversity of rotaviruses circulating in human populations and, more importantly in the context of public health, animal strains have been found to be the cause of infections in humans (see section 1.15). The new rotavirus vaccines will have to be designed accordingly to target those areas where unusual strains circulate locally.

1.15 Animal strains and the zoonotic potential of human rotavirus infection

The great diversity of human rotaviruses has been contributed to by possible transmission from animal rotaviruses, either as whole viruses (Gentsch *et al.*, 1993) or by contributing individual genes during reassortment in mixed infections (Nakagomi *et al.*, 1989). Animal rotaviruses are even more flexible and exhibit a greater number of possible G/P combination than human rotaviruses. There are a number of serotypes that

are found both in animals and humans, such as G3 which is found in cats, dogs and monkeys) (Das *et al.*, 1993; Mochizuki *et al.*, 1997), G9 which has been identified in lambs and pigs (Arista *et al.*, 1997; Fitzgerald *et al.*, 1995; Ramachandran *et al.*, 1996; 1998) and P[6] which is found in pigs (Leite *et al.*, 1996; Ramachandran *et al.*, 1996). In some cases the genetic relatedness determined through nucleotide sequencing has suggested an animal rotavirus as the likely origin of human isolates (Browning *et al.*, 1992; Gentsch *et al.*, 1993; Santos *et al.*, 1999).

1.16 Seasonal and climatic variation of human rotavirus infection

Rotavirus is distributed throughout the world and diarrhoeal illness associated with the virus has been detected in every country it has been sought. In temperate climates a typical pattern of infection is observed with epidemic peaks in the cooler months. However, the usual seasonal pattern of rotavirus infection observed in temperate climates does not occur uniformly in other climates, such as the tropics, where infections occur regularly throughout the year (Cook, 1990).

In Melbourne, Australia, in a 13-year study conducted from April 1980- March 1993, 4,637 children from 0–14 years of age were admitted with gastroenteritis for treatment in a large paediatric hospital. Group A rotaviruses occurred in 39.6% of children, 55% of those were aged 12-23 months and 18.7% were aged under 6 months. As is typical for a temperate climate, rotavirus was responsible for a peak in admissions during the winter months (Barnes *et al.*, 1998).

In a large Australian-wide study investigating 4,634 children less than 5 years of age admitted to hospital in a number of major urban centres from 1993-1996, contrasting epidemiological patterns were noticed. In all temperate climates, rotavirus incidence peaked during winter (Bishop *et al.*, 2001). Centres caring for aboriginal communities, however, no relationship between rotavirus incidence and climate was evident, with widespread dissemination of few rotavirus strains over distances of more than 1,000km (Bishop *et al.*, 2001; Fischer *et al.*, 2002a). The genetic complexity of strains, based on electropherotypes, was greatest in centres with larger populations. Identical

electropherotypes appeared each winter in more than one centre, indicating the spreading of some strains from west to east and from east to west across the continent. In Japan, a recent study showed the usual winter rotavirus peak existed in cooler months (January to February) from 1974-1981 have shifted from January to March in recent time during a twenty one year survey (Suzuki *et al.,* 2005). Winter rotavirus peak was also observed in France (Chikhi-Brachet *et al*., 2002).

1.17 Surveillance

Due to the high incidence of severe gastroenteritis illness caused by rotavirus and the loss of lives in poorer countries across the world, the development of a global rotavirus vaccine has been sought with much expectation and interest. The extent of genetic and antigenic diversity among human rotaviruses, and the possibility of animal strains contributing genes to human strains, mean that continuous surveillance of human as well as animal rotaviruses is essential to establish the efficacy of candidate rotavirus vaccines against the wide variety of circulating rotaviruses in various parts of world (Barnes, 1999; Kapikian *et al.*, 2001).

While there have been numerous studies investigating the distribution of rotavirus serotypes in various locations worldwide, the following studies highlight the need for continual surveillance to detect changes in serotype prevalence. In a national rotavirus surveillance program in carried out in ten different regions of China during 1998-2000, 41% of the 3,177 faecal samples from children with acute diarrhoea investigated were rotavirus positive. Serotype G1 was the predominant type (72.6%) followed by G3 (14.2%), G2 (12.1%), G4 (2.5%), G9 (0.9%) and untypeable isolates (0.7%) (Cubitt *et al.*, 2000; Fang *et al.*, 2002). The results indicated that although the common rotaviruses were predominant, the diversity of strains was much greater than apparent from previous studies.

During routine surveillance of diarrhoea caused by rotavirus in Calcutta, India, the emergence of a novel type of human rotavirus, serotype G12, was reported. Three rare human serotype G12 strains were detected from diarrheic samples from children aged

less than 8 months. The VP7 genes of the serotype G12 strains and their deduced amino acid products showed maximum homology (97 to 99% at the nucleotide level and 98% at the amino acid level, respectively) with those of two recently reported serotype G12 strains from the United States and Thailand, but lesser homology with those of the prototype G12 strain, L26 (CDC., 1999a; Das *et al.*, 2003). A recent study conducted in Nepal determined a higher incidence of G12 (20%) rotaviruses infecting young children along with an unusual strain G11P[25] (Uchida *et al*., 2006). In Brazil, serotype G5 are commonly isolated in greater numbers, >10% as reported in many studies (Araüjo, *et al*., 2002; Gentsch, *et al.,* 1996; Linhares, 2000).

The epidemiological characteristics of the recent introduction of serotype G9 strains worldwide may suggest that pre-existing immunity towards other rotavirus serotypes was not sufficient in preventing infection and proliferation of these strains (Cubitt *et al.*, 2000; Iturriza-Gomara *et al.*, 2000; Das *et al.*, 2003; Iturriza-Gomara *et al.*, 2000). Therefore, as with influenza vaccines, the formulation of rotavirus vaccines used in different parts of world may need regular updating to adapt their composition to that of locally or regionally circulating prevalent strains.

1.18 Rotavirus vaccines

Due to the significance of rotavirus infection, its enormous health burden worldwide and, in particular, the staggering death toll in developing countries, the development of an effective rotavirus vaccine has been a major priority. Researchers around the world have been involved in rotavirus vaccine development and the recent licensure of two human rotavirus vaccines was highly anticipated. Development of rotavirus vaccines suffered a huge setback in 1999 when the first rotavirus vaccine, Rotashield (a tetravalent formulation containing reassortants of the rhesus rotavirus strain) was licensed in the US but later withdrawn from the market following increased reports of the risk of intussusception among vaccinated children (CDC., 1999a; Glass and Parashar, 2006).

Despite this setback, after six years of intense research, two new rotavirus vaccines, Rotarix® from Glaxo SmithKline and Rotateq ® from Merck successfully completed phase III trials and have been licensed in many parts of the world, including Australia (see below). Rotarix is a monovalent vaccine, containing a multiply passaged attenuated G1P[8] strain. The molecular basis for the attenuation of this strain is not clear but very large-scale safety studies have demonstrated that the vaccine does not cause diarrhoea or any other enteric disease (Arvin and Greenberg, 2006; De Vos *et al.*, 2004; Dennehy, 2005; Vesikari *et al.*, 2004a). Importantly, this single G and P serotype appeared to have conferred protection against at least serotype G9 and probably several other G types (Arvin and Greenberg, 2006; O'ryan *et al.*, 2005). Rotateq employs a "modified Jennerian" vaccine approach and is based on the bovine strain, WC3, into which human VP7 and VP4 genes have been introduced by genetic reassortment (Clark *et al*., 2006).

These two vaccines are different in formulation and administration schemes, yet the safety of both has been demonstrated and no association with intussusception has been observed in large phase III trials involving more than 60,000 children. These vaccines have been demonstrated to be highly efficacious against severe rotavirus gastroenteritis caused by the most prevalent serotypes (Clark *et al.*, 2004; O'ryan *et al.*, 2005). Rotarix has been effective against 86% of severe diarrhoea caused by rotavirus (Arvin and Greenberg, 2006). Rotateq was highly effective against any rotavirus-associated diarrhoea in 75% of cases and 90% protective against severe diarrhoea (Clark and Offit, 2004b). Despite the promising outlook for these two second generation rotavirus vaccines, the risk of intussusception needs to be closely monitored post-licensure. Recently, both Rotarix and Rotateq were licensed in Australia (Newall *et al.*, 2006) and in many European countries. Rotateq has been licensed in the USA and Finland and some other countries (Linhares and Villa, 2006). However, the availability of these new vaccines in developing countries, where they are needed most, is a major challenge due to the costs involved. Their successful delivery will require joint cooperation and collaboration among governments, vaccine manufacturers and national and international non-governmental organisations.

Despite the availability of the two new rotavirus vaccines, the development of alternative candidate rotavirus vaccines continues. At the National Institute of Allergy and Infectious Disease, NIH, USA, Kapikian and colleagues are involved in developing an alternative reassortment vaccine based on bovine rotavirus, UK strain. This vaccine aims to provide antigenic coverage against the four common serotypes G1, G2, G3 and G4 and two common P types; P[4] and P[8]. However, due to growing reports of uncommon types circulating worldwide, eight additional reassortment vaccines, each of which bears a single human or bovine VP7 gene encoding G5, G8, G9 or G10 specificity and VP4 encoding P[8], have been developed (Barnes, 2002; Hoshino *et al.*, 2003). In Australia, a small randomised double blind controlled phase II trial of a human rotavirus vaccine candidate, RV3, have been carried out This vaccine induced protection in only 43% of the infants vaccinated and while these infants were protected against rotavirus disease in the subsequent winter epidemic, the immunogenicity of this vaccine needs to be improved (Barnes, 2002; Wu *et al.*, 2003).

1.19 Summary

Rotavirus causes a considerable amount of health problems both in developed and developing countries. Hospitalisation resulting from acute severe diarrhoea in developed countries leading to significant financial burdens on public health systems, and the high level of fatalities in developing countries means that rotavirus disease is a global issue. More than 90% of children have experienced at least one episode of rotavirus-related diarrhoea by the age of 5 years. The scenario in underdeveloped world is alarming, with high rates of childhood death. Rotavirus infection cannot be prevented simply by adherence to good hygiene and sanitation; therefore prevention of the disease by vaccination is seen as the only practical means of control. Due to the diverse nature of rotavirus and its ever-changing genetic nature, the epidemiological study and surveillance of rotavirus are vital. There are different serotypes circulating in a community at any given time and the types of strains circulating change in subsequent years. The emergence of new types has been reported worldwide and they pose a serious threat to control of the disease. The recent emergence of serotype G9 worldwide as a cause of severe disease is an important example of this. Therefore, this

study aims to investigate the genetic and epidemic nature of this type in order to understand the origin and evolution of this serotype. There are also reports of unusual strains causing human disease which may have originated from animal or bird rotaviruses through genetic reassortment. The present efforts in developing an effective rotavirus vaccine need to consider the diverse nature of the pathogen and vaccines should aim to target the new emerging types and unusual strains. However, the ultimate aim of controlling this global disease is likely to depend on the availability of vaccines in regions of the world which suffer the greatest burden of rotavirus infection.

1.20 Aims of this study

The major aim of this study is to use genetic and antigenic detection and analysis methods to study the epidemiology of rotaviruses infecting children in Melbourne, Australia. In particular, this study aims to:

1. Determine the distribution and prevalence of rotavirus G- and P-genotypes rotavirus strains isolated in Melbourne, Australia.
2. Determine the extent of diversity among serotype G9 strains circulating in Melbourne over a six-year period (1997-2002) by sequence and phylogenetic analysis of genes encoding structural and non-structural proteins.
3. Examine the antigenic properties of serotype G9 isolates and consider the implications for current efforts to develop and implement new rotavirus vaccine strategies.
4. Compare the Australian serotype G9 isolates to standard strains and strains from other parts of the world in order to understand the diverse nature of this serotype from a global perspective.

Chapter Two

Materials and Methods

2.1 Chemicals, reagents and commercial kits

The materials used in this study are listed below. Full details on the preparation and sterilisation (where appropriate) of these reagents are provided in Appendix I. All the reagents and chemicals used in this study were purchased from Sigma, BDH and Oxoid and were of Analytical Grade (AR) and Molecular Grade (where applicable) unless otherwise mentioned.

2.1.1 Commercial kits

Table 2.1: List of commercial kits, enzymes and reagents used in this study

Name of Kit	Supplier	Purpose
ABI Prism BigDye Terminator Cycle Sequencing Kit v.3	Applied Biosystem	DNA Sequencing
AMV Reverse Transcriptase	Promega	For RT- PCR
Anti-DIG Antibody	Roche Diagnostics	For Probe detection
Blocking Reagent	Roche Diagnostics	Northern Hybridisation
CDP-Star	Roche Diagnostics	For Probe Detection
DIG 11-dUTP	Roche Diagnostics	For Probe Preparation
DNA Isolation Kit	AppliChem	Purification of PCR Products
DNA Markers; 3kb, 1kb & 100bp	Promega	For Size Estimation of PCR Products
Hydroxyapatite (HA); Type I	Sigma	For RNA Purification
Taq Polymerase in buffer B	Promega	For PCR
Total Genome Labelling Kit	Roche Diagnostics	For Probe Preparation

2.1.2 Buffers, reagents and solutions used in this study

A number of buffers were supplied with the kits for use in PCR, DNA purification and labelling of probes. In addition, following buffers were prepared:

Table 2.2: List of buffers, reagents and solutions used in this study

Name of Buffer, Reagent and Solution	Composition
20xSSC buffer	3M NaCl, 0.3M Sodium citrate
3x Sample buffer	30% (v/v) Glycerol, 0.1% (w/v) Bromophenol blue in Upper Tris buffer
BigDye Sequencing buffer	1.4mM $MgCl_2$, 60mM Tris-HCl (pH 7.5), 3M Sodium acetate, pH 5.2
Detection Buffer	0.1M Tris pH 9.5, 0.1M NaCl
EDTA	0.5M
Elution solution for RNA extraction	200mM Potassium phosphate, pH 6.8
Ethidium bromide	10mg/mL
Formaldehyde	6M
High SDS hybridisation buffer	7% (w/v) SDS, 5x SSC, 2 % (w/v) Blocking reagent, 0.1% (w/v) N-Lauryl Sarcosine, 5mM Sodium phosphate, pH 7.0 and 50% (v/v) Formamide
KCl	1M
Lower Tris buffer for PAGE	1.5 M Tris, pH 8.8
Maleic acid buffer	0.1M Maleic acid, 0.15M NaCl, pH 7.5
$MgCl_2$	100mM
PAGE Running buffer	0.3%(w/v) Tris base and 1.44% (w/v) glycine
PCR buffer	10mM Tris-hydrochloride [pH8.3], 40mM KCl, 1.5mM $MgCl_2$, 0.2mM each of dATP, dGTP, dTTP, dCTP and 2.5 unit of *Taq* polymerase enzyme
Phenol-chloroform-isoamyl alcohol	25:24:1
RT-PCR buffer	10mM Tris-HCl [pH8.3], 50mM KCl, 1.5mM $MgCl_2$, 7.0 µl of DMSO, 0.2mM each of dATP, dCTP, dGTP, dTTP
Sodium acetate	1M
TAE buffer	40mM Tris-acetate pH 8.0, 10mM EDTA
TNC buffer	0.01M Tris-HCl pH 7.5, 0.01M $CaCl_2$, 0.15M NaCl
Tris-HCl	1M, pH 8.3
Upper Tris buffer for PAGE	0.5M Tris, pH 6.8
Washing buffer for hybridisation	1xMaleic acid buffer, 0.3% (v/v) Tween 20, pH 7.5
Washing buffer for RNA extraction	10mM Potassium phosphate, pH 6.8

2.2 Collection and preparation of faecal specimens

The samples investigated in this study were collected from two different sources

1. Monash Medical Centre (MMC), Melbourne, Australia

Rotavirus positive samples were collected from the Monash Medical Centre, Melbourne, where primary screening for the presence of group A rotavirus was conducted in the Department of Microbiology using a commercially available Latex Agglutination test (Rotalex, Orion Diagnostics, England). The positive samples were collected and stored at -20^{o}C. These samples were then transferred to our laboratory and stored at -70^{o}C until further use.

2. The Royal Children's Hospital (RCH), Melbourne, Australia

The Enteric Virus Research Group (EVRG) at the Murdoch Children Research Institute, Royal Children's Hospital is a World Health Organisation Rotavirus Reference Laboratory. This laboratory collects and analyses samples from all the major centres around Australia. Seventy nine serotype G9 rotaviruses isolated from 1997-2002 were selected for further analysis, this selection was based upon different electropherotype patterns (e-type) on PAGE, and availability of sufficient amounts of specimens for analysis, 29 samples representing each of different e-types were selected for detailed analysis.

2.2.1 Preparation of 10% faecal homogenates of stool specimens

All the faecal samples selected for this study were stored at –70^{o}C until used. 10% (w/v) faecal homogenates were made in TNC buffer by homogenisation for 30 seconds using a vortex mixer. The homogenate was then centrifuged at 10000g for 1 minute to remove large particulate matters and fibrous materials. The supernatant was collected and stored at – 70^{o}C until required.

2.2.2 Determination of serotype G type of rotavirus specimens collected at Monash Medical Centre by enzyme immuoassay

The samples collected at MMC were G-serotyped by EIA. The serotyping EIA was carried out using a 'sandwich' system with MAbs specific for serotypes G1, G2, G3, G4

and G9 (Kirkwood *et al*., 2005). The specific MAbs used for serotype determination were RV4:1, RV4:2 and RV4:3 (G1 specific), RV5:3 (G2 specific), RV3:1 (G3 specific), ST3:1 (G4 specific) and F45:1 (G9 specific). The MAbs used in this study are described in Table 2.3 and were obtained from the Enteric Virus Research Group. The EIA method is described below and the test format is summarised in Table 2.4.

EIA immunoplates were coated with 100 μL of polyclonal antisera diluted in freshly made 1X PBS pH 7.2. Each column was coated with specific polyclonal antisera as detailed in the plate layout (Fig 2.1). The appropriate dilution of each polyclonal serum was determined, and found to range from 1:1000 to 1:10,000. Polyclonal antisera representing each of the standard G rotavirus types were used. The plates were sealed with adhesive plate sealer (ICN-Flow) and incubated at 37^{o}C for 1.5 hours in a water bath.

The plates were then washed 3 times with PBS/0.05% Tween 20, allowing 3 minutes for each wash, and blotted onto paper towel to remove liquid between each wash. Seventy five microliter (μL) of freshly made 0.5% casein in PBS/0.05%Tween20 was added to each well. Twenty five μL of 10% faecal homogenates were loaded into assigned wells. Positive tissue culture antigens controls representing the common rotavirus G serotypes were used in each assay. Negative controls were added to designated wells. The plate was sealed with adhesive plate sealer (ICN-Flow) and incubated overnight at 4^{o}C. The plate layout with the distribution of MAbs, positive controls, negative controls and samples is depicted in Table 2.4. The reagents and their preparation are listed in Appendix I.

After overnight incubation, the supernanatnt was aspirated from all wells and the plate was washed 3 times with PBS/0.05% Tween 20 allowing 3 minutes for each wash. After each wash the plate was blotted onto absorbent paper towel. One hundred μL of diluted monoclonal antibodies were added to the appropriate wells which contained appropriate polyclonal antibody. The plate was sealed and incubated at 37^{o}C water bath for 2.5 hours. After incubation the plate was washed with PBS/0.05% Tween 20 3 times with each wash of 3 minutes duration. The plate was blotted and 100 μl of diluted horseradish peroxidase-conjugated sheep antimouse immunoglobin conjugate (Silenus)

was added to each well. The appropriate dilution was determined by checkerboard titration using antibody dilutions that ranged between 1:5000 and 1:50,000. The plate was again sealed and incubated at 37^{o}C for 1.5 hours in water bath. After incubation, the plate was washed 3 times with diluted PBS/0.05% Tween 20 and blotted dry.

Table 2.3 Serotype specificity, subgroup specificity and antigenic regions recognised by various monoclonal antibodies (MAbs)[#]

MAbs	**G serotype specificity**
RV4:1	1
RV4:2	1
RV4:3	1
RV5:3	2
RV5:5	2
RV3:1	3
ST3:1	4
F45:1	9
MAbs	**Group / subgroup specificity**
255/60	Subgroup I
631/9	Subgroup II
101BC5C7	Rotavirus group A Mab

^aa- amino acid; *Nd-not determined. Table compiled from [#]Kirkwood *et al*, 2005.

Detection was carried out by adding 100 µL of TMB substrate buffer to all wells. The plate was incubated for 10 minutes at room temperature and reaction was stopped by addition of 50 µL of 2M H_2SO_4 (BDH). All plates were read in a Titertek Multiscan MCC/340 EIA plate reader using a 450nm filter. Specimens were assigned as positive for rotavirus when the absorbance exceeded 0.2 or was at least twice the absorbance of the background control. Samples were assigned a G serotype based on the positive result with specific monoclonal antibodies as described in Fig 2.1

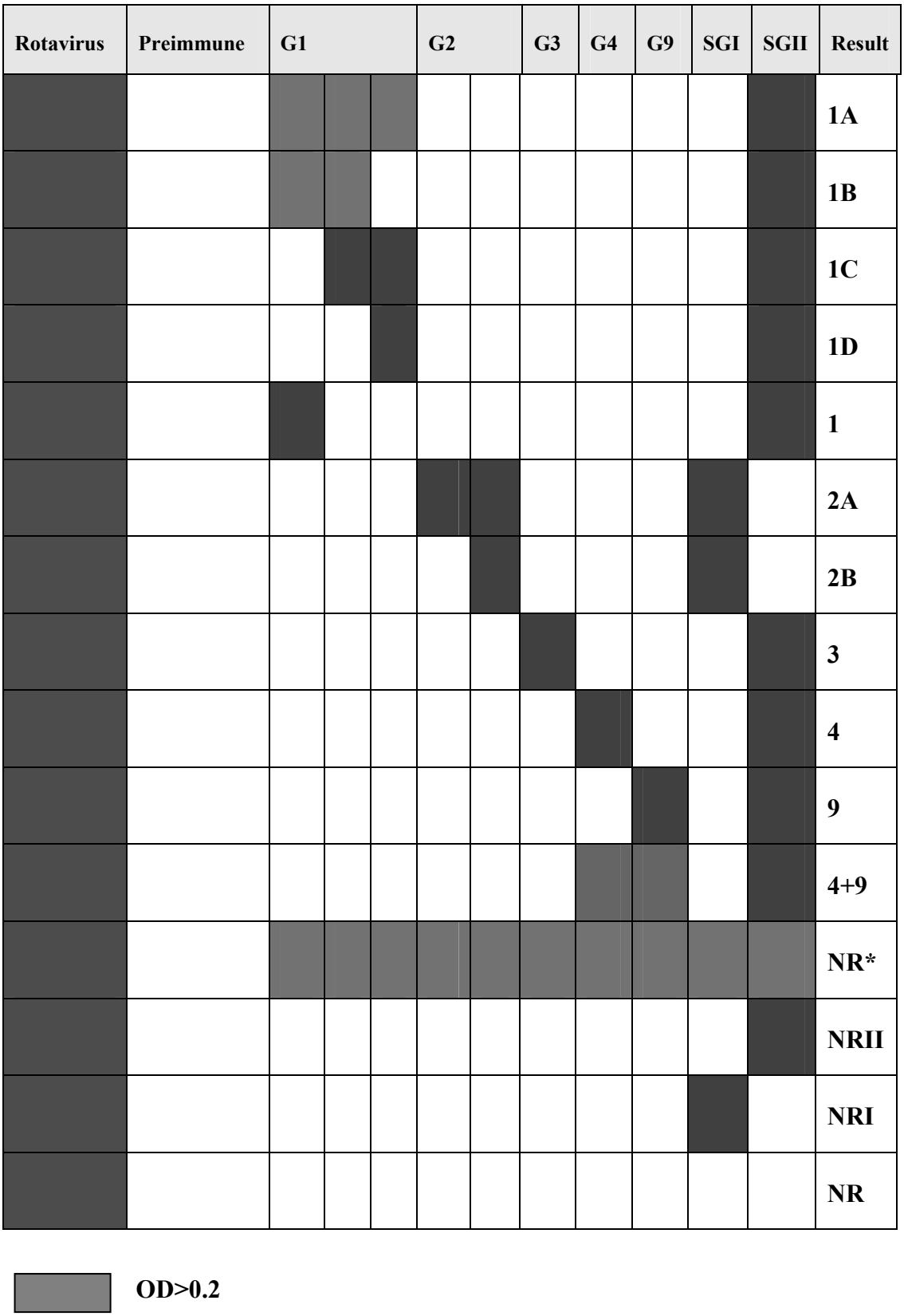

Figure 2.1: EIA results: range of possible G Serotypes obtained using typical monoclonal antibodies. Darkened squares represent a positive result (OD>0.2) and G type designation is indicated. The last column listed all of the G typing results using MAbs listed in Table 2.1. An asterisk represents samples with mixed reactivities.

Table 2.4: Sample Plate Layout for polyclonal antiserum, monoclonal antibodies, test and control specimens (Table adapted from Kirkwood *et al*. (2005))

	1	2	3	4	5	6	7	8	9	10	11	12
Poly clonal serum	RabαSA11	Preimmue	Rabα RV4	Rabα RV4	Rabα RV4	Rabα RV5	Rabα RV5	Rabα RV3	Rabα ST3	Rabα F45	Rabα SA11	Rabα SA11
Mabs A	101B-C5C7	101B-C5C7	RV4:1	RV4:2	RV4:3	RV5:1	RV5:3	RV3:1	ST3:1	F45:8	255/60	631/9
B												
C												
D												
E												
F												
G												
H												

2.2.3 Serological analysis using G9 specific monoclonal antibodies in enzyme immunoassay

The antigenic variation of all serotype G9 isolates were investigated by determining their reactivity to a panel of G9-specific N-MAbs derived against the prototype strain F45. The method employed was similar to that of G serotyping method (section 2.2.3), with the only difference being that all wells were coated with polyclonal antiserum raised to strain F45, then seven serotype G9 specific Mabs were used to determine the reactivities.

2.3 Extraction of genomic RNA and polyacrylamide gel electrophoresis (PAGE) of rotavirus RNA

2.3.1 Extraction of rotavirus RNA from 10% faecal homogenates

Rotavirus genomic RNA was extracted from 10% faecal extract (section 2.2.2) made in TNC buffer as previously described by Gouvea *et al*. (1991) with modifications by Kirkwood *et al.* (2005). A 500 μL sample of faecal homogenate was mixed with 50 μL

of 10% (w/v) SDS, 50 µL of 1M sodium acetate (pH 5.6) and 500 µL of phenol-chloroform -isoamylalcohol (25:24:1) and vortex mixed for 10 seconds then centrifuged at 10000g for 2 minutes. The upper aqueous phase was recovered and mixed with 50 µL Hydroxyapatite (HA) type I (Sigma) to purify the genomic RNA from other faecal impurities. After vortexing for 10 seconds and centrifugation at 10000g for 1 minute, the supernatant was discarded. The pellet was washed with 500 µl of washing solution twice. The washed pellet was then eluted in 50 µL of elution solution, mixed by vortexing, centrifuged at 10000g for 1 minute and the supernatant containing purified dsRNA was recovered and stored at -20^{o}C.

2.3.2 Polyacrylamide gel electrophoresis of rotavirus dsRNA

PAGE was performed largely as per Laemmli (1970) without SDS in the buffers. Polyacrylamide gels composed of an upper stacking gel and a lower resolving gel were prepared. The lower resolving gel contained 12% (w/v) acrylamide (Sigma), 0.33% (w/v) methylene bisacrylamide (Sigma) and 375 mM Tris-HCl (pH 8.8). Polymerisation was initiated by the addition of 0.03% (w/v) ammonium persulphate and 0.025% (v/v) N,N,N',N'-tetramethylenediamine (TEMED) (BioRad), and the gel was cast between clean glass plates 0.75mm apart. The gel was overlaid with 50% (v/v) isopropanol (BioRad) to produce a level interface and allow polymerisation. Following polymerisation the isopropanol solution was removed and the gel was rinsed three times with distilled water.

The upper stacking gel consisted of 4% (w/v) acrylamide, 0.12% (w/v) methylene bisacrylamide and 125 mM Tris-HCl (pH 6.8). Ammonium persulphate (0.3% (w/v)) and TEMED (0.1% (v/v) were added and the solution was overlaid onto the lower gel. Appropriate combs were inserted and the gel was allowed to polymerise. Following polymerisation, the gel assembly was positioned into the electrophoretic tank and running buffer was added to the top and bottom reservoirs. Then 10 µL of genomic RNA samples were prepared by mixing with 3x sample buffer (1.5 µL) and loaded into the designated wells and electrophoresis was carried out at a fixed current of 30 mA for 3 hours at 4^{o}C in a vertical slab apparatus using a discontinuous reservoir system (Kirkwood *et al*., 2005).

After electrophoresis, the gel was carefully removed from between the glass plates. The stacking gel was discarded and silver staining of the gel was carried out according to method described by Dyall-Smith and Holmes (1984). In brief, the gel was fixed in 50 mL of fixer solution (10% (v/v) ethanol, 0.5% (v/v) acetic acid) for 30 minutes by gentle rocking. The fixing solution was then aspirated off and the gel was rinsed with sterile distilled water. It was then immersed in freshly prepared 0.011M silver nitrate solution for 40 minutes and gently rocked. The silver nitrate solution was removed by aspiration and the gel washed 5 times with sterile distilled water. A reducing solution (0.1M formaldehyde and 0.75M NaOH) was added for 10-20 minutes during which time the RNA bands became visible on the gel. The gel was rinsed with distilled water and stored in 5% (v/v) acetic acid solution until photographed.

2.4 Determination of VP7 genotype using reverse transcription-polymerase chain reaction (RT-PCR) amplification

Genotyping by reverse transcription-polymerase chain reaction (RT-PCR) was employed for those samples where dual serotypes were demonstrated by EIA and also for those samples which serotype could not be determined by EIA (non typeable). In order to determine the VP7 genotype, initial first round amplification with plus and minus sense primers was carried out by RT-PCR followed by a second round of PCR amplification with nested multiplex, serotype specific pooled primers (cocktail of primers Table 2.5), following the method described by Gouvea *et al.* (1990).

2.4.1 First round amplification

Primers Beg9 and End9 (Table 2.5) were used in the first round amplification to produce full length VP7 cDNA of 1062 bp. These primers are highly conserved among human group A rotaviruses. Five μL of extracted dsRNA were added to tubes containing 94 μL of RT- PCR buffer and 500ng of primers Beg9 and End9. The reaction mix was mixed briefly and heated at 97 °C for 4 minutes and quickly quenched on ice. Enzymes [12.5 U of avian myeloblastosis virus reverse transcriptase (AMV-RT; Promega) and 2.5 U of DNA *Taq* Polymerase (Promega)] were added to each tube, then

samples were mixed briefly and centrifuged for 5 seconds at 10000g in a bench microcentrifuge to ensure all the buffer was at the bottom of the tube. Samples were subjected to an initial reverse transcription step of 42^{o}C for one hour then 35 cycles of PCR, each consisted of 1 minute at 94^{o}C, 2 minutes at 42^{o}C and 2 minutes at 68^{o}C. All RT-PCR steps were carried out in a Master Cycler (Eppendorf).

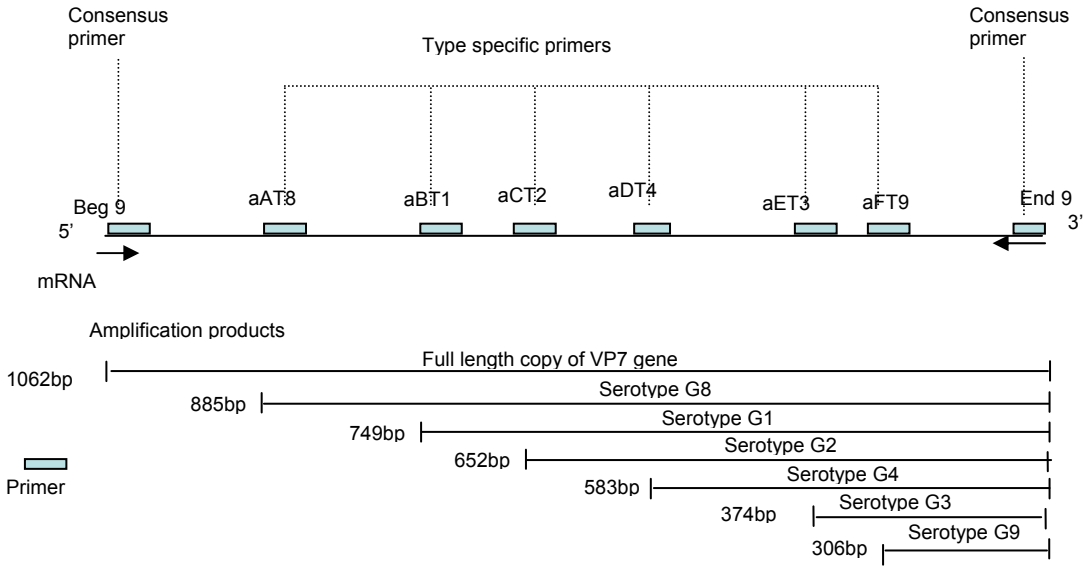

Figure 2.2: Diagrammatic representation of the VP7 gene, showing the location of the consensus regions within group A rotavirus, the regions conserved within types, and the location of the oligonucleotide primers (Table 2.5) used for rotavirus G- genotyping (Adapted from Gray and Desselberger, 2000).

Table 2.5. Oligonucleotide Primers used in this study

Primer	Sequence(5'-3')	Nucleotide Position (sense)	Purpose	Serotype specificity	Reference
VP7 End Primers					
Beg 9	GGCTTTAAAAGAGAGAATTTCCGTCTG	1-28 (+)	G type, sequencing	VP7 Full length	Gouvea *et al.*, 1990
End 9	GGTCACATCATACAATTCTAATCTAAG	1062-1036, -	G type, sequencing	VP7 Full length	Gouvea *et al.,* 1990
RVG 9	GGTCACATCATACAATTCT	1062-1044,-	G type	VP7 Full length	Gouvea *et al.,* 1990
VP7 Serotyping Primers					
aAT8	GTCACACCATTTGTAAATTCG	178-198,+	G typing	G8	Gouvea *et al.,* 1990
aBT1	CAAGTACTCAAATCAATGATGG	314-335,+	G typing	G1	Gouvea *et al.,* 1990
aCT2	CAATGATATTAACACATTTTCTGTG	411-435,+	G typing	G2	Gouvea *et al.,* 1990
aDT4	CGTTTCTGGTGAGGAGTTG	480-498,+	G typing	G4	Gouvea *et al.,* 1990.
aET3	CGTTTGAAGAAGTTGCAACAG	689-709	G typing	G3	Gouvea *et al.,* 1990
aFT9	CTAGATGTAACTACAACTAC	757-776,+	G typing	G9	Gouvea *et al.,* 1990
VP7 Sequencing Primers					
CK5	ATGGTATTAAATATACCACA	53-72,+	sequencing	VP7 gene	Kirkwood *et al*, 1996
G9.1	CAGCAAACAGATGAAGCGAATAAATGG	574-600,+	sequencing	VP7 gene	Kirkwood *et al*, 1996
G9.2	CCATTTATTCGCTTCATCTGTTTGCTG	463-489, -	sequencing	VP7 gene	Kirkwood *et al*, 1996
VP4 End Primers					
Con3	TGGCTTCGCCATTTTATAGACA	11-32,+	P typing	Full length/ P type	Gentsch *et al.,*1992.

Table 2.5. Oligonucleotide (Contd.)

Con2	ATTTCGGACCATTTATAACC	868-887,-	P typing	Full length	Gentsch *et al.,* 1992
VP4 Serotyping Primers					
2T-1	CTATTGTTAGAGGTTAGAGTC	474-494,-	P typing	P4	Gentsch *et al,* 1992.
3T-1	TGTTGATTAGTTGGATTCAA	259-278,-	P typing	P6	Gentsch *et al.* , 1992
1T-1	TCTACTTGGATAACGTGC	339-356,-	P typing	P8	Gentsch *et al,* 1992.
4T-1	TGAGACATGCAATTGGAC	385-402,-	P typing	P9	Gentsch *et al,* 1992.
5T-1	ATCATAGTTAGTAGTCGG	575-594,-	P typing	P10	Gentsch *et al,* 1992.
NSP1 Primers					
Beg 5	CTAGATGTAGAAT(G/A)TTCACG	885-905,+	probe, sequencing	Partial NSP1	Maunula and Bonsdorff, 2002
End 5	TAGGCGCTACTCTAGTG	1524-1550,-	probe, sequencing	Partial NSP1	Maunula and Bonsdorff, 2002
5Beg1	AAATGTAGAAATGAATGTATGA	402-423,+	sequencing	Partial NSP1	This study
5End1	C/TGAG/TGTTAT/CTAATTTGGTTT	949-968,-	sequencing	Partial NSP1	This study
5.1	TTTGAAAAGTCTTGTGGAA	1-19,+	sequencing	Partial NSP1	This study
5.2	ATCATAATACCCAAATAC	480-497,-	sequencing	Partial NSP1	This study
NSP4 Primers					
10.1	GGCTTTTAAAAGTTCTGTTCC	1-21,+	sequencing	NSP4	Kirkwood *et al.,* 1999
10.2	GGTCACACTAAGACCATTCC	731-750, -	sequencing	NSP4	Kirkwood *et al.,* 1999

2.4.2 Second round PCR amplification

The genotyping PCR was performed by multiplex nested PCR using the product of first round as template and genotype specific primers. The pool of primers is listed in Table 2.5. Ninety five μL of PCR buffer and five μL of first round RT-PCR product were mixed and briefly centrifuged, and PCR amplification was carried out for 35 cycles of 94^{o}C for 1 minute, 42^{o}C for 2 minutes and 68^{o}C for 1 minute. The amplified gene products were analysed in a 1% (w/v) agarose gel (see below).

2.4.3 Agarose gel electrophoresis of PCR products

Electrophoresis of cDNA products generated by PCR was carried out in a horizontal gel apparatus using 1.0-1.5 % (w/v) agarose gels prepared and submerged in Tris-acetate-EDTA buffer (Appendix I) containing 0.5μg/mL ethidium bromide (BioRad). Two μL of 6x loading dye (50% (w/v) sucrose, 1% (w/v) bromophenol blue in 0.2M EDTA [pH8.0], (Promega) were added to 10 μL aliquots of each cDNA product and electrophoresed at 100V for an hour. A 100bp or 1500 bp DNA marker (Promega) was used to allow size determination of each PCR product. Bands were visualised over a UV-transilluminator and gels were photographed using a computer linked camera using DigiDoc software (UVA Inc). As depicted in Fig 2.2, each specific rotavirus G genotype produced cDNA of a characteristic size.

2.5 VP4 genotyping of rotavirus isolates

The P genotype of rotavirus samples was carried out using the method previously described by Gentsch *et al.* (1992) which involved a first round amplification by RT-PCR, then a second round multiplex PCR amplification using nested, serotype specific primers (Table 2.5).

2.5.1 First round amplification

Primers Con2 and Con3 (Table 2.5) were used in the first round RT-PCR amplification to produce an 876 bp PCR product, which includes sequences encoding the VP8* fragment of VP4. These primers correspond to highly conserved sequnces among human rotavirus strains from VP4 genotypes [4], [6], [8], [9] and [10] (Gentsch *et al*., 1992). Five µL of purified dsRNA (section 2.3.1) were added to tubes containing 94 µl of RT-PCR buffer with 500 ng of primers Con2 and Con3. The samples were mixed then denatured at 97°C for 4 minutes in a heating block before quenching on ice for 1 minute. Enzymes (12.5 U of AMV-RT and 2.5 U of *Taq* Polymerase) were added to each tube. Samples were mixed and centrifuged briefly at 10,000g in a bench microcentrifuge to ensure all the buffer was at the bottom of the tube. Samples were subjected to an initial reverse transcription step of 42°C for 1 hour, followed by 35 cycles of PCR, which consisted of 1 minute at 94°C, 2 minutes at 42°C and 2 minutes at 68°C. All RT-PCR steps were conducted in a MasterCycler. First round PCR products were electropherosed in a 1% (w/v) agarose gel (section 2.4.3).

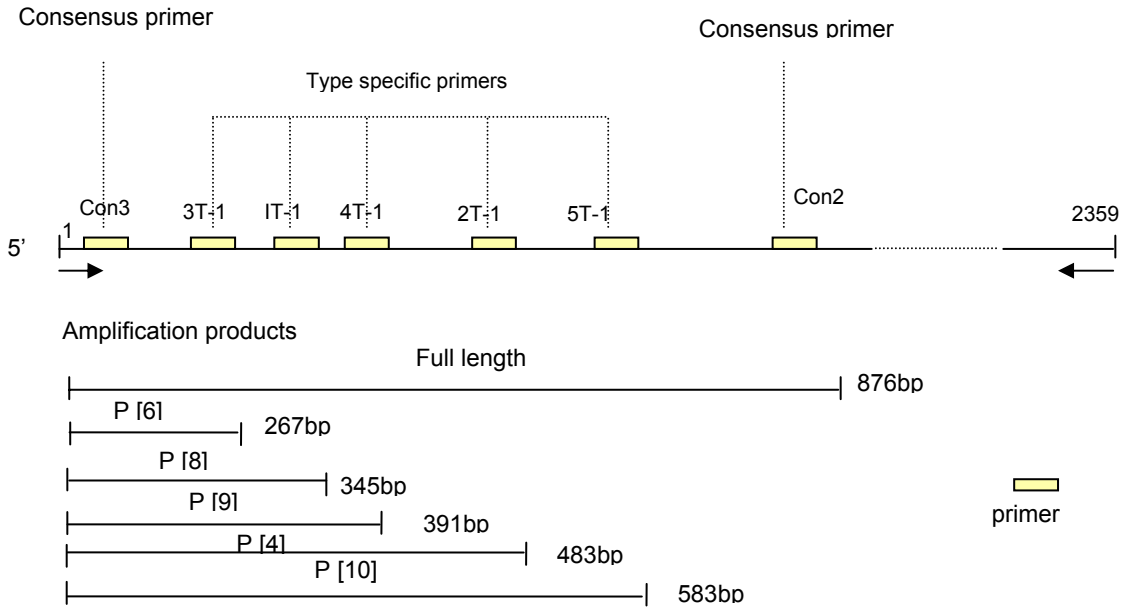

Figure 2.3. Diagrammatic representation of the VP4 gene, showing the location of the consensus regions within group A rotavirus, the regions conserved within types, and the location of the oligonucleotide primers (Table 2.5) used for genotyping of five P types. (Adapted from Gray and Desselberger, 2000)

2.5.2 Second round amplification with semi nested PCR

The second round genotyping PCR was performed using a multiplex nested PCR approach and the product of the first round PCR as template. Five minus-sense, genotype-specific primers (IT-1, 2T-1, 3T-1, 4T-1and 5T-1; Table 2.5) were used in conjunction with the plus-sense consensus primer Con3 in the second PCR amplification step to produce products of specific lengths. The primers sequences and positions are listed in the Table 2.5.

In the second amplification step, 1 to 5 μL of the first amplification product (depending on amount of first round amplification product visible in a 1% (w/v) agarose gel) were mixed with 95- 99 μL of reaction mixture. The PCR buffer and a cocktail of primers containing 100 ng each of the five genotyping primers and 500 ng of Con3 were used. The samples were subjected to 35 cycles of PCR using identical temperatures and times as described earlier (section 2.5.1). Following amplification, 10 μL of the PCR product were analysed by agarose gel electrophoresis as described earlier (section 2.4.3).

2.6 PCR amplification of gene segments 5 and 10

The method utilised to obtain cDNA corresponding to gene segments 5 and 10 was identical to that described above for the first round G genotyping amplification (section 2.4.1) except that gene 5 or gene 10 specific primers (Table 2.5) were used.

The full length of gene 5 (1569 bp) was amplified in three overlapping segments. A 665 bp segment corresponding to region 885-1569 bp was amplified using primers Beg 5 and End 5 described by Maunuala and Bonsdorff (2002) (Table2.5). Region 402-968 bp (556 bp) was amplified using primers 5Beg1 and 5End1. The 5' of gene 5 (497 bp) was amplified using primers 5.1 and 5.2.

The full length of gene segment 10, encoding NSP4 protein, was obtained by RT-PCR as a 750 bp fragment using the primers 10.1 and 10.2 (Kirkwood *et al.,* 1999) that were complementary to the 5' and 3' end of the each RNA strand of gene segment 10.

2.7 Northern Hybridisation Analysis

For genogroup analysis, Northern hybridisation was carried out by employing the technique previously described by Palombo and Bishop (1996).

2.7.1 Electrophoresis of target RNA

Total dsRNA extracted was separated by electrophoresis in 10% (w/v) polyacrylamide gels for two and half hours at 30 mA (section 2.3.2). The gels were stained with ethidium bromide for 10 minutes then viewed over a UV-transilluminator to ensure the separation of genes and RNA profile of each sample. The RNA was then photographed using a digital camera and DigiDoc software (UVA Inc).

2.7.2 Transfer of dsRNA to nylon membrane

The technique employed here was adapted from Palombo and Bishop (1996). The dsRNA in the gel was denatured in 100mM NaOH for 20 minutes on rocking platform, followed by neutralisation for 15 minutes in 200mM NaAc [pH 5.6], then in 50mM NaAc [pH 5.6] for an additional 15 minutes. The gel containing the denatured dsRNA was rinsed with sterile distilled water, equilibrated in TBE buffer (Appendix I) for 30 minutes then placed onto a positively charged nylon membrane (Roche Diagnostics). Whatman 3M filter paper was placed on either side of the membrane and gel. The entire set up was then placed into a Mini Electric Transfer apparatus (BioRad). The transfer apparatus was positioned into the tank and immersed into transfer buffer (TBE). The transfer was carried out at constant voltage of 30V for one hour at 4^{o}C with agitation of buffer facilitated by a magnetic stirrer. After transfer, the nylon membrane was air dried and the RNA was fixed by UV-crosslinking for 5 minutes.

2.7.3 Preparation of digoxigenin (DIG)-labelled whole genome probe of rotavirus dsRNA

Whole genome probe was prepared by labelling total dsRNA extracted from the serotype G9 standard strain, F45. Purified total RNA (30 μL) was labelled using the Total Genome Labelling Kit (Roche Diagnostics) following the manufacturer's

instructions. Five μL of labelling reagent was added to 30 μL of F45 total genome RNA. The mixture was heated at 85^{o}C for 30 minutes and labelling was stopped by adding 5 μL of 2M H_2SO_4.

The principle involved in the reaction is that the cis-platinium DIG Chem-Link reagent is capable of binding to the N7 position of guanosine and adenosine bases (Roche Diagnostics). DIG-labelled probes that hybridised to a target sequence are detected with an alkaline phosphatase conjugated anti-DIG antibody. If the blot is incubated with a suitable reagent, like CDP-Star or CSPD, phosphatase activity is detected by a chemiluminescence reaction which can be revealed by exposing the blot to imaging equipment, such as ChemiDoc XRS (BioRad).

2.7.4. Determination of probe conentration

A 10-fold serial dilution of probe was prepared. One μL each of dilution was loaded onto a positively charged nylon membrane. UV- crosslinking was carried out by exposing the nylon membrane to a UV transilluminator for 5 minutes (Kirkwood *et al.*, 2005). The membrane was washed in 0.1M Maleic acid buffer (Appendix I) for 5 minutes. The detection method was carried out as described in section 2.7.6. A probe concentration of 25 ng/mL was used for the hybridisation assay.

2.7.5 Prehybridisation and hybridisation

Prehybridisation was carried under stringent conditions in a hybridisation oven (Hybaid Ltd, UK) using 50 mL Falcon tubes. The nylon membrane was prehybridised at 52^{o}C for 2-4 hours in high SDS hybridisation buffer (Appendix I). The probe was denatured at 94^{o}C for five minutes and quickly quenched on ice then briefly spun in a bench microcentrifuge to collect all liquid at the bottom of the tube. It was then mixed with 20 mL of high SDS hybridisation buffer. The prehybridisation buffer was discarded and the membrane was placed into the fresh hybridisation buffer containing probe. Hybridisation was carried out at 52^{o}C overnight. After hybridisation, the probe was recovered and stored at -20^{o}C for future use.

2.7.6 Post hybridisation washing of the membrane and detection of bound probe

Following hybridisation, the membrane was washed twice at room temperature for 5 minutes in a low stringency buffer (Appendix I) to remove any unbound probe from the membrane. The membrane was then washed twice in a high stringency buffer (Appendix I) at 52^{o}C for 15 minutes to remove any non-specifically bound probe.

The bound probe was detected by using the chemiluminescence detection technique. The detection method was carried out using anti-DIG antibody conjugated to alkaline phosphate and the chemiluminiscent substrate CDP star (Roche Diagnostics). Briefly, the membrane was equilibrated for 5 minutes in washing buffer (Appendix I). The membrane was then incubated with 1% blocking buffer (Roche Diagnostics) for 1 hour at room temperature. The blocking buffer was replaced with anti-DIG antibody conjugated to alkaline phosphatase diluted 1:20,000 in blocking buffer for 1 hour at room temperature. After two 15 minutes washes with washing buffer (Appendix I), the nylon membrane was equilibrated in detection buffer (Appendix I) for 5 minutes. All the steps were carried out at room temperature on a rocking platform. The chemiluminescent substrate CDP star, diluted 1:100 in detection buffer, was directly added onto the nylon membrane for 5 minutes. The membrane was blotted on filter paper to remove excess buffer before placing in a plastic bag and sealed. The membrane was exposed to ChemiDoc XRS (BioRad) without any filter for 15 minutes or until the desired bands were visible, and the bands were analysed using Quantity One software (BioRad).

2.8 Slot blot analysis

2.8.1 Blotting of dsRNA onto nylon membrane

Five µL of dsRNA of all isolates, including standard strains RV4, RV5, RV3, ST3 and F45, were mixed with 45 µL of sterile distilled water and denatured at 97^{o}C for 3 minutes and quickly quenched on ice. These samples were then loaded onto a positively charged nylon membrane which had been immersed in sterile distilled water for five

minutes. The denatured RNA samples were blotted onto the membrane using a Slot Blot Manifold (BioRad). The RNA samples were then UV-crosslinked for five minutes.

2.8.2 Preparation of gene segment 5 probe of samples R1 and R14

Two isolates, designated R1 and R14, were selected to further investigate the presence of various alleles of gene segment 5 among serotype G9 isolates. Northern slot blot experiments were carried out by preparing probes derived from either R1 or R14 using primers Beg 5 and End 5 described in section 2.6. The gene segments were amplified by RT-PCR employing the same method as described in section 2.4. The PCR products were gel purified as described in 2.9.1. cDNA was labelled by the incorporation of DIG-11-dUTP (Roche Diagnostics) during PCR. The PCR buffer contained 0.5 μl of 20 mM dTTP and 1 μL of 20mM DIG-11-dUTP alongwith other dNTPs. PCR was carried out for 35 cycles, as described in section 2.4.2. The concentrations of the probes were determined as described in section 2.7.4.

2.8.3 Prehybridisation and hybridisation

Prehybridisation, hybridisation and detection of bound probe were carried out as described in section 2.7.5 except that the hybridisation temperature was increased from 52^{o}C to 54^{o}C for more specific binding of the probe and also to reduce background. The high stringency washes were carried out at 54^{o}C.

2.8.4 Detection of bound probe

The detection of the bound probe was carried out by using chemiluminescence substrate CDP Star employing the technique described in section 2.7.6.

2.8.5 Stripping of the bound probe

Where the membrane was to be reprobed, the first probe was stripped from the membrane using buffer that contained 50% (v/v) formamide, 0.1% (w/v) SDS and 0.1xSSC. The membrane was placed into stripping buffer and stripping of the probe was

carried out at 70°C for one hour. The membrane was then rinsed in sterile deionised water for 1 minute. To determine that the probe has been successfully removed, the membrane was examined on the ChemiDoc XRS for the absence of any bands. Prehybridisation and hybridisation with a second probe were carried out as described above (section 2.5).

2.9 Nucleotide sequence determination

2.9.1 Purification and quantitation of cDNA

A DNA Purification Kit (Ampli Chem) was used to purify PCR products (cDNA) from 1% (w/v) agarose gels. The PCR product was analysed by electrophoresis as described in section 2.4.3. Following electrophoresis, the cDNA band of interest was excised from the agarose gel and mixed with 3 volumes of 6M NaI in a 1.5 mL polypropylene tube. The mixture was incubated at 55°C for five minutes, to dissolve the agarose. Ten μL of the glass powder provided with the kit was then added to the dissolved agarose and mixed well. To improve the binding efficiency of DNA to the glass powder, the sample was incubated at 55°C for 5 minutes with regular mixing. The mixture was then centrifuged at 10000g for 30 seconds. The supernatant was removed and the pellet was rinsed with washing solution provided with the kit. The washing was repeated two more times. DNA was then eluted from the glass powder pellet into 20 μL sterile Milli Q water by incubating at 55 °C for five minutes with regular mixing. One μL of purified cDNA was analysed by agarose gel elctrophoresis (section 2.4.3).

To estimate the concentration of the purified cDNA samples, the intensity of the DNA in agarose gel was compared with DNA standards of known concentration. The appropriate concentration of the DNA was then used directly in sequencing reactions.

2.9.2 Sequencing reaction

Sequencing reactions were performed according to the principle of Sanger *et al.* (1977) and was conducted using the ABI Prism BigDye Terminator Cycle Sequencing Ready

Reaction Kit v3 (Applied Biosystems). Sequencing reactions were typically conducted in 20 μL final volumes containing 1 μL of the commercial BigDye solution, 1.5mM $MgCl_2$, 3.7mM Tris-HCl (pH 9.0), 3.2 pmoles of primer and ~50 ng of purified DNA. Thermal cycling was undertaken in a Mastercycler (Eppendorf). The sequencing reaction was denatured initially at 96^{o}C for 10 mins followed by 35 cycles, each of 96^{o}C for 10 seconds, 55^{o}C for 5 seconds and 60^{o}C for 4 minutes. The sequencing reaction was then subjected to post reaction cleanup with 0.2mM $MgSO_4$. The dried labelled sequencing samples were then sent to the Australian Genomic Research Facility in Melbourne, Australia for electrophoresis and analysis.

Alternatively, some sequencing reactions were also carried out at Baker Heart Research Institute, Melbourne, Australia by the technical staff. Pure DNA samples and primers were submitted to the institute. The following protocol was performed; the purified DNA was labelled with the BigDye terminator Chemistry v3.1 and labelled samples were analysed with 3100 Genetic Analyzer, which is an automated, high throughput, capillary electrophoresis system used for analysing fluorescently labelled DNA fragments. The estimated DNA was then labelled with the BigDye v 3.1 with the appropriate primers and sequencing buffer. Post reactions clean up of the products was carried out as follows: five μL of 125mM EDTA and 60 μL of 100% ethanol were added to the labelled sample and mixed by inversion several times. The mixture was allowed to precipitate at room temperature for fifteen minutes. The mixture was then centrifuged at 3260 rpm for 30 minutes in a Beckman GS-6R centrifuge. Then 60 μL of 70% ethanol was added and the mixture was centrifuges for fifteen minutes. All the liquid was then removed by inverting the tube and the pellet was air dried. Fifteen μL of HiDi Formamide was added to each tube, vortex mixed and centrifuged at 2000 rpm for 1 minute. The sample was then heated at 95^{o}C for 5 minutes and quickly quenched on ice for 2 minutes. The samples were loaded into automated DNA sequencer, 3100 Genetic Analyzer, for analysis.

2.10 Sequence analysis

Nucleotide sequence data were analysed using the molecular biology software program, BioEdit (www.mbio.ncsu.edu/BioEdit). The sequence data were compared to other rotavirus gene sequences available on GenBank and were aligned using Clustal W program in Biology Workbench version 3.2 (Thompson *et al*., 1994). Final alignments were visually examined and adjusted manually using data from both positive and negative strands.

2.10.1 Phylogenetic relationships of sequences

The aligned sequences were imported into the genetic analysis program MEGA version 3 (Kumar, 2004). Phylogenetic analysis was carried out using the phylogenetic tools available in MEGA 3 (www.megasoftware.net) (Kumar, 2004) using the UPGMA method to understand the evolutionary events and diversity of rotavirus genetic changes. Rooted phylogenetic trees were constructed and the robustness of the data evaluated by carrying out 1000 bootstrap replications.

Chapter Three

Molecular Epidemiology of Rotavirus

3.1 Introduction

The great impact of rotavirus related illness has provided the impetus for the development of a safe and effective vaccine to control the disease caused by this virus. The success of a rotavirus vaccine will depend greatly on its ability to exhibit both homotypic and heterotypic protection against the various serotypes of rotavirus circulating worldwide. To monitor the circulation of various strains of rotavirus in the community, a well established surveillance system and continued epidemiological studies of the virus in any given geographical location are essential. Rotavirus is a double stranded RNA virus with characteristic 11 segments of genomic RNA and because of the high error rate of RNA polymerase, extensive genetic variation is possible. Various techniques have been developed to determine the extent of variation among rotavirus isolates. For example, a traditional approach where the electrophoretic migration patterns of viral genomic dsRNA segments (electropherotyping) is used to classify rotaviruses into two major groups, the long electropherotype and the short electropherotype (Kapikian *et al.*, 2001). Electropherotyping provides information about changes in rotavirus strains both within and between annual epidemics in the same population (Holmes, 1996). Around the world, it is still considered to be a very useful diagnostic and epidemiological tool with relatively low cost and good sensitivity (Holmes, 1996). Modern molecular methods such as Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and genome sequencing have revealed greater diversity among rotavirus strains from around the world than previously thought (Estes *et al.*, 2001). Information about the molecular epidemiology of rotaviruses obtained collectively by classifying rotavirus strains into electropherotypes, G-types and P-types, coupled with gene sequence data, provides the true picture of the degree of strain diversity among rotaviruses globally.

In this section, the epidemiology of rotavirus strains from children admitted to one hospital in Melbourne, Australia was investigated. Samples were collected from January 2002 to December 2002. Fifty rotavirus positive samples were obtained from the Monash Medical Centre (MMC), Melbourne, from initial stool examinations of more than 700 children suffering from acute gastroenteritis in 2002. The initial

detection of rotavirus was carried out at the MMC using a rapid Latex Agglutination test (Rotalex, Orion Diagnostics, England). Positive specimens were then transferred to Swinburne University for this study. The main aim was to determine the various rotavirus strains circulating in Melbourne during the study period. These isolates were classified employing methods, such as determination of G serotype by EIA, determining electropherotypes, and RT-PCR to determine the G- and P-genotypes.

3.2 Results

3.2.1 Incidence of rotavirus in Melbourne in infants and young children in 2002

The detection and age distribution of rotavirus from infants and young children suffering from acute gastroenteritis during 2002 is shown in Figure 3.1. The majority of children suffering from rotavirus infection were aged between 7 and 18 months. The incidence declined as the age of children increased. More than 60% (31/50) of children infected with rotavirus diarrhoea were < 2 years of age with peak prevalence between the ages of 1 and 1.5 years (28%). The frequency was less in children <6 months (12%) and over 3 years (8%) of age.

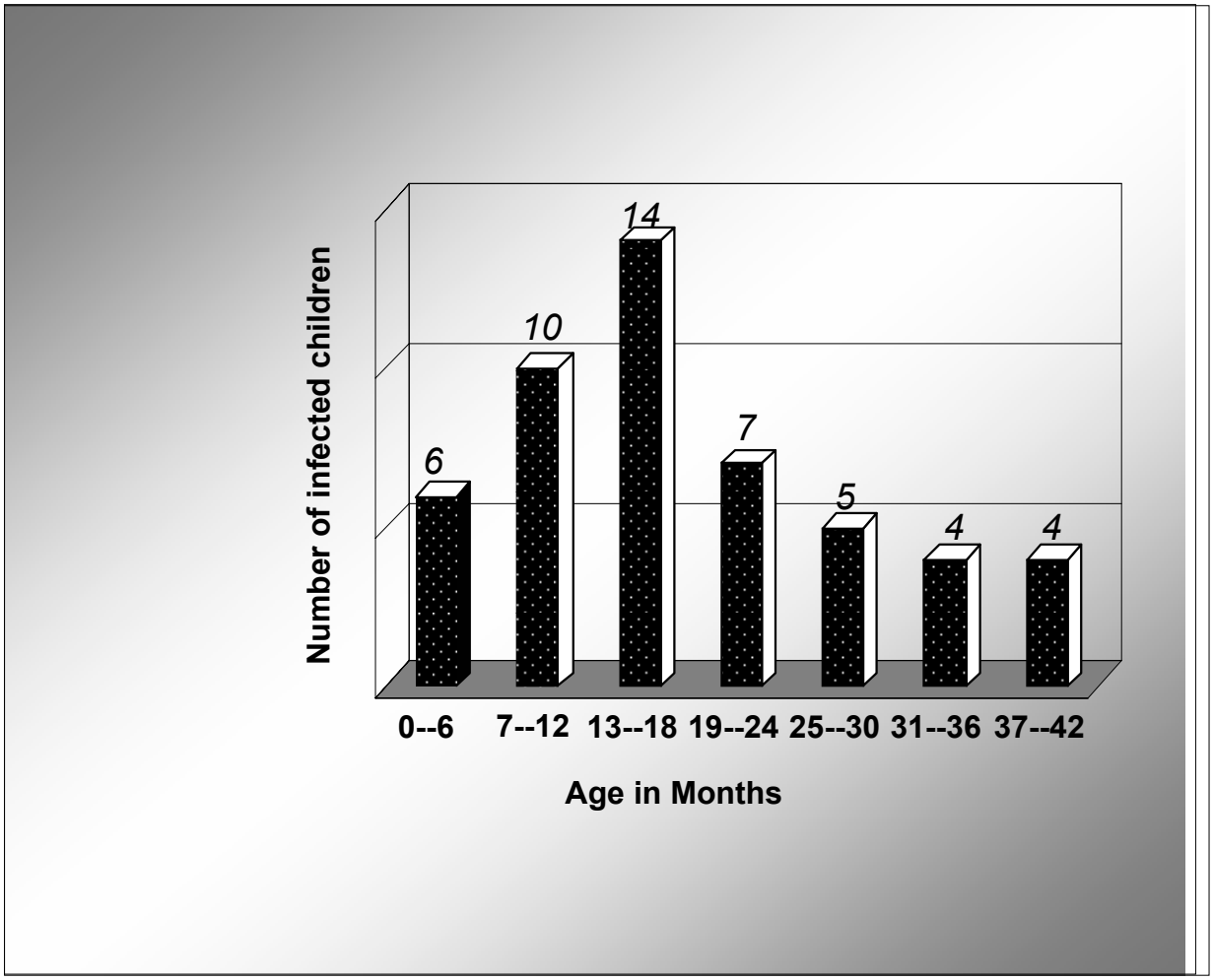

Figure 3.1 Number and age distribution of children suffering from acute gastroenteritis caused by rotavirus in 2002. The maximum number of infections occurred inchildren aged between 7-18 months. Rotavirus occurrence decreased as the age increased**.** The number s on the histogram indicates the total cases.

3.2.2 Monthly distribution of rotavirus in Melbourne, 2002

Rotavirus occurrence was unevenly distributed throughout the year. A rise in prevalence was observed during cooler months, starting in June and peaking during August and September (Fig 3.2). There was a decline in the numbers of rotavirus cases at the start of summer and no rotavirus was isolated in the months of March, April and May in 2002.

The majority of isolates were reported in August and September (30% and 28% respectively) which comprised about 60% of total occurrence. The winter peak, during

July to October, is consistent with the so called 'winter diarrhoea' caused by rotavirus in many temperate regions around the world (Bishop, 1996).

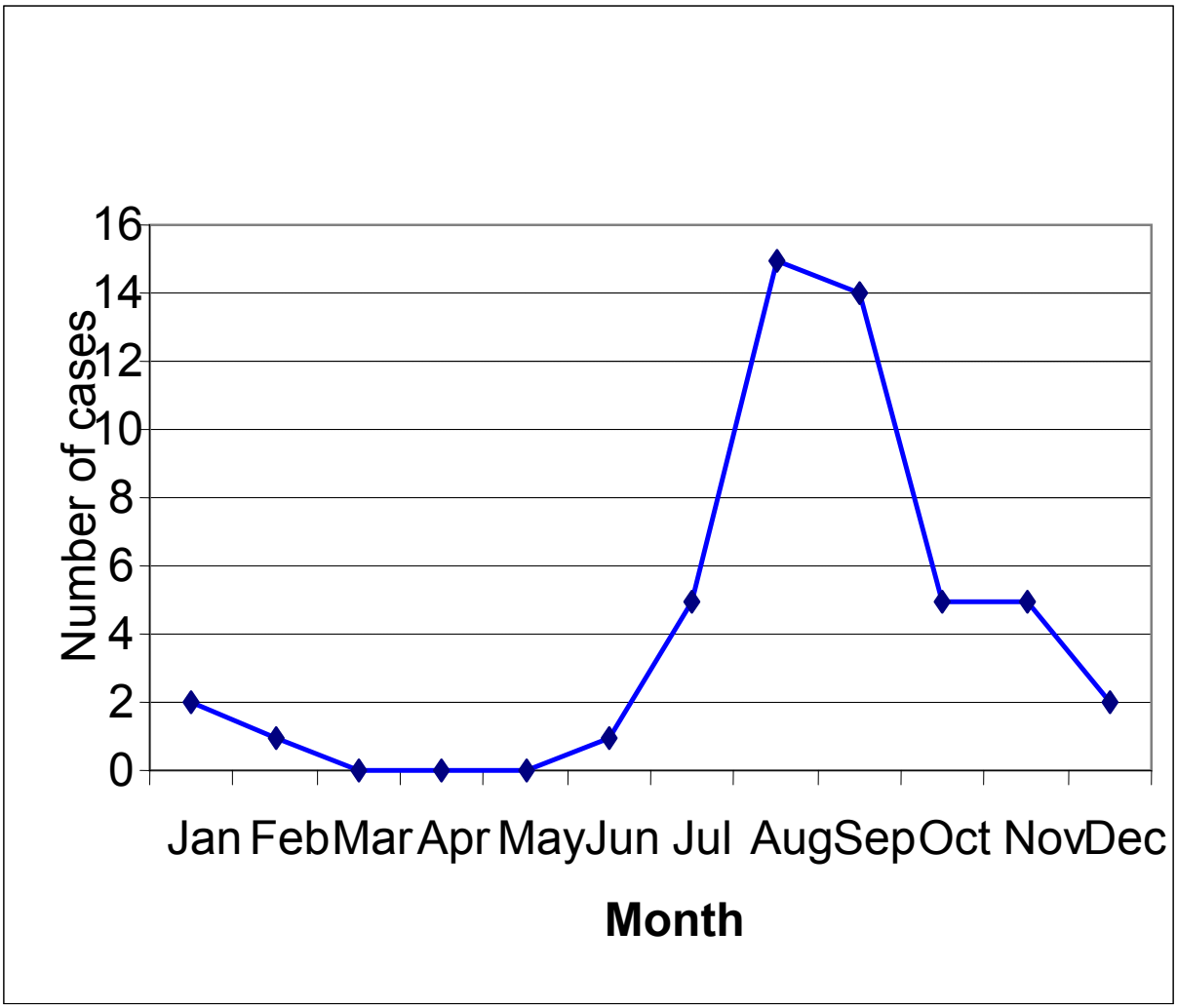

Figure 3.2 Monthly distribution of rotavirus detection in diarrhoeic children hospitalised at the Monash Medical Centre, Melbourne, Australia in 2002.

3.2.3 G serotyping of rotavirus isolates by Enzyme Immunoassay (EIA)

The G serotyping EIA results showed the presence of various G types of rotavirus in Melbourne in 2002. Faecal extracts of rotavirus positive samples were prepared and subjected to G serotyping and subgrouping by using specific MAbs in an EIA assay (Kirkwood *et al*., 2005). The results revealed that serotype G9 was the most prevalent type detected in that year, followed by serotypes G1 and G3. Serotype G2 was not

detected. Initially, EIA results revealed that the majority of serotype G9 samples exhibited dual serotype specificities of both G4 and G9. These samples were subjected to genotyping by semi-nested RT-PCR, following the method described in Section 2.4. The results showed that all but one of the isolates with dual specificities belonged to genotype G9 (Section 3.2.5). The distribution of G serotypes and their corresponding subgroup specificities are listed in Table 3.1. Serotype G4+G9 isolates accounted for 60% of samples, followed by G1 (16%) and G3 (10%). Mixed infections (G1+G3, G1+G9, G3+G9) were isolated in three patients (6%) and six isolates remained non-typeable. Most of the samples belonged to subgroup II (82%) whereas samples with mixed infections and non-reactive specimens had both subgroup I and II specificities (18%). The details of all results are summarised in Table 3.2

Table 3.1G serotype and subgroup specificities of rotavirus positive samples as determined by EIA

G-serotype	No. of positive isolates*	Subgroup specificity
G1	7	II
G3	4	II
G4+G9	30	II
Mixed	3	I, II
Non-Reactive	6	I, II

* details of these isolates are listed in Table 3.2

3.2.4 Polyacrylamide Gel Electrophoresis of rotavirus RNA

To determine the electrophoretic RNA profile (electropherotype) of rotavirus isolates, genomic RNA was extracted, purified and analysed by PAGE. Thirty seven samples exhibited a clear and characteristic human group A electropherotype, with most of the isolates exhibiting long patterns (Fig 3.3). Thirteen specimens did not yield RNA that was visible on the gel. Two isolates, including one mixed infection (G1+G3) exhibited short patterns (Fig 3.3, lanes 33 and 37) and were non-typeable by both EIA and RT-PCR. Eight different electropherotype patterns were observed on two separate gels (Fig 3.3) and three samples had mixed infections (Fig 3.3, lanes 29, 30 and 37).

The electropherotype patterns of the serotype G9 rotavirus strains revealed two different electropherotype profiles (lanes 21 and 22). Furthermore, serotypes G1 and G9 exhibited similar electrophoretic patterns (lanes 6 and 7). Taking electropherotypes and G serotype together, seven G1 and twenty five serotype G9 isolates exhibited two different E-types. Of the three mixed samples, MK37 (lane 29) and MK38 (lane 30) had long E-types whereas sample MK46 (lane 37) had both a short and a long pattern. The results are summarised in Table 3.2.

3.2.5 G-genotyping of rotavirus isolates by RT- PCR

Genotyping of all isolates that exhibited dual serotype specificities (G4+G9) and those that differed in their E-types was determined using VP7 specific primers (Table 2.5) in a semi-nested multiplex RT-PCR method as described in section 2.4. The majority of these samples belonged to genotype G9 except for one isolate (MK14) which had G4 specificity. Of the six non-typeable isolates, only one specimen could be typed as genotype G9. The remaining five isolates could not be typed despite showing good concentrations of RNA in the PAGE gel (Fig 3.3, lanes 14, 15, 16, 33, 34). All the RT-PCR (first round) and semi nested multiplex PCR (second round) products obtained were analysed by electrophoresis on 1% agarose gels with a known size DNA marker to estimate the size of the amplified product. Full length amplification (first round) of the VP7 gene produced a 1062 bp product, as expected, and second round amplification yielded a 306 bp product, which is the expected size for serotype G9 (Fig 3.4).

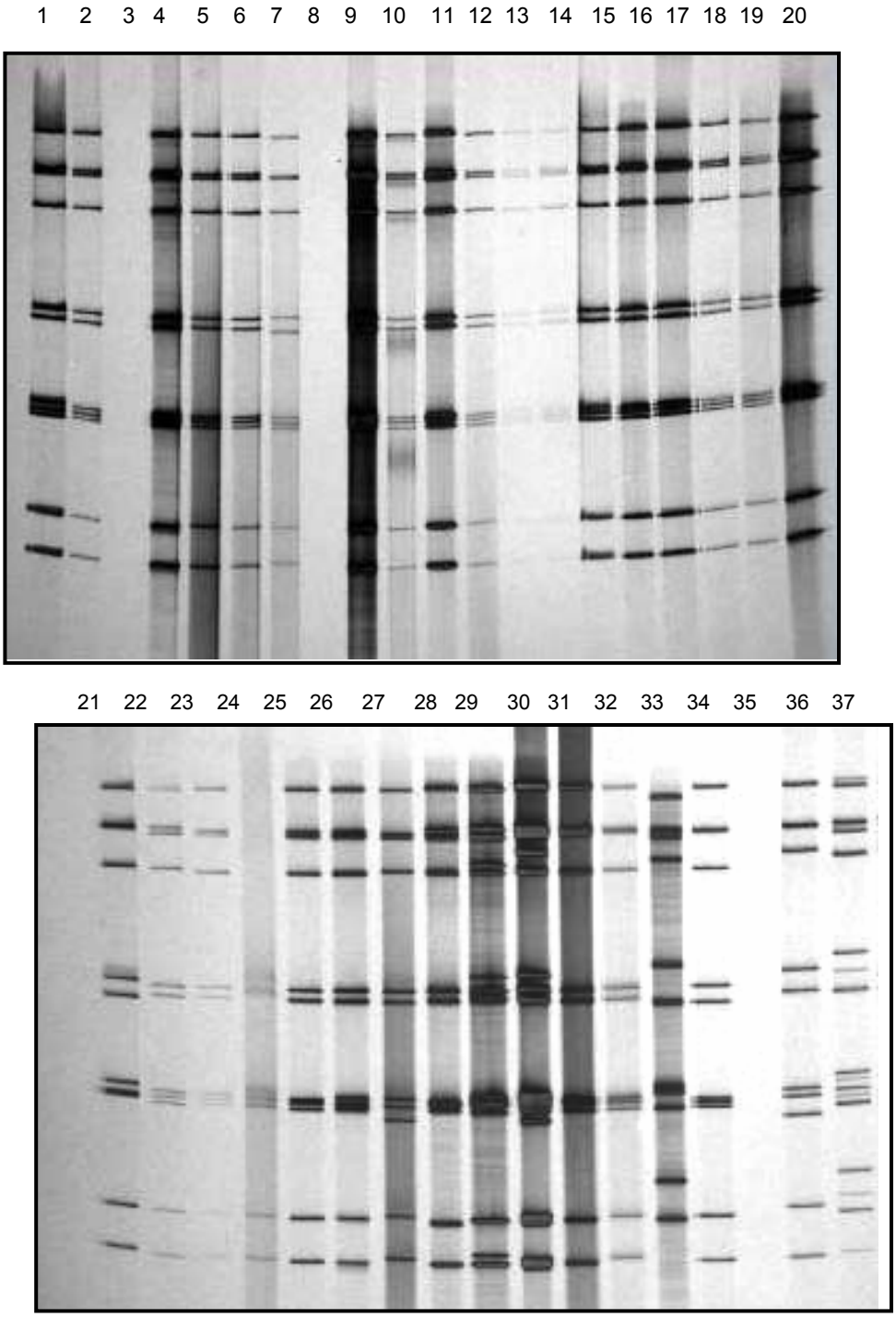

Figure 3.3 Polyacrylamide gel electrophoresis of representative strains of rotaviruses isolated from the Monash Medical Centre, Melbourne. All the isolates, except MK42 (lane 33) and one mixed sample (lane 37) displayed long patterns. Lanes 3, 8 & 35 did not have sufficient RNA whereas lanes 13 and 14 showed faint RNA profiles. There were mixed infections in samples in lanes 29, 30 and 37. The identity of samples in each lane is listed in Table 3.2.

The overall findings of G typing by both EIA and RT-PCR were that seven specimens (14%) belonged to serotype G1, 4 specimens (8%) were serotype G3 and 1 specimen was serotype G4. Serotype G9 was the predominant type with 27 specimens (56%). Ten percent of specimens were non-typeable and 3 specimens (6%) had mixed infections (Table 3.1).

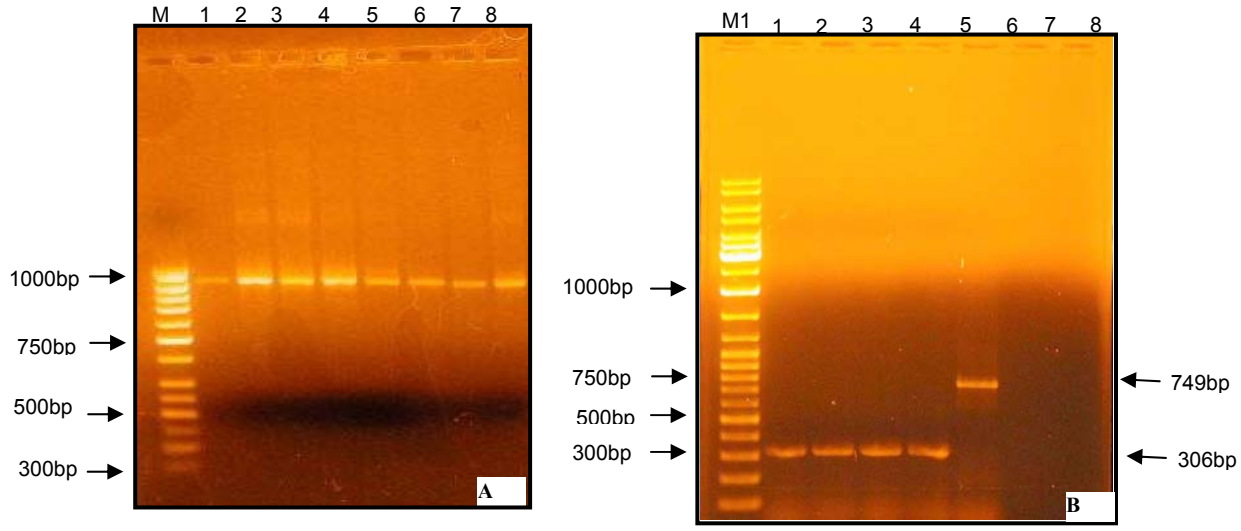

Figure 3.4. RT-PCR amplification of VP7 gene for G typing of selected rotavirus isolates using primers described in Table 2.5 (A) first round amplification, (B) second round amplification. M and M1 are 100bp and 3000 bp DNA markers; Lanes 1-4 contain samples MK15-MK18, lane positive control G1 and lanes 6-8 contain samples MK19, MK26 and MK36, respectively.

3.2.6 Optimisation of RT-PCR conditions for un-typed samples

As seen in Fig 3.4B, specimens MK19, MK26 and MK36 did not yield any product following the standard PCR protocol despite producing the full length product in the first round of amplification (Fig 3.4A, lanes 6-8). The inability of these samples to amplify may have been due to presence of inhibitors and the crude nature of the clinical specimens (stool). Optimisation of PCR conditions for non-typeable samples was then carried out as described. With the modifications in concentration of $MgCl_2$, some of the isolates could be successfully amplified (Fig 3.5). The best results were obtained when the $MgCl_2$ concentration was increased from 1.5mM to 2.5mM, the annealing temperature was best suited at 42^{o}C and the RNA template was optimal at 5 μL RNA per 100 μL reaction mix per sample. Under these conditions, above specimens (MK19, MK26 & MK36) could be successfully typed as genotype G9 (Fig.3.5).

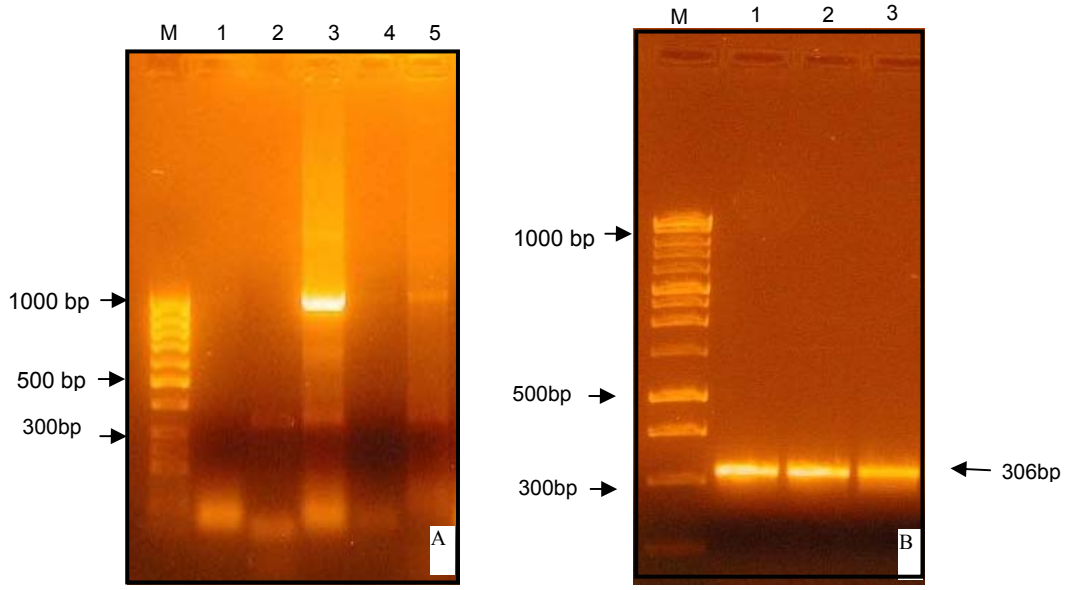

Figure 3.5. (A) Optimisation of RT-PCR conditions of specimen MK36 by varying the concentration of 100mM $MgCl_2$ and altering annealing temperature in 100 μL reaction mix. Lanes 1-2 have sample MK36 amplified with 1.5mM $MgCl_2$ at annealing temperature of 40°C and 42°C respectively. Lane 3 consists of samples with 2.5mM $MgCl_2$ at annealing temperature of 42 °C. Lanes 4 and 5 have the same sample amplified with 2mM $MgCl_2$ and annealing temperature of 38 °C and 40 °C respectively. M is the 100 bp DNA marker. B) Second round amplification of samples MK19, MK26 and MK36 in lanes 1-3 respectively with 2.5mM of $MgCl_2$ and annealing temperature of 42 °C. M is the 100 bp DNA marker.

3.2.7 VP4 Genotyping (P-type) of rotavirus by RT-PCR

Randomly selected serotype G1 and G9 rotavirus specimens were P genotyped employing the method described in Section 2.5 and the VP4 primers described in Table 2.5 (Fig 3.6). After the first round amplification, a PCR product of size 876 bp was obtained. Following second round amplification, a product of 345 bp was obtained therefore classifying the samples into P genotype [8] (Gentsch *et al.*, 1992). Of fifteen samples analysed, only eleven were typed as P[8], the remaining could not be typed despite having identical electropherotypes. The detail of these samples are listed in Table 3.2

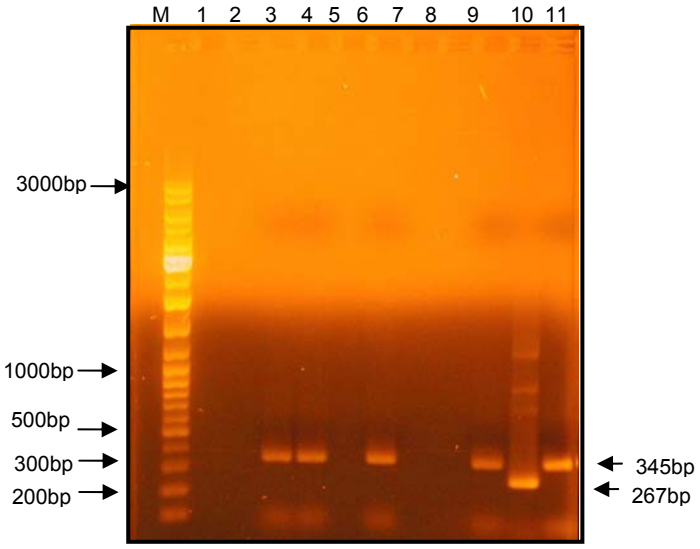

Figure 3.6 Genotyping of randomly selected serotype G9 isolates into P genotype by semi-nested PCR as described in section 2.5 and using primers described in Table 2.5. M is the DNA marker; lanes 1-9 contain samples MK5, MK6, MK12, MK17, MK18, MK20, MK23 MK25 and MK45 respectively, lanes 10 and 11 contains positive controls P[6] ands P[8] respectively.

In summary, employing typing methods such as EIA, G-serotyping and G-genotyping, P-genotyping and PAGE analysis, the following observations were made and a summary of combined results is listed in Table 3.2. Most of the rotavirus isolates in this study had dual serotype specificities with the G typing by EIA i.e. G4+G9. However, G-genotyping with RT-PCR indicated that most of these isolates belonged to a single genotype, G9. Of fifty rotavirus specimens analysed, three showed mixed infections. Five samples could not be typed by either serotyping or genotyping methods. One sample had a short pattern on PAGE and was non-typeable. Isolate MK46 was a mixed infection (G1+G3) with rotaviruses of short and long patterns. Serotype G9 isolates exhibited two different E-types, suggesting differences in their genomes. The VP4 genotype of all isolates analysed was of P[8] specificity.

Table 3.2. Summary of rotavirus typing results of all isolates**.**

Isolate number	G type by EIA	Sub-group	G type by PCR	P type by PCR	E-type	Position in gel Fig 3.3 (lane)
MK1	G4+G9	II	G9	ND*	2	1
MK2	G1	II	ND	P[8]	ND	-
MK3	G3	II	ND	ND	ND	-
MK4	G4+G9	II	G9	NP^	2	2
MK5	G4+G9	II	G9	NP	2	3
MK6	G1	II	ND	P[8]	2	4
MK7	G4+G9	II	G9	ND	2	5
MK8	G3	II	ND	ND	ND	-
MK9	G1	II	ND	P[8]	1	6
MK10	G9	II	ND	P[8]	2	7
MK11	G3	II	ND	ND	ND	-
MK12	G4+G9	II	G9	P[8]	1	8
MK13	G4+G9	II	G9	P[8]	2	9
MK14	G4+G9	II	G4	ND	3	10
MK15	G4+G9	II	G9	ND	ND	-
MK16	G4+G9	II	G9	ND	2	11
MK17	G4+G9	II	G9	P[8]	2	12
MK18	G4+G9	II	G9	NP	2	13
MK19	G1	II	ND	ND	ND	-
MK20	NR@	II	G9	P[8]	2	14
MK21	NR	II	NT	ND	5	15
MK22	NR	II	NT	ND	6	16
MK23	G4+G9	II	G9	NP	ND	-
MK24	G4+G9	II	G9	ND	1	17
MK25	G9	II	ND	P[8]	2	18
MK26	G4+G9	II	G9	ND	1	19
MK27	G4+G9	II	G9	ND	1	20
MK28	G4+G9	II	G9	ND	2	21
MK29	G4+G9	II	G9	ND	2	22
MK30	G4+G9	II	G9	ND	2	23
MK31	G3	II	ND	ND	4	24
MK32	G4+G9	II	G9	ND	2	25
MK33	G4+G9	II	G9	ND	ND	26
MK34	G4+G9	II	G9	ND	ND	-

MK35	G4+G9	II	G9	ND	2	27
MK36	G4+G9	II	G9	ND	1	28
MK37	G1+G9	II	G1,G9	ND	mixed	29
MK38	G3+G9	II	G3, G9	ND	mixed	30
MK39	G4+G9	II	G9	ND	2	31
MK40	NR	II	NT[#]	ND	ND	-
MK41	G4+G9	II	G9	ND	2	32
MK42	NR	I	NT	ND	8	33
MK43	NR	II	NT	ND	7	34
MK44	G4+G9	II	G9	ND	2	35
MK45	G1	II	ND	P[8]	2	36
MK46	G1+G3	II	G1,G3	ND	mixed	37
MK47	G4+G9	II	G9	ND	ND	-
MK48	G1	II	ND	P[8]	ND	-
MK49	G1	II	ND	ND	ND	-
MK50	G4+G9	II	ND	ND	ND	_

[*]ND: not determined; [#]NT: non-typeable;^ no product, [@] non-reactive

3.3 Discussion

Determining the variety of electrophoretic patterns, and the G type and P type distribution of rotaviruses will help to understand the diversity of strains diversity circulating in Melbourne. The data will assist in the management of the disease and help evaluate the effectiveness of the two new rotavirus vaccines, RotaTeq and Rotarix, which have shown promising results in clinical trials and have been licensed in many countries (Arvin and Greenberg, 2006). In Australia, the Rotarix vaccine has been licensed recently (Newall *et al.*, 2006). The effectiveness and success of these vaccines will depend greatly on their ability to control the diverse population of rotavirus and, therefore, epidemiological studies will greatly assist in their evaluation.

Group A rotavirus is the major group that causes infection in humans around the world. In Australia, >50% of acute gastroenteritis is caused by rotavirus group A alone leading to approximately 10,000 children being hospitalised every year (Carlin *et al.*, 1998) and

thirteen rotavirus related illnesses resulted in death from 1990-2002 (Newall *et al.*, 2006). This study was therefore focused on determining the serotype and genotype distribution among group A rotaviruses circulating in Melbourne in 2002. Characterisation of rotavirus strains isolated from children less than 5 years of age identified the presence of three different G serotypes in 2002. Five rotavirus specimens could not be typed by any of the methods utilised, which underlines the limitations and difficulties of the typing of clinical specimens despite improvement in detection techniques. Several factors are believed to be responsible for this: (i) presence of inhibitors in faecal specimens could hamper the genotyping of rotavirus by RT-PCR, (ii) the constantly mutating genome could lead to point mutations occurring in the PCR primer binding regions of the target gene which may prevent amplification (Martella *et al.*, 2004; Rahman *et al.*, 2005), (iii) mutations in the antibody binding epitopes may prevent binding with the typing MAbs (Dyall - Smith *et al.*, 1986).

A combination of typing methods, including RNA pattern analysis by PAGE, VP7 serotyping by EIA and genotyping by RT-PCR, allowed classification of 45 of the 50 rotavirus isolates studied. Classification of these isolates revealed the presence of eight different E-types and four different G-serotypes: G1, G3, G4 and G9. The P genotyping of selected serotype G9 isolates revealed the presence of genotype P[8]. Epidemiological studies have demonstrated that four rotavirus G-types (G1-G4) and two P serotypes (P[4] and P[8]) are the most frequent VP7 and VP4 types associated with human rotavirus infection globally, and thus, are the targets for vaccine development (Hoshino and Kapikian, 2000; Martella *et al.*, 2005). Importantly, the determination of serotype G9 rotavirus in greater numbers (Table 3.1) in this study indicates the dominance of a single serotype in 2002 in Melbourne. A recent study conducted at National Rotavirus Surveillance Centre, Australia, reported a significant rise in number of this serotype in Australia which was responsible for 74.7% of total cases of rotavirus related illnesses. The same study also determined a sharp increase in serotype G9 rotavirus numbers in Melbourne that accounted for 65% of total cases of rotaviruses detected in 2002 (Kirkwood *et al.*, 2003) compared to 11% in 2000 and 40% in 2001 (Kirkwood *et al*., 2002; Masendycz *et al.*, 2001). Similar findings have been reported in many other countries revealing the extent of serotype G9 dominance worldwide in recent years (Arista *et al.*, 2004; Hoshino *et al.*, 2005; Santos, 2005). This

study has contributed to understanding the distribution of various G serotype rotavirus in Melbourne during a one year period, and the greater numbers of isolates belonging to serotype G9 (58%) which is epidemiologically significant. As reviewed in section 1.18, the success of one of the recently licensed rotavirus vaccines, Rotarix, which is an attenuated monovalent vaccine (G1P[8]), will greatly depend on its ability to protect against other rotavirus serotypes, including dominant serotype G9 strains, co-circulating in the community. Therefore, pre-vaccination and post-vaccination epidemiological data, such as those determined in the present study, will play a vital role in determining the success of this and other vaccines.

In the present study, rotavirus infection was most common in children aged between 1 and 1.5 years of age, even though children as young as six months were also reported to be suffering from acute rotavirus related diarrhoea needing hospitalisation. However, children younger than six months seem to be immune, possibly due to acquired maternal protective antibodies (Bishop, 1996). The detection of rotavirus was maximal during the cooler months, from July to September in 2002, agrees with that commonly seen in many other temperate countries like the USA, the UK, France and Japan (Carlin *et al.*, 1998; Cook, 1990; Desenclos *et al.*, 1999; Shears and Wright, 1995). One explanation for this seasonal peak during cooler month is that the cold weather can have physiological effects in the host that favours and aggravates the symptoms caused by rotavirus infection (Bishop, 1996). In this study, five non-typeable isolates were identified. Moreover, three specimens had mixed infections (6%), which is epidemiologically important since it is believed that genetic reassortment is facilitated in mixed infections, contributing to the greater diversity of rotavirus populations by providing the means for natural reassortment and contributing to virus evolution (Das *et al.*, 2002). Where mixed infections are more common, such as in Bangladesh (23%) (Ramachandran *et al.*, 2000), virus evolution appears to proceed at a more rapid rate compared to those countries where mixed infections are not as prevalent, such as in the United States (Griffin *et al.*, 2000; Reidy *et al.*, 2005). Also, surveys in urban areas in developing countries yield a greater variety of electropherotypes than developed countries due to densely populous environments (Masendycz *et al.*, 1994). Interestingly, in any centre the dominant electropherotype usually changes from year to year and seldom persists for more than two seasons (Bishop, 1996), which further

underlines the complexity of rotavirus epidemiology. Therefore, the continued monitoring of the distribution of G-serotypes and E-types in Melbourne in order to determine the existence of any trends in the reappearance or disappearance of the particular types of virus strains will be of great importance. The epidemiological studies conducted at the National Rotavirus Network laboratory in Melbourne has determined such changing trends in Australia including Melbourne (Kirkwood *et al.*, 2002; 2003; 2004a; Masendycz *et al.*, 2001) which will help to determine the most suitable rotavirus vaccine for this region.

The prevalence of serotype G9 in Melbourne concurs well with the greater incidence of this serotype in other parts of the country (Kirkwood *et al.*, 2004; Kirkwood *et al.*, 2003). Previous epidemiological surveys based on the determination of G-serotype have indicated that genetic diversity can exist with serotypic diversity and more detailed analysis of a single serotype needs to be determined in order to establish the basis for such variation. The Melbourne serotype G9 isolates exhibited two different dominant E-types which indicate genetic variation among these isolates and therefore it is necessary to determine the genetic evolution, origin and diversity among this type. The success of new vaccines in controlling the prevailing serotype G9 rotaviruses remains to be seen.

Chapter Four

Genetic and Antigenic Variation in Major Structural Protein, VP7, of Serotype G9 Rotaviruses Collected From 1997-2002

4.1 Introduction

Fifteen G-serotypes and twenty six P-types of rotavirus have been reported worldwide, of which ten G- and eleven P-types have been reported in humans (Rahman *et al.*, 2005a; Santos, 2005). Over the past thirty years the majority of rotavirus serotypes identified in epidemiological studies have belonged to the four major G-types, G1-G4 and thus vaccines were developed to protect against these serotypes. However, recent epidemiological studies have reported the emergence of G-types such as G5, G6 and G8 in Brazil and Malawi (Cunliffe *et al.*, 1999; Gouvea *et al.*, 1999; Leite *et al.*, 1996; Santos *et al.*, 1998) and G9, G10 and G12 in India and Brazil (Arias *et al.*, 1996; Araujo *et al.*, 2001; Samajdar *et al.*, 2006; Santos *et al.*, 1998). Over the past ten years serotype G9 has become common with a worldwide distribution (Kirkwood *et al.*, 2003; Santos and Hoshino, 2005) During the last decade, there have been increasing reports of incidence of serotype G9 rotavirus in Australia, Bangladesh, India, the United Kingdom, Brazil, Italy, Nigeria and the United States (reviewed by Santos and Hoshino, 2005). In Australia, serotype G9 rotavirus was first reported in Sydney, Perth and Melbourne in 1997 (Palombo *et al.*, 2000). The emergence and persistence of serotype G9 rotavirus in recent years highlights the unique evolutionary phenomenon of rotavirus. In Australia, serotype G9 rotavirus was identified as the major type causing infections in 2000 and 2001, being the dominant type in many cities around the country (Kirkwood *et al.*, 2004). A recent outbreak of serotype G9 rotavirus in Central Australia, where >98% of total incidence of rotavirus was due to this serotype, highlights the impact of this type (Kirkwood *et al.*, 2004a). Serotype G9 rotavirus has been identified in Melbourne and around Australia for the past six years. Given the results of the previous chapter which showed a dominance of serotype G9 during a single year, the remainder of this study focussed on a detailed investigation of the antigenic and genetic diversity of serotype G9 rotaviruses collected over a six-year period in Melbourne.

There are several mechanisms whereby rotavirus can undergo genetic alterations. These include: (i) sequential point mutations in genes coding for immunologically important rotavirus proteins, (ii) reassortment of genes between human strains, (iii) introduction of animal rotavirus genes into human strains, (iv) genomic rearrangement (Bishop, 1996).

Two neutralising proteins are located on the outer capsid of rotavirus: VP7, a glycoprotein, confers G-serotype specificity, and VP4, the spike protein, confers P-serotype specificity. VP7 contains six antigenic regions: region A (amino acids (aa) 87-101), B (aa 143-152), C (aa 208-221), D (aa 65-76), E (aa 189-190) and F (aa 235-242) (Dyall-Smith and Holmes, 1984; Kirkwood *et al*., 1993). Investigations of these regions have provided valuable understanding of the antigenic properties of VP7 in rotaviruses when analysed using various MAbs directed against this protein in EIA (Ciarlet *et al.*, 1997). Antigenic regions on the VP4 protein has been mainly associated with the cleaved protein product, the VP8* region, between residue 93 and 208 (Fuentes-Panana *et al.*, 1995).

Several studies have described the genetic and antigenic variation within serotypes G1, G2, G3 and G4 (Estes *et al.*, 2001; Kapikian, 2001; Palombo, 1993). Information about the sequence and antigenic divergence of the VP7 protein of serotype G9 rotaviruses is limited. The aim of this study, therefore, was to study the genetic and antigenic changes in Melbourne serotype G9 isolates and investigate the evolution and origin of this serotype. For this purpose, serotype G9 isolates obtained over a six year period (1997-2002) from children admitted to the Royal Children's Hospital, Melbourne, and were analysed.

4.2 Results

4.2.1 RNA profiles of serotype G9 isolates

The RNA electropherotype profiles of seventy nine serotype G9 rotaviruses collected over a period of six years, from 1997-2002, were determined by PAGE. These seventy nine isolates exhibited twenty eight different gene migration patterns (E-type) (Fig 4.1). All of the isolates analysed exhibited long patterns in regards to the mobility of gene segment 11. One isolate was selected from each of the different electropherotypes for the further study and they were designated R1-R29. The year of isolation of these selected representative samples are listed in Table 4.1. With the possible exception of gene segment 4, all other gene segments exhibited variation in the RNA pattern.

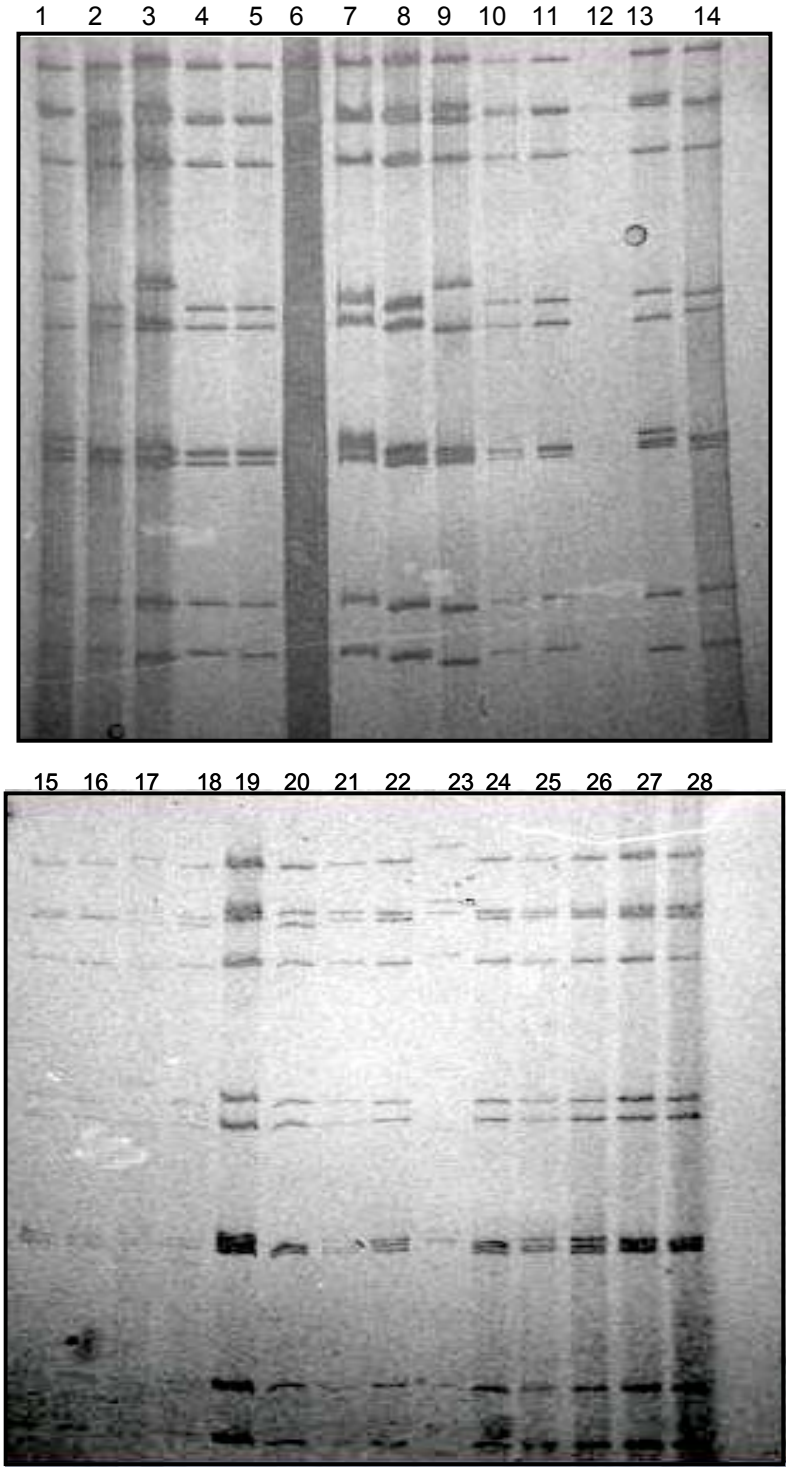

Figure 4.1 Electropherotypes of representative serotype G9 isolates collected from 1997-2002 from the Royal Children's Hospital, Melbourne. Lanes 1-14 shows samples R1-R8, R10-R15 respectively, lanes 15-28 show samples R16-R29 respectively. The sample in lane 12 was faint possibly due to degraded RNA. Isolate R9 did not yield any RNA and is not shown in the gel.

Table 4.1 Representative serotype G9 isolates collected in Melbourne from 1997-2002

Isolate number	Month and year of isolation	Position (lane) in Fig 4.1
R1	February 1997	1
R2	June 1999	2
R3	August1999	2
R4	August 1999	4
R5	September 1999	5
R6	September 1999	6
R7	October 1999	7
R8	October 1999	8
R9	June 2000	-*
R10	August 2000	9
R11	September 1999	10
R12	September 1999	11
R13	January 2001	12
R14	April 2001	13
R15	May 2001	14
R16	June 2001	15
R17	February 2001	16
R18	May 2001	17
R19	July 2001	18
R20	April 2001	19
R21	September 2001	20
R22	February 2002	21
R23	June 2002	22
R24	July 2002	23
R25	August 2002	24
R26	September 2002	25
R27	August 2002	26
R28	September 2002	27
R29	July 2002	28

* not present in Fig 4.1.

4.2.2 VP7 genotyping of isolates by RT-PCR and semi nested PCR

Of seventy nine serotype G9 isolates collected, twenty eight isolates were selected based on their different electropherotypes for further analysis. These twenty eight samples were G-genotyped by RT-PCR and semi-nested PCR (section 2.4) using primers described in Table 2.5. The RT-PCR product of the full length VP gene (first round) was of the expected size of 1062bp (Fig 4.2). The VP7 genotype was determined for all isolates and all yielded a product of 306bp (Fig 4.2) in second round PCR, which is characteristic of genotype G9 rotavirus (Fig 2.2).

4.2.3 Antigenic analysis of serotype G9 isolates by EIA

VP7 specific MAbs drived from the serotype G9 prototype strains F45 and WI61 (F45:1, F45:2, F45:5, F45:7, F45: 8, F45:9, WI61:1) were used to determine the antigenic reactivities of Melbourne serotype G9 isolates. The EIA procedure was carried out as described by Kirkwood *et al.* (2005). Comparison of the EIA results identified six different antigenic groups, designated as monotypes G9a to G9f, based on the combinations of positive reactivities with these MAbs (Table 4.2).

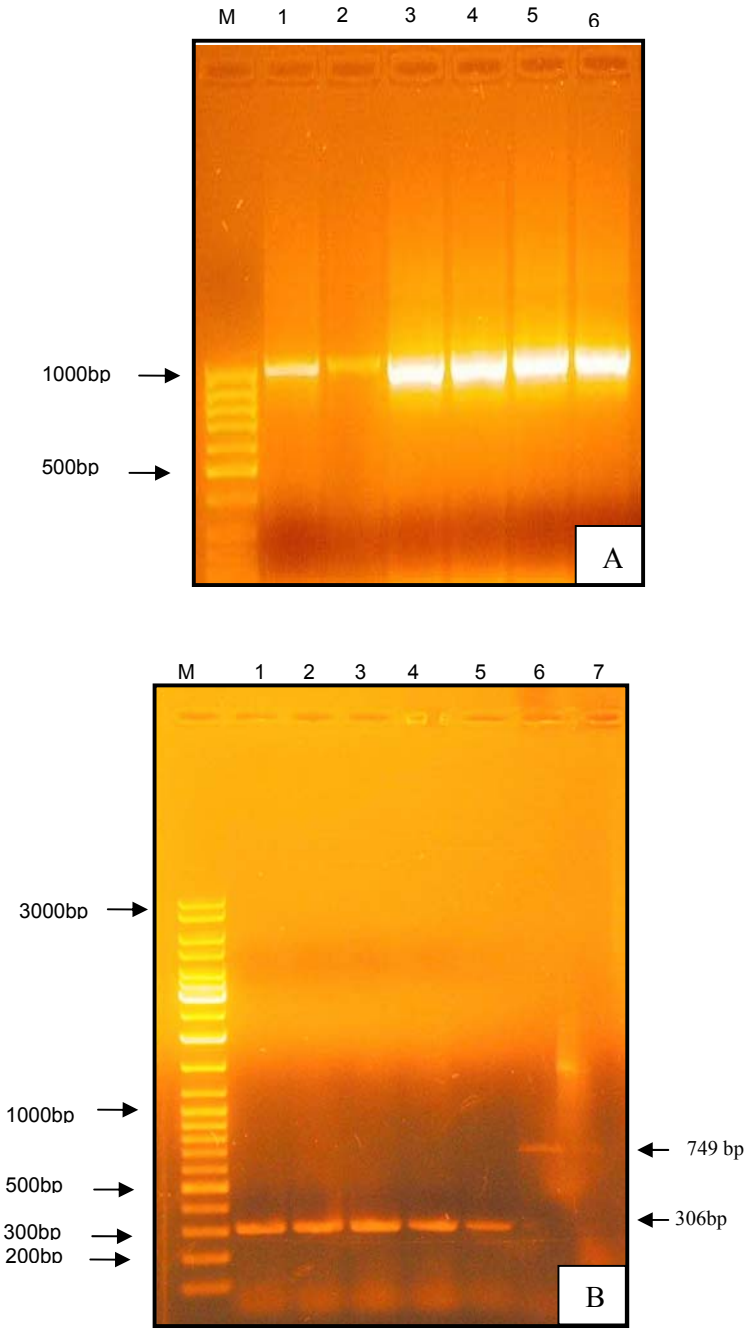

Figure 4.2 VP7 genotyping of selected serotype G9 isolates. Lanes 1-5 contain samples R1-R4 and R14 and positive control serotype G1 respectively. M is the 3kb DNA marker. Gel A contains cDNA produced by the first round amplification by RT-PCR and gel B contains corresponding second round semi-nested PCR products. Lane 6 contains positive control serotype G1 (749bp).

Table 4.2 Serotype G9 monotypes of Melbourne serotype G9 isolates determined by EIA with monoclonal antibodies derived from strains F45 and WI61.

	MAb reactivity*							
Monotype	**F45:1**	**F45:2**	**F45:5**	**F45:7**	**F45:8**	**F45:9**	**WI61:1**	**Representative isolates**
G9a	-	+	-	-	-	-	+	R1
G9b	+	+	+	+	+	-	+	R2
G9c	-	+	+	+	+	+	+	R3
G9d	+	+	+	+	+	+	+	R4
G9e	-	+	+	-	-	-	+	R26
G9f	+	+	+	-	+	-	+	R22

*Positive reaction was indicated by absorbance reading of ≥0.2 at 450nm.

Five isolates (R1, R5, R11, R19 and R20) reacted with two MAbs (F45:2 and WI61:1) and they were termed monotype G9a. Ten isolates (R2, R6, R7, R14, R15, R16, R23, R27, R28 and R29) belonged to monotype G9b and reacted to all MAbs except F45:9. Monotype G9c comprised only two isolates (R3 and R13) and did not react with MAb F45:1. Monotype G9d contained four isolates (R4, R8, R17, R18 and R25) that showed positive reactivities to all seven MAbs. Monotype G9e comprised three isolates (R10, R24, and R26) which showed positive reactivities with MAbs F45:2, F45:5 and WI:61 and monotype G9f contained three isolates (R12, R21 and R22) that reacted with all the MAbs except F45:7 and F45:9.

4.2.4 Nucleotide and putative amino acid sequences determination of the VP7 gene

The complete VP7 gene coding sequences of selected samples from each monotype were determined. The sequence data were analysed and aligned using the program BioEdit version 7 (www.mbio.ncsu.edu/BioEdit). The coding region of these isolates was 978 bp long and contained one open reading frame encoding a protein of 326 amino acids (Fig 4.3). The aligned DNA sequences of these isolates are shown in Appendix II. Sequence alignments of the deduced VP7 protein from each monotype and the standard strain, F45 (Fig 4.3) revealed amino acid substitutions in various regions of the protein (Table 4.3) including antigenic regions A, C, D, E and F (Fig 4.3).

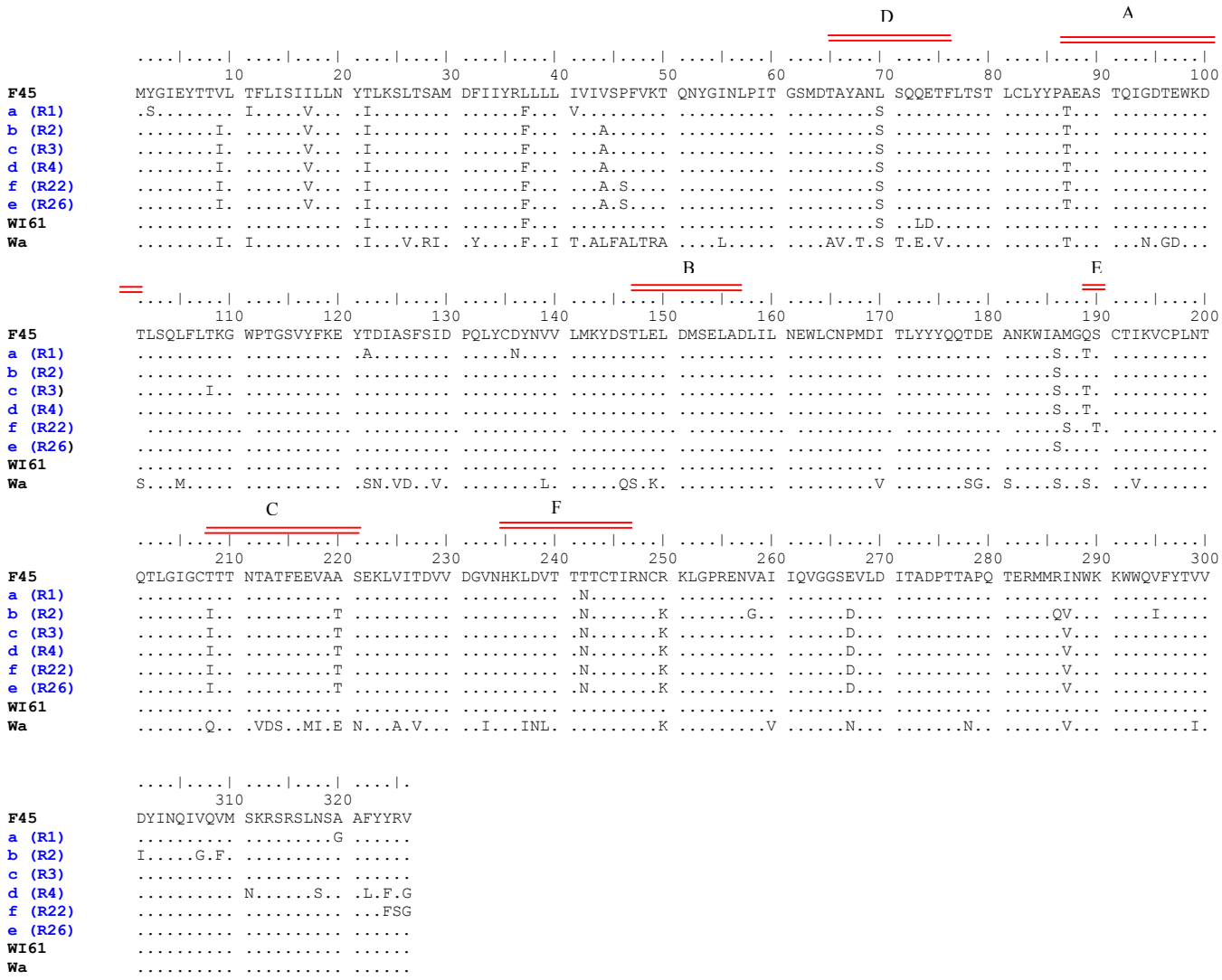

Figure 4.3: Alignment of deduced VP7 amino acid sequences of Melbourne isolates (in blue letters) and prototype strains F45 and WI61 using Clustal W software. The sequence of the prototype serotype G9, F45 is shown in full, while only amino acid substitutions of other strains are indicated. Dots above the sequence signify well conserved positions. Antigenic regions are indicated by red double lines above the sequence. The sequence of the prototype serotype G1 strain, Wa, is included for comparison

Table 4.3 Variations in antigenic regions of VP7 protein of serotype G9 monotypes when compared with the VP7 protein of the G9 prototype strain, F45. The position of the substituted amino acid is indicated in parentheses followed by the substituted amino acid.

G9 monotype	Antigenic Regions (Amino Acids)					
(Isolate)	A (87-101)	B (143-152)	C (208-221)	D (65-76)	E (189-190)	F (235-242)
a (R1)	A (87) →T	None	None	L (70) →S	Q (189) →T	T (242) →N
b (R2)	A (87) →T	None	T (208) →I A (220) →T	L (70) →S	none	T (242) →N
c (R3)	A (87) →T	None	T (208) →I A (220) →T	L (70) →S	Q (189) →T	T (242) →N
d (R4)	A (87) →T	None	T (208) →I A (220) →T	L (70) →S	Q (189) →T	T (242) →N
e (R26)	A (87) →T	None	T (208) →I A (220) →T	L (70) →S	none	T (242) →N
f (R22)	A (87) →T	None	T (208) →I A (220) →T	L (70) →S	Q (189) →T	T (242) →N

The sequence data determined in this study were also compared with global serotype G9 isolates and with those of prototype serotype G9 strains F45, AU32 and WI61. The glycosylation site at position 69-71 was conserved among all isolate sequenced. No other additional potential glycosylation sites were observed.

Melbourne isolate R1 (isolated in 1997) exhibited more genetic variation than any other isolates sharing only 89% nucleotide and 93% deduced amino acid sequence identity with the rest of the isolates analysed and 88% nt identity with prototype strain, F45. All of Melbourne isolates with the exception of R1 exhibited 96-99% nucleotide and amino acid identity with one another when compared by the program CLUSTAL W of PHYLIP version 3.5c (Thompson *et al.*, 1994)).

Nine variable regions (VR) of rotaviruses have been determined, with six regions present in the mature VP7 gene. Sequences of the VP7 genes of Melbourne serotype G9 isolates showed mutations in most of the VR as compared to the prototype serotype G9 strain, F45 (Table 4.4). The sequence comparisons of these isolates with the prototype serotype G9

strains are shown in Fig 4.3 and amino acid substitutions in antigenic and variable regions are listed in Table 4.3 and Table 4.4 respectively. The amino acid differences among Melbourne serotype G9 isolates, representative global serotype G9 isolates and prototype serotype G9 isolates are shown in Table 4.5. The only regions conserved among all six monotypes were antigenic region B and VRs 2, 5 and 7.

4.2.5 Correlation between monotypes and the sequences of VP7 antigenic regions

To investigate the correlation between monotypes and the genetic variation among Melbourne serotype G9 isolates, the VP7 genes of representative isolates from each antigenic group were compared. The sequence comparisons revealed that monotype G9b and G9c shared 98% nucleotide and 96% deduced amino acid identity. Similarly, monotype G9c and G9d shared 97% nucleotide and 96% amino acid identity. The only isolate that showed significant nucleotide variation was monotype G9a (isolate R1), which shared only 89% nucleotide identity with all other monotypes but had 95% amino acid identity. When the antigenic regions were compared among the monotypes, no correlation with the monotype was identified. The amino acid differences among these monotypes are listed in Table 4.3 and their antigenic regions are compared in Fig 4.3.

All isolates sequenced showed amino acid substitutions at numerous positions in the antigenic regions but these did not correlate with their MAbs reactivities. For example, G9c and G9d had amino acid substitutions at exactly the same positions in the major antigenic regions (at aa 42, 70, 87, 189, 208 and 220) and yet they reacted differently with the monoclonal antibody F45:1; monotype G9c did not bind with the antibody, whereas monotype G9d did (Table 4.2). These two isolates however, differed in positions outside the antigenic regions (at aa 109, 301, 307, 309). Likewise, monotypes G9b and G9e showed different EIA results; G9b reacted with the MAbs F45:1, F45:7 and F45:8 while G9e did not bind yet they both showed similar substitutions in the antigenic regions at positions 42, 70, 87, 208 and 220 (Table 4.2 and Table 4.3).

Table 4.4: Amino acid substitutions in the variable regions of VP7 protein of isolates when compared with the prototype strain, F45. The position of amino acid substitution is shown in parentheses followed by substituted amino acid.

G9 Monotype (Isolate)	Variable regions (amino acids)								
	1 (9-20)	**2 (25-32)**	**3 (37-52)**	**4 (65-76)**	**5 (87-100)**	**6 (119-132)**	**7 (141-150)**	**8 (208-224)**	**9 (235-242)**
a (R1)	I (17)→V	none	L (37) →F I (42)→V	L (70) →S	A (87) →T	T (122) →A	none	none	T (242) →N
b (R2)	V (9) →I	none	L (37) →F V (44)→A	L (70) →S	A (87) →T	None	none	T (208) →I A (220) →T	T (242) →N
c (R3	V (9) →I	none	L (37) →F V (44)→A	L (70) →S	A (87) →T	None	none	T (208) →I A (220) →T	T (242) →N
d (R4)	V (9) →I	none	L (37) →F V (44)→A	L (70) →S	A (87) →T	None	none	T (208) →I A (220) →T	T (242) →N
e (R26)	V (9) →I	none	L (37) →F V (44)→A P (46) →S	L (70) →S	A (87) →T	None	none	T (208) →I A (220) →T	T (242) →N
f (R22)	V (9) →I	none	L (37) →F V (44)→A P (46) →S	L (70) →S	A (87) →T	None	none	T (208) →I A (220) →T	T (242) →N

Note: Variable regions 5, 7, 8, 4 and 9 are equivalent to antigenic regions A, B, C, D and F respectively.

Table 4.5 The percentage of nucleotide and amino acid differences among VP7 genes of Melbourne serotype G9 isolates (in blue) and global serotype G9 isolates. Strain Wa (Serotype G1) was included to show high nucleotide and deduced amino acid variation with serotype G9 isolates.

% of nucleotide and amino acid substitution between isolates[#]

	F45	R1	R2	R3	R4	R22	R26	WI61	AU32	Wa	OM67	OM46	116E	MW47
F45		4.3	6.1	4.9	7.4	5.8	4.6	1.5	0.6	20.8	3.2	3.2	7.7	4.6
R1	9.8		6.1	4.3	6.7	5.2	4.6	3.9	4.9	20.5	1.2	1.2	8.6	4.6
R2	10.5	10.5		2.4	4.6	3.3	2.1	5.8	6.7	20.8	4.9	4.9	9.2	2.1
R3	10.6	10.5	1.7		3.2	1.5	0.9	4.6	5.5	19.3	3.6	3.6	8.6	0.9
R4	11.1	10.6	3.1	2.3		2.7	3.3	7.0	7.9	21.7	6.1	6.1	10.1	3.3
R22	10.5	9.8	1.8	1.1	1.8		1.2	5.5	6.4	19.9	4.6	4.6	9.2	1.8
R26	10.1	9.9	1.1	1.1	2.4	0.9		4.3	5.2	19.1	3.3	3.3	8.2	0.6
WI61	1.1	9.6	10.1	10.1	10.7	9.9	9.6		2.1	20.2	2.7	2.7	7.3	4.3
AU32	0.2	10.1	10.7	10.7	11.3	10.6	10.3	1.2		20.8	2.6	2.6	8.2	5.2
Wa	23.4	23.8	23.3	22.7	23.3	22.5	22.5	23.1	23.4		19.6	19.6	21.7	19.3
OM67	9.3	0.5	9.9	10.5	10.6	9.8	9.4	9.1	9.5	23.5		0	7.3	3.3
OM46	9.3	0.5	9.9	10.5	10.6	9.8	9.4	9.1	9.5	23.5	0		7.3	3.3
116E	9.8	9.9	11.3	11.4	11.8	11.3	10.9	10.1	10.1	23.4	9.4	9.4		8.2
MW47	10.3	9.6	1.5	1.5	2.7	1.6	0.6	9.8	10.5	22.7	9.1	9.1	10.7	

% of amino acid difference (above the line)

% of nucleotide difference (below the diagonal line)

[#]Values determined by Pairwise distance in MEGA from nucleotide and deduced amino acid sequences alignment between isolates, using complete deletion. Melbourne serotype G9 rotaviruses sequenced in this study are indicated in blue.

4.2.6 Phylogenetic analysis of VP7 gene with other Australian serotype G9 isolates

In order to investigate the evolutionary relatedness of Melbourne serotype G9 isolates, a phylogenetic tree was drawn from nucleotide sequences of VP7 coding regions of isolates representing the six different monotypes (G9a-G9f) sequenced in this study, together with VP7 sequences of serotype G9 isolates from Australian cities such as Alice Springs, Perth, Sydney, Brisbane and Melbourne sequenced previously by Kirkwood *et al*. (2003)(Fig 4.4). These serotype G9 isolates comprised a seven year collection dating from the first serotype G9 strain isolated in 1997 to most recent strain isolated in 2003. The results revealed that isolate R26 clustered with the Melbourne strains previously sequenced. Of interest was that isolate R2 (isolated in 1999) shared close homology with an outbreak strain isolated in Perth and Alice Springs (2001) (Kirkwood *et al*., 2004). Two of the Melbourne serotype G9 isolates representing monotypes G9d (R4, isolated in 1999) and G9f (R22, isolated in 2002) formed a separate branch. Interestingly, isolate R1 detected in 1997, the year of the first detection of serotype G9 in Melbourne, exhibited considerable variation from the rest of Melbourne serotype G9 isolates analysed as well as other Australian isolates and formed a separate branch (Fig 4.4).

The degree of nucleotide identity among Melbourne serotype G9 isolates sequenced in the present study was >98% except for isolate R1, which had <89% sequence identity with these. The nucleotide sequence identities of serotype G9 isolates from this study compared with other Australian serotype G9 isolates ranged from 96-98% and deduced amino acid identities ranged from 96-99%. The Central Australian outbreaks strain, OB G9.1, had high overall amino acid identity of 97-98% with serotype G9 isolates investigated in the present study. However, amino acid substitutions at positions 40 (Leu →Phe) and at 68 (Ala →Val) were unique to the outbreak strain. Again, isolate R1 shared only 88-89% nucleotide sequence identity and 93-96% deduced amino acid identity with other Australian serotype G9 isolates analysed. Nucleotide sequence identity among six Melbourne isolates from previous studies (Kirkwood *et al*., 2003) ranged from 98-99% and deduced amino acid identities ranged from 99-100%. All nucleotide sequences obtained in this study are aligned with other serotype G9 isolates and are shown in Appendix II.

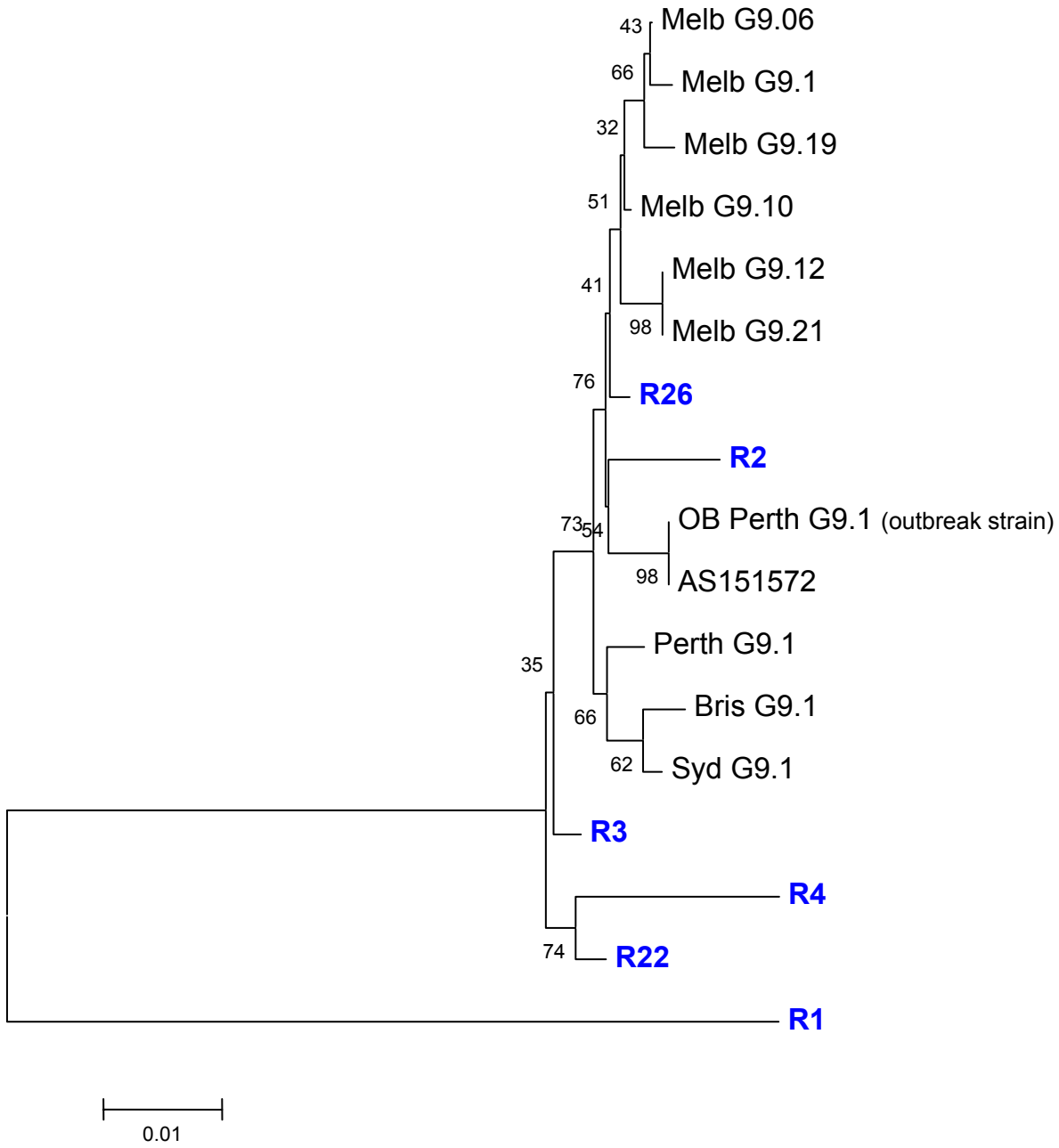

Figure 4.4 Phylogenetic tree of coding regions of VP7 genes of Melbourne serotype G9 isolates determined in this study (in blue bold) and other Australian serotype G9 strains. Australian serotype G9 isolates Melb G9.1, G9.6, G9.10 G9.12, G9.19, Syd G9.1, OB Perth G9.1, Bris G9.1 were obtained from the GenBank database (accession numbers AY307085-AY307094 respectively). Scale shows the genetic distance and the bootstrap values are shown in respective branches. The tree was generated using the Neighbor-Joining method in MEGA 3.1.

4.2.7 Phylogenetic analysis of Australian serotype G9 isolates with global and reference serotype G9 strains

The phylogenetic relationships among Australian serotype G9 isolates and representative global serotype G9 strains, including the prototype strains F45 and WI:61, were investigated (Fig 4.5). This analysis revealed the presence of three different phylogenetic lineages and some sub-lineages. All Australian serotype G9 isolates, including isolates sequenced in this study, clustered within lineage 3 together with other contemporary global serotype G9 isolates, but distinct from the prototype serotype G9 strains. They shared 96-99% nucleotide sequence identity with other lineage 3 isolates compared to only 88% identity with lineage 1 and 87% identity with lineage 2. A comparative analysis of Melbourne serotype G9 isolates with the worldwide serotype G9 strains revealed these serotype G9 isolates are more closely related with representative strains from Asian regions such as Thailand, Japan, Korea and Taiwan (Fig 4.5) exhibiting identities of 98-99%. In comparison, the US strains US1205 and US1212 exhibited slightly lower amino acid identity (96%).

One Melbourne isolate (R1) however, exihibited highest sequence identity with two US strains, Om46 and Om67 (99% nucleotide and deduced amino acid identities), and formed a distinct sub-lineage withing lineage 3.

4.2.8 Comparison of VP7deduced amino acid sequences

When comparing the Melbourne serotype G9 isolates to global isolates, an amino acid substitution (Pro→Ser) at position 46 was noteworthy as this substitution was also seen for Asian strains 02-22 (S Korea), CMH045 (Thailand), 00-2509 (Japan) but not observed in strains from other countries or prototype G9 strains. However, an amino acid substitution at position 189 (Gln→Thr) was distinctive in serotype G9 isolates

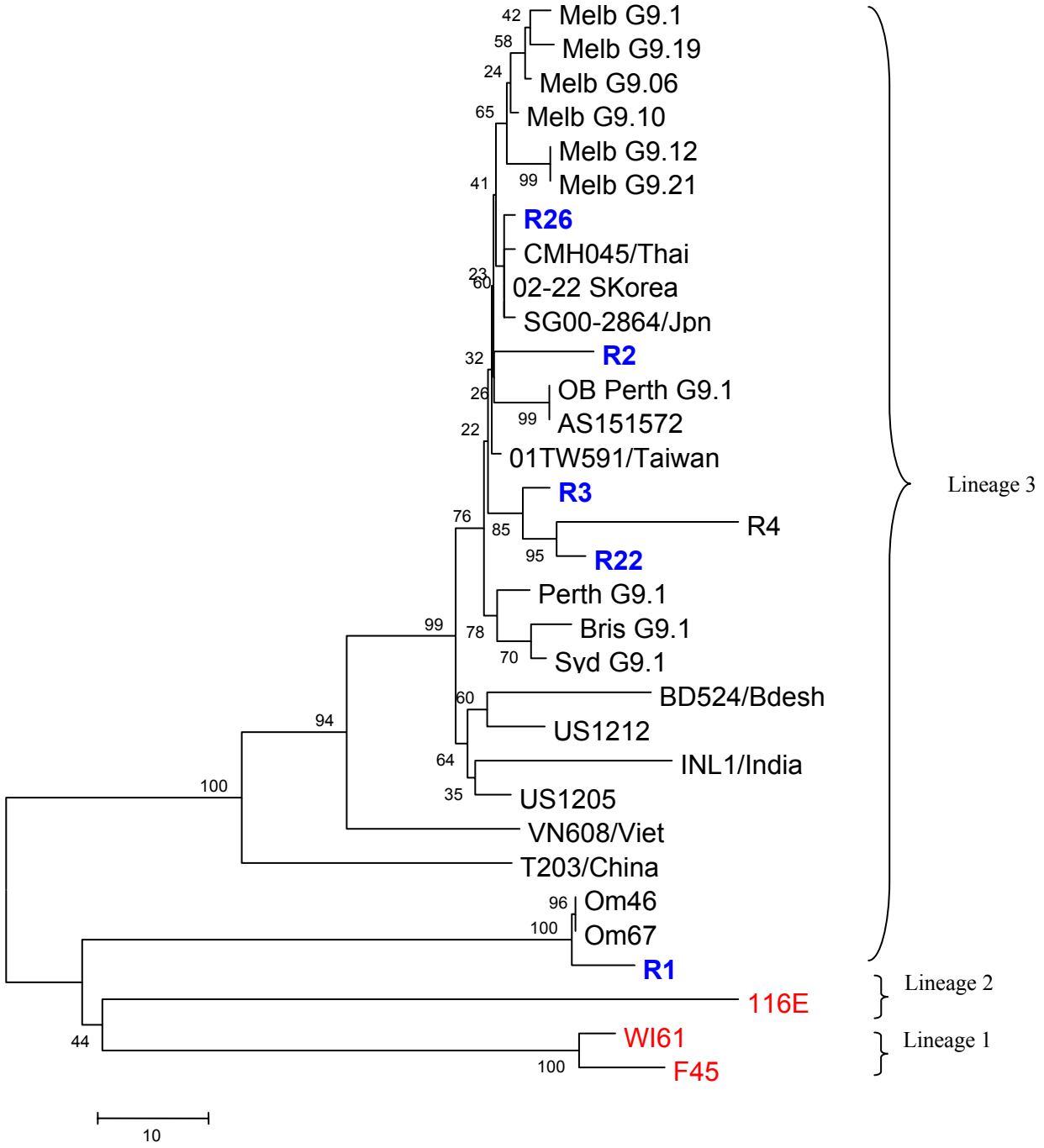

Figure 4.5 Phylogenetic tree of Melbourne isolates (in blue) compared with global serotype G9 isolates (in black) and prototype strains (in red). Sequences of global G9 and prototype strains were derived from Genbank and their accession numbers are; F45 (AB180970), WI61 (AB180969), CMH3045 (AY699292), US1205 (AF060487), US1212 (AJ250272), OM67 (AJ491179), OM46 (AJ491181), VN608 (AB091777), T203 (AY003871), CS96-40(AY165008), INL1 (AJ250277), BD524 (AJ250543), 02-22 (AY879296), 116E (L14072), 01TW591 (AY165008), SG00-2864 (AB091751) . Australian G9 isolates Melb G9.1, G9.6, G9.10 G9.12, G9.19, Syd G9.1, OB Perth G9.1, Bris G9.1 accession numbers AY307085-AY307094 respectively. The phylogenetic tree was drawn using the neighbor-joining method in software MEGA 3.1. Scale shows the genetic distance and the bootstrap values are shown in respective branches.

sequenced in this study and was not present in other worldwide serotype G9 strains. Of significance were the amino acid substitutions in the antigenic regions A (Ala → Thr, at position 87) and F (Thr →Asn, at position 242) among all recent serotype G9 isolates.

Isolate R1 exhibited more substitutions than any other serotype G9 isolates sequenced in the present study. Other than the substitution in antigenic regions (Table 4.3) this isolate also showed additional changes at positions 2 (Tyr →Ser), 11 (Thr →Ile), 41 (Ile →Val), 122 (Thr →Ala), 137 (Asp →Asn) and at 320 (Ala →Gly) not shown by any other serotype G9 strains with the exception of two US strains Om46 and Om67 isolated in 1996. Isolate R1 had only 88-89% nucleotide identity and 95% amino acid identity with other serotype G9 strains including prototype strains, F45 and WI61 but shared 99% nucleotide and deduced amino acid identity with these two US strains.

4.2.9 VP4 genotyping of selected serotype G9 isolates

The VP4 genotypes of six representative rotavirus isolates, R1-4, R22 and R26, representing monotypes G9a-G9f, respectively, were determined by RT-PCR and semi-nested PCR as described in section 2.5 (Fig 4.6). All of the six isolates produced a cDNA product of 876 bp in the first round PCR amplification with the conserved VP4 primers. Second round PCR amplification yielded a product of 345 bp in all serotype G9 isolates that corresponded to the expected size for P genotype [8] (Fig 4.6).

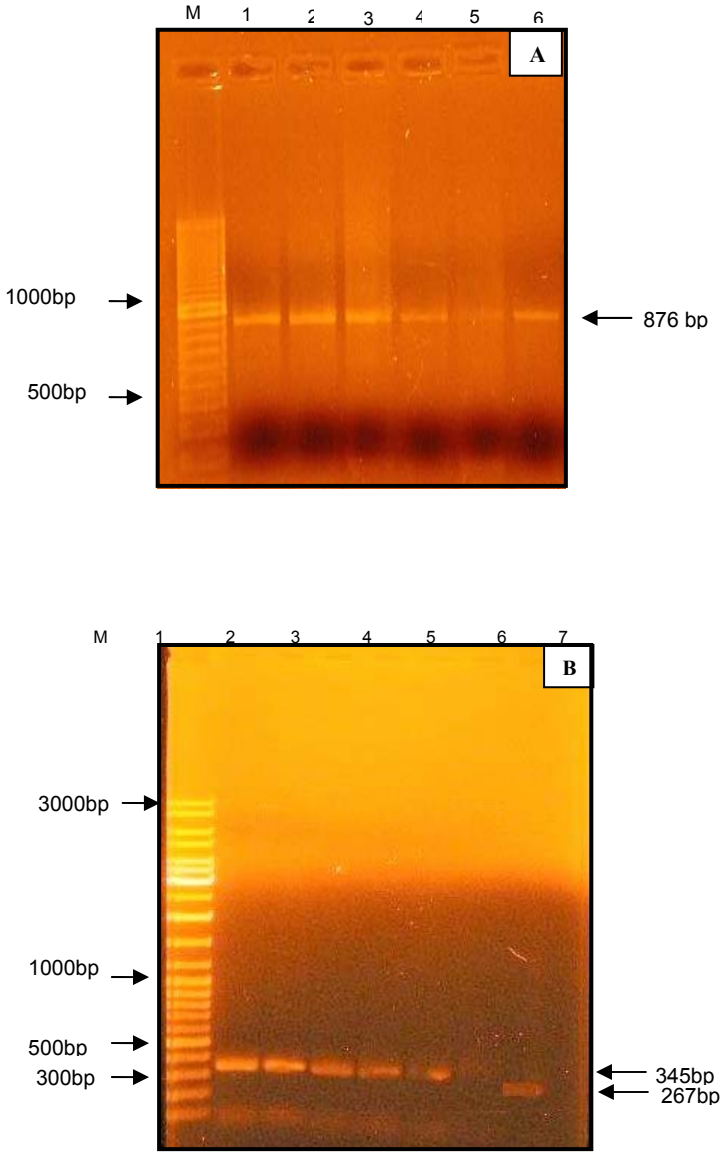

Figure 4.6 VP4 genotyping of selected Melbourne serotype G9 isolates by two step amplification; (A) RT-PCR and (B) corresponding semi-nested PCR products. M is the 3000bp DNA marker. Lanes 1-6 contain isolates R1-R4, R22 and R26 respectively (345bp), lane 7 contains positive control P[6] (267 bp).

4.2.10 Northern hybridisation of serotype G9 rotaviruses using whole genome probe derived from prototype serotype G9 strain, F45

To study the genetic relatedness of all gene segments of serotype G9 isolates with the prototype G9 strain, F45, Northern hybridisation was carried out on the all twenty eight representative Melbourne serotype G9 isolates using a whole genome probe derived from strain F45. Homologous reactions were identified as bands on a nylon membrane. The specificity of the F45 whole genome probe is illustrated in Fig 4.7 as the probe hybridised to all eleven RNA segments of the parent virus (lane 1).

A high level of genomic relatedness of all twenty eight Melbourne serotype G9 isolates was observed with the prototype F45 strain since the probe hybridised with all RNA segments of these local serotype G9 isolates. In all but two serotype G9 isolates, all gene segments hybridised strongly with the probe. In two isolates (R1 and R14) gene segment 5 showed limited homology (Fig 4.7, lanes 2 and 3). The result indicated that Melbourne serotype G9 isolates belonged to the same genogroup as the prototype strain, F45.

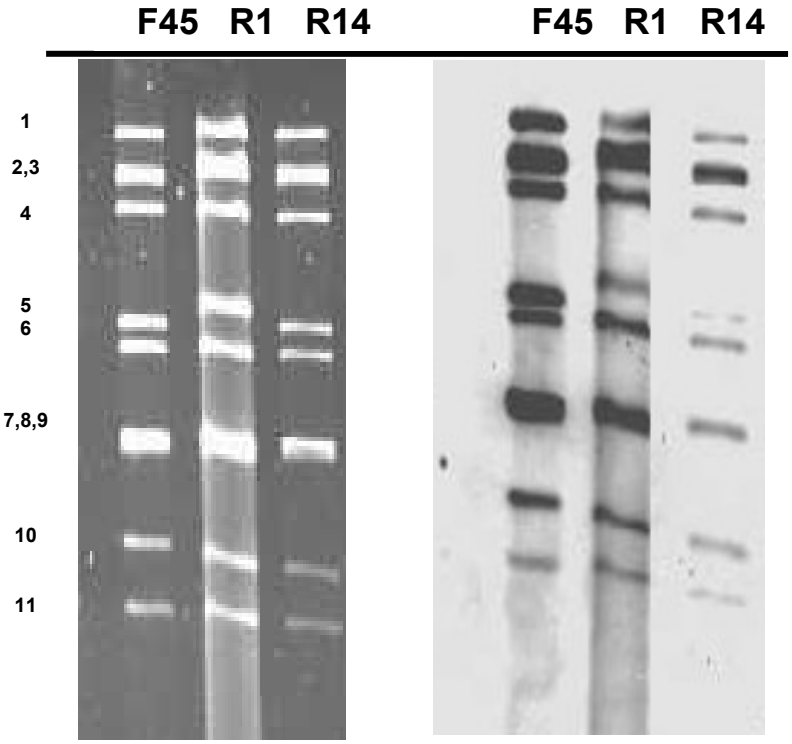

Figure 4.7 Northern hybridisation analysis of serotype G9 rotaviruses using a whole genome probe derived from F45. RNA on the left panel was stained with ethidium bromide, while the right panel shows segments hybridising with the F45 probe. Numbers indicate the gene segments.

4.3 Discussion

The study of rotavirus evolution will greatly assist in assessing the risk of emerging new rotavirus serotypes. Therefore, the success of any vaccine in preventing severe rotavirus infection will depend on its efficacy against a range of rotavirus isolates, including major existing strains as well as emerging strains. The emergence and persistence of serotype G9 rotavirus has had a major impact on health care services in Australia as demonstrated in Central Australia by a severe outbreak of rotavirus gastroenteritis in 2001 (Kirkwood *et al.*, 2004). Because of the extent of spread and dominance of serotype G9 isolates worldwide, this serotype has been incorporated in the vaccine formulations of Merck (Matson, 2006). The development of rotavirus vaccines is based primarily on the VP7 protein due to its antigenic properties. Therefore, information regarding variation in the antigenic sites on the VP7 protein capable of inducing protective antibodies is critical. In this study, the antigenic properties of the VP7 protein of serotype G9 rotavirus isolates collected in Melbourne was investigated using a panel of serotype G9-specific VP7 MAbs. Six different antigenic groups, designated as monotypes G9a-G9f, were identified among 28 serotype G9 isolates analysed. Thirty one percent of serotype G9 isolates belonged to monotype G9b, and these isolates reacted with all of the MAbs except F45:9. Isolates belonging to monotype G9d reacted with all of the seven MAbs and represented 17% of total isolates tested. Sequence comparisons indicated that these two monotypes exhibited only one amino acid substitution in antigenic region E. In previous studies, antigenic variants resistant to MAb F45:9 selected a gene substitution at position 96 (Thr→Ala). Whether a change in antigenic region E can influence this motif is unclear (Kirkwood *et al*., 1993). However, G9c, G9d and G9f had identical antigenic regions yet they reacted differently with MAbs F45:1, F45:7 and F45:9 respectively (Table 4.1 and 4.2). Similarly, G9b and G9e had identical antigenic regions even though they reacted differently with MAbs F45:1, F45:7, F45:8 and F45:9. No single antigenic site or amino acid could be attributed to the lack of MAb reactivities and, therefore, no correlations could be made between antigenic properties and sequences of antigenic regions. A number of substitutions were observed in the deduced amino acid of VP7 (Fig 4.3) which could possibly be responsible for the resistance to antibody bindings, however, no conserved differences were observed.

In a recent study, the antigenic characterisation of other Australian serotype G9 rotaviruses revealed similar findings, where three different antigenic groups of serotype G9 isolates were defined when analysed with MAbs F45:1, F45:8 and WI61:1 (Kirkwood *et al.*, 2003). Antigenic diversity has been reported previously in clinical isolates of serotypes G1 identified from Melbourne (Diwakarla and Palombo, 1999). Similarly, various antigenic variants were also reported in serotype G1 isolates from a nineteen years collection in Italy (Arista *et al.*, 2006).

There could be several factors affecting the binding with the MAbs. (i) interaction of the VP7 protein with the other outer capsid protein VP4 (Chen *et al.*, 1992). Interactions between the two neutralising proteins have been reported to alter the antigenic properties of the virus (Dunn *et al.*, 1994; Mendez *et al.*, 1996; Prasad *et al.*, 1990). (ii) mutation in antibody binding regions significantly alters immunodominant antigenic sites, thus inhibiting the binding of the majority of neutralising antibodies by altering the conformation of the protein and ultimately changing antigenic recognition site involving many epitopes in the region (Shaw *et al.*, 1988). No such mutations that could be attributed to the neutralising properties of MAbs were observed in this study. (iii) another factor affecting MAb binding is glycosylation of VP7. Alteration of a glycosylation site can have a direct effect in the antigenic properties of VP7 protein (Caust *et al.*, 1987). However, no mutations were seen in potential glycosylation sites of serotype G9 isolates. Hence, it can be predicted that possible interaction with VP4 protein may be responsible for the altered VP7-specific MAbs binding seen in this study. To confirm this, sequence analysis of the VP4 gene and analysis of the possible sites of interaction between the two outer capsid proteins is needed. Studies with heterologous combinations of VP4 and VP7 have shown that subtle changes in the phenotypic features of VP4 could be attributed to the specific interactions between these two outer capsid proteins (Chen *et al.*, 1992). Whether such a phenomenon exists in viruses with the same G/P combination is unknown. Two of the serotype G9 specific MAbs; F45:2 and WI61:1, reacted with all of serotype G9 isolates analysed in this study suggesting that either of these MAbs could be used successfully to identify serotype G9 in EIA.

There are three genogroups of human rotavirus (Wa-like, DS-1 like, AU-1 like) determined using RNA-RNA hybridisation under stringent conditions (Nakagomi *et al.*, 1989).

This technique has led to the identification of gene substitutions and reassortment between rotaviruses of different genogroup (Bishop, 1996). The Northern hybridisation analysis carried out in this study revealed that all Melbourne serotype G9 isolates belonged to the same genogroup as the prototype G9, F45 (Wa-like) as all gene segments of these isolates, with the exception of isolates R1 and R14, showing significant homology. Gene segment 5 of isolates R1 and R14 did not hybridised with the probe which prompted a detailed investigation of the variation of gene segment five among Melbourne serotype G9 isolates, as described in the next chapter (Chapter Five).

The phylogenetic analyses showed that Melbourne serotype G9 isolates are closely related to the contemporary serotype G9 isolates from other locations (Fig 4.5). Sequencing studies of emerging serotype G9 isolates from various countries have revealed that strains detected around the mid 1990s do not share the cognate genes of strains detected a decade ago (Gentsch *et al.*, 2005; Santos and Hoshino, 2005). This finding indicates that the modern serotype G9 rotaviruses are not direct descendents of the previous strains but could have been introduced into recent circulation through genetic reassortment (Gentsch *et al.*, 2005; Iturriza-Gomara *et al.*, 2000; Ramachandran *et al.*, 2000). The findings of the present study are in agreement with this suggestion.

Chapter Five

Variation among Non-Structural Proteins, NSP1 and NSP4, of Serotype G9 Rotaviruses

5.1 Introduction

There are six structural proteins and six non-structural proteins encoded by the 11 segments of the rotavirus genome (Estes *et al.*, 2001). Structural proteins are involved in viral morphology and have roles in viral attachment, while the non-structural proteins are responsible for the RNA binding and have roles in viral replication, gene packaging and morphogenesis of the virus (Desselberger, 2000) and are essential for the formation of infectious virions (Hua *et al.*, 1994).

Of the non-structural proteins, NSP1, which is encoded by gene sement 5, shows a high degree of sequence variation among strains from various animals and humans (Taniguchi *et al.*, 1996). NSP1 is unique in regards to its variable length in different strains. For example, it is 486 amino acids long in the human strain Wa whereas it contains 495 amino acids in the simian strain SA11 and is poorly conserved among rotavirus of various species (Hua *et al.*, 1993; Mitchell and Both, 1990). This protein contains a cysteine rich sequence near its amino terminus; C-X_2-C-X_8-C-X_2-C-X_3-H-X-C-X_2-C-X_5-C, detected in NSP1 of all group A rotaviruses analysed to date and is associated with RNA binding activity (Hua *et al* ., 1993; 1994; Patton, 2001). NSP1 has been associated with RNA binding activity and is believed to be responsible for host range restriction and the inability of viruses from one species to replicate efficiently in another species (Ramig, 2004). However, Dunn *et al.* (1994) showed the high rate of gene reassortment between human and bovine strains of gene segment 5 as compared with another three genes, VP4, VP6 and VP7. In another study, phylogenetic analysis showed that NSP1 amino acid sequences fell into species specific groups with the exception that human and porcine strains were included in a single group (Kojima *et al.*, 1996). Despite the huge diversity of NSP1 gene sequences among various animal and human isolates, correlations with the overall genomic relatedness of a particular genogroup have been observed (Dunn *et al.*, 1994; Kojima *et al.*, 1996; Palombo and Bishop, 1994). These observations imply that interaction between NSP1 and other cytoplasmic proteins are host species-specific, however, the fundamental structure of NSP1

of all rotaviruses are similar (Taniguchi *et al.*, 1996). The first 150 amino acids of NSP1 show a greater degree of homology than the rest of the sequence (Taniguchi *et al.*, 1996).

NSP4, which is encoded by gene segment 10, is a multifunctional protein; it is involved in morphogenesis of the virus by attaching with the endoplasmic reticulum (ER) of the host cell (Ball *et al.*, 1996), it acts as an intracellular receptor for double-layered virus as it buds into the ER (Estes *et al.*, 2001), and is a viral enterotoxin that has been shown to induce age-dependent diarrhoea and thus functions as a virulence factor in the host (Ball *et al.*, 1996). However, unlike other non-structural protein, it does not bind RNA. Sequence analyses have identified five genetic groups of NSP4, genotypes A-E (Borgan *et al.*, 2003; Estes *et al.*, 2001; Lin and Tian, 2003) represented by reference strains Wa (genotype A), KUN (genotype B), AU-1 (genotype C), EW (genotype D) and PO-13 (genotype E) (Ball *et al.*, 2005; Lin and Tian, 2003). Sequence diversity of NSP4 is observed mostly in the cytoplasmic domain which interacts with the structural proteins, VP4, VP6 and VP7 (Estes *et al.*, 2001).

As determined by Northern hybridisation (Section 4.2.10), some of the Melbourne serotype G9 rotaviruses carried a gene segment 5, that was genetically different to that of the prototype strain, F45, suggesting variation in this gene. Therefore, this non-structural protein was investigated in detail to examine the extent of genetic diversity among Melbourne serotype G9 isolates collected over the six year period. Another protein, NSP4 was also investigated in this study because of its important role in the pathogenesis and morphogenesis of the virus.

5.2 Results

Northern hybridisation analysis of all Melbourne serotype G9 isolates demonstrated that gene segment 5 of two isolates (R1 and R14) displayed limited homology with an F45 derived probe (section 4.2.10) which prompted further investigation of this gene. To extend this analysis, two additional probes were prepared from the gene 5 of isolates R1

and R14 and these were used in hybridisation experiments with all serotype G9 isolates collected in this study to determine the extent of variation in this gene.

5.2.1 Slot Blot analysis

A DIG-labelled probe was prepared from partially amplified segment 5 of R1 (665 bp, nt 885-1550) by PCR using primers as described by (Maunula and Bonsdorff, 2002). RNA from all serotype G9 clinical isolates and standard strains was denatured and blotted onto a positively charged nylon membrane and hybridised at 54^{o}C using similar hybridisation conditions as used for the F45 whole genome (section 2.7.). No isolate shared significant homology with the R1 probe, except three (R28, MK37 and MK38) that showed partial homology (Fig 5.1). None of the standard reference strains i.e. RV4 (G1), RV5 (G2), ST3 (G3), RV3 (G4) and F45 (G9) had homology with the probe (Fig 5.1).

Similarly, a DIG-labelled probe was prepared from partially amplified segment 5 of R14 (665 bp, nt 885-1550) and was hybridised to the Melbourne serotype G9 isolates. Only 11 out of the 27 RCH isolates (R2, R4, R5, R7, R15 R20, R21, R26, R27, R28 and R29) and two MMC serotype G9 isolates shared significant homology with this probe (Fig 5.2). Insufficient RNA was available for isolates R6, R9, R23, MK37 and R120. Interestingly, isolate R28 shared homology with both the probes, while thirteen of 27 specimens (R3, R8, R10, R11, R12, R13, R16, R17, R18, R19, R22, R24 and R25) did not share any homology with either of the probes. These results suggest the presence of four gene 5 alleles among Melbourne serotype G9 isolates; one group that showed homology with isolate R1, a second group of isolates that shared homology with isolate R14, a third group which carried a gene 5 that was different to those carried by R1 and R14 and a fourth group containing isolate R28 which had a distinctive gene 5 as it shared homology with both the probes.

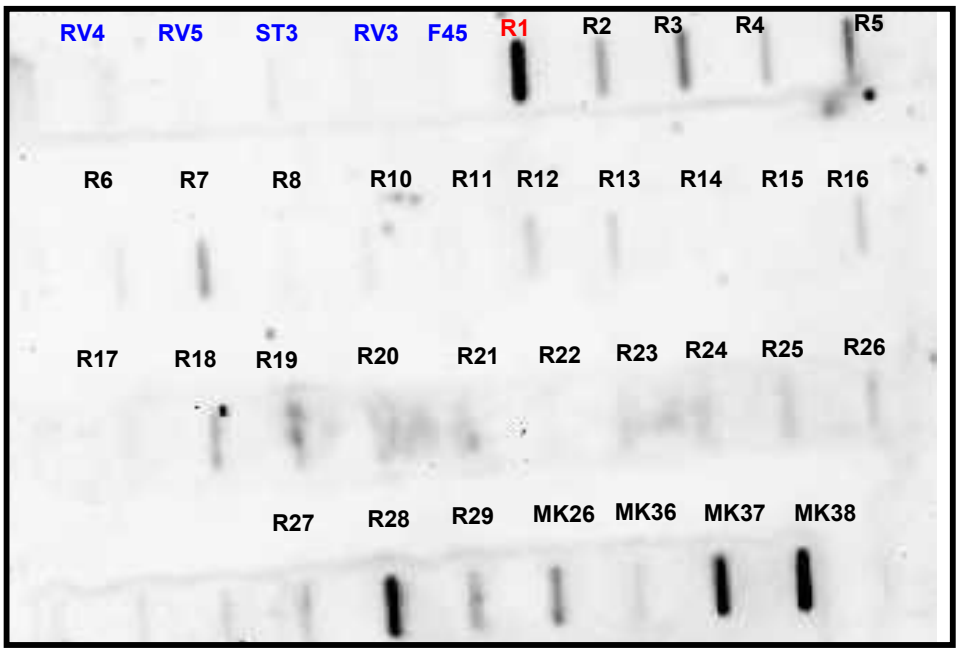

Figure 5.1 Slot blot hybridisation of Melbourne serotype G9 rotaviruses and standard strains (in blue) using a DIG-11-dUTP labelled gene 5 probe of isolate R1. The isolate from which the probe was derived is shown in red. Insufficient RNA was available for isolate R9 and it was not included in this analysis.

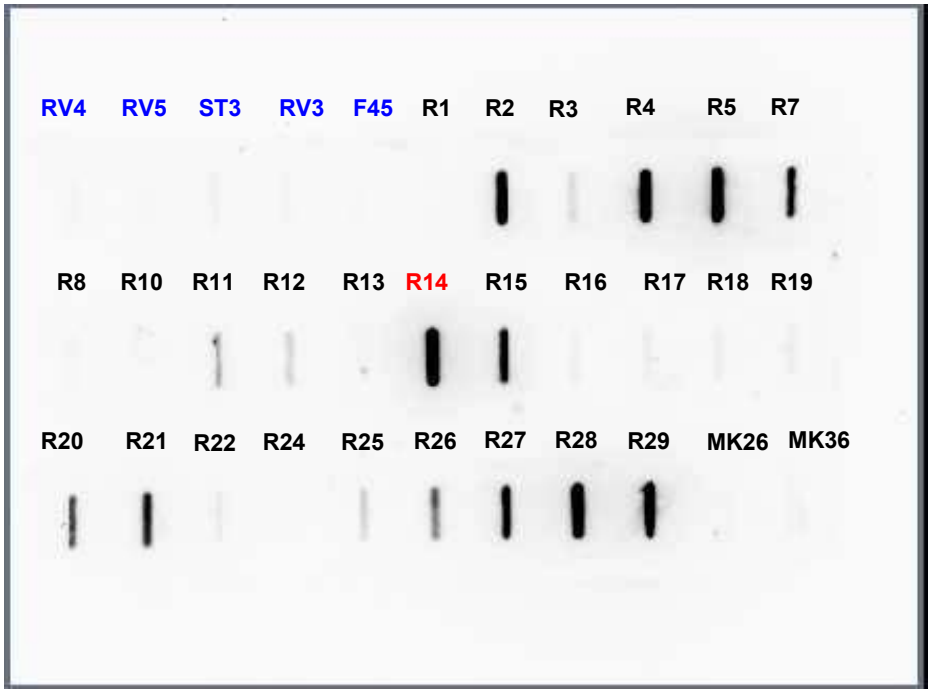

Figure 5.2 Slot blot hybridisation of Melbourne G9 rotaviruses and standard strains (in blue) using a DIG-11-dUTP labelled gene 5 probe of isolate R14. The isolate from which the probe was derived is shown in red. RNA samples were not available for isolates R6, R9, R23, MK37 and MK38.

5.2.2 Sequencing of gene segment 5

To further investigate the degree of genetic diversity in gene 5, a number of isolates were selected for sequence analysis. Three isolates, R1, R11and R14, were selected as they represented the different gene 5 alleles. Four additional isolates, R4, R19, MK26 and MK36, were selected as they represented isolates detected in years 1999, 2001, 2000 and 2002, respectively. Despite several attempts, isolate R28, that showed homology with both R1 and R14 probes, could not be amplified. A 665bp region (nt 885-1550) of the gene segment was amplified from these seven isolates using primers described in Table 2.5 (Fig 5.3).

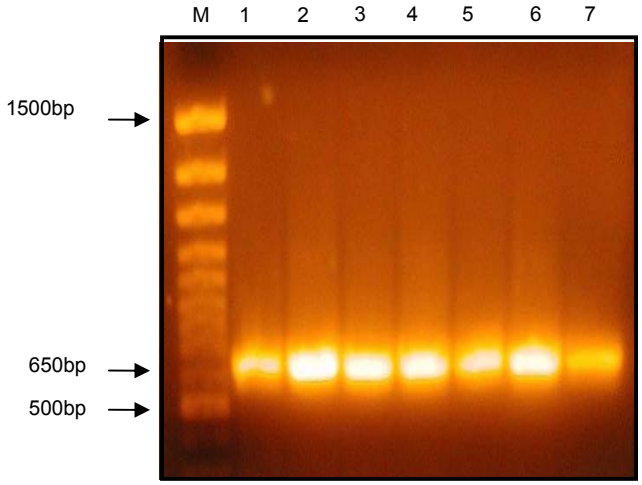

Figure 5.3 Amplification of a 665 bp region of gene 5 of selected specimens by RT-PCR using primers Beg 5 and End 5. M is 1500bp DNA marker; lanes 1-7 have specimens R1, R4, R11, R14, R19, MK26 and MK36 respectively.

Partial gene 5 sequences (nt 885-1550, encoding amino acids 294-486) of the isolates (R1, R4, R11, R14, R19, MK26 and MK36) were determined. Nucleotide sequence comparison and alignment of these isolates was carried out by Clustal W (Fig 5.4). Isolate R1 exhibited significant nucleotide sequence variation, sharing only 79-82% sequence identity with other Melbourne serotype G9 isolates. It had 82% nucleotide sequence identity with prototype strain F45. In contrast, isolate R14 exhibited up to 98% nucleotide sequence identity with other samples, but similarly showed only 82% sequence identity with strain F45. The nucleotide sequence identity between R1 and R14 was only 79%.

The deduced amino acid sequences of these isolates were also compared. Isolate R1 exhibited 77-79% amino acid identity with the other Melbourne isolates and 78% amino acid identity with prototype strain, F45 (Table 5.1). Isolate R14 showed 97-99% amino acid sequence identity with the other Melbourne serotype G9 isolates but only 80% identity with prototype strain, F45 and 82% identity with isolate R1. Isolates MK26 and MK36 shared 100% amino acid identity with each other (Table 5.1).

Several conserved amino acid substitutions were observed in Melbourne serotype G9 isolates when compared with the prototype strain F45. Amino acid change at positions 301 (Leu →His), 307 (Met →Val), 357 (Val →Ser), 371 (Cys →His) and 381 (Asn →Asp) were some of the changes observed among all isolates analysed in this study. The amino acid alignment of these isolates is shown in Appendix II. Thus, gene 5 of isolate R1 had significant sequence divergence from the rest of the Melbourne isolates. Surprisingly, it had only 12 amino acid differences with the prototype serotype G1 strain, Wa compared to 39 with the prototype serotype G9 strain, F45.

A phylogenetic tree of partial gene 5 sequences was constructed using sequences from prototype rotavirus strains isolated from geographically diverse regions using 100 bootstrap replicates (Fig. 5.5). The tree showed a clear divergence of the Melbourne serotype G9 gene 5 sequences. Isolate R1 clustered together with Wa and other human serotype G1 sequences, whereas the gene 5 of the remainder of Melbourne serotype G9 isolates formed a separate cluster, indicating that they were closely related, but distinct from the the gene 5 of strain F45, the prototype G9 rotavirus.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        940        950        960        970        980        990       1000       1010       1020
F45             CTAATGAAAC CAAATTATAT GACATCAAAT CATAGAGCAT CGGCTACTGA GGTACATAAT TGCAAATGGT GTTCGACCAA TAGTAGCTAT
R14             .AT..A.... ........G. A......... ......A... .A..G..... ...G.....C ..T....... .C..A.TT.. ..AC..T...
R4              .AT..A.... ........G. A......... ......A... .A..G..... ...G.....C ..T....... .C..A.TT.. ..ACG.T...
M100            .AT..A.... ........G. A......... ......A... .A..G..... ...G.....C ..T....... .C..A.TT.. ..ACG.T...
R1              .....C.... .......... A..C..G... ......A... .A........ A.....C... .....G.... ....AGTT.. ..A...T...
R11             .AT..A.... ........G. A......... ......A... .A..G..... ...G.....C ..T....... .C..A.TT.. ..ACG.T...
M117            .AT..A.... ........G. A......... ......A... .A..G..... ...G.....C ..T....... .C..A.TT.. ..ACG.T...
R19             ---------- --......G. A......... ......A... .A..G..... ...G.....C ..T....... .C..A.TT.. ..ACG.T...


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1030       1040       1050       1060       1070       1080       1090       1100       1110
F45             ATTGTATGGA ATGATTTTAG AGTTAAGAAG ATATATGATA ATATCTTGAA TTTTTTACGA GCTTTAGTTA AATCAAATGT TAATGTTGGA
R14             .C........ .......... .A........ .......... .C..T..C.. ....C..... ........C. ........TC ....A.....
R4              .C........ .......... .A........ .......... .C..T..C.. ....C..... ........C. ........TC ....A.....
M100            .C........ .......... .A........ .......... .C..T..C.. ....C..... ........C. ........TC ....A.....
R1              .C........ .......... ...G.....A .......... ....T..T.. ....C..... .......... .G........ ....A.C...
R11             .C........ .......... .A........ .......... .C..T..C.. ....C..... ........C. ........TC ....A.....
M117            .C........ .......... .A........ .......... .C..T..C.. ....C..... ........C. ........TC ....A.....
R19             .C........ .......... .A........ .......... .C..T..C.. ....C..... ........C. ........TC ....A.....


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1120       1130       1140       1150       1160       1170       1180       1190       1200
F45             CACTGCTCAT CACAAGAAAA GATATATGAA TGTGTTGAAA ATATTCTAGA TATATGTGAT AATGAGAAAT GGAAAACATC GGTAACAGAA
R14             ..T..T..G. .G..G..... .......... CA.A..A..G ..G....G.. .G.C...... G....A.... ......TGG. ....G.G...
R4              ..T..T..G. .G..G..... .......... CA.A..A..G ..G....G.. .G........ G....A.... ......TGG. ....G.G...
M100            ..T..T..G. .G..G..... .......... CA.A..A..G ..G....G.. .G........ G....A.... ......TGG. ....G.G...
R1              ..T..T.... ....G..... A......... .A.A.....G .......GA. CG.G...... .G.A.A.... .......G.. AA...T.A..
R11             ..T..T..G. .G..G..... .......... CA.A..A..G ..G....G.. .G........ G....A.... ......TGG. ....G.G...
M117            ..T..T..G. .G..G..... .......... CA.A..A..G ..G....G.. .G........ G....A.... ......TGG. ....G.G...
R19             ..T..T..G. .G..G..... .......... CA.A..A..G ..G....G.. .G........ G....A.... ......TGG. ......G...


                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                        1210       1220       1230       1240       1250       1260       1270       1280       1290
F45             ATATTCAATT GTCTAGAACC AGTAGAACTT AATGCTGTTA ACTATGTTTT ATTTAATCAT GAAGTAAATT GGGATGTTAT TAATATATTA
R14             .....T.... .......... .......... ...A...... .A......C. G......... ..G....... .......... ....T.....
R4              .....T.... ..T....... .......... G..A...... .A......C. G......... ..G....... .......... ....T.....
M100            .....T.... ..T....... .......... G..A...... GA......C. G......... ..G....... .......... ....T.....
R1              G.T..T.... .......... .......... G..AA...C. .G........ ......C... ...A...... .......... ....G....G
R11             .....T.... ..T....... .......... G..A...... .A......C. G......... ..G....... .......... ....T.....
M117            .....T.... ..T....... .......... G..A...... GA......C. G......... ..G....... .......... ....T.....
R19             .....T.... ..T....... .......... ...AA..... .A......C. G......... ..G....... .......... ....T.....
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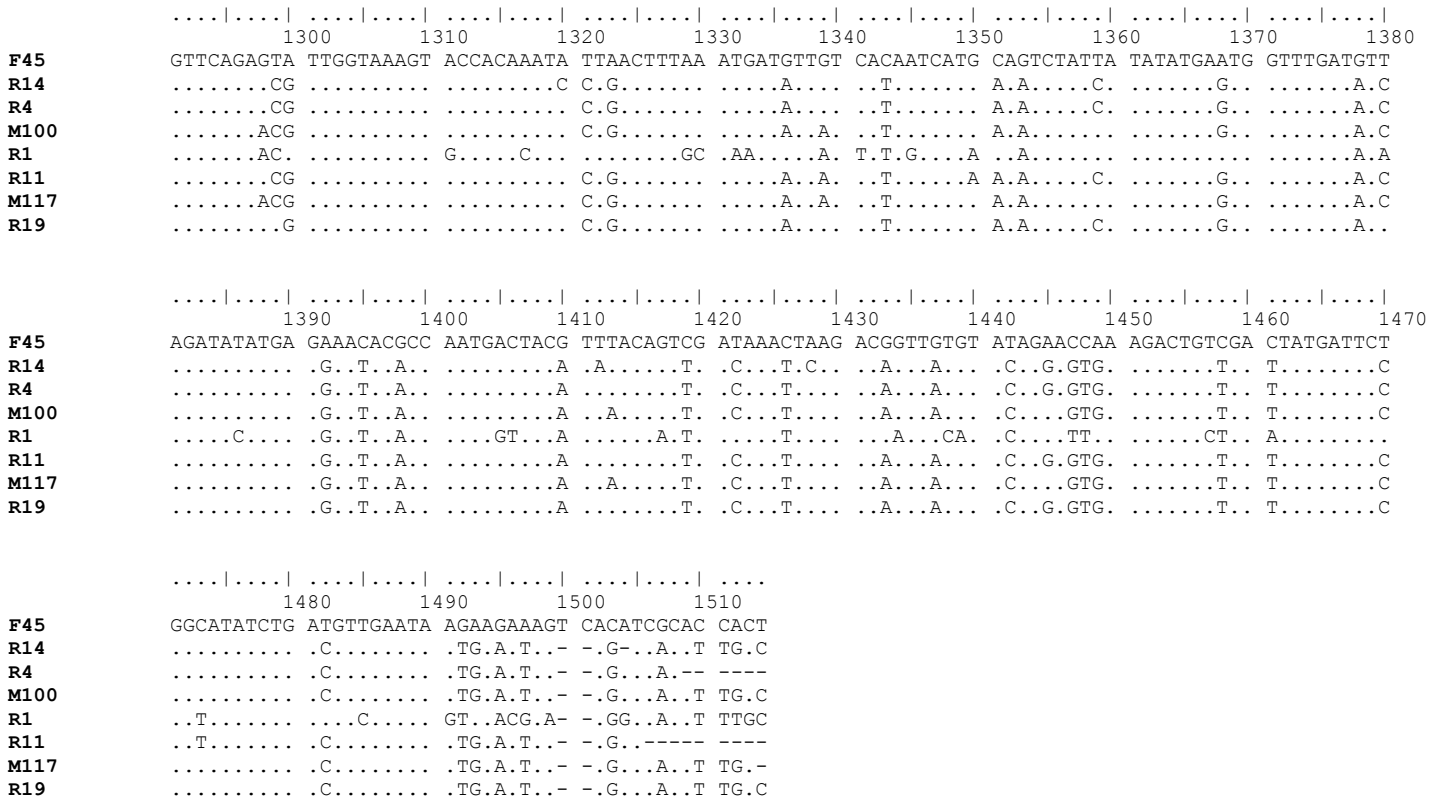

Fig 5.4 Alignment of partial (nt 931-1514) nucleotide sequences of NSP1 genes of Melbourne serotype G9 isolates. The sequence of F45 is indicated and nucleotide substitutions of other strains are indicated. Dots signify conserved positions. Dashes indicate sequences not available.

Table 5.1 Percentage of nucloetide and amino acid differences between NSP1 genes from Melbourne serotype G9 (in bold) and other standard strains

% of amino acid difference above line

% of nucleotide difference below line

	F45	**R1**	**R4**	**R11**	**R14**	**R19**	**MK26**	**MK36**	M37	ST3	I321	Hochii	OSU	K8	WA	B223
F45		15.81	13.26	13.26	13.16	12.75	13.77	13.77	13.26	10.71	15.30	13.77	5.61	15.81	13.77	74.48
R1	17.11		17.34	17.34	18.36	17.34	17.34	13.34	18.36	16.32	11.73	5.10	14.79	10.20	4.59	74.48
R4	15.04	16.92		0.00	2.04	1.02	2.04	2.04	6.12	3.57	16.83	15.38	12.75	14.79	15.81	74.48
R11	15.55	16.41	0.51		2.04	1.02	2.04	2.04	6.12	3.57	16.83	15.38	12.75	14.79	15.81	74.48
R14	15.21	17.26	1.19	1.70		2.04	3.57	3.57	6.12	4.59	17.85	16.83	12.75	18.81	16.83	74.48
R19	14.52	16.92	0.85	1.36	1.70		3.06	3.06	5.10	4.08	17.34	15.81	12.24	15.30	15.81	74.48
MK26	15.38	16.58	1.02	1.19	2.22	1.88		0.00	8.16	5.61	17.85	16.32	14.28	16.83	16.83	73.97
MK36	15.28	16.58	1.02	1.19	2.22	1.88	0.00		8.16	5.61	17.85	16.32	14.28	16.83	16.83	73.97
M37	14.70	18.29	6.49	6.66	7.08	5.98	7.52	7.52		5.61	17.34	17.34	12.75	15.83	16.83	73.46
ST3	13.50	16.75	4.78	5.29	5.64	5.29	5.81	5.81	5.12		16.83	14.79	10.20	14.79	14.79	75.00
I321	14.87	10.25	16.23	16.58	16.48	15.89	16.41	16.41	17.60	17.26		8.10	15.83	7.14	8.67	75.00
Hochii	14.87	3.93	15.89	15.38	16.23	15.89	15.89	15.89	17.60	15.89	8.20		12.75	7.14	1.53	73.97
OSU	8.02	14.70	14.18	14.01	15.89	13.67	14.87	14.87	14.18	13.50	15.89	13.60		14.79	12.75	74.48
K8	15.72	8.37	15.04	15.21	15.33	14.70	15.72	15.72	15.89	15.21	8.03	6.15	15.89		7.65	74.48
WA	14.87	3.24	15.55	15.04	15.89	15.55	15.55	15.55	17.26	15.72	8.20	1.02	13.60	6.15		74.48
B223	64.44	65.64	64.27	64.10	63.76	63.76	64.27	64.27	63.07	63.41	64.44	65.47	64.44	65.65	65.81	

[#]Values determined by Pairwise distance in MEGA from nucleotide (below line) and deduced amino acid (above line) sequence alignments between isolates, using pairwise deletion. Melbourne G9 rotaviruses sequenced in this study are indicated in bold letters.

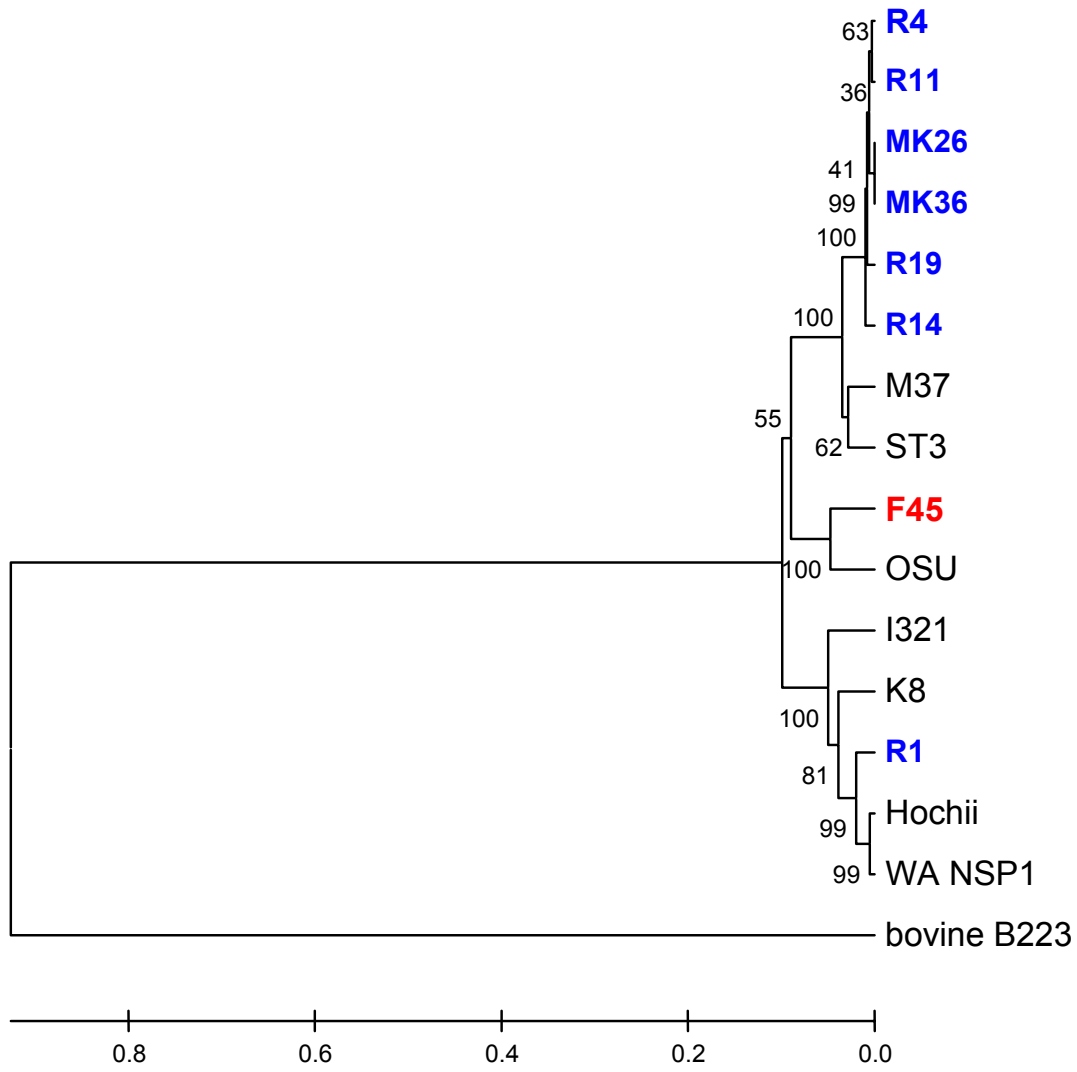

Figure.5.5. Phylogenetic tree of partial (nt 931-1516) NSP1 gene sequences of Melbourne serotype G9 isolates (in blue) and standard rotavirus strains. The tree was constructed using the neighbor-joining method. Bootstrap values (100 replicates) are shown at the branch nodes. The scale bar is proportional to genetic distance. The isolates in bold indicate the Melbourne serotype G9 isolates sequenced in this study. The sequence of the standard serotype G9 strain, F45 (in red), was determined in this study, while sequences for the other strains were obtained from the GenBank database: OSU (accession number D38153), Wa (L18943), Hochii (Z12106), K8 (D38152), I321 (U08418), ST3 (U11492), M37 (U11491) and B223 (Z12105).

5.2.3 Full length NSP1 gene sequence of isolates R1 and R14

Isolates R1 and R14 represented different alleles of gene 5 of serotype G9 rotaviruses based on the hybridisation results described in section 4.2.10. The complete gene 5 sequences of these two isolates were determined using a series of primers described in Table 2.5. Both genes had a coding region of 1461 bp in length representing a single open reading frame encoding a protein of 486 amino acids in length. Nucleotide sequence comparisons showed that isolate R1 shared 97% identity with the serotype G1 prototype strain, Wa, but had only 88% sequence identity with isolate R14. Isolate R14 showing 92% nucleotide sequence identity with the standard strains M37 and ST3, which are neonatal strains belonging to serotype G1 and G3, respectively.

Comparisons of deduced amino acid sequences showed isolates R1 and R14 shared only 88% identity. Isolate R1 had 96% amino acid identity with strain Wa whereas isolate R14 exhibited 87% identity. The consensus cysteine rich region (residues 42-72), which is involved in RNA binding, was conserved in both the isolates (Fig 5.6). Similarly, proline residues at eight positions, involved in folding of the NSP1 protein, were conserved as were the conserved histidine residues (Fig 5.6) suggesting that the RNA binding domain of the protein was functionally conserved. Also there were conserved amino acid substitutions at various positions, for example at amino acid 253 (Leu→Phe), 267 (Ser→Arg), as compared to strain Wa (Fig 5.6).

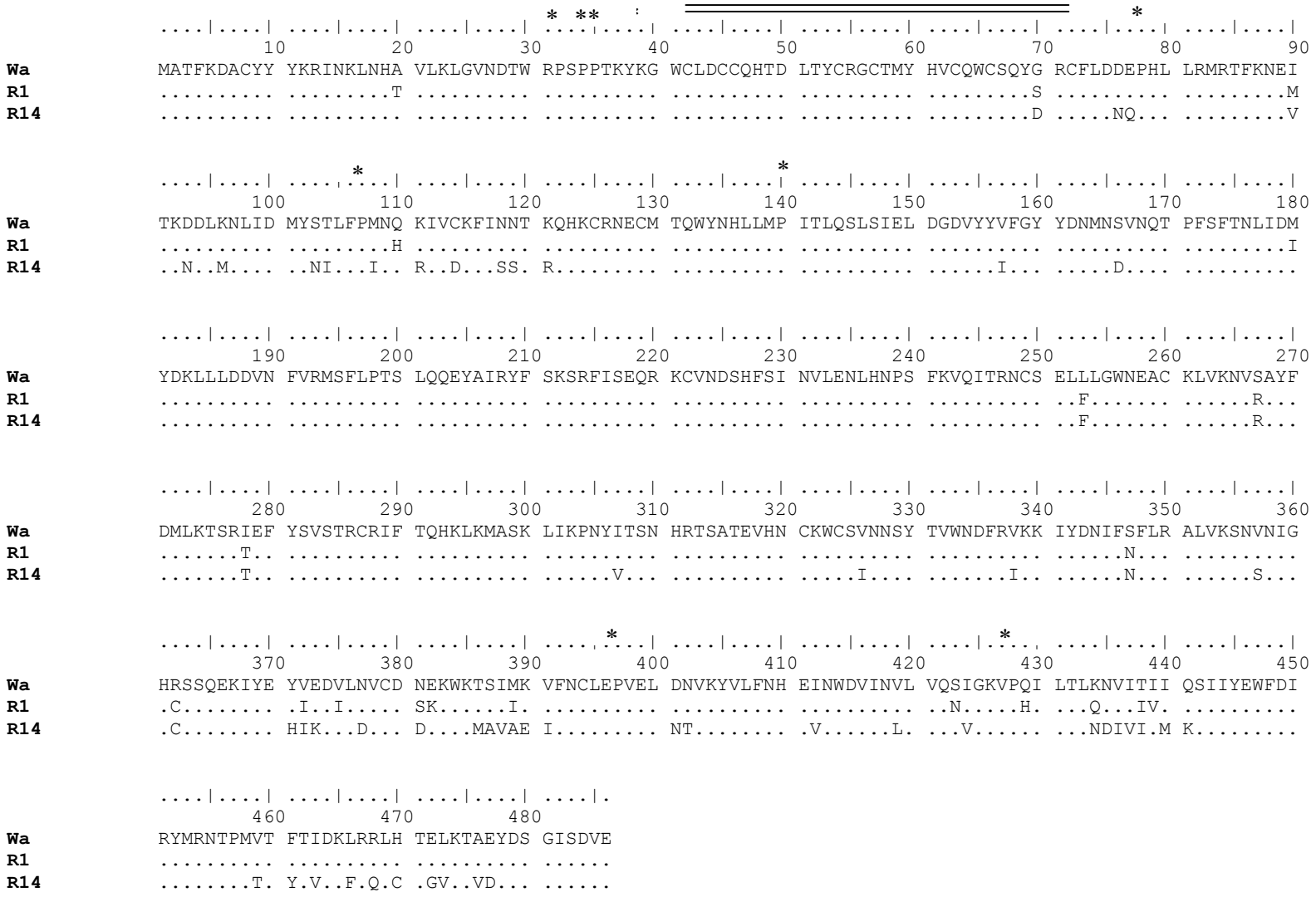

Fig 5.6 Alignment of deduced NSP1 amino acid sequences of Wa, R1 and R14 using Clustal W software in Bio Edit (www.bioedit.com). The sequence of the Wa NSP1 is shown in full, while only amino acid substitutions of R1 and R14 are indicated. Dots signify conserved positions. * indicates the conserved proline regions and the conserved cysteine region is indicated with double lines.

5.2.4 Sequencing of the NSP4 gene of serotype G9 rotaviruses

To investigate the genetic variation of the NSP4 protein among the Melbourne serotype G9 rotaviruses, full length genes of seven randomly selected isolates (R1, R2, R11, R14, R22, R24 and R26) were amplified using primers 10.1 and 10.2 and the resulting PCR product of 750 bp was obtained (Fig 5.7). The cDNA was gel purified and sequenced using the same set of primers.

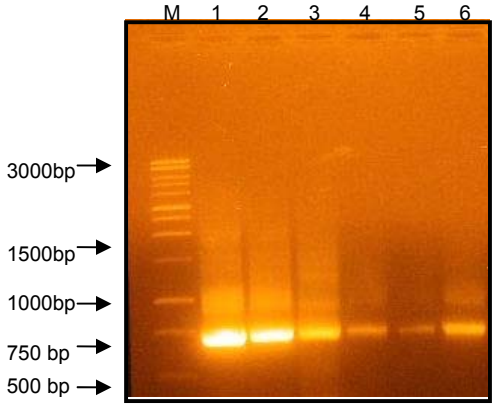

Figure 5.7. Amplification of selected samples using primers 10.1 and 10.2 by RT-PCR. M is DNA marker. Lanes 1-6 contain isolates R1, R2, R11, R14, R22 and R24 respectively. R26 is not shown gel.

The complete nucleotide sequence of the NSP4 coding regions were obtained for all seven isolates with the exception of R22 and R24 where only partial sequences were obtained (the first 77 and 79 amino acids, respectively, of these two isolates were not determined) and sequence comparisons revealed that the NSP4 from all Melbourne isolates shared 97% nucleotide and 96-100% amino acid identity, except for isolate R24 which exhibited only 78% nucleotide and 83% deduced amino acid identity with rest of Melbourne isolates. Isolate R1 shared 96% deduced amino acid identity to the NSP4 protein of strain Wa. The distinct NSP4 gene in R24 that shared only 79-83% nucleotide and deduced amino acid identities with the other Melbourne serotype G9 isolates as well as strains representing other genotypes (Wa, AU-1, KUN and PO-13) suggested that this isolate may possess an NSP4 of a novel genotype. Pairwise comparisons of deduced amino acid sequences of serotype G9 isolates with strains from all major NSP4 genotypes revealed fewer amino acid substitutions (5-7) between Melbourne serotype G9 isolates and strain Wa, suggesting that they belonged to the same NSP4 genotype as Wa, i.e. genotype 1. However, isolate R24

differed considerably from the rest of the Melbourne isolates analysed (11-13% amino acid substitutions, Table 5.2, Fig 5.8) and other standard strains (11-68% amino acid substitutions) suggesting it may be a distinct genotype.

Table 5.2. Percentage of NSP4 amino acid differences between rotaviruses of different genotypes (Wa, KUN, AU-1 and PO13 representing genotype 1-3 and 5 respectively) and Melbourne serotype G9 isolates (in blue)

Percentage of amino acid substitutions between isolates[#]										
	Wa	**R1**	**R14**	**R2**	**R11**	**R26**	**R24**	**R22**	KUN	AU-1
PO13										
Wa										
R1	3.42									
R14	4.00	5.14								
R2	2.85	4.00	4.00							
R11	2.85	4.00	4.00	0.00						
R26	3.42	4.57	4.57	0.57	0.57					
R24	12.00	12.57	12.00	12.00	12.57	11.42				
R22	2.85	3.42	2.28	0.57	0.57	0.00	10.85			
KUN	13.71	16.57	17.14	14.28	14.28	14.85	11.42	10.28		
AU-1	14.85	16.57	17.71	15.42	15.42	15.42	13.71	10.28	15.42	
PO13	66.28	66.28	66.85	66.85	66.85	66.28	68.00	33.71	64.57	65.71

[#]Values determined by Pairwise distance in MEGA from deduced amino acid sequence alignments between isolates, using Pairwise deletion. Melbourne serotype G9 rotaviruses sequenced in this study are indicated in bold letters.

5.2.5 Phylogenetic analysis of NSP4 gene

Phylogenetic analysis of the NSP4 sequences of Melbourne serotype G9 isolates and those from standard strains Wa, KUN, AU-1 and PO-13 (representing different NSP4 genotypes) revealed that six of the seven Melbourne serotype G9 isolates clustered together into the Wa genotype, with isolate R1 most closely related to Wa (Fig. 5.9). Isolate R24, however, formed a separate branch suggesting that this isolate belongs to a different NSP4 genotype. Deduced amino acid sequences revealed variation in the three of the four antigenic sites and in the VP4 binding domain of NSP4 (Fig 5.8, Table 5.3). Importantly, the enterotoxigenic region (114-135), the two N-linked high mannose glycosylation sites and transmembrane domains were conserved in all the isolates analysed except for isolate R24 which exhibited considerable variation within these regions (Fig 5.8, Table 5.3).

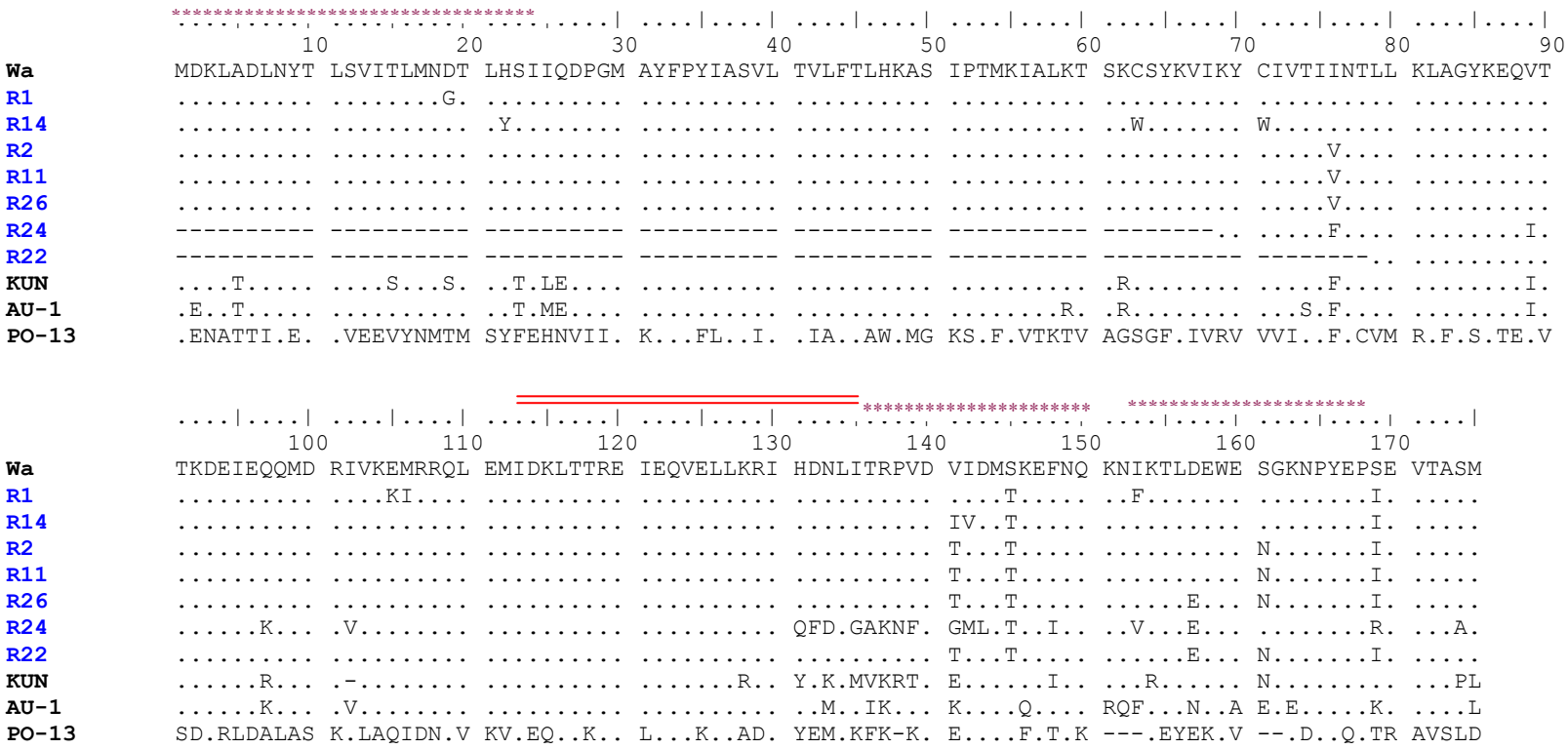

Figure 5.8 Alignment of NSP4 amino acid sequences of Melbourne serotype G9 isolates (in blue) compared with standard strains obtained from the GenBank database, accession numbers are given in parentheses; Wa (AF093199), KUN (D88829**)**, AU-1(D89873) and PO-13(AB009627). The sequence of the Wa NSP4 is shown in full, while only amino acid substitutions of other strains are indicated. Sequence identity is indicated with dots, - indicates unavailable sequences. The enterotoxigenic region is shown with the red double lines. Antigenic regions are shown with asterisks.

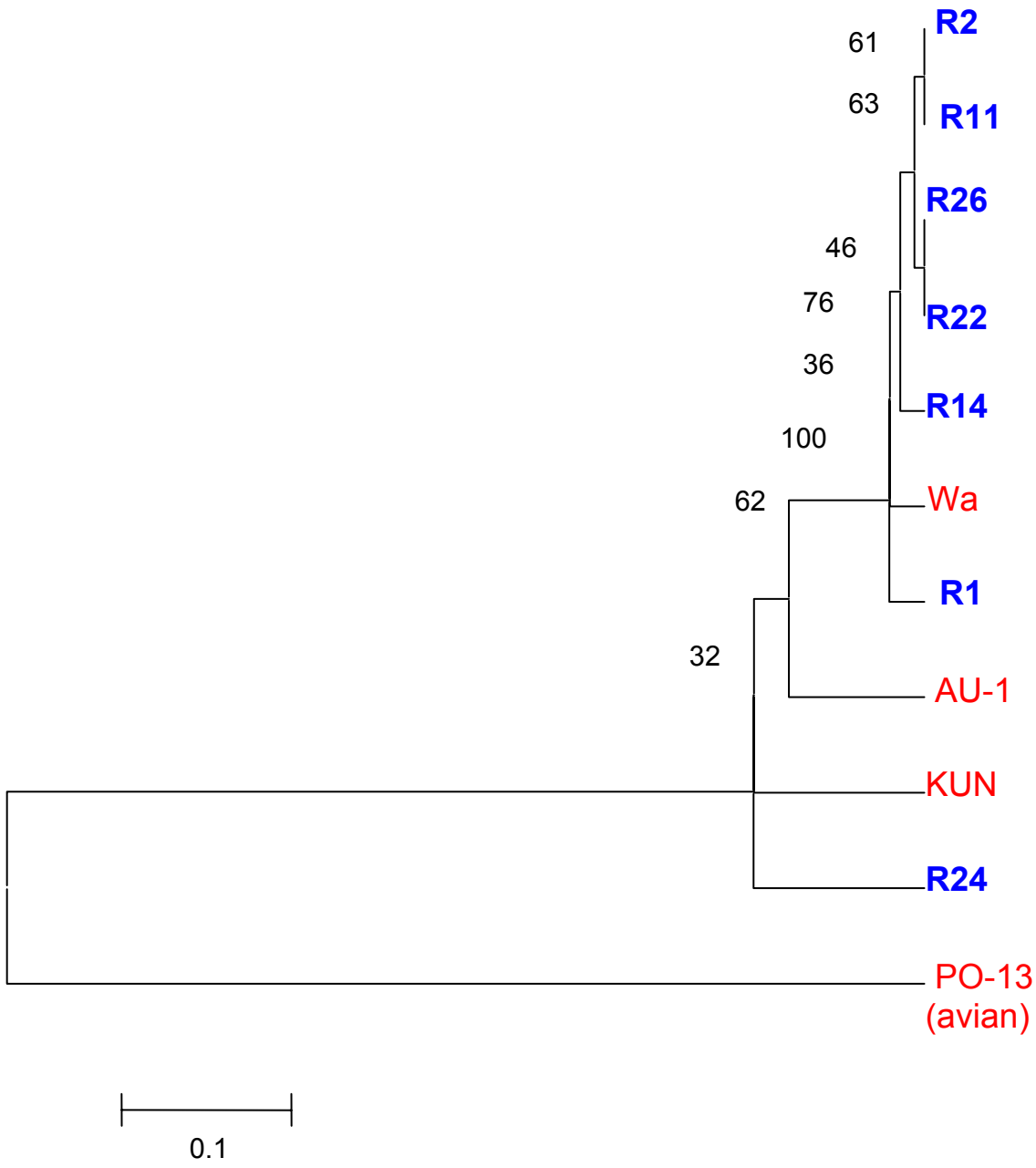

Figure 5.9 Phylogenetic tree of NSP4 gene sequences of serotype G9 and standard rotaviruses. The tree was constructed using the neighbor-joining method. Bootstrap values (100 replicates) are shown at the branch nodes. The scale bar is proportional to genetic distance. The isolates in blue bold indicate the Melbourne isolates sequenced in this study. Sequences for the other strains were obtained from the GenBank database: Wa (accession number AF093199), KUN (D88829), PO-13 (AB009627) and AU-1(D89873).

Table 5.3. Amino acid sequence differences in the antigenic sites (as) and VP4 binding region of NSP4 of rotavirus serotype G9 isolates.

Isolate	Positions of antigenic sites and VP4 binding region of NSP4 protein			
	AS I (aa 151-169)	**AS II (aa 136-150)**	**AS IV (aa 1-24)**	**VP4 binding region (aa 112-148)**
R1	Ile(153) →Phe Ser (169)→Ile	Ser(145)→Thr	Asp(19)→Gly	Ser(145)→Thr
R2	Ser(161)→Asn Ser(169) →Ile	Val(141)→Thr Ser(145)→Thr		Ser(145)→Thr
R11	Ser(161)→Asn Ser(169) → Ile	Val(141)→Thr Ser(145)→Thr		Ser(145)→Thr
R22	Ser(161)→Asn Asp(157)→Glu Ser (169) →Ile	Val(141) → Thr Ser(145)→Thr	Not sequenced^	Ser(145)→Thr
R26	Ser(161)→Asn Asp(157)→Glu Ser(169) →Ile	Val(141)→Thr Ser(145)→Thr		Ser(145)→Thr
R24	Ile(153)→Val Asp (157) → Glu Ser(169) → Arg	Thr (136) → Ala Arg(137) → Lys Pro(138) → Asn Val(139) → Phe Val (141) → Gly Ile(142) → Met Asp(144) → Leu Ser(145) → Thr Phe(148) → Ile	Not sequenced^	His(131) → Gln Asp(132) → Phe Asn(133) → Asp Ile(135) → Glu Ile(142) → Met Asp(144) → Leu Ser(145) → Thr Phe(148) → Ile

Sequence changes indicate the variation found in a particular isolate in the specified amino acid compared to the residue commonly found in that position in other isolates.^ the first 77-79 amino acids of isolates R24 and R22 respectively, could not be determined.

5.3 Discussion

While previous studies have investigated variation within structural genes of serotype G9 rotavirus, the results of the present study reveals that the genes encoding non-structural proteins NSP1 and NSP4 exhibit variation among serotype G9 rotaviruses isolated in Melbourne.

Gene 5 encodes NSP1, the least conserved protein, which is an RNA-binding protein that accumulates in host cytoplasm during virus replication, though it is not involved in virus replication (Silvestri *et al.*, 2004). However, the protein is associated with the cytoskeleton fraction of infected cells and is needed for efficient cell-to-cell spread of the virus (Graff *et al.*, 2002; Hua *et al.*, 1993; Patton, 2001). Recent work published by Barro and Patton (2005) reported a possible pathogenic role for NSP1 where it interferes with host innate immunity by degrading interferon and hence is a critical virulence determinant of the virus. Hybridisation and sequence analysis indicated the presence of different alleles of gene 5 among serotype G9 isolates circulating in Melbourne, Australia.

The NSP1 gene of the serotype G9 isolate R1 exhibited a high level of identity with the prototype G1 strain, Wa (96%) but only 79% to prototype G9 strain F45 and only approximately 80% amino acid identity with the other serotype G9 isolates analysed in this study. The high level of genetic identity between R1 and Wa is suggestive of reassortment between rotaviruses of different serotypes. The variation of NSP1 could have resulted in important phenotypic differences among serotype G9 rotaviruses. Therefore, variation in the RNA binding site of the protein, an important functional region of NSP1 that contains a cysteine-rich region and zinc fingers (Taniguchi *et al.*, 1996) was examined. Two selected isolates, R1 and R14 (representing different alleles of gene 5), exhibited amino acid sequence conservation within this region. This result suggested that one of the essential functions of this protein is maintained despite considerable sequence variation. All eight proline residues responsible for introducing bends in NSP1 polypeptides (Kojima *et al.*, 1996) were found to be conserved in these isolates.

The presence of different NSP1 alleles among serotype G9 rotaviruses investigated in this study prompted an investigation of another non-structural protein, NSP4. This multifunctional protein is the first viral enterotoxin described. It is thought to mediate cell signalling after being secreted from infected cells by increasing calcium levels leading to chloride secretion in adjacent uninfected cells (Ball *et al.*, 1996; Morris *et al.*, 1999; Tian *et al.*, 1996). It acts as an intracellular receptor for single-shelled particles, thus playing an important role in morphogenesis of the virus (Ball *et al.*, 2005) and has recently been shown to bind to the extracellular matrix proteins laminin and fibronectin (Boshuizen *et al.*, 2004). The various functional regions of the NSP4 protein have been mapped, with region 114-135 found to be able to induce diarrhoea and hence acting as an enterotoxic region. The region 87-145 is the site of interaction with the extracellular matrix (Boshuizen *et al.*, 2004). NSP4 is also found to induce heterotypic antibody responses during infection (Ray *et al.*, 2003).

At least three alleles of rotavirus NSP4 represented by the standard strains Wa, KUN and AU-1 have been identified (Kirkwood and Palombo, 1997; Horie *et al.*, 1997; Ball *et al.*, 2005), with sequence diversity of up to 18% between any of these alleles and only 5.5% within each allele. Isolates in this study exhibited 4-5% sequence diversity with the exception of isolate R1 which was significantly different (>20%). A study of serotype G9 rotaviruses from the USA found that strains isolated in the same year were more closely related than those isolated in different years regardless of geographical distribution (Kirkwood *et al.*, 1999). The phylogenetic analysis of NSP4 of Melbourne serotype G9 rotaviruses showed that representative samples clustered together with each other and closely grouped with the Wa NSP4 allele. The sequence analysis revealed amino acid substitutions in several important regions of the protein. The variation in the antigenic sites may have implications on the efficacy of vaccines as it has been suggested that NSP4-specific antibodies may have neutralising effects to enterotoxin and may thus reduce diarrhoeal symptoms (Ball *et al.*, 1996). Two important functional domains of NSP4, the transmembrane domain and the enterotoxin domain, were conserved among Melbourne isolates.

Isolate R1 exhibited significant variation compared to other isolates analysed in this study. One explanation could be that R1, isolated in 1997 (the first year in which

serotype G9 rotaviruses were identified in Australia), represented the initial serotype G9 strains that emerged in Australia, perhaps imported from overseas, and was replaced by other strains of a different genetic constellation in subsequent years. The distinct NSP4 gene of isolate R24 indicates that this isolate belongs to a different genogroup from the other Melbourne serotype G9 viruses, affirming the presence of several alleles of NSP4 gene among Melbourne serotype G9 rotaviruses.

In conclusion, considerable diversity was identified in two non-structural proteins, NSP1 and NSP4, within serotype G9 isolates in Melbourne. Nevertheless, most of the isolates formed a distinct lineage and clustered together when compared with global serotype G9 isolates. However, isolate R1 showed considerable genetic variation in NSP1 and isolate R24 had a remarkably different NSP4 genotype than the rest of Melbourne isolates analysed in this study. These results further emphasise the genetic complexity possible with serotype G9 strains, often oserved in other locations around the world.

Chapter Six

General Discussion

6.1 Burden of Rotavirus infection in young children

Rotavirus causes significant morbidity and mortality worldwide in children less than five years of age. The global death toll due to severe infection caused by rotavirus accounts for 610,000 childhood deaths a year (Parashar *et al.*, 2006). Rotavirus causes more diarrhoea related deaths than any other viral or bacterial pathogen, the scenario is worse in developing countries where more than 80% of total rotavirus related mortality occurs due to inadequate treatment and management of the disease. In Australia, rotavirus related hospitalisation is estimated to be 10,000 a year, which accounts for over $26 million in direct medical costs (Carlin *et al.*, 1998; 1999). Thirteen rotavirus related deaths were reported in Australia during 1990-2002 (Newall *et al.*, 2006). Almost all children are infected with rotavirus in the first few years of life (Bishop, 1996). An Australian study showed that the majority of rotavirus related hospitalisation occurs in first two years of life (Carlin *et al.*, 1998) suggesting rotavirus gastroenteritis is age-related. Also, rotavirus related gastroenteritis reportedly causes more severe gastroenteritis than that caused by other enteric pathogens (Albano *et al.*, 2006).

The epidemiological study carried out in this thesis (Chapter 3) determined that the majority of children infected with rotavirus were aged between 6-35 months, with the maximum number of children aged between 7- 24 months (66%). This is consistent with other studies showing the prevalence of rotavirus gastroenteritis in children less than five years of age (reviewed in Section 1.7.3). It is believed that virtually every child will experience at least one case of rotavirus gastroenteritis during the first few years of life. The rate of rotavirus infection gradually decreases with the increased age (Bishop, 1996), which was also observed in this study. This thesis did not find rotavirus infection in any newborn babies of less than 6 months of age. This is possibly due to the fact that maternal antibodies confer protection in newborns against rotavirus illness (Bishop, 1996; Pickering *et al.*, 1995; Ramachandran *et al.*, 1998a).

6.2 Epidemiology of human rotavirus: emergence of serotype G9

The epidemiological study (Chapter 3) revealed that three different serotypes (G1, G3 and G9) were causing infections in children in Melbourne and that these exhibited as

many as eight different electropherotypes. Most importantly, the presence of serotype G9 rotavirus in significant numbers (58%) indicated the dominance of this single type in 2002. These results support other findings of a gradual increase of serotype G9 rotavirus in Australia (Kirkwood *et al.*, 2002; 2003; 2004a*;* Masendycz *et al.*, 2001).

To prevent the huge global health burden of rotavirus illness, prevention by vaccination is urgently sought since public awareness, improved hygiene and sanitation have failed to control the incidence of rotavirus in both developed and developing countries (Glass, 2006a). For the effectiveness of any vaccine to be determined, the epidemiology of rotavirus plays an important role and such studies are carried out worldwide to monitor the circulation of rotavirus in the community. Molecular epidemiology of rotavirus has demonstrated considerable strain diversity among human and animal populations (Estes *et al.*, 2001; Holmes, 1996). The degree of variation has been determined for the viruses within and between annual epidemics in the same human population and among strains circulation globally and has revealed that rotavirus epidemiology is more complex than that of influenza viruses (Holmes, 1996). The complex nature of rotavirus epidemiology is demonstrated by the various G/P combinations detected and by the ability of the virus to undergo frequent genetic reassortment in nature (Hoshino *et al.*, 2005). Monitoring of rotavirus strain diversity is essential in order to implement effective rotavirus vaccines and evaluate the success of new vaccines. Until a decade ago, global rotaviruses mainly constituted four major G-types (G1-G4) and two major P types (P4 and P8). However, since the mid 1990s, the emergence of serotype G9 rotavirus in human populations has been reported worldwide (reviewed by Santos and Hoshino, 2005) (Fig 6). Similar findings have been reported in Australia (Palombo *et al.*, 2000; Kirkwood *et al.*, 2003) (Fig 1.15) and are also supported by the epidemiological study conducted in this thesis (Fig 6.1c).

Retrospective analysis of the distribution of G serotypes worldwide from the last 10 years revealed a unique pattern of rotavirus epidemiology. In Fig 6.1a, the distribution of rotavirus serotypes from global collections until the year 1996 showed the presence of four major serotypes of rotavirus G1-G4 with G1 being the predominant type (Gentsch *et al.*, 1996). In a similar study published eight years later, the global distribution of serotypes of rotavirus revealed a similar trend with major four types G1-

G4 being present and serotype G1 rotavirus still the predominant type (Fig 6.1b) (Hoshino *et al.*, 2004). However, there was the emergence of serotype G9 rotavirus in later years comprising approximately 4% of total rotavirus isolates around the world. Consistent with this analysis, the results of this study also revealed a higher incidence of serotype G9 rotaviruses in Melbourne in 2002 (Fig 6.1c). The dominance of serotype G9 rotavirus was shown by the fact that it constituted an astounding 58% of total rotaviruses isolated that year.

Due to improved identification and typing methods the presence of non-typeable has also decreased from 14% to 5% of global collections (Fig 6.1 a, b). This highlights the importance of continued strains surveillance and epidemiology of rotavirus in order to monitor the changing dynamics of rotavirus serotype distribution. The epidemiological studies will help assess the success of newly available rotavirus vaccines in controlling the diverse types of virus in the community. Alternatively, depending upon the types of strains circulating in one community at certain time, the rotavirus vaccine will need to be tailored to cater for the particular community based on its epidemiological data, as practised for influenza vaccines.

The epidemiologcal, genetic and antigenic characterisation of rotavirus is critical in order to understand the evolution of this virus. Serotype G9 rotavirus has been reported to exhibit considerably greater diversity compared to other serotypes (Santos and Hoshino, 2005), suggested to be the result of the ability of strains of this serotype to undergo higher rates of reassortment with gene segments of rotaviruses belonging to other serotypes (Fang *et al.*, 2002; Hoshino *et al.*, 2002; Ramachandran *et al.*, 2000).

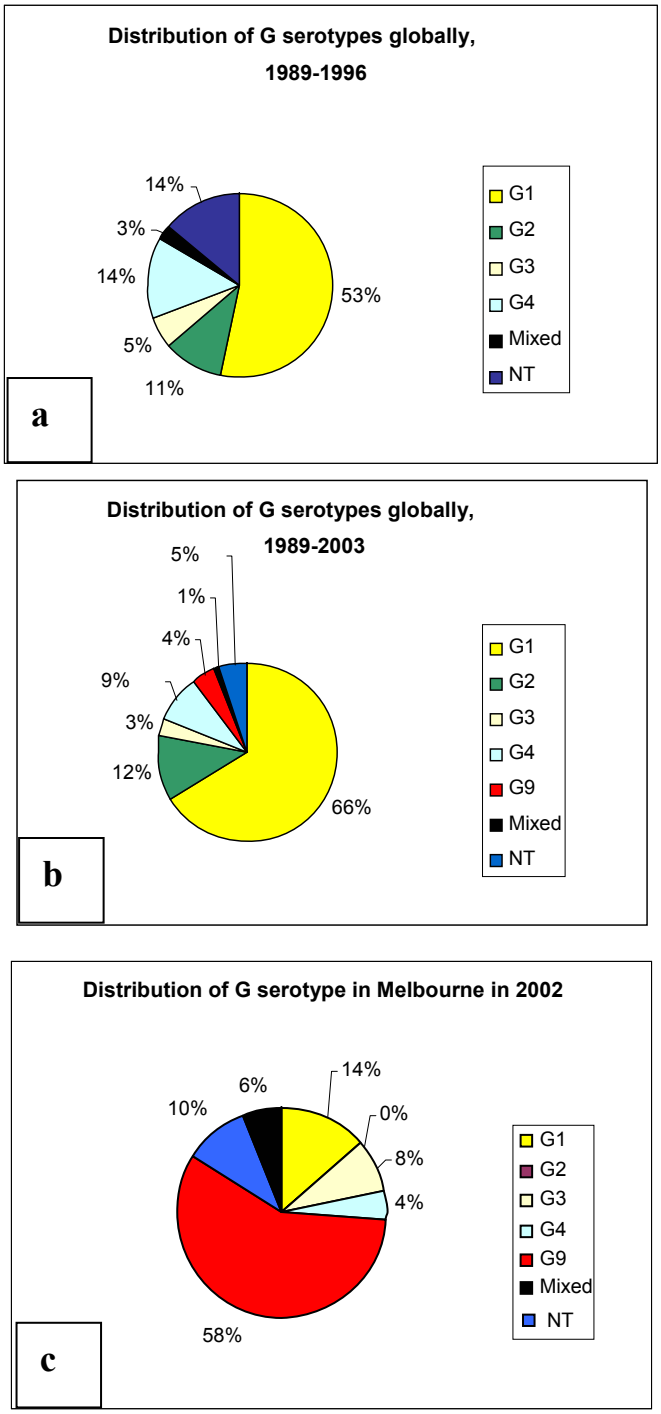

Figure 6.1 Distribution of rotavirus G serotypes from 1989-1996 (a) and 1989-2004 (b) globally. Note the emergence of serotype G9 rotavirus (4%, red slice of pie chart) in Fig b. Also of importance is the presence of 14% non-typeable isolates in 1996 and only 5% in 2004. Both the data were adapted from Gentcsh *et al*. (1996) and Hoshino *et al*. (2004). G1 was the most dominant serotype in both cases. Results obtained in this study of the distribution of G serotypes in Melbourne in 2002 are from a one year collection (c). Note the dominance of serotype G9 rotavirus (red slice) (58%) followed by G1 (14%) and non-typeable isolates (10%) in Melbourne.

The results of the present study have demonstrated that serotype G9 rotaviruses exhibited two different electropherotypes during a one year period, suggesting genetic differences among this serotype. However, some similarities were observed among all isolates, i.e. they all belonged to subgroup II, had long electropherotypes and, of the isolates tested, all had VP4 P[8] specificity.

6.3 Higher incidence of rotavirus in cooler months in Melbourne, Australia

Rotavirus infection is commonly referred to as "winter virus and causes winter diarrhoea in Australia and in many temperate regions of the world, as demonstrated by a unique peak in rotavirus infections observed in the cooler months (Bishop, 1996). In this thesis, rotavirus infection also appears to peak during the cooler months in Melbourne (between July to September) and was at its lowest during the warmer months between February and May (Fig 3. 2). This confirms the findings by similar studies previously conducted in Australia with a slight difference in the peak months. Barnes *et al.* (1998) showed a clear peak during May to September with the number of patients admitted during this period being approximately double the number of those admitted during the warmer months in Australia (October-April). The winter peak of rotavirus detection was consistently seen in 12 of 13 epidemic seasons (1980-1993) of the study period, however, no such seasonal pattern was observed for other enteric viruses such as adenovirus or calcivirus in the same study. There is no clear explanation given to such a pattern. It is, however, possible that during the colder months, children tend to stay together in close groups indoors which could result in more efficient spread of the virus. In many parts of the world, a typical winter peak is observed in temperate regions compared to the tropical regions where no such distinct peak is observed. Since the initial part of the investigation in this thesis was conducted in a period of one year, no such correlation could be made with previous or later seasons. The winter peak has also been seen in the US, France, and Japan (Chikhi-Brachet *et al.*, 2002; Jin *et al.*, 1996; Suzuki *et al.*, 2005). However, the usual seasonal pattern of rotavirus infection observed in temperate climates does not occur as uniformly in tropical climates such as those occurring in Brazil, Bangladesh and India (Broor *et al.*, 2003; Cook, 1990; Kale *et al.*, 2004).

6.4 Non-typeable rotaviruses and mixed infections

Ten percent of rotavirus isolates investigated in this study (Chapter 3) could not be assigned a serotype by either EIA or RT-PCR. Some explanations for this finding could be; (i) insufficient viral antigens present in those samples for recognition by MAbs, (ii) insufficient RNA present in the sample for amplification using serotype specific primers, (iii) possible genetic and antigenic changes in the primer binding regions or MAbs specific epitopes, and (iv) presence of new, unidentified serotypes. During a 16 year study in Australia, Bishop *et al.* (1991) determined that 11- 46% of rotavirus isolates were non-typeable. Recently, the total number of non-typeable isolates has reduced significantly in Australia (5%) (Kirkwood *et al.*, 2006) possibly due to the introduction of molecular typing methods and sequence analysis of non-reactive specimens. However, there is still a need to improve the molecular techniques to address the various limitations of current typing techniques discussed above. Non-typeable rotaviruses may indicate the presence of uncommon viruses that could pose a challenge for the new vaccines as these strains may have the potential to escape vaccine induced immunity and become epidemic strains. It is thus important to monitor the changing patterns of various rotavirus serotypes with special attention to non-typeable strains.

The isolates investigated in this study exhibited three cases (6%) of mixed infection where two different serotypes (G1+G9, G3+G9 and G1+G3) of rotavirus were infecting an individual patient (Table 3.2). The reports of National Rotavirus Surveillance Program in Australia have identified lower incidences of mixed infections of 3.2% in 2003, 1.3% in 2004 and 0.9% in 2005 Australia-wide (Kirkwood *et al.*, 2003a; 2004a; 2006), which are slightly less as compared to the results obtained in this study. Around the world, mixed infections are commonly reported albeit at low frequency (Chen *et al.*, 2005; Hoshino *et al.*, 2005; Nielsen *et al.*, 2005) except in a few countries (see below). Mixed infections are believed to be responsible for the generation of wider serotype diversity among rotaviruses by mechanisms such as gene reassortment and genetic recombination (Santos *et al*., 2005) and can also potentially lead to the formation of novel reassortant strains (Das *et al.*, 2002). Where mixed infections are more common, such as in Bangladesh (23%) (Ramachandran *et al.*, 2000) and Ireland (28.5%) (Reidy

et al., 2005), virus evolution appears to proceed at a more rapid rate compared to those countries where mixed infections are less prevalent, such as in the United States (Griffin *et al.*, 2000; Reidy *et al.*, 2005).

6.5 Determining epidemiology of rotavirus using various detection methods

As discussed earlier, rotaviruses are highly diverse and three different mechanisms are commonly used to determine genetic variation and epidemiology of the virus, and these were used in this thesis. Firstly, electropherotyping of genomic RNA of isolates and the characteristic migration patterns of the 11 gene segments of rotavirus in PAGE (electropherotype) have contributed much important information regarding genetic diversity (Nakagomi and Nakagomi, 1996); (i) extensive heterogeneity occurs in the electropherotypes of rotavirus isolates, (ii) two distinct electropherotypes of rotavirus, "short" and "long" patterns, are readily identified on the basis of relative mobility of gene segments 10 and 11, and (iii) during outbreaks, one strain is predominant at any time. Secondly, Northern hybridisation is used to determine how such heterogeneity in electropherotypes reflects diversity at the nucleotides sequence level based on RNA-RNA hybridisation. The overall genomic relatedness of RNA segments under stringent hybridisation conditions allows isolates to be classified into 'genogroups'. Three major genogroups have been described for human rotavirus isolates (Wa, DS-1 and AU-1) (Nakagomi and Nakagomi, 1996). Finally, sequence analysis of various genes of rotavirus has revealed much more diversity than previously thought.

6.5.1 Eight different electrophoretic patterns of Melbourne rotavirus isolates

Electropherotyping has been recognized as a powerful and economical method for epidemiological studies of rotaviruses (Bishop, 1996) and it is widely used in most studies of rotavirus epidemiology carried out worldwide (Chen *et al*., 2005; Coulchi *et al*., 2002; Das *et al*., 2002). Analysis carried out in this thesis revealed the presence of many different RNA migration patterns and highlights the great diversity of rotaviruses collected over a one year period at Monash Medical Centre (MMC) in Melbourne. In

this study, the serotype G9 rotaviruses examined exhibited several different electropherotypes, with one isolate displaying a pattern similar to that of serotype G1 isolates and of fifty isolates studied, eight different electropherotypes were determined. Serotype G9 isolates collected at the Royal Children's Hospital (RCH) in Melbourne over a six year (1997-2002) period revealed twenty nine different electropherotypes suggesting considerable genome variation among this serotype. Bishop (1996) examined virus isolates from different epidemic seasons and found extensive genomic variation in the viruses circulating in large communities, with the co-circulation of a number of viruses of differing electropherotypes.

It can be concluded from the above findings that rotaviruses of the same serotype are capable of exhibiting different electropherotypes; and that isolates of different serotypes can exhibit similar electropherotypes. Electropherotyping is thus still an important tool to determine the extent of overall variation in a single serotype and also to determine the predominance of one epidemiological strain circulating in the community. The results obtained in this thesis demonstrated that one particular long electropherotype was the dominant type present (Fig 3.4 lane 2, 3, 4 and 5) among serotype G9 isolates collected at MMC. The diverse electropherotypes highlights the presence of numerous co-circulating strains in one location, as reported worldwide where co-circulating rotavirus strains are highly variable, change quickly over time in the same site, and differ to those reported at other sites at the same time (Desselberger *et al.*, 2001; Kapikian *et al.*, 2001). In most localities, there is a sequential pattern of appearance of electropherotypes, with predominant strains being replaced annually (Bishop *et al.*, 1991). The reason behind such an enormous level of diversity could be attributed to genetic drift and accumulation of point mutations in the genome of rotavirus, a common phenomenon seen in most RNA viruses such as influenza viruses, while gene rearrangement and reassortment between co-circulating strains of rotaviruses also contribute. Studies conducted by the National Rotavirus Surveillance Network in Australia at the Royal Children's Hospital on a yearly basis for the past 10 years suggest considerable variation of rotaviruses circulating in Melbourne, with many different serotypes co-circulating at the same time, numerous electropherotypes (up to 46) and different serotypes exhibit similar electropherotypes (Masendycz *et al.*, 2001; Kirkwood *et al*., 2002; 2003a; 2004; 2005).

6.5.2 High genetic relatedness of serotype G9 isolates

Northern hybridisation is commonly used to determine the genogroup of the virus based on overall genomic RNA relatedness to standard viruses (Nakagomi and Nakagomi, 1996). Investigations of the molecular epidemiology of rotaviruses by this technique under stringent conditions in this study produced following observations. Most of the serotype G9 rotaviruses analysed belonged to same genogroup of strain F45, from which whole genome probe was derived. All of the 11 gene segments of twenty eight representative serotype G9 rotaviruses hybridised with the probe except for two isolates, R1 and R14 which had limited homology with the probe in particular gene segments. The hybridisation conditions allowed sequences with up to 22% mismatch to hybridise. Even though the majority of isolates belonged to the same genogroup as F45 and showed considerable similarity in overall genomic RNA constellation, including serotype G- and P- determining genes, there was evidence of reassortment of gene segment 5 in two of the Melbourne serotype G9 isolates. Genetic analysis helped to determine the presence of different alleles of this particular gene.

The genetic constellations of unusual rotavirus isolates are readily determined using hybridisation techniques and are thus commonly used in many laboratories. In this thesis, the determination of unusual gene segments 5 among serotype G9 rotaviruses demonstrates the usefulness of this technique. However, the actual extent of variation among other gene segments, including changes in their functional and structural domains could only be determined by gene sequencing.

6.5.3 Mutations in gene sequences of serotype G9 strains

Rotaviruses are constantly undergoing genetic mutations and the accumulation of such mutations could result in significant genetic and antigenic drift. Sequencing of genes was carried out to determine such evolutionary processes as well as to determine the subtle changes in the rotavirus genome and to determine the identity of unusual strains which could not be typed by other methods such as serotyping by EIA or Northern hybridisation. The molecular characteristics of new and emerging viruses and their phylogenetic relationships to other viruses provide valuable information regarding the

origin of such strains. In this thesis, phylogenetic analyses of VP7 genes provided crucial evidence of the genetic evolution of serotype G9 rotaviruses. Many of the viruses analysed appear to not be direct descedndant of earlier serotype G9 strains but have been introduced into circulation recently, as the sequences of earlier serotype G9 strains varied significantly to those of the recent isolates analysed in the present study. Gene reassortment was observed for the NSP1 gene among serotype G9 isolates in this study, where some isolates exhibited close gene homology with the NSP1 gene of a serotype G1 strain and significant gene variation with the prototype serotype G9 strains. NSP4 gene sequence analysis also revealed high sequence identity with serotype G1 isolates and one isolate (R24) may belong to a new genotype. These results highlight the unique evolutionary phenomenon of reassortment in regards to the generation of diverse rotavirus population in nature.

6.6 Genome variation in serotype G9 rotaviruses

Given the incidence of serotype G9 rotavirus in one location in a single year (2002), this thesis thus carried out an extended analysis of this serotype among isolates collected by the National Rotavirus Surveillance Network laboratory at the Royal Children's Hospital in Melbourne over a greater period of time. From a collection of seventy nine serotype G9 rotaviruses isolated from children hospitalised with severe infection from 1997-2002, twenty nine representative isolates exhibiting different electropherotypes were examined in detail. The emergence and persistence of serotype G9 rotaviruses in Australia (Section 1.13, Fig 1.14), in particular in Melbourne, prompted an investigation of their genetic evolution and origin.

6.7 Antigenic characterisation of serotype G9 rotaviruses

Two outer capsid proteins VP7 and VP4 independently elicit the production of neutralising antibodies in the host. Therefore a binary system for the classification of these two surface proteins, i.e. P-type for VP4 and G-type for VP7, has been established (Hoshino and Kapikian, 1996). VP7 is a glycoprotein and constitutes 30% of total viral structural protein, is a major component of outer capsid and is the second most abundant protein (Kapikian *et al.*, 2001). VP4 constitutes 1.5% of total viral protein, forms a

series of short spikes on outer capsid and has a proteolytic cleavage site (Kapikian *et al.*, 2001). Because neutralising antibodies play an important role in protection against rotavirus, the outer capsid protein, VP7, has been targeted in research and development of vaccines (Hoshino, 1996). The antigenic regions of VP7 are well characterised and are identified as six major neutralising epitope regions (A-F) (Dunn *et al.*, 1993; Kirkwood *et al.*, 2003). However, such antigenic specificities of VP4 protein have not been assigned for all rotavirus strains.

VP7 contains of one to three potential glycosylation sites and two hydrophobic domains. Analysis of amino acid sequences of the various serotypes of the VP7 protein has revealed nine discrete regions that are divergent among different serotypes but are highly conserved within rotaviruses of same serotypes (Hoshino and Kapikian, 1994). In this thesis, the VP7 amino acid sequence analysis revealed variation in seven out of nine variable regions of the VP7 protein, contradicting earlier observations that these regions are highly conserved within G-types. The significance of these changes in the overall conformation of the VP7 protein needs to be assessed to further investigate their effect on the antigenicity and other functions of the protein.

The antigenic properties of serotype G9 isolates studied in this thesis were determined by EIA using a panel seven different VP-7 specific MAbs derived from two prototype serotype G9 strains, F45 and WI61. There was considerable variation observed in the reactivities of these isolates against these MAbs and a total of six different antigenic groups (monotypes) were determined. To locate differences in the VP7 gene that correlated with these monotypes, one representative isolate from each monotype was selected and the full length VP7 gene was sequenced.

The sequences in the antigenic regions of these isolates revealed many changes; all but one region showed one or more amino acid substitutions. However, no correlation between monotype and genotype could be made. Nevertheless, this finding further contributes to information regarding the extent of antigenic variation among serotype G9 rotaviruses. Previously, VP7 sequence variation has been determined among other serotypes such as G1 and G4 in Melbourne (Palombo *et al.*, 1993; 1993a). Most importantly, G9 isolates showed resistance to neutralisation by these MAbs which may

be attributed to possible mutations in the other regions that may directly or indirectly influence MAbs binding. There were several amino acid substitutions outside the antigenic regions of VP7 proteins for example, at amino acid position 46 (Pro →Ser), at 108 (Thr →Ile), at 136 (Asp →Asn), at 186 (Ala →Ser) and at 267 (Glu →Asp). However, the significance of these substitutions in resistance to MAb binding is not apparent. Moreover, there are many other factors that may have influenced the MAbs binding activities of these isolates.

6.7.1 Factors affecting the binding of VP7-specific MAbs

Neutralisation epitopes on VP7 are considered to be highly dependent on the conformation of the VP7 protein and any mutations may adversely affect the neutralisation by the VP7-specific MAbs (Gunn *et al.*, 1985). Thus the amino acid changes observed above may be responsible for the negative reactivities against the MAbs. Also, there are two neutralisation proteins of the rotavirus outer capsid, VP7 and VP4, which independently induce the production of neutralising antibody. Interactions between these two proteins have been reported (Prasad *et al.*, 1990) with the proposed site being at position 180-183 (Zhou *et al.*, 1994) and it has been shown that these interactions may alter the antigenic properties of the virus (Mendez *et al.*, 1996; Dunn *et al.*, 1994). VP7 can also influence the VP4-mediated receptor binding specificities of rotaviruses or vice versa (Dunn *et al.*, 1994; Mendez *et al.*, 1996). Furthermore, interaction between these two proteins is believed to be a key factor in the binding of the MAbs and their neutralisation (Chen *et al.*, 1992). Thus resistance to neutralisation against the MAbs by serotype G9 isolates in this study could be attributed to the interaction between these two outer surface proteins.

Another factor affecting MAb binding is glycosylation of the VP7 glycoprotein (Caust *et al.*, 1987), which contains one to three potential sites for N-linked glycosylation. Mutations in the glycosylation sites have direct effects on the antigenic properties of VP7 (Caust *et al*., 1987). Mutations resulting in the creation of a new glycosylation site have previously resulted in significant resistance to neutralisation by hyperimmune antiserum (Caust *et al.,* 1987; Kapikian *et al.*, 2001). It was also observed that a mutation in antigenic region C (Asp →Asn) at position 211 caused a dramatic reduction

in the ability of polyclonal antiserum to neutralise virus. One explanation attributed to such a reaction was the addition of new glycosylation site, so it is possible that antibody binding is inhibited by attached carbohydrate (Dyall-Smith *et al.*, 1986). In this thesis, mutation in the glycosylation site at aa 22 (Thr →Ile) resulted in loss of glycosylation in the serotype G9 isolates analysed. However, this glycosylation site was present in prototype strain F45. The effect of this change in antibody recognition site is not clear, as this region is located far from the antigenic regions in the linear sequence. In this thesis, one amino acid substitution was seen in antigenic region A at position 87 (Ala →Thr) and two amino acids substitution was observed in antigenic regions C at positions 208 (Thr →Ile) and 220 (Ala →Thr). The alteration in these regions may form an altered conformational antigenic site which could result in the failure of recognition by N-MAbs, as observed by Taniguchi and Urasawa (1985).

Shaw *et al*. (1988) observed that mutations in antibody binding regions significantly alters immunodominant antigenic sites, thus inhibiting the binding of the majority of neutralising antibodies present in hyperimmune antiserum by altering the conformation of the protein and ultimately changing antigenic recognition sites involving many epitopes in the region (Shaw *et al.*, 1988). Similar findings have been reported for the influenza virus. Single amino acid mutation in the hemagglutinin of influenza A virus has been shown to alter the receptor binding specificity of the virus (Aytay and Schulze, 1991). However, no particular amino acid substitutions observed in this study that could be correlated with the resistance to MAb binding by serotype G9 isolates.

6.8 Sequence analysis of VP7 gene of serotype G9 rotaviruses

As described earlier (Section 4.2.7), sequencing of the VP7 genes of various serotype G9 rotaviruses revealed the presence of at least three phylogenetic lineages (Fig 4.5). Lineage 1 consists of serotype G9 isolates detected in the mid 1980s in the USA and Japan (representative strains WI61 and F45, respectively), lineage 2 contains a serotype G9 isolate detected in India in1994 (strain 116E) and lineage 3 comprises most of the recent serotype G9 isolates circulating worldwide at present (reviewed by Santos and Hoshino, 2005). All of serotype G9 isolates analysed in this study clustered together into lineage 3, demonstrating they are closely more related with to contemporary G9

isolates than to strains isolated earlier. The Melbourne serotype G9 isolates exhibited only 87-88% nucleotide sequence identity with lineage 1 strains WI61 and F45 and 87% nucleotide identity with lineage 2 strain 116E. They shared >97% nucleotide sequence identity with other lineage 3 isolates. The amino acid sequence identities with lineage 1 and 2 were 94% and 91%, respectively but >97% with other lineage 3 viruses.

One Melbourne G9 isolate, R1, however, exhibited less homology with other contemporary serotype G9 isolates sharing only 88-89% nucleotide and 95% amino acid sequence identities. This isolate shared 89% and 88% nucleotide sequence and 95% and 93% amino acid sequence identities with linage 1 and lineage 2 isolates, respectively. Interestingly, isolate R1 exhibited higher sequence identity with two US isolates, Om46 and Om67 (>98%) than with any other isolate available in the GenBank database. Interestingly, these two strains were outliers in the serotype G9 strain phylogenetic analysis carried out recently (Laird *et al.*, 2003) suggesting they may represent the distant progeny of the original G9P[8] population that has been evolving since the 1980s, or alternatively, these strains may have been derived by the independent introduction into the human population of an isolate with a VP7 gene that is not a direct descendant of the serotype G9 isolates from the 1980s. The high degree of homology of R1 with these two US serotype G9 strains suggests that they might have a common progenitor, since R1 was the first serotype G9 isolated in Melbourne in 1997 and the two US strains were detected in 1996.

Sequencing studies of emerging serotype G9 rotaviruses from various countries have revealed that serotype G9 strains detected around the mid 1990s do not share the cognate genes of serotype G9 isolates detected a decade ago (Gentsch *et al.*, 2005; Santos and Hoshino, 2005). This finding indicates that recent serotype G9 rotaviruses are not direct descendents from the previous serotype G9 strains but could have been introduced into circulation through genetic reassortment (Gentsch *et al.*, 2005; Iturriza-Gomara *et al.*, 2000; Ramachandran *et al.*, 2000). These studies have also shown that subtle differences in the antigenic composition of the VP7 protein may exist among different phylogenetic lineages within a given G- type. Laird *et al*. (2003) determined the presence of other minor lineages among serotype G9 strains isolated from the US and India from 1993-2001.

The presence of six monotypes among Melbourne serotype G9 rotaviruses further highlights the greater level of antigenic variation. Rotavirus monotypes appear to have epidemiological significance. The change from monotype 1a to 1c for G1 rotaviruses has been associated with the maintenance of serotype G1 for 8 years as the predominant serotype causing infection resulting in hospitalisation of children with severe rotavirus gastroenteritis in Australia (Coulson and Kirkwood, 1991) and a 19 year analysis of serotype G1 in Italy revealed several antigenic types disappeared and reappeared at certain interval of times (Arista *et al.*, 2006) demonstrating the unique evolutionary phenomenon of rotavirus in nature. A similar trend could be predicted for serotype G9 to extend and maintain its dominance in the community in the future given the fact that this serotype is more prone to undergo genetic changes than any other type (Laird *et al.,* 2003) and controlling the spread of this type might pose challenge for rotavirus vaccine.

6.9 Sequence variation and implications for vaccine efficacy

The sequence analysis of the VP7 protein of Melbourne serotype G9 rotaviruses demonstrated sequence variation when compared with the prototype G9 strains, F45 and WI61. Further, amino acid substitutions in four of five antigenic regions of VP7 with strain F45 indicated a high level of genetic changes in recent serotype G9 rotaviruses which could result in antigenic drift. Moreover, most of Melbourne serotype G9 isolates shared >97% nucleotide and deduced amino acid sequences homology with one another except for R1. Isolate R1 had nucleotide sequence homology of 89% and deduced amino acid homology of 95% with the above prototype G9 strains and with the rest of serotype G9 isolates analysed in this thesis. Except for isolate R1, all of Melbourne serotype G9 isolates analysed here shared nucleotide and deduced amino acid sequence identity of 97-99% suggesting the recent serotype G9 rotavirues isolated in recent years (1999-2002) are more closely related with one another than to strains isolated earlier such as strain F45 (isolated in 1985), WI61 (isolated in 1984) and 116E (isolated in 1994), sharing only 89-95% sequence identity.

Due to its ubiquitous nature, rotavirus has been called ‘the democratic virus’ because all children in the world are infected in the first few years of life, in both developing and developed countries, living in either temperate or tropical areas. The presence of

rotavirus infection globally means that improvements in water quality, food hygiene, or sanitation are unlikely to alter the incidence of the disease. Rotaviruses are spread by the faecal-oral route. Until recently, no interventions have proven to be effective in preventing the spread of rotavirus infection (Glass, 2003). Previously, a rotavirus vaccine, Rotashield, was licensed in the US in 1998. Unfortunately, due to its possible link with the cases of intussusception, it was withdrawn from the market (CDC, 1999a). Since that time researchers around the world have been engaged in developing a safe and effective rotavirus vaccine.

The two rotavirus vaccines, RotaTeq and Rotarix, that showed excellent efficacy in phase III clinical trials conducted in the US and Mexico respectively and have been licensed in several countries (Arvin and Greenberg, 2006). Both vaccines have recently been licensed in Australia (Newall *et al*., 2006) and in a number of other countries (Linhares and Villa, 2006), which heralds new hope in preventing the huge health burden caused by this virus worldwide. Rotarix is an attenuated monovalent vaccine derived from a G1P[8] strain which has shown sufficient protection against other rotavirus serotypes including serotype G9 (Franco *et al.*, 2006). However, it has been suggested that correlates of protection after vaccination may vary depending on the type of vaccine for e.g. heterologous protection may not be adequate for the homologous vaccine and in multivalent vaccine such as RotaTeq (a pentavalent reassortant vaccine) , homologous protection may not be sufficient (Franco *et al*., 2006).

Furthermore, the changes in antigenic regions of the VP7 protein determined in this thesis may have implications in regards to the ability of the virus to escape the host immune system as well as the immune response induced by the vaccine. The efficacy of these vaccines in preventing rotavirus infection caused by new and emerging types and its ability to reduce high rates of hospitalisation of young children due to rotavirus disease, especially in developing countries where 2000 children die every day (Glass and Parashar, 2006), remains to be seen.

To escape the host defence mechanisms, viruses exhibit different approaches which constitute significant hurdles to vaccine development. One of these approaches, genetic variation, results in selection of mutations in antibody binding epitopes that allow the

virus to escape the host immune system (Gulati *et al.*, 2002). The tendency of influenza virus to undergo such genetic evolution has resulted in a number of global pandemics. The most disastrous and devastating was that caused by the emergence of swine-like H1N1 subtype which resulted in > 20 millions deaths worldwide in 1918-1919, the worst pandemic ever recorded. The possibility of a pandemic of avian influenza H5N1 is greatly feared. Due to the error prone nature of RNA polymerase, mutations accumulate in the surface proteins of the virus over a period of time, which are then no longer recognised by the protective antibodies produced by the host. Thus, there is a need for the reformulation of vaccine against influenza every year (Lee and Air, 2006). Whether a similar approach will be required for rotavirus vaccines is not known. Future studies, therefore, should continue to monitor strain diversity in vaccinated populations and epidemiological data should report the emergence of new rotavirus types through well established rotavirus surveillance systems worldwide.

6.10 Non-structural genes of serotype G9 rotaviruses show further genetic variation

Non-structural proteins (NSP) play important roles in rotavirus replication and gene packaging. There are six non-structural proteins present in rotavirus, NSP1-6, encoded by various gene segments. Northern hybridisation analysis revealed significant variation of gene segment 5 among serotype G9 isolates (Chapter Five). Gene segment 5 encodes NSP1, which is an RNA binding protein involved in genome packaging. Various studies have reported that NSP1 is not required for the virus replication as strains with a rearranged gene 5 that produced a truncated NSP1 have been isolated from both animals and children with diarrhoea (Desselberger, 1996; Graff *et al.*, 2002). However, the truncated NSP1 protein has been associated with small plaque phenotypes in cell cultures (Graff *et al*., 2002). Also, NSP1 is responsible for promoting cell to cell spread of rotavirus as shown by the use of gene 5-specific short interfering RNAs (si RNA) in plaque assays (Barro and Patton, 2005). These factors imply that the protein signifies a critical virulence determinant of the virus (Barro and Patton, 2005). Recently, it has been demonstrated that NSP1 is capable of interfering with cellular interferon activity in host cells therefore destroying the innate immunity of the host (Barro and Patton, 2005; Graff *et al*., 2002); this is an important function of rotavirus

protein NSP1 and variation in this gene as determined in this study may have implications in regards to this function.

Although rotavirus gene segment 5 is believed to be highly variable in sequence, the encoded protein, NSP1, is conserved among rotaviruses infecting the same host (Kojima *et al.*, 1996). A comparison of 22 NSP1 sequences from various host species showed a higher level of host specificity than VP7 and VP4. Gene 5 nucleotide sequences from viruses of the same species were found to exhibit greater homology than those from different species (Dunn *et al.*, 1994) suggesting that this protein was involved in host range restriction. However, in various studies, when interspecies relatedness was examined, a high degree of amino acid identity between the human strain Wa and the porcine strain OSU (86%) and between the human strain AU-1 and the bovine strain UK (87%) was observed (Dunn *et al.*, 1994; Kojima *et al.*, 1996; Nakagomi and Kaga, 1995). In this thesis, the sequence identities of gene 5 among isolates were 93-96%. However, isolate R1 shared only 79-84% with rest of the Melbourne serotype G9 isolates but had 96% sequence identity with the serotype G1 strain Wa and 86% identity with the porcine strain OSU. These findings are suggestive of natural gene reassortment among different serotypes.

Prompted by the detection of variation in the NSP1 gene, another important non-structural protein of rotavirus, NSP4, was also analysed in this thesis to determine the extent of genetic variation in this protein. NSP4 has multifunctional properties; it is involved in the morphogenesis of the newly made subviral particles by transporting them to the endoplasmic reticulum (ER) (Tian *et al.*, 1996), and also acts as an intracellular receptor (Tian *et al.*, 1996), it is a first viral enterotoxin reported and induces age-dependent diarrhoea (Ball *et al.*, 1996). Expression of NSP4 in insect cells increases intracellular calcium levels which is harmful to eukaryotic cells (Tian *et al.*, 1996). Five different genotypes (genotype 1-5) of NSP4 gene has been reported in rotaviruses based on their sequence analysis (Lin and Tian, 2003; Ball *et al.*, 2005). The NSP4 sequence analysis of serotype G9 isolates in this thesis revealed considerable sequence variation and the presence of more than one genotype. Importantly, the enterotoxigenic region of the protein (aa 114-135) was conserved among all isolates,

except for isolate R24, suggesting that the pathogenic function of this protein was well conserved

NSP4 has shown to induce protective immunity in mice when immunised with this protein (Ball *et al*., 2000). There are four antigenic sites have been described in NSP4 protein (I-IV). Sequence analysis exhibited changes in regions I, II and IV. The overall nucleotide sequence identity among serotype G9 isolates in this study was 94-96%. One isolate, R24, however differed considerably with the rest of the Melbourne serotype G9 isolates, sharing only 77% nucleotide and 79% amino acid identities. This isolate also had only <77% nucleotide and <80% amino acid identities with NSP4 genotype 1, strain Wa. Isolate R24 had only <77% nucleotide and amino acid homologies with the reference strains belonging to genotype 1-5 and other Melbourne serotype G9 isolates. However, despite repeated attempts, the full length genes of VP7 and NSP4 of this isolate could not be amplified and sequenced. Therefore, cultivation of the virus in tissue culture is required to fully characterise this isolate in future. Nevertheless, based on the sequence data obtained, it can be predicted that this isolate may belong to new NSP4 genotype. Phylogenetic analysis revealed that most of the Melbourne isolates clustered together in one branch except for isolate R1, which was more closely related to strain Wa (≥96% nucleotide and amino acid sequence identities) and isolate R24, which formed a separate branch.

6.11 Strong evidence of gene reassortment found among serotype G9 rotaviruses

Group A rotaviruses can be classified into lineages based on antigenic and genetic differences of the outer capsid proteins, VP7 and VP4 (Franco and Greenberg, 2000). These two proteins play vital roles in protective immunity and are thus targets for the development of the new rotavirus vaccines (Banyai *et al.*, 2005). Since rotaviruses exhibit great strain diversity and various combinations of G and P antigens, the regular monitoring of virus types in circulation is essential for the effectiveness of any vaccine to be determined. So far, 42 different G/P combinations have been determined for rotavirus (Santos and Hoshino, 2005). Genetic reassortment between viruses with different G and P types has contributed significantly to the wide diversity of the virus.

Distinct genotypic combinations of serotype G9 strains formed by VP4, VP6, and VP7 gene reassortment between long and short E-type isolates were found in a Bangladeshi study, suggesting that intergenogroup reassortment of VP4 and VP7 genes occurred at a high rate (Ahmed *et al.*, 1991) in mixed infections. In the US, two common serotype G9 genome constellations have been found in regards to their combined E-type, subgroup and P type specificities: constellation A (long pattern, subgroup II and G9P[8]) and C (short pattern, subgroup I and G9P[6]) (Laird *et al.*, 2003). The collection of serotype G9 rotaviruses examined in this study belong to constellation A, since all isolates exhibited long patterns, had subgroup II specificity and belonged to P [8]. In Australia serotype G9 isolates have so far been reported in association with P[8] (Kirkwood *et al.*, 2003a) as compared to other studies where serotype G9 isolates have been associated with other P types such as P[6] and P[4] (Laird *et al.*, 2003; Ramachandran *et al.*, 2000). The VP7 gene sequences of representative serotype G9 isolates revealed that all but one isolate had high degrees of nucleotide and amino acid sequence identity with one another and contemporary serotype G9 strains but greater divergence from the earlier serotype G9 strains (Fig 4.5), which supports the finding that recent serotype G9 isolates are more closely related to one another than to reference serotype G9 strains (Santos and Hoshino, 2005). Strain Om46 and Om67 exhibited marked variation suggesting these two isolates were more closely related to prototype strains isolated in the mid 1980s (Laird *et al.*, 2003), a feature of isolate R1 examined in this thesis.

One important observation of serotype G9 rotaviruses is thier combination with various P types in nature. Serotype G9 rotaviruses have been associated with P[8], P[6], P[4], P[9], P[11] and P[19]. The wide variety of serotype G9 strains that have been isolated suggests that this serotype may be unusual in its ability to reassort with other strains with different VP4 genes which has therefore lead to the efficient spread of serotype G9 rotavirus worldwide (Santos and Hoshino, 2005). A recent study reported that serotype G9 rotavirus causes more severe infection than any other serotype but the reason for this is not clear (Linhares *et al.*, 2006). Analysis of the VP7 genes of G9 rotaviruses isolated from various locations in Australia, including Melbourne, suggested that there was minimal variation (<1% nucleotide diversity) in this gene (Kirkwood *et al.*, 2003a). The results in this study revealed slightly higher (2-3% nucleotide diversity) sequence

variation in this gene in most of the serotype G9 isolates sequenced. However, isolate R1, the first serotype G9 isolated in Melbourne, exhibited significant gene sequence diversity (11%) as compared to other serotype G9 rotaviruses isolated in Australia. Clearly, further genetic analysis of Australian serotype G9 viruses is warranted. The occurrence of this serotype as the cause of an outbreak in Central Australia (Kirkwood *et al.*, 2004) further underlines the epidemiologic importance of this serotype.

The ability of the G9 viruses to reassort readily with other serotypes is thought to be an important factor contributing to this diversity (Hoshino *et al.*, 2005). The results of this study contribute to evidence of the diverse nature of G9 rotaviruses. The genetic variability observed in a number of gene segments encoding structural proteins can now be extended to include genes encoding non-structural proteins. The presence of several NSP1 and NSP4 gene alleles within isolates from a single geographic location allows speculation that there may be an even higher degree of variation in global isolates of serotype G9. It would be of interest to analyse these genes further, considering the pathogenic role played by these proteins. Given the unusual genetic features of serotype G9 rotaviruses, continued surveillance and genetic analysis needs to be carried out to provide information about the changes occurring in circulating strains in future. The potential impact on public health and implications for the effectiveness of rotavirus vaccines need to be considered. Monitoring the temporal changes in various genes may help to understand the nature and pattern of rotavirus evolution and also report the emergence or introduction of new types in human populations.

6.12 Summary

This thesis describes the molecular characterisation of serotype G9 rotaviruses isolated from hospitalised children in Melbourne from 1997-2002. Serotype G9 has emerged worldwide, was first isolated in Melbourne in 1997 and since its first isolation, a gradual increase in its incidence, Australia-wide, has been reported. A total of seventy nine serotype G9 rotaviruses were reported in Melbourne during that period. Based on the different electropherotypes, twenty nine representative isolates were then chosen for detailed analysis. As demonstrated by variation in genomic RNA migration patterns in PAGE, these isolates exhibited considerable gene variation. Based on the Northern hybridisation analysis, the NSP1 gene of these isolates was found to exist as more than two alleles that were significantly different to the cognate gene of the prototype strain, F45. This finding contributes important information about the extent of variation in genes encoding non-structural proteins of serotype G9 rotaviruses, which has not been recognised previously. The phylogenetic analysis of NSP1 revealed that most of Melbourne serotype G9 isolates shared a close genetic relationship with one another and formed a separate branch containing most of the other global serotype G9 strains. An exception was isolate R1 which was more closely related to the prototype serotype G1 strain, Wa. This result suggests gene reassortment between rotaviruses of different serotypes in nature. Sequence analysis of the NSP4 gene exhibited that most of Melbourne serotype G9 isolates shared close identity with one another and belonged to genotype 1. However, variation was observed in the antigenic regions of the protein which may have functional implications. One isolate, R24, which exhibited significant sequence variation as compared to other cognate genes available on the GenBank database and other Melbourne serotype G9 isolates, may belong to a new genotype. Isolate R1, the first serotype G9 rotavirus detected in Melbourne in 1997, exhibited greater levels of NSP1 and NSP4 sequence variation compared to more recent Melbourne and other Australian serotype G9 isolates. Moreover, this isolate had 99% VP7 sequence identity with two US serotype G9 strains, Om46 and Om67, suggesting this isolate may have been imported into Australia from overseas.

The antigenic analysis of Melbourne serotype G9 isolates based on the major neutralisation protein, VP7, revealed six different antigenic groups (monotypes). A

representative isolate from each group was chosen and their full length VP7 gene was sequenced to determine the location of mutations in amino acids responsible for resistance to neutralisation by the MAbs and also to investigate the degree of variation in the entire gene, especially in coding regions. The sequence analyses revealed several amino acids changes in the antigenic regions among serotype G9 isolates. However, no correlation could be made between the amino acid changes and resistance to neutralisation by MAbs. Phylogenetic analysis revealed the overall close similarity of Melbourne serotype G9 isolates with the recent contemporary global serotype G9 rotaviruses. However, significant differences with the prototype strains and other global serotype G9 isolates detected in the mid 1980s (when serotype G9 was first detected) were observed.

In conclusion, there was considerable genetic variation among serotype G9 isolates in Melbourne in the genes investigated (NSP1, NSP4 and VP7). This thesis provides new information about the level of genetic diversity in non-structural proteins of serotype G9 rotaviruses, as previous studies were mainly focussed on structural proteins. Most importantly, the extent of antigenic and genetic variation determined in this thesis highlights the complex evolutionary pattern of rotavirus which could pose a major challenge for the new vaccines.

6.13 Further studies

Analysis of other gene segments of serotype G9 isolates, including more recent and earlier isolates, will be able to provide more information in regards to the evolution of this virus. Two isolates identified in this study, R1 and R24 should be further assessed by adapting these to cell culture and fully characterising them in order to determine their origin and genetic evolution. These two isolates were genetically diverse compared to other serotype G9 rotaviruses in Melbourne. The genetics of all other gene segments encoding for structural proteins such as VP1, VP2, VP3 and VP6 needs to be analysed to examine the evolutionary changes occurring in these proteins. Detailed investigation of other non-structural proteins (NSP2, NSP3, NSP5 and NSP6) is also warranted to fully understand the extent of genetic variation in these proteins. The evolutionary

process of rotaviruses in other serotypes also needs to be assessed by carrying out similar studies and determine genetic factors responsible for the emergence of new strains in the community. Large amounts of sequence data would be required to analyse the molecular evolutionary mechanism of different serotypes. The effect of the new vaccines in controlling and preventing rotavirus illness and their impact on rotavirus epidemiology would be interesting to observe.

Glossary

The definitions are taken from concise dictionary of Biomedicine and Molecular Biology, 2nd Edition by Pei-Show Juo, CRC Press.

Acute a disease that has a rapid onset and persists for a relatively short period of time.

Å an angstrom unit. 10^{-1}nm or 10^{-4}micron or 10^{-7}mm.

Allele one of two or more alternative forms of a given gene that control a particular characteristic occupying corresponding loci on the homologous chromosomes.

Amino terminus referring to the amino terminus of a protein or polypeptide that is the terminus where the first amino acid is incorporated during mRNA translation.

Antigen a substance that when introduced into the body stimulates the production of an antibody. Antigens include toxins, bacteria, foreign blood cells, and the cells of transplanted organs.

Asymptomatic presenting no symptoms of disease.

Atrophy a wasting, progressive degeneration and loss of function of any part of body.

Attenuated reduced or weakened, as in strength, value, or virulence.

Autokinase spontaneous or voluntary activity or movement.

Bootstrap being or relating to a process that is self-initiating or self-sustaining.

Capped 5'end methylated guanosine added posttrancriptionally to the 5' end of an mRNA.

Cluster a grouping of a number of similar things.

Codon sequence of three adjacent nucleotides (triplet) on mRNA that specifies a given amino acid.

Congenital present at the time of birth.

Consensus agreement in the judgment or opinion reached by a group as a whole.

Conserve to maintain (a quantity) constant during a process of chemical, physical, or evolutionary change.

Cytoskeleton the complex network of microtubules, microfilaments in the cytoplasm that provide structure to the cytoplasm of the eukaryotic cell and plays an important

role in cell movement and maintaining the characteristic shape of the cells.

Domain the structural and functional portion of a polypeptide that folds. independently of the other portion of the protein; a region of a protein with tertiary structure. 2. Regions of H or L chains of the immumoglobulin molecule that are folded.

EIA Abbreviation for enzyme immuno assay.

Electrophoresis a method of separating charged molecules or particles according to their charge, size and shape as they migrate through a medium in an electric field.

Endoplasmic reticulum a network of membranous tubules and flattened sacs in the cytoplasm of eukaryotic cells that is composed of ribosome-studded (tough ER)and ribosome free (smooth ER) regions.

Enteric virus virus infecting intestine.

Enterocyte cells of intestinal epithelium.

Enterotoxin a group of bacterial or viral exotoxins that act on the intestine mucosa and perturb ion and water transport systems causing vomiting and diarrhoea.

Enzyme bioactive protein that catalyses the biochemical reactions in the living cell.

Epidemiology the science that deals with the interrelationship among pathogens, environments, and host populations.

Erythema redness or swelling of the skin or mucous membrane.

Etiology the science that deals with causal agents of disease.

Fibronectin a dimeric protein produced by mast cells and macrophages. It is a chemotactic factor and plays an important ole in tissue repair and cell to cell adhesion.

Gastroenteritis inflammation of stomach and intestine.

Gene substitution the replacement of one allele by another allele of the same gene.

Gene a segment of the DNA molecule that encodes a functional product, e.g. Polypeptide chain.

Genetic drift changes in genotype or gene frequencies from generation to generation in a population as a result of random processes.

Genetic recombination the combination of two different molecules to produce a third molecule that is different from either of the original two.

Genogroup a group of organism belonging to same gene.

Genome the complete set of genes of an organism.

Genotype the genetic constitution of a cell or organism.

Glycoprotein the protein that has carbohydrate attached.

Glycosylation covalent attachment of carbohydrate molecule to a polypeptide or polynucleotide.

Guanyltransferase an enzyme that helps in guanylation of heterologous RNA containing 2 or 3 phosphate at their 5' terminal (source: www.biologyonline.org).

Hemagglutinin antibody or substance that causes agglutination of erythrocytes.

Heterotypic a combination of group that does not form like or common or same type.

Homotypic a combination of group that form like or common or same type.

Hybridisation formation of genetically different parents, formation of double-stranded structure from two single stranded molecules, e.g. DNA:DNA, DNA:RNA, and RNA:RNA.

Hyperimmune serum serum from an animal that has received repeated antigen injections that contains a high concentration of polyclonal antibodies against that antigen.

Immunocompetent cells any cell that participate in the immune response, e.g., lymphocytes and antigen presenting cells.

Immunogenic the ability of a substance to elicit an immune response.

In situ in the normal, natural, original or appropriate position.

In vitro outside the organism, e.g., an experiment performed in the test tube.

In vivo occurring within the living organism.

Infectious capable of producing a disease in a susceptible host.

Inhibitor substances that repress a chemical or biological action.

Innate immunity the defenses acquired at birth that is independent of the exposure To specific antigens. The efficiency of innate immunity cannot be improved by repeated infection or vaccination.

Interferon a family of glycoproteins produced by peripheral blood leukocytes upon

exposure to interferon inducer e.g. virus, double stranded RNA or bacterial products into functional three-dimensional structures.

Intracellular inside or within cells.

Intussusceptions invagination, especially an in folding of one part of the intestine Into another.

Jejunum the middle portion of the small intestine between duodenum and ileum.

Lamina propria loose connective tissue of a mucous membrane, binding the epithelium to underlying structures and holding blood vessels serving the epithelium.

Laminin protein component of the basement membrane.

Lineage the descent from a common progenitor, the ancestry of a cell during development or evolution.

Methyltransferase any of several enzymes that catalyse the transfer of methyl group from one compound to another (source: www.dictionary.com).

Monoclonal antibody homogeneous immunoglobulin derived from a single clone of cells.

Monotype a single type of organism.

Morphogenesis the growth and differentiation of cells to form tissues or organs.

Morphology the science that deals with the structures and forms of living organisms.

Mutation a process by which a gene undergoes change, e.g. change of nucleotide sequence in DNA, leading to an inheritable change in phenotype or alteration in the product encoded by the gene.

Nosocomial infection an infection acquired while in the hospital.

Nucleotide the basic building block of nucleic acids, it contains a nitrogenous base (a purine or pyrimidine), a pentose (a ribose or a deoxyribose) and a phosphate.

Oligomer a polymer that consists of only a small number of monomeric units, e.g. short chain nucleotide (oligonucleotide), short chain polysaccharide (oligosaccharide), or short chain peptide (oligopeptide).

ORF acronym for open reading frame (in a DNA sequence).

PAGE acronym for Polyacrylamide Gel Electrophoresis.

Pathogenesis the mechanism of an organism to cause disease.

Phosphoprotein proteins that contain phosphate groups.

Phylogenetic referring to the classification system based on the evolutionary relationships of organisms.

Polyacrylamide Gel an electrophoresis gel formed by polymerisation of N,N-methylene bisacrylamide and acrylamide in the presence of polymerising agents.

Polymerase chain reaction a technique that employs a repetitive cycle of DNA amplification using heat-stable DNA polymerase from *Thermus aquaticus* to eliminate the need to add fresh enzyme after each heat denatures cycle.

Primer a short sequence of RNA or DNA that serves as starting point for synthesis of DNA.

Probe a sequence of labeled DNA or RNA that is used to locate and identify the Sequence on a blot by hybridisation under optimal conditions of a salt concentration and temperature.

Prototype a type that was first of the kind.

Radioactive labeling a radioactive element used to label a compound to follow the course of the labeled compound in a biological system.

Reassortment a virus produced containing genome segments and proteins from different viruses.

Receptor protein located either on the cell surface (membrane receptor) or within the cytoplasm (cytoplasmic receptor) that bind ligand initiating signal transmission and cellular activity.

Recombinant the progeny resulting from genetic recombination where the phenotype of the recombinant differs from that of the parent.

Recombination break and ligation of DNA or RAN fragments.

Reverse transcription a process by which an RNA molecule is used as a template for synthesis of a single stranded DNA copy or DNA-RNA hybrid.

Rhinitis inflammation of nasal mucous membrane.

RNA polymerase the enzyme catalyses the synthesis of RNA from triphosphate ribonucleic acid using DNA as template.

Sequence referring to the linear order of the different monomeric units in a polymer, e.g. amino acid sequence in a protein or nucleotide sequence in a nucleic acid.

Serotype a type of classification based on the variation of surface epitopes of microorganisms, serologically distinguishable member of the same species.

Species category of biological classification ranking immediately below genus.

Structural protein proteins that serve as the structural component of cells or tissues.

Substitution a reaction that replaces a molecule or group of molecules by another molecules.

Surveillance A type of observational study that involves continuous monitoring of disease occurrence within a population.

***Taq* DNA polymerase** a heat-stable DNA polymerase from *Thermus aquaticus.*

Template the macromolecular mold for the synthesis of another macromolecule in a complementary fashion, e.g. DNA serves as template for the synthesis of RNA.

Transcription the process by which the information contained in the DAN is copied into a single-stranded RNA molecule by the enzyme RNA polymerase.

Transmembrane protein proteins that span the membrane with peptide chains Exposed on both sides.

Vaccine a nonvirulent antigenic preparation used for vaccination to stimulate the recipient's immune defense mechanisms against a given pathogen.

Villi a small, vascular, hairlike projection from the surface of a membrane, e.g., inner mucous membrane of the intestine.

Virion the complete mature infectious virus particle.

Virulence the degree of pathogenicity or ability to cause disease.

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Appendix I:

Table 1 Buffers and reagents used in the EIA

Buffers/Reagents	Preparation
Diluent for Faecal extract: TNC buffer	0.2 M Tris HCl, 8.5g NaCl, 1.1g $CaCl_2$ in 50mL D/W : Adjusted pH to 7.5. Stored at 4^{o}C.
5X Phosphate Buffer Saline (PBS) pH 7.2	200g NaCl, 5g KCl, 36g Na_2HPO_4 (anhydrous), 6g KH_2PO_4, pH adjusted to 7.2 with NaOH, made up to 5 L. Diluted 1:5 to make working solution (1XPBS).Store at RT
Washing Buffer	1X PBS, pH7.2 + 0.05% (v/v) Tween 20. Stored at RT.
0.2M Sodium Acetate	8.203g in 500 mL distilled water. Stored at RT
0.2M Citric Acid	8.44g in 200 mL distilled water. Stored at 4^{o}C.
Conjugate	Horseradish peroxide conjugated sheep anti mouse immunoglobin (Silenus, DAH) (1:1000 dilution used).
TMB solution	0.1g Tetramethyl benzidine (Sigma) 10mL Dimethylsulphoxide. Stored at 4^{o}C.
TMB substrate buffer (per plate)	D/W -5mL, 0.2MSodium Acetate-5mL, 0.2M Citric acid-50 µL, 30% (v/v) Hydrogen peroxide-1.2 µL, TMB Solution 100 µL

Table 2 Buffers and reagents used in PAGE and Northern hybridisation analysis

Buffer	Preparation
Lower Tris (pH 8.8)	18.17g Tris in 100 mL of distilled water, pH adjusted with 6N HCl, autoclaved and stored at RT.
Upper Tris (pH 6.8)	6.06g of Tris in 100mL of D/W, pH adjusted with 6N HCl, autoclaved and stored at room temperature.
Running buffer (x10)	To make 1 litre: 30g Tris, 144g Glycine, autoclaved and stored at room temperature. 1x buffer used for analysis.
Sample Buffer	To make 10 mL: 3 mL (v/v) Glycerol, 0.1g Bromophenol blue dissolved in 7mL upper Tris, autoclaved and stored at 4^{o}C.
Silver nitrate 0.011M	To make 100 mL: 0.186g silver nitrate, dissolved in 100 mL distilled water.
Reducing solution	To make 100 mL: 300 µl Formaldehyde (Sigma), 3g NaOH pellets, mixed to dissolved, prepared freshly.

Buffers	Preparation
High SDS hybridisation buffer	To make 100 mL: 50mL formamide, 7g SDS, 50mM sodium phosphate[pH 7.0], 2g blocking reagent (Roche Diagnostics Diagnostics), 5 mL 20 XSSC, 0.1g N-larylsarcosine. Autoclaved and stored at -20^{o}C.
Maleic Acid buffer	To make 500 mL: 5.8g Maleic acid, 4.37g NaCl, pH 7.5 adjusted with solid NaOH. Autoclaved and stored at room temperature.
20xSSC	To make 250 mL: 43.8g NaCl, 22.05g Sodium citrate pH 7.0 adjusted with 14N HCl. Autoclaved and stored at room temperature.
Washing Buffer	To make 500mL: 5.8g Maleic acid, 4.37g NaCl; pH adjusted with solid NaOH to 7.5; 0.3% (v/v) Tween 20 Autoclaved and stored at room temperature
Detection Buffer	To make 250mL: 3.02g Tris, 1.45g NaCl; pH adjusted to 9.5 with 6N HCl. Autoclaved and stored at room temperature.
10% Blocking buffer	To make 10 mL: 1g Blocking reagent (Roche Diagnostics) in10 mL Maleic acid buffer. Autoclaved and stored at 4^{o}C.

Table 3 Agarose Electrophoresis buffers

TAE buffer (50X)- to make 500 mL.		**TBE Buffer (10X)-to make 1000mL**	
Tris base	121g	Tris base	121g
Glacial Acetic acid	25.5 mL	EDTA	7.4g
EDTA (0.5M, pH 8.0)	50 mL	Boric Acid pH adjusted with NaOH to 8.3.	53.4g

For PAGE analysis

To make 10 mL of separating gel (lower gel):

Distilled water	4.16mL
Lower Tris (18.17 %(w/v)Tris, pH 8.8)	2.5 mL
Acrylamide-bisacrylamide (Bio Rad)	3 mL
Ammonium per sulphate (10% w/v))	200 µL
TEMED	7 µL

To make 10 mL of stacking gel:

Distilled water	3.1 mL
Acrylamide (40%)	0.375 mL
Upper Tris (6.06% (w/v) Tris, pH 6.8)	1.5 mL
TEMED	8 µL
Ammonium per sulphate (10% (w/v))	200 µL (freshly prepared).

Typical RT-PCR buffer (First round amplification): G type

For 1 sample:

D/W	77.5 µL
1M Tris	1.0 µL
1M KCl	4.0 µL
100mM $MgCl_2$	1.5 µL
DMSO	7.0 µL
dNTP mix(20mM each)	1.0 µL
Primer plus sense	1.0 µL (50µM)
Primer minus sense	1.0 µL (50µM)

The master mix was mixed well by vortexing and in a tube 94 µL of the master mix prepared and 5 µL of extracted rotavirus RNA was added. After mixing, it was heated at 97^{o}C for 4 minutes and quickly quenched on ice. 0.5 L of *Taq* polymerase (12 U) and 0.5 µl of AMV reverse transcriptase (2.5 U) was added. Vortexed quickly to mix and spun briefly before placing it in thermocycler.

Typical PCR buffer (Second round amplification):

Primers specific for G1, G2, G3, G4, G8 and G9 as described by Gouvea *et al*. (1990) were mixed with concentration of 12 μM each.Consensus primer RVG9 was used with same concentration.

D/W	79.5 μL
1M Tris	1.0 μL
1M KCl	4.0 μL
100mM $MgCl_2$	1.5 μL
DMSO	7.0 μL (optional)
dNTP mix(20mM each)	1.0 μL
Primer RVG9	1.0 μL (12μM)
Primer mix(G1-G9)	1.0 μL (50μM)
Taq polymerase	0.5 μL

The reaction mix was vortex and 1 μL of template from first round was used.

Similar approach was employed for P type amplification.

Table 5 Dilutions of F45 Monoclonal antibodies in 0.5% PBS+0.05% Tween 20

Monoclonal antibodies	First dilution	Second dilution in 0.5% casein PBS T20 (casein buffer)
F45:1	1/100	9.6 μL in 7.2 mL casein buffer
F45:2	none	4.8 μL in 7.2 mL casein buffer
F45:5	1/100	22.5 μL in 7.2mL casein buffer
F45:7	1/100	45 μL in 7.2 mL casein buffer
F45:8	1/100	45 μL in 7.2 mL casein buffer
F45:9	1/100	48 μL in 7.2 mL casein buffer
WI61:1	1/100	72 μL in 7.2 mL casein buffer

Table 6 List of Equipment used

Equipment	**Model no.**	**Company**
Agarose Electrophoresis tank	Minnie gel unit	Bioscience
Vortex		Ratek
Rocker	Platform rocker	Bio Line
Acrylamide electrophoresis tank	Minnie Protein II Cell	Bio Rad
UV transilluminator		UVP, Upland USA
Centrifuge	Mini Spin	Eppendorf
PCR thermal Cycler	Master Cycler	Eppendorf
Chemiluminescence detection	ChemiDoc XRS	Bio Rad

Appendix II

VP7 gene (coding region) alignment using Clustal W

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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                   10         20         30         40         50         60         70         80         90        100
F45       ATGTATGGTA TTGAATATAC CACAGTTCTA ACCTTTTTGA TATCAATCAT TTTGTTGAAT TATACACTAA AATCATTAAC TAGTGCAATG GACTTTATAA
R1        ....C..... .......... .......... .T........ .......TG. ...A...... ....T.T... .......... ......G... ..........
R2        .......... .......... ....A..... ......C... .......AG. ...A.....C ....T.T... .....C.... ......G... ..........
R3        .......... .......... ....A..... .......... .......AG. ...A.....C ....T.T... .....C.... ......G... ..........
R4        .......... .......... ....A..... ......C... .......AG. ...A.....C ....T.T... .....C.... ......G... ..........
R26       .......... .......... ....A..... ......C... .......AG. ...A.....C ....T.T... .....C.... ......G... ..........
R22       .......... .......... ....A..T.. ......C... .......AG. ...A.....C ....T.T... .....C.... ......G... ..........

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  110        120        130        140        150        160        170        180        190        200
F45       TTTATAGACT CCTTTTGCTT ATCGTCATTG TGTCACCATT TGTCAAGACG CAAAATTATG GAATTAATTT ACCAATCACT GGCTCCATGG ATACGGCATA
R1        ....C...T. T.....A... G.T..... .A........ ...G..A..A .......... .......C.. ......T... ..T..T.... ....A..G..
R2        ........T. T.....A... ..T..T.... CA.....T.. ...T..A..A .......... .......... ...G...... .......... ....A.....
R3        ........T. T.....A... ..T..T.... CA.....T.. ...T..A..A .......... .......... ...G...... .......... ....A.....
R4        ........T. T.....A... ..T..T.... CA.....T.. ...T..A..A .......... .......... ...G...... .......... ....A.....
R26       ........T. T.....A... ..T..T.... CA...T.T.. ...T..A..A .......... .......... ...G...... .......... ....A.....
R22       ........T. T.....A... ..T..T.... CA...T.T.. ...T..A..A .......... .......... ...G...... .......... ....A.....

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  210        220        230        240        250        260        270        280        290        300
F45       TGCAAACTTA TCACAGCAAG AGACATTTTT AACTTCAACG TTATGTTTAT ATTACCCTGC TGAAGCATCA ACTCAAATTG GAGATACAGA ATGGAAAGAC
R1        ...G....C. ..G..A.... .......C.. .........A .......... ....T...A. .........G .......... .......... ......G..T
R2        ......T.C. .......... .A........ G......... C....C.... ....T...A. A......... .......... .......G.. ......G..T
R3        ......T.C. .......... .A........ G......... C....C.... ....T...A. A......... .......... .......G.. ......G..T
R4        ......T.C. .......... .A........ G......... C....C.... ....T...A. A......... .......... .......G.. .........T
R26       ......T.C. .......... .A........ G......... C....C.... ....T...A. A......... .......... .......G.. ......G..T
R22       ......T.C. .......... .A........ G......... C....C.... ....T...A. A......... .......... .......G.. ......G..T

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  310        320        330        340        350        360        370        380        390        400
F45       ACTCTGTCTC AATTGTTCTT GACTAAAGGG TGGCCAACTG GGTCAGTCTA TTTTAAAGAA TATACTGATA TCGCTTCATT TTCTATTGAT CCACAACTTT
R1        .......... ....A..T.. .......... .......... .A..G..T.. .......... ...G.C..C. .......... ...A..C... ..G.......
R2        ........C. ....A..... .......... .......... .A........ .......... ..C....... .......... C..A...... ..........
R3        ........C. ....A..... ..T....... .......... .A........ .......... ..C....... .......... C..A...... ..........
R4        ........C. ....A..... .......... .......... .A..C..... .......... ..C....... .......... C..A...... ..........
R26       ........C. ....A..... .......... .......... .A........ .......... ..C....... .......... C..A...... ..........
R22       ........C. ....A..... .......... .......... .A........ .......... ..C....... .......... C..A...... ..........

          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  410        420        430        440        450        460        470        480        490        500
F45       ATTGCGATTA TAATGTTGTG TTAATGAAGT ACGATTCAAC ATTAGAGTTA GACATGTCTG AACTAGCTGA TTTGATTCTA AACGAATGGT TATGTAATCC
R1        ....TA.C.. .......... ..G....... .T........ ......A... ..T....... ....G..... .......... ..T....... ..........
R2        ....T..... .........A C.G....... .T........ G......C.. ..T....... ..T....... ...A...... ..T....... .......C..
R3        ....T..... .........A C.G....... .T........ G......C.. ..T....... ..T....... ...A...... ..T....... .......C..
R4        ....T..... .........A C.G....... .T........ G......C.. ..T....... ..T....... ...A...... ..T....... .......C..
R26       ....T..... .........A C.G....... .T........ G......C.. ..T....... ..T....... ...A...... ..T....... .......C..
R22       ....T..... .........A C.G....... .T........ G......C.. ..T....... ..T....... ...A...... ..T....... .......C..
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          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  510        520        530        540        550        560        570        580        590        600
F45       GATGGATATA ACACTATATT ATTATCAACA AACAGATGAA GCGAATAAAT GGATAGCGAT GGGACAATCT TGTACAATAA AAGTGTGTCC GTTGAATACG
R1        A......... ...T....C. .......G.. .......... .......... .....T.A.. ...GAC.... ..C....... ....A..... A.........
R2        A......... ...T..... .......G.. .......... .......... .....T.... ......G... .....C.... ....A..... A.........
R3        A......... ...T..... .......G.. .......... .......... .....T.... ...GAC.... .....C.... ....A..... A.........
R4        A......... ...T..... .......G.. .......... .......... .....T.... ...GAC.... .....C.... ....A..... A.........
R26       A......... ...T..... .......G.. .......... .......... .....T.... ......G... .....C.... ....A..... A.........
R22       A......... ...T..... .......G.. .......... .......... .....T.... ...GAC.... .....C.... ....A..... A.........


          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  610        620        630        640        650        660        670        680        690        700
F45       CAGACTTTAG GAATAGGCTG TACAACTACA AATACAGCAA CATTTGAGGA AGTGGCCGCA AGTGAAAAAT TAGTGATAAC TGATGTTGTT GATGGTGTGA
R1        ..A..A..G. .......A.. ......C... ........G. .......... ......A... ..C..G.... .......... C..C...... ..........
R2        .......... .......T.. ..TT..C... ........G. .......A.. G.....TA.. .......... ....A..... C.......... ..........
R3        .......... .......T.. ..TT..C... ........G. .......A.. G.....TA.. .......... ....A..... C.......... ..........
R4        .......... .......T.. ..TT..C... ........G. .......A.. G.....TA.. .......... ....A..... C.......... ..........
R26       .......... .......T.. ..TT..C... ........G. .......A.. G.....TA.. .......... ....A..... C.......... ..........
R22       .......... .......T.. ..TT..C... ........G. .......A.. G.....TA.. .......... ....A..... C.......... ..........


          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  710        720        730        740        750        760        770        780        790        800
F45       ATCATAAACT AGATGTAACT ACAACTACTT GTACTATTAG AAATTGTAGA AAATTAGGAC CAAGAGAGAA TGTGGCAATT ATACAAGTTG GCGGCTCAGA
R1        .......... T......... ....AC..C. .C..G..... .......... ..G....... .......A.. .......... .......... ..........
R2        .C........ T.....G... ....A...C. ....A..... G.......AG ..G....... .......A.. ..GA..G... ........C. .T........
R3        .C........ T.....G... ....A...C. ....A..... G.......AG ..G....... .......A.. ...A..G... ........C. .T........
R4        .C........ T.....G... ....A...C. ....A..... G.......AG ..G....... .......A.. ...A..G... ........C. .T........
R26       .C........ T.....G... ....A...C. ....A..... G.......AG ..G..G.... .......A.. ...A..G... ........C. .T........
R22       .C........ T.....G... ....A...C. ....A..... G.......AG ..G....... .......A.. ...A..G... ........C. .T........


          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                  810        820        830        840        850        860        870        880        890        900
F45       AGTGCTAGAT ATTACAGCAG ACCCAACCAC TGCGCCACAA ACTGAACGTA TGATGCGAAT AAATTGGAAA AAATGGTGGC AAGTTTTTTA TACGGTAGTA
R1        ...A...... ........G. .T.....T.. ...A.....G ........C. .......... ..........G .......... ....C..... ...A......
R2        T...T..... ........G. .T.....T.. ...A..... .......... ......A.G. ..........G .......... ..A....C.. ..........
R3        T...T..... ........G. .T.....T.. ...A..... .......... ........G. ..........G .......... .......C.. ..........
R4        T...T..... ........G. .T.....T.. ...A..... .......... ........G. ..........G .......... .......C.. ...A......
R26       T...T..... ........G. .T.....T.. ...A..... .......... ........G. ..........G .......... .......C.. ..........
R22       T...T..... ........G. .T.....T.. ...A..... .......... ........G. ..........G .......... .......C.. ..........


          ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| .
                  910        920        930        940        950        960        970        980
F45       GATTATATCA ATCAGATTGT TCAAGTTATG TCCAAAAGAT CGCGATCGTT GAATTCAGCA GCTTTCTACT ATAGAGTCTG A
R1        ........T. .......... A......... .....G..G. .A..G..... A.......G. .....T.... .........-- -
R2        AT......T. .........G G...T..... .......... .C..G..T.. A......... .....T.... ....G..T-- -
R3        ........T. .......... G......... .......... .A..G..A.. A......... .....T.... ....G..T-- -
R4        ........T. ........T. C.......G. AA........ .A.TT..A.. CTC....... ...C.A.... TC....GA-- -
R26       ........T. .......... G......... .......... .A..G..A.. A......... .....T.... ....G..T-- -
R22       ........T. .......... G......... .......... .A..G..A.. A......... .....T.... T...C.GA-- -
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Partial NSP1 protein alignment using Clustal W

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          300        310        320        330        340        350        360        370        380        390
F45              FTQRKLQIAS KLMKPNYMTS NHRASATEVH NCKWCSTNSS YIVWNDFRVK KIYDNILNFL RALVKSNVNV GHCSSQEKIY ECVENILDIC DNEKWKTSVT
R1               ...H..KM.. ..I....I.. ...T...... ......V.N. .T........ ......F... .........I .......... .YI.D..NV. .SK.....II
R4               --.H..K... .HI....V.. ...T...... ......I.NG .T......I. ......F... .......S.I .......... .HIKDV..V. .D....MA.A
R11              ...Y..K... .HI....V.. ...T...... ......I.NG .T......I. ......F... .......S.I .......... .HIKDV..V. .D....MA.A
R14              ...H..KM.. ..I....V.. ...T...... ......I.N. .T......I. ......F... .......S.I .......... .HIKDV..V. .D....MA.A
R19              ---------- -----..V.. ...T...... ......I.NG .T......I. ......F... .......S.I .......... .HIKDV..V. .D....MA..
MK26             ---------- -HI....V.. ...T...... ......I.NG .T......I. ......F... .......S.I .......... .HIKDV..V. .D....MA.A
MK36             ---------- -HI....V.. ...T...... ......I.NG .T......I. ......F... .......S.I .......... .HIKDV..V. .D....MA.A
M37              ...H..K... .HI....V.. ..KT...... ......I.N. .T........ ......F... .......A.. .......... .YIKDV..V. .D....IA..
ST3              ...H..KM.. .HI....V.. ...T...... ......I.N. .A........ ......F... .......A.. .......... .HI.DV..V. .D.....A..
I321             ......K.V. NSI....IV. ..K....... ......V.N. .T......I. .V....F... .........I .......... .Y.K.V.NV. .T.R.E...M
HOCHI            ...H..KM.. ..I....I.. ...T...... ...L..V.NN .T........ ......FS.. .........I .......... .Y..DV.NV. ........IM
OSU              ......K... ..I....I.. ...T...... ......I... .T........ ......F... .......... .......... ........V. ..........
K8               ...C..KM.. ..I....I.. ..KTL..... ......V.N. .T......I. ......F... .........I .......... .Y.KDV.NV. .D.R....IM
WA               ...H..KM.. ..I....I.. ...T...... ......V.N. .T........ ......FS.. .........I ..R....... .Y..DV.NV. ........IM
Bovine B22       ..IY..N.I. ..V....IF. ..GLC.LD.N .....KIDNH .EI.....LR ...N.MM..I .......T.. .....H.LV. K.ISSVFIVW KI...ND..R

                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....
                          400        410        420        430        440        450        460        470        480
F45              EIFNCLEPVE LNAVNYVLFN HEVNWDVINI LVQSIG-KVP QILTLNDVVT IMQSIIYEWF DVRYMRNTPM TTFTVDKLRR LCIEPKTVD- YDSGISDVE
R1               KV........ .DN.K..... ..I......V ...N..-... H....QN.II VI........ .I........ V...I..... .HT.L..AE- .........
R4               .......... .DT.K..... .........L ....V.-... .......I.I ..K....... .I........ .........Q ..TGV....- .........
R11              .......... .DT.K..... .........L ....V.-... .......III .IK....... .I........ .........Q ..TGV....- .........
R14              .......... ..T.K..... .........L ....V.-... .......I.I ..K....... .I........ ..Y....F.Q ..TGV....- .........
R19              .......... ..N.K..... .........L ....V.-... .......I.I ..K....... .I........ .........Q ..TGV....- .........
MK26             .......... .DT.R..... .........L ...NV.-... .......III ..K....... .I........ ..L......Q ..T.V....- .........
MK36             .......... .DT.R..... .........L ...NV.-... .......III ..K....... .I........ ..L......Q ..T.V....- .........
M37              .......... ..N.K.A... .........L ....VD-.A. .......I.I ..K....... .I........ .......... ..TGV....- .........
ST3              .......... .D..K..... .........L ....V.-... .......I.I ..K....... .I........ .........Q ..TGV....- .........
I321             .........D .DD.K..... ..I......V ..N...-... .....KN.L. ......S... .I........ A...I..... .HT.L...E- ....V....
HOCHI            KV........ .DD.K..... ..I......V ......-... .....KN.I. .I........ .I........ V...I..... .HT.L..AE- .........
OSU              K...Y..... .......... .........V ......-... ........T. .......... .TK....... .......... ..TGS....- .........
K8               .......... .DD.K..... ..I......V ..H...-... .....EN.I. .......... .I........ V...I..... .YTGL...E- .......I.
WA               KV........ .DN.K..... ..I......V ......-... .....KN.I. .I........ .I........ V...I..... .HT.L..AE- .........
Bovine B22       TL.EY..... I.H.E...LD ..LS.EMSGV IM.IMNG... R..SFD..KK ..GA...D.. ......E..V IVS.TNE..K .NKDNNLM.G ..YEL..I.
```

Multiple NSP1 protein (amino acid 290-489) sequence alignment using Clustal W. Dots signify conserved region. – indicate unavailable sequence.

Appendix III

Publication arising from this thesis:

Genetic Variation of NSP1 and NSP4 Genes among Serotype G9 Rotaviruses Causing Hospitalization of Children in Melbourne, Australia, 1997–2002.

Kiran Shah,1 Carl D. Kirkwood,2,3 Mrinal Bhave,1 and Enzo A. Palombo

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