CHAPTER 2: LITERATURE OVERVIEW **2**

2.1 Introduction

Since a link between rotavirus and gastroenteritis was 40 years ago (Bishop et al., 1973, Flewett et al., 1973), this virus is now recognized to be the leading cause of severe dehydrating gastroenteritis among children under the age of five. In excess of 80% of the nearly half a million annual rotavirus related deaths occur in the developing countries of Asia and Africa where access to proper medical care is limited (Madhi et al., 2010, Msimang et al., 2013, Mwenda et al., 2010, Sanchez-Padilla et al., 2009, Tate et al., 2012). Studies have indicated that natural rotavirus infection normally shields against severe disease (Anderson and Weber, 2004, Velazquez et al., 1996). Typical symptoms of rotavirus infection include dehydrating diarrhoea, nausea, fever as well as vomiting and abdominal cramping. Currently there is no specific treatment for rotavirus disease and the most effective action involves intravenous or oral rehydration.

The mature rotavirus particle is roughly 80 nm in diameter without the VP4 spike (100 nm with VP4 spike) and when viewed under the electron microscope has a wheel-like appearance, therefore "*rota*" (latin. Wheel) was used to name the virus (Flewett et al., 1974). As members of the *Reoviridae* family, rotaviruses are non-enveloped particles, consisting of 11 double-stranded RNA (dsRNA) genome segments with a total size of about 18 550 base pairs. Ten of these genome segments encode for a single viral protein, including 6 structural proteins (VP1-4, VP6 and VP7) and 4 non-structural proteins (NSP1-4). Genome segment 11 encodes two non-structural proteins, NSP5 and NSP6. The genome is encapsulated in a triple-layered particle.

The most prevalent rotavirus strain infecting humans and other mammals is rotavirus A (Heiman et al., 2008, Matthijnssens et al., 2010). Group A rotaviruses include the AU-1 (G3P[8]), DS-1 (G2P[4]) and Wa (G1P[8]) genogroups. The AU-1 genogroup is fairly uncommon globally, in contrast to rotaviruses of the DS-1-like and Wa-like genotype constellations that occur widespread among humans and various animal species (Matthijnssens and van Ranst, 2012).

Reverse genetics is an innovative molecular biology tool that enables the manipulation of specific viral genomes at the cDNA level in order to generate certain mutants or artificial viruses. Plasmid-based reverse genetic systems have been developed for many animal RNA viruses, including paramyxoviruses, bunyaviruses, coronaviruses, picornaviruses, bornaviruses, flaviviruses, orthoreoviruses, orthomyxoviruses, and rhabdoviruses (Kobayashi et al., 2010, Satterlee, 2008). This ground-breaking technology has led to the generation of valuable evidence regarding the replication and pathogenesis of viruses. Unfortunately, extrapolating these reverse genetics systems to rotavirus proved to be much more difficult. No selection-free (comprehensive) reverse genetic system exists for rotavirus.

Many questions regarding the rotavirus replication cycle and innate immune response still remains unanswered. A rotavirus reverse genetics system may provide us with the tools to answer some of these elusive questions.

2.2 Historical look at gastroenteritis and rotavirus

Acute gastroenteritis is one of the most common diseases amongst humans worldwide. Every year, an estimated 1 billion diarrhoea cases are reported, of which 2.4 – 5 million are fatal (Black et al., 2010, Bryce et al., 2005, Linhares, 2000, Wilhelmi et al., 2003). In the 1800s and 1900s, this disease was popularly referred to as typhoid or cholera morbus and the term gastroenteritis was only first used in 1825. Throughout the late 19th century and early 20th century, researchers still believed that the most prominent cause of gastroenteritis was bacterial. The first report of an epidemic gastroenteritis illness caused by a viral agent was published in 1929 by the American physician, Zahorsky (Zahorsky, 1929). Zahorsky termed this sickness ‘hyperemesis hiemis’ or ‘winter vomiting disease’. In the midst of the Second World War, Light and Hodes inoculated calves with faecal filtrate from a newborn with diarrhoea to produce the same state in calves (Light and Hodes, 1943). Unfortunately, the causative agent could not be adapted to cultured cells. Nearly 25 years later, in 1968, faecal samples were collected from students and teachers showing symptoms of acute diarrhoea and vomiting at an

elementary school in Norwalk, Ohio, USA. However, all efforts to identify the pathogen responsible for the Norwalk outbreak were in vain until Kapikian and co-workers discovered viral particles in faecal matter of a volunteer infected with a purified stool sample isolated from a Norwalk outbreak patient in 1972 (Agus et al., 1973, Kapikian et al., 1972). Electron microscopy identified particles measuring between 27 – 32 nm and this agent was named the Norwalk virus. The first rotavirus to be described was the simian agent 11 (SA11) which was isolated from a *Cercopithecus* monkey at the National Institute of Virology (Johannesburg, South Africa) by Dr. Hubert Malherbe in 1958 (Malherbe and Strickland-Cholmley, 1967). In 1973, Bishop and her colleagues identified a viral agent in the duodenal mucosa of infants with gastroenteritis (Bishop et al., 1973). In the following year, 1974, Thomas Henry Flewett observed that rotavirus particles resemble a wheel when observed through an electron microscope and proposed the name *rotavirus* (*rota* in Latin) (Flewett et al., 1974). Rotavirus as a name was officially recognised by the International Committee on Taxonomy of Viruses in 1979. Serotypes for rotavirus were only first defined in 1980s by Birch and co-workers (Birch et al., 1988, Coulson et al., 1987). A major breakthrough came the following year when rotavirus Wa, isolated from an infant stool sample, was adapted to replicate in cultured cells (Wyatt et al., 1980). This was followed by a whole range of rotavirus strains being successfully adapted to cell cultures. Adapted strains made it much easier to study the rotavirus replication cycle and develop vaccine strategies.

2.3 Rotavirus genome and protein structure

The rotavirus genome consists of 11 dsRNA genome segments that are encapsulated in a triple-layered particle. Ten of the 11 genome segments encode for a single viral protein, including 6 structural proteins (VP1-4, VP6 and VP7) and 4 non-structural proteins (NSP1-4) (***Table 2.1***). The eleventh genome segment encodes for two non-structural proteins, NSP5 and NSP6 (Estes and Kapikian, 2007) (***Figure 2.1***). These genome segments range from ~ 660 – 3300 bp in size and the electrophoretic pattern of group A rotaviruses are composed of four high molecular weight dsRNA segments (genome segments 1 – 4), two mid length segments (genome segments 5 and 6) and 5 smaller segments (genome segments 7 – 11) (Figure 2.2). Every genome segment

contains a 5'-terminal cap, while the open reading frame of every genome segment is flanked by untranslated regions (Imai et al., 1983, Patton, 1995, Pizarro et al., 1991). The complete dsRNA genome is believed to be packed in the centre void of the viral particle in conical spirals (Pesavento *et al*., 2001).

Reassortment can occur between rotavirus strains of different genogroups during co-infection owing to the segmented nature of the rotavirus genome (Nakagomi and Nakagomi, 1991). The exchange of genome segments between different rotavirus strains are known as reassortment and may lead to the generation of novel phenotypes in rotaviruses. Reassortment has been characterised in genome segments 5 (NSP1), 6 (VP6), 7 (NSP3), 8 (NSP2), 9 (VP7), and 10 (NSP4) (Estes and Kapikian, 2007, Schnepf et al., 2008). Genome segment 11 (NSP5/6) seems to be most prone to rearrangement (Schnepf et al., 2008). On the other hand, genome segment recombinations are partial duplications of individual genome segments (Jere *et al*., 2011). This occurrence can take place between two strains of the same genotype or between strains of different genotypes (Parra *et al*., 2004, Jere *et al*., 2011). Reports on the discovery of genome segment recombination that contributes to rotavirus diversity has been uncommon thus far.

Table 2.1: The 11 rotavirus genome segments, encoded viral proteins and their functions based on the human rotavirus Wa.

RV Genome Segment (encoded protein)#	Length (bp)	Length (and position) of ORF	Molecular weight of viral protein (kDa)	Location	Description of viral protein	Function of viral protein
Segment 1 **(VP1)**	3302	3267 (19-3285)	125	Core	RNA-dependent RNA polymerase	RNA-dependent RNA polymerase; replicase and transferase activities; 3'-mRNA binding; together with VP3 forms transcription complex
Segment 2 **(VP2)**	2717	2673 (17-2689)	102	Core	Viral core shell protein	Viral core shell protein ; binds non-specifically to ssRNA and dsRNA; serves as a platform for VP1 and is required for replicase activity
Segment 3 **(VP3)**	2591	2508 (50-2557)	97	Core	Guanylyl-transferase	Guanylyltransferase and methyltransferase; complex for minus-strand synthesis of viral dsRNA; non-specific ssRNA binding; part of transcription complex with VP1
Segment 4 **(VP4)**	2360	2328 (10-2338)	88	Triple Layered Particle	Protease-sensitive viral spike protein	Outer capsid protease-sensitive viral spike protein; attachment to cell, role in virulence, P-type neutralising antigen; cleraved by trypsin into VP5* and VP8*
Segment 5 **(NSP1)**	1567	1460 (32 - 1492)	59	Cytoplasm	Interferon antagonist	E3 ubiquitin ligase which is able to bind to various IFN regulation factors (IRF3/5/7) and mark them for proteasomal degradation; is associated with the cytoskeleton
Segment 6 **(VP6)**	1356	1194 (24-1217)	45	Double Layered Particle	Intermediate viral capsid shell	Middle viral capsid shell; stabilizes the core; Anchor for VP7 and VP4; group specific antigen
Segment 7 **(NSP3)**	1059	933 (35-967)	37	Cytoplasm	Translation enhancer	Virus specific 3'-mRNA binding; role in translational regulation and shut-off of host cell protein synthesis
Segment 8 **(NSP2)**	1059	954 (47-1000)	35	Cytoplasm	NTPase	non-specific RNA binding; helix destabilisation actions; acts as an NTPase, major component of the viroplasm
Segment 9 **(VP7)**	1062	981 (49-1029)	37	Triple Layered Particle	Structural viral glycoprotein	Outer capsid structural viral glycoprotein; G-type neutralization antigen; endoplasmic reticulum transmembrane calcium-binding
Segment 10 **(NSP4)**	750	528 (42-569)	20	Cytoplasm	Enterotoxin	Enterotoxin; role in outer capsid assembly; role in virulence; transcription regulator, interaction with VP6
Segment 11 **(NSP5/6)**	664	593 (22-615) [NSP5] 278 (80-358) [NSP6]	NSP5: 22 NSP6: 11	Cytoplasm	Phosphoprotein	Major component of the viroplasm; autokinase activity of the *O*-linked glycosylated Phosphoprotein; NSP5: phosphoprotein; NSP6: interaction with NSP5

[#] The size of the genome segments and encoded proteins were based on the prototype human rotavirus Wa RVA/Human-tc/USA/WaCS/1974/G1P[8].

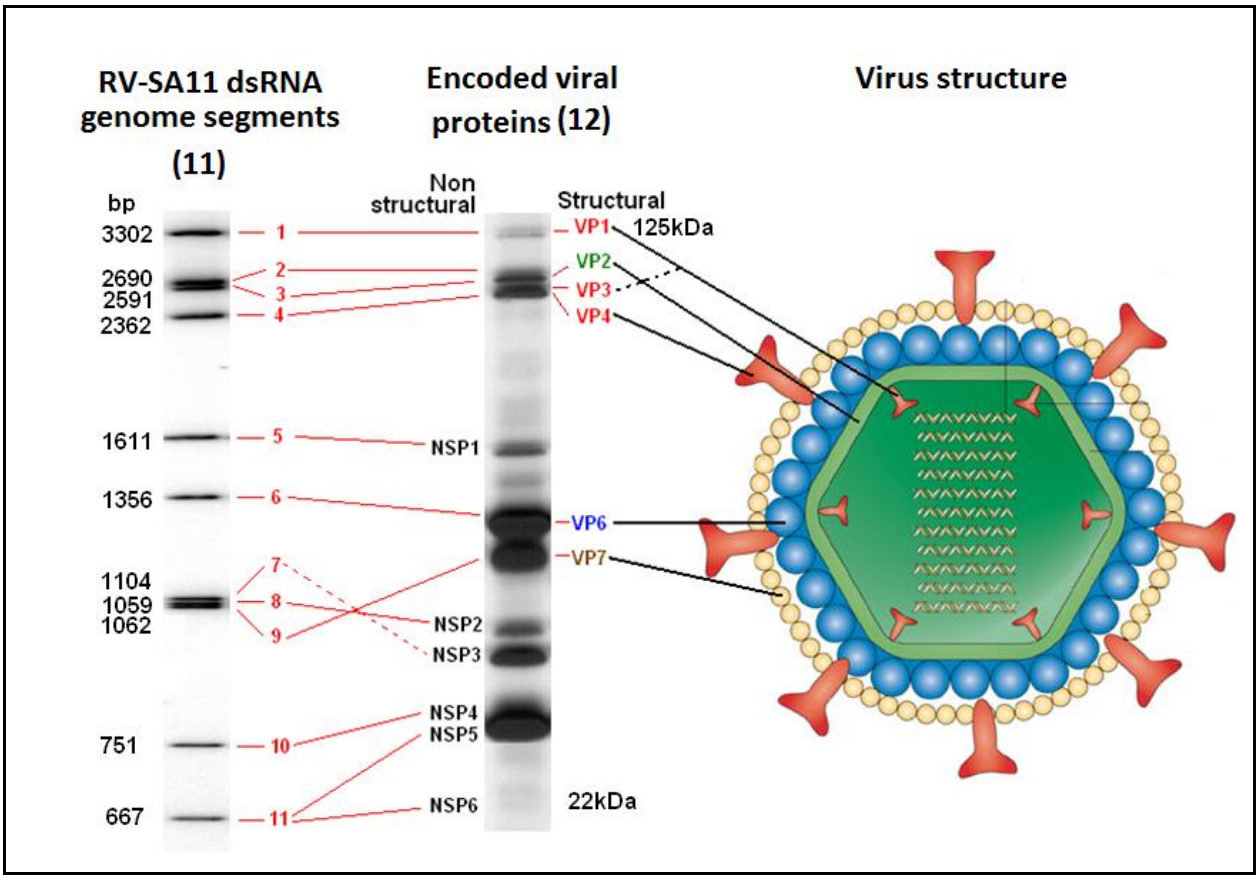

Figure 2.1: ***Diagrammatic illustration of the genome and proteins organization of rotavirus SA11.*** *The illustration shows the migration pattern of all 11 dsRNA genome segments of rotavirus SA11 on a polyacrylamide gel and the viral proteins encoded by the specific genome segments. The schematic diagram shows the structure and organization of a rotavirus SA11 particle. Original figure by Robert F. Ramig, Ph.D.*

2.4 Rotavirus particle structure

The rotavirus genome is encapsulated in a triple-layered particle. The inner capsid particle consists of the RNA-dependent RNA polymerase (VP1), the RNA capping enzyme (VP3) and genomic double-stranded RNA (dsRNA), all encapsulated in the VP2 protein lattice. VP1 and VP3 together form the so-called five-fold vertex which is anchored in VP2 with their N-terminal tethers (McDonald and Patton, 2011) (***Figure 2.2A***).

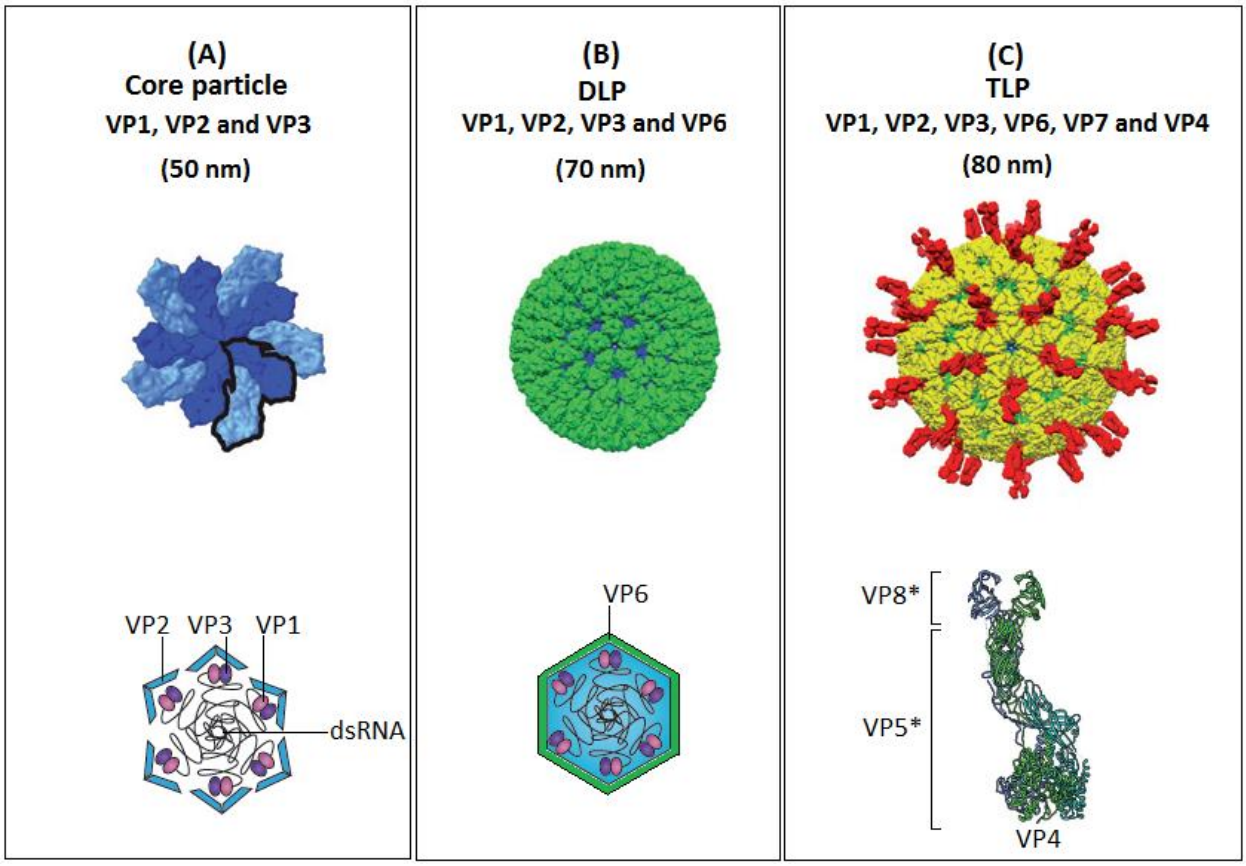

Figure 2.2: ***The structural architecture of rotavirus.*** *(A) An illustration of the core particle of rotavirus consisting of VP1, VP2 and VP3. (B) The double-layered particle (DLP) with VP6 illustrated in green, stabilizing the inner core. (C) The infectious rotavirus triple-layered particle coated with the glycoprotein VP7 (yellow) and the protease sensitive spike protein VP4 (red). VP4 is cleaved* in vitro *by exogenous protease into subunits VP5* and VP8*. Adapted from Trask et al. 2012.*

The core particle (inner capsid particle) is surrounded by a middle protein layer (VP6) and the fragile VP2 inner shell is thought to be stabilized by the binding of VP6 (***Figure 2.2B***). This complex, consisting of VP1, VP2, VP3 and VP6, is known as the double-layered particle (DLP). It has been shown that VP6 interacts with both the inner (VP2) and outer (VP7) capsid subunits (Charpilienne et al., 2002, Mathieu et al., 2001, McClain et al., 2010). Apart from playing a vital role in particle stabilization, the peripentonal channels of VP6 also serve as the scaffold for the rotavirus spike protein VP4 (Trask et al., 2012).

The outer layer consists of the glycoprotein VP7 and the protease sensitive spike protein VP4 which form the infectious triple-layered particle (TLP) (Estes and Kapikian, 2007) (***Figure 2.2C***). *In vitro*, the cell-surface receptor binding protein, VP4, is cleaved by exogenous protease into subunits VP5* and VP8*. This spike-like protein protrudes through the outer capsid (VP7) and is responsible for the attachment of the triple-layered rotavirus particle onto the cell membrane and subsequent penetration (Estes and Kapikian, 2007). VP8* forms the 'head' (N-terminal fragment) of the spike. The VP5* subunit extends from VP6, through the outer VP7 shell and forms the 'body' (C-terminus fragment) of the VP4 spike. The mature particle is approximately 80 nm in diameter (Crawford et al., 1994, McClain et al., 2010, Settembre et al., 2011).

2.5 Rotavirus evolution and classification

Rotavirus infections are common among animals and humans (Estes and Kapikian, 2007). Certain rotaviruses are zoonotic and have the potential of being transmitted between animals and humans. Rotaviruses are classified under the *Reoviridae* family which encompasses two subfamilies, called *Sedoreovirinae* and *Spinareovirinae,* and a total of 15 genera (***Table 2.2).*** The family consists of viruses containing a dsRNA genome consisting of between 9–12 linear segments (Estes and Kapikian, 2007). Rotaviruses belong to the subfamily *Sedoreovirinae* and contain, furthermore, the genera *Seadornavirus, Orbivirus, Phytoeovirus, Cardoreovirus* and *Mimoreovirus*. The following unique biological and morphological properties describe the rotavirus genus as reviewed in (Estes and Kapikian, 2007, Trask et al., 2012).

1. The addition of a exogenous protease activates rotavirus infectivity in cultured cells
2. Rotavirus replicates in the cytoplasm of the host cell and all the enzymes required for replication are provided by the virus itself;
3. Rotavirus transcripts have dual roles - the plus sense ssRNA transcripts act as mRNA for viral protein translation and templates for dsRNA synthesis;
4. No dsRNA is found in the cytoplasm and the complete viral replication cycle takes place within viroplasms;
5. Intracellular calcium is a crucial regulator of rotavirus assembly;

6. Premature double-layered particles acquire the outer capsid proteins (VP7 and VP4) by budding through the ER membrane;
7. The mature triple-layered particle is released through cell lysis or a budding process

Table 2.2: Classification of dsRNA viruses (Group III)

Family	Genera	Number of genome segments	Genome size
Hypoviridae	Hypovirus	1	~ 9 000 – 13 000 bp
Birnaviridae	Aquabirnavirus	2	~ 6 000 bp
	Avibirnavirus	2	~ 6 000 bp
	Blosnavirus	2	~ 6 000 bp
	Entomobirnavirus	2	~ 6 000 bp
Partitivirdae	Partitivirus	2	~ 4 000 bp
	Alphacryptovirus	2	~ 4 000 bp
	Betacryptovirus	2	~ 4 000 bp
	Cryspovirus	2	~ 4 000 bp
Picobirnaviridae	Picobirnavirus	2	~ 1 700 – 2 500 bp
Cystoviridae	Cystovirus	3	~ 6 000 bp
Chrysoviridae	Chrysovirus	4	~ 12 300 bp
Reoviridae	Aquareovirus	11	~ 30 500 bp
Spinareovirinae	Coltivirus	12	~ 29 000 bp
	Cypovirus	10	~ 25 000 bp
	Dinovernavirus	9	N/A
	Fijivirus	10	~ 27 000 – 30 000 bp
	Idnoreovirus	10 - 11	~ 27 000 – 30 000 bp
	Mycoreovirus	11 - 12	~ 23 000 bp
	Orthoreovirus	10	~ 23 000 bp
	Oryzavirus	10	~ 26 000 bp
Sedoreovirinae	Cardoreovirus	11	N/A
	Orbivirus	10	~ 19 200 bp
	Mimoreovirus	11	~ 25 400 bp
	Phytoreovirus	12	~ 26 000 bp
	Rotavirus	11	~ 18 500 bp
	Seadornavirus	11	~ 21 000 bp
Endornaviridae	Endornavirus	Linear dsRNA	~ 14 000 – 17 600 bp
Totiviridae	Giardiavirus	Linear dsRNA	~ 6 200 bp
	Leishmaniavirus	Linear dsRNA	~ 5 300 bp
	Totivirus	Linear dsRNA	~ 4 600 – 6 700 bp
	Victorivirus	Linear dsRNA	~ 4 600 – 6 700 bp

Table compiled from the online databases. Universal Database for the International Committee on Taxonomy of Viruses (ICTV) (http://www.ncbi.nlm.nih.gov/ICTVdb/ICTVdB/) and the Viral zone database (http://www.expasy.org/viralzone)

Rotaviruses are further classified into several serogroups (A – H) (see ***Figure 2.3*** for phylogenetic relationship of serogroups). Group A rotaviruses is one of the economically more important rotavirus groups and can be found in humans, variouse mammal species as well as birds.

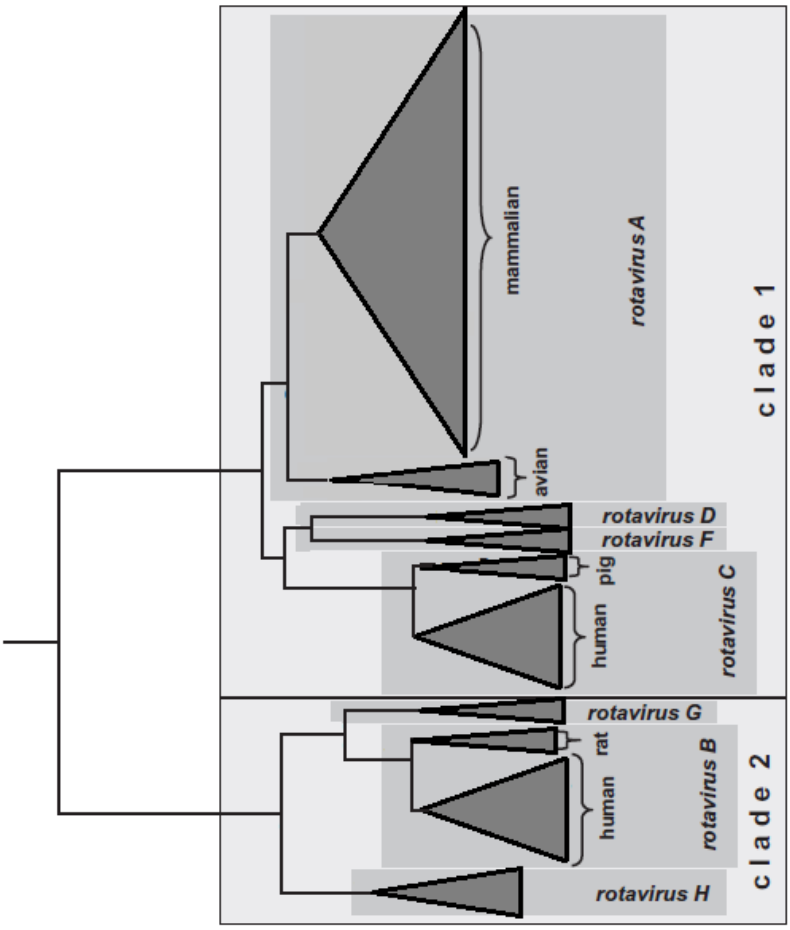

Figure 2.3: ***Phylogenetic relationship of rotavirus serogroups A – H based on all 11 genome segments.***

Type A rotaviruses are further classified using a binary system, G- and P-types, which refer to glycoprotein VP7 encoded by genome segment 7, 8 or 9 (depending on strain) and the protease sensitive VP4, encoded by genome segment 4. The most prevalent rotavirus A strains found in humans are the genotypes G1, G2, G3, G4, G9 and G12 in combination with P[4], P[6] and P[8] (Heiman et al., 2008, Matthijnssens et al., 2010). Group A rotaviruses include the AU-1 (G3P[8]),

DS-1 (G2P[4]) and Wa (G1P[8]) genogroups. The AU-1 genogroup is uncommon globally, in contrast to the DS-1-like and Wa-like genogroups that occur widespread among humans and various animal species (Matthijnssens and van Ranst, 2012). Phylogenetic analysis of rotavirus whole genome sequences indicated that DS-1-like strains are descendant from bovine rotaviruses, while Wa-like strains share a common ancestor with porcine rotaviruses (Matthijnssens et al., 2008a). The DS-1 strain was isolated in Washington D.C. (USA) in 1976 in from a gastroenteritis patient (Kalica et al., 1981). This strain (G2P[4] serotype) has a short electropherotype and is the prototype of DS-1-like strains. The Wa strain was also one of the first rotaviruses to be successfully adapted to cultured cells (Wyatt et al., 1980). Although the fact that the Wa strain is one of the best-studied human rotaviruses, the Wa reference strain sequence is a composite sequence of various Wa variants largely isolated from infant stool samples determined using Sanger sequencing (Heiman et al., 2008). There are also no evolutionary studies done on the Wa strain in order to determine how serial passaging the original virus, isolated in 1974, in animal and cultured cells has effected its nucleotide and subsequent amino acid sequences. The need exists to determine the consensus sequence of rotavirus Wa strain derived from a single rotavirus Wa population using next generation sequencing technology.

Serogroups B and C of rotavirus are also prevalent in humans and animals but not as common. Group E rotaviruses are predominant in pigs, while groups D, F and G have been isolated in chickens and other birds (Pedley et al., 1986, Trojnar E et al., 2013). Group H rotaviruses can be found in humans and pigs (Matthijnssens et al., 2011).

Historically, rotaviruses were typically classified using the electropherotype or dual typing classification systems. Electropherotyping is used to classify rotaviruses based on their dsRNA migration patterns when separated by PAGE (Jiang, 1985). Due to their size difference, the 11 dsRNA genome segments of rotaviruses have unique migration patterns when ran on a PAGE gel. Although electropherotyping is still popular as a classification technique today, the technique has never been adopted as a taxonomical tool. The main reasons for this are the fact

that SDS-PAGE analysis cannot be utilized to identify point mutations, reassortments and/or recombinations (Estes and Kapikian, 2007).

The dual typing classification system were initially used to differentiate group A rotaviruses by serotyping based on the reactivity of their outer capsid proteins (VP4 and VP7) to neutralising antibodies (Offit and Blavat, 1986). As mentioned before, type A rotaviruses are further classified using a binary system, G- and P-types. glycoprotein VP7 encoded by genome segment 9 and the protease sensitive VP4, encoded by genome segment 4. Thus far, 37 P and 27 G genotypes have been identified (Matthijnssens et al. 2011, Matthijnssens and van Ranst, 2012, Trojnar et al., 2013,). Though the dual typing system is still widely used in epidemiological studies, its use is principally limited to classifying only rotavirus A strains. This classification system can also not be employed to examine recombination and reassortment events in all 11 rotavirus genome segments because it is restricted only to outer capsid encoding genome segments (genome segment 4 and 9) (Matthijnssens et al., 2008a).

Matthijnssens and co-workers suggested a novel classification system based on the whole genome sequence of all 11 rotavirus genome segments in order to obtain a more complete picture of rotavirus strain diversity (Matthijnssens et al., 2008b). Nowadays, whole genome characterization has become the sought after procedure for viral strain characterization as next generation sequencing technology becomes more widely available and affordable. Next generation sequencing is able to generate large amounts of data, making the assembly of a consensus sequence much more reliable. The easily accessible public sequence databases contain massive amounts of sequencing data, facilitating complex analysis and strain comparisons. Additionally, in 2009 Maes and co-workers proposed a classification system for group A rotaviruses that simplifies the identification of all 11 genome segments. They developed an easy to use, web-based tool, RotaC (http://rotac.regatools.be) taking into account all the new classification guidelines (Maes et al., 2009). Accordingly, the RotaC designation Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx (where x represents the number of the genotypes) representing VP7, VP4, VP6, VP1, VP2, VP3, NSP1, NSP2, NSP3, NSP4, and NSP5

encoding genome segments, is used to classify rotavirus strains. The letters (G-P-I-R-C-M-A-N-T-E-H) represent the description of the viral proteins, for instance, “G” for the glycosylated VP7 and “P” represents the protease sensitive VP4.

2.6 Replication cycle of rotavirus

The rotavirus conducts its life cycle primarily in the cytoplasm of mature epithelial cells of the small intestine (apex of villi), never moving to the nucleus like DNA viruses and some RNA viruses (Estes and Kapikian, 2007). A bovine rotavirus strain takes about 20 – 24 hours to complete a full replication cycle in human intestinal cells (McCrae and McCorquodale, 1987). However, most of the knowledge we have on the replication cycle of rotavirus is derived from research done in African green monkey kidney cultured cells (MA104). Contrary to human intestinal cells, rotavirus replication is rapid in MA104 cells, about 10 – 12 hours (McCrae and McCorquodale, 1987). This is an indication that the binding, entry, uncoating and viral release may significantly differ in various cell lines and different rotavirus strains. The lack of a selection-free (comprehensive) reverse genetics system for rotavirus is hampering our progress in understanding of this process. In the following discussion, the rotavirus replication cycle will be summarised from the known literature (see ***Figure 2.4*** for a schematic summary of the rotavirus replication cycle).

2.6.1 Rotavirus attachment to host cell

Rotavirus cell attachment is a complex, multistep process which requires several specific interactions between the virus and epithelial cell surface molecules (Estes and Kapikian, 2007, Lopez and Arias, 2004). The exact cellular receptors for rotavirus binding differ from strain to strain. Animal strains bind to sialic acid containing receptors (***Figure 2.4A***) to infect polarized cultured intestinal cells (Fiore et al., 1991), while all human rotaviruses instigate infection of polarized intestinal cells by a sialic acid independent mechanism (Ciarlet and Estes, 1999, Haselhorst et al., 2009). Originally, it was believed that cell receptors for rotavirus attachment were only found at mature enterocytes at the tips of villi (Bastardo and Holmes, 1980).

However, there is also evidence of the extra-intestinal spread of rotavirus, signifying that other host cells may also be vulnerable to rotavirus infection (Blutt et al., 2003). The capsid of the rotavirus' triple layer particle is composed of two proteins - the spike protein VP4 and outer capsid protein VP7. Although the exact attachment method is not well understood, it is clear that cell attachment cannot be achieved without these two proteins (Crawford et al., 1994). *In vitro*, VP4 is cleaved by the exogenous protease, trypsin (or trypsin-like proteases such as furin), into subunits VP5* and VP8* which is responsible for the attachment of the triple-layered particle onto the cell membrane and the subsequent penetration. Studies done in the MA104 cells found that rotavirus binding is most likely a two-step process. The first binding step is the rapid association of VP8* with a sialic acid containing receptor. This is followed by a second but slower binding step consisting of the association with VP5* to heat shock protein 70 and binding of VP7 to one or more of the following receptors: N-linked glycoproteins, gangliosides and/or proteins associated in so called lipid rafts (***Figure 2.4B***) (Coulson et al., 1997, Guerrero et al., 2000, Mendez et al., 1999). There is also strong evidence that α2β1, αxβ2, and α4β1 integrins and heat-shock protein 70 may also act as stage 2 receptors (Coulson et al., 1997, Hewish et al., 2000, Lopez and Arias, 2004).

2.6.2 Rotavirus cell penetration and uncoating

It is believed that rotavirus cell entry is an intricate multistep process, primarily involving the spike protein VP4 and the outer capsid protein VP7 (Mendez et al., 1999). After rotavirus attachment, VP5* and VP7 binds to heat shock protein 70 and specific integrins depending on the cell type (Coulson et al., 1997, Hewish et al., 2000). Virus cell entry takes approximately 60-90 minutes and seems to penetrate the cell membrane directly after interaction with a plasma membrane receptor (Nandi et al., 1992). Uncoating of the spike protein and outer capsid is thought to take place in endocytic vesicles due to the low Ca^{2+} presence which promotes capsid solubilisation (***Figure 2.4C***) (Ruiz et al., 1997). These capsid fragments may also play a role in disrupting the endocytic vesicle's membrane, leading to the release of double layered particles (DLP) into the cytoplasm for viral mRNA transcription (Ruiz et al., 1997). During rotavirus

infection the host cells becomes suspended in the S phase during the cell cycle, delaying apoptosis during early infections (Bagchi et al., 2010, Frias et al., 2012).

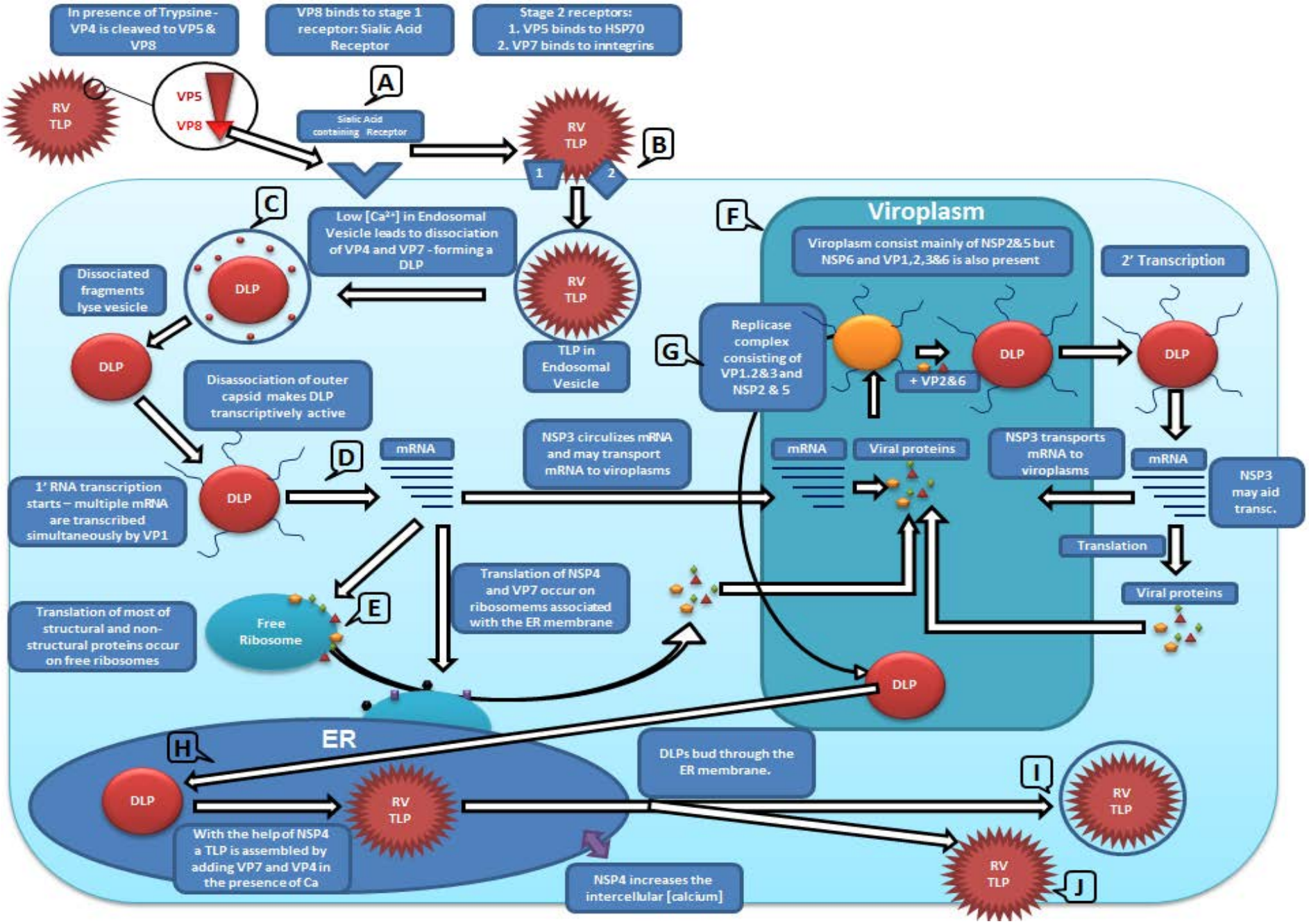
In presence of Trypsine - VP4 is cleaved to VP5 & VP8
VP8 binds to stage 1 receptor: Sialic Acid Receptor
Stage 2 receptors:
1. VP5 binds to HSP70
2. VP7 binds to inntegrins
RV TLP
VP5
VP8
A
Sialic Acid containing Receptor
RV TLP
1
2
B
C
Low [Ca²⁺] in Endosomal Vesicle leads to dissociation of VP4 and VP7 - forming a DLP
Dissociated fragments lyse vesicle
DLP
RV TLP
TLP in Endosomal Vesicle
F
Viroplasm
Viroplasm consist mainly of NSP2&5 but NSP6 and VP1,2,3&6 is also present
2' Transcription
G
Replicase complex consisting of VP1.2&3 and NSP2 & 5
+ VP2&6
DLP
DLP
Disassociation of outer capsid makes DLP transcriptively active
DLP
D
mRNA
NSP3 circulizes mRNA and may transport mRNA to viroplasms
mRNA
Viral proteins
NSP3 transports mRNA to viroplasms
mRNA
NSP3 may aid transc.
1' RNA transcription starts – multiple mRNA are transcribed simultaneously by VP1
Translation
Viral proteins
Translation of NSP4 and VP7 occur on ribosomems associated with the ER membrane
Translation of most of structural and non-structural proteins occur on free ribosomes
Free Ribosome
E
DLP
ER
H
DLP
RV TLP
With the help of NSP4 a TLP is assembled by adding VP7 and VP4 in the presence of Ca
DLPs bud through the ER membrane.
I
RV TLP
RV TLP
J
NSP4 increases the intercellular [calcium]

Figure 2.4: ***Schematic illustration of the replication cycle of rotavirus.*** *The replication cycle illustration is mainly based on studies conducted in MA104 cultured cells using the simian rotavirus, SA11. The rotavirus spike protein, VP4, is cleaved in the presence of trypsin to produce VP5* and VP8*. (**A,B**) VP8* attaches to specific receptors on the cell membrane.*

*The virus is transported to the endosome through endocytosis. (**C**) A reduction in the intracellular calcium concentration triggers the dissociation of the VP7 layer and the DLP is released into the cytoplasm. The polymerase complex (comprised of VP1 and VP3) is stimulated by the uncoating event. (**D**) Transcription of capped (+) RNAs from each of the 11 dsRNA genome segments is initiated. (**E**)The (+) RNAs can either function as mRNAs for viral protein synthesis by host cell ribosomes or as templates for synthesis of (–) RNA for genome replication. (**F**) Non-structural protein 2 (NSP2) and NSP5 interact to form the main structure of the viroplasms. Assembly and replication is thought to take place in the viroplasms. (**G**) dsRNA is synthesised by VP1 within the inner VP2 core. VP6 then assembles onto the VP2 core to stabilise it and to form the DLP. NSP4 increases intracellular calcium and recruits VP4 and the DLP to the ER membrane. (**H**) The NSP4-VP4-DLP complex then buds into the ER. (**I**) The ER membrane is detached followed by the attachment of VP7 to the DLP to form a TLP. (**J**) The mature virus is either released through exocytosis or cell lysis.*

2.6.3 Transcription of viral mRNA

All the enzymes required for transcription and replication are provided by the virus. The exact mechanisms which activate transcription are unknown. However, the low Ca^{2+} concentration in the endocytic vesicles may activate the viral polymerase (Ruiz et al., 2000). DLPs seem to be the transcriptively active particle and transcription takes place within this subdivision of the virus. It is speculated that the initial transcription phase takes place within the cytoplasm (***Figure 2.4D***) (Estes and Kapikian, 2007, Lawton et al., 1997). All transcripts are full length capped, non-polyA-tailed positive strands of positive polarity, encoded by the negative strand of dsRNA genome(Guglielmi et al., 2010, Patton et al., 2004). The viral transcripts are synthesized by the viral RNA-dependant RNA polymerase, a component of the core particle's VP1 protein. Each VP1 complex is responsible for the transcription of a single gene segment in symmetry with the other 11 genome segments (McDonald and Patton, 2011). VP1 and VP3 together form a complex which is attached to the inner capsid protein VP2. These three proteins (VP1, VP2 and VP3) make up the active replication core (McDonald and Patton, 2011). It is thought that genome segment transcription takes place within this virion in close approximation to VP1 and VP3. The VP2 inner capsid contains type 1 channels through which synthesised mRNA can exit the transcription complex (Lawton et al., 2000). VP3 is responsible for capping mRNA and has both guanylyltransferase and methyltransferase activities. The plus sense ssRNA transcripts act as mRNA for viral protein translation and templates for dsRNA synthesis (Chen et al., 1994). The synthesis tempo of transcripts seems to be size dependent, with the smaller transcripts being transcribed more rapidly and in greater amounts (Ayala-Breton et al., 2009b). Rotavirus mRNA is present in the cytoplasm 1 hour post infection and can be utilized in the translation process (Patton et al., 2004). Rotavirus counters innate immune response of the host cell by utilizing NSP1. This non-structural protein is able to degrade various inteferon transcription factors and by down regulating the RNA sensitive retinoic acid-induced gene (RIG-I) (Barro and Patton, 2007, Qin et al., 2011).

2.6.4 Translation of viral mRNA

Translation of capped viral mRNA that accumulated in the cytosol is facilitated by cellular systems and it is thought that most of the structural- and non-structural proteins are synthesized on the free ribozymes (***Figure 2.4E***). On the other hand, the VP7 and NSP4 are translated on ribosomes associated with ER membranes (Estes and Kapikian, 2007). All 11 rotavirus plus strand RNAs vary in size from 0.7 to 3.1 kb and contain a single open reading frame except for genome segment 11, which encodes for two proteins (NSP5/6). Capped, positive stranded RNAs lacking 3'-poly(A) tails are translated to either one of the six structural proteins or six non-structural proteins (Silvestri et al., 2004). The 3' consensus sequence of cytosolic plus strand ssRNAs seems to be particularly important and is recognised by NSP3 (Poncet et al., 1994). The non-structural protein NSP3 is thought to play an important role in the translation of capped viral mRNA. The N-terminus of NSP3 interacts with viral mRNA's 3'-consensus sequence while NSP3's C-terminus interacts with cellular eIF4G, severely hindering the translation of host cell mRNA. It is widely believed that this ability of NSP3 to circularize mRNA enhances the translation of the viral genome (Deo et al., 2002, Groft and Burley, 2002, Piron et al., 1998, Poncet et al., 1993, Poncet et al., 1994, Vende et al., 2000). Contrary to this notion, Montero and co-workers showed that NSP3 is not required for viral protein synthesis. In their experiment, NSP3 was knocked down using RNAi which resulted in normal viral protein synthesis and uninterrupted cellular protein translation (Montero et al., 2006). NSP3 may not be as important for viral translation as initially thought and may play a more significant role in impairing cellular protein synthesis. The 3' consensus sequence of plus strand ssRNAs is also essential for polymerase recognition (Lu et al., 2008). Core associated VP1s probably facilitate incorporation of plus strand ssRNAs into the core particle (Guglielmi et al., 2010). It has been hypothesized that following VP1 activation, the 3' consensus sequence functions as a possible initiator of minus-strand ssRNA synthesis within the core particle (Wentz et al., 1996). The synthesis of minus-sense strand ssRNA and genome replication must take place in a protected and secluded environment. Rotavirus morphogenesis takes place in electron-dense, membrane-free, cytoplasmic inclusions bodies, better known as viroplasms (***Figure 2.4F***). These inclusion bodies can be detected 3-4 hours post-infection and NSP2, NSP5 and VP2 are

the main actors in the formation of viroplasms and have been shown to form empty viroplasm if co-expressed (Fabbretti et al., 1999, Wentz et al., 1996). Lipid droplets are also recruited by nascent viroplasms and may be an integral part of these structures (Cheung et al., 2010). NSP2 appears to serve as the framework for the replicase complex while it is speculated that NSP5 may act as facilitator between NSP2, VP1 and VP3 (Berois et al., 2003, Jayaram et al., 2004). According to another model, assembly of DLPs take place within the viroplasms. The so called pre-core (consisting of VP1, VP3, NSP3, NSP5 and dsRNA genome) will unite with the inner capsid protein, VP2, and the middle capsid protein VP6 (Gallegos and Patton, 1989). An elegant study by Eichwald and co-workers found that the cellular microtubule network plays an important role in the fusion of viroplasms to the perinuclear area and that rotavirus infection acetylates tubulin to induce microtubule network stabilisation (Eichwald et al., 2012). This strategy, to subvert the microtubule transport network of the host cell in order to enable viral replication and to facilitate their invasion into other adjacent cells, can be observed in other viruses as is the case of adenoviruses (Radtke et al., 2006), herpes viruses (Douglas et al., 2004) and African swine fever virus (Alonso et al., 2001). The exact spectrum of influence and function of these inclusion bodies are still not known but these viroplasms seem to be central to rotavirus replication.

2.6.5 Genomic RNA Replication and Packaging

RNA replication and packaging of viral genome segments into newly synthesized core particles take place simultaneously in viroplasms (Patton and Gallegos, 1990). dsRNA is believed to be synthesized within a replicase complex consisting of structural proteins VP1, VP2 and small amounts of VP6 (***Figure 2.4G***). NSP1, NSP2, NSP3 and NSP5 are also implicated in this process (Gallegos and Patton, 1989). Genomic dsRNA is observed 2–4 hours post infection in equimolar amounts, despite large size variations, suggesting that this is a highly regulated process (Ayala-Breton et al., 2009b, Patton et al., 2004). Synthesized mRNA complexes can exit the viroplasm for translation in the cytoplasm. The exact RNA encapsulation mechanism is not known yet and there are three main hypotheses:

A pre-core replication complex consisting of VP1, VP2, VP3 and mRNA is formed. This complex may serve as a nucleation site for virion assembly where the 11 segments of rotavirus positive RNA associates with the polymerase complexes (VP1 and VP3) possibly assisted by NSP2 and/or NSP5. A VP2 shell may then amass around the assorted complex to form the core particle (Crawford et al., 1994, Labbe et al., 1991). A second model suggests that empty cores are formed first and that mRNA only associates with this complex later on (Patton and Spencer, 2000). In the core-filling model, the inner shell protein (VP2) is assembled containing the polymerase complexes VP1 and VP3, but lacking nucleic acid. The 11 positive RNA strands are then individually inserted into the core, perhaps aided by NSP2 and/or NSP5 of the viroplasm. When core packaging is finished, the particle is transported out of the viroplasm (McDonald and Patton, 2011). The third model proposes that NSP2 and NSP5 associate with cellular lipid droplets to form a viroplasm-like structure (Cheung et al., 2010). These structures may then act as a platform for the replication complex (VP1, VP3 and single stranded positive RNA) as well as VP2 and VP6. Viroplasm fusion then takes place and DLPs are assembled within this inclusion body (Lawton et al., 2000).

2.6.6 Rotavirus Assembly

A unique feature of rotavirus's life cycle, compared to other viruses in the *Reoviridae* family, is the transportation of the DLP from the viroplasm to the ER by membrane budding (***Figure 2.4H***) (Trask *et al*., 2012). In the ER, the DLP loses the budding envelope and requires a new layer of protein, ultimately forming the mature TLP by assembling VP4 and VP7 to the DLP. NSP4 is crucial for the budding of the subviral DLP into the ER (Au et al. 1993). NSP4 is also a calcium agonist and mobilises the intracellular calcium for effective budding of DLPs during infection (Morris and Estes, 2001, Tian et al., 1994). This non-structural protein consists of three domains, H1, H2 and H3 (Bergmann et al., 1989). The H1 domain is found in the ER lumen where it forms intra-molecular disulphide bonds (Bergmann et al., 1989). The H2 domain anchors NSP4 in the lipid bi-layer of the ER. The C-terminal H3 domain of NSP4 associates with the rotavirus DLP and mediates the transportation of this particle over the ER membrane by acting as an intercellular receptor (Taylor et al., 1993). This interaction of the DLP with NSP4

tetramers is able to distort the ER membrane, consequently allowing the DLP/VP4 complex to "bud" into the ER. After budding, VP4 and VP7 assembled onto the DLP to form the mature, triple layered virus particle (***Figure 2.4I***) (Hyser et al., 2012, Trask and Dormitzer, 2006). Interestingly, silencing NSP4 had profound consequences on rotavirus protein localization and replication in infected cells (Lopez et al., 2005a, Silvestri et al., 2005).

2.6.7 Virus release

Although the exact mechanism of virus release remains unclear, it is known that the bulk of rotaviruses are released by host cell lysis (***Figure 2.4J***) (Altenburg et al., 1980, Estes and Kapikian, 2007)**.** Evidence also suggests that VP4 may act as a remodelling agent by destabilizing the brush border membrane, allowing rotavirus to exit (Gardet et al., 2006). The secretion of rotavirus SA11 from kidney epithelial cells has also been described (Musalem and Espejo, 1985).

Certain key aspects regarding rotavirus replication are still not completely understood. For example, the mechanism in the ER responsible for the addition of VP4 is not known. The transport mechanism for ssRNA and viral proteins into the viroplasm is also not completely understood. The exact factors of the innate immune response being suppressed by NSP1 must still be identified and it is not known if there are any additional viral suppression mechanisms. The development of a comprehensive rotavirus reverse genetics system will greatly aid with the illumination of some of these elusive replication cycle questions.

2.7 Rotavirus pathogenesis

Our knowledge of rotavirus pathophysiology is largely based on studies done on animal models. Rotaviruses infect and complete their replication cycles in non-dividing, mature enterocytes of the villi. Rotavirus infection results in diarrhoea by modifying the function of the small intestinal epithelium.

NSP4 seems to be one of the main role players in inducing diarrhoea in rotavirus infections. During infection, NSP4 is able to mobilise the intracellular calcium from the ER (Morris and Estes, 2001). This escalation in the calcium concentration leads to the disruption of the cytoskeletal network of microvilli, decline of digestive enzyme expression on the epithelial surface and consequently necrosis (Ramig, 2004). These cellular processes, triggered by NSP4, contribute to the malabsorption associated with diarrhoea. It has also been shown that rotavirus infection increases plasma membrane permeability in enterocytes due to an increase in sodium and a decrease potassium (del Castillo et al., 1991). This may also contribute to malabsorption. NSP4, as an enterotoxin, could also induce diarrhoea by manipulation of the enteric nervous system (Ball et al., 1996, Lundgren et al., 2000). Virus-induced ischemia (intestinal damage resulting from a virus infection) may be an alternative mechanism. Viral infections weaken villi and negatively affect the absorptive ability of enterocytes resulting in osmotic diarrhoea (Osborne et al. 1991).

2.8 Immune response to rotavirus infections

The specific mechanisms responsible for rotavirus immunity in infected hosts are poorly understood. However, it is clear that a rotavirus infection elicits the acquired- (humoral), innate- (non-specific) and cellular immune response (Desselberger and Huppertz, 2011, Offit et al., 1993).

2.8.1 Acquired immunity

Although infant rotavirus infections typically provoke a homotypic humoral immune response, re-infections often elicit both homotypic and heterotypic antibody responses and are usually asymptomatic (Estes and Kapikian, 2007, Velazquez, 2009). Homotypic protection is thought to be determined by the level of mucosal IgA antibodies. Contrary, heterotypic protection seems to be reliant on the type of rotavirus strain and viral infection dose (Feng et al., 1994). It seems like both asymptomatic and symptomatic rotavirus infections leads to comparable degrees of protection (Velazquez, 2009). Intracellular rotaviruses are thought to be inactivated by IgA.

Several of the rotavirus proteins seem to be involved in the generation of antibodies (Svensson et al., 1987). The outer capsid proteins, VP4 and VP7, are both associated with heterotypic and homotypic immune responses (Yuan et al., 2004). VP6 prompts protective antibodies while the rotavirus non-structural protein NSP4 is coupled with the homotypic immune response (Zhou et al., 2011).

2.8.2 Innate immune response

Most viral infection leads to the activation of a complex cascade of host cell signalling pathways that are required for mounting an effective antiviral offensive. The innate immune system is the cell's first line of defence against viruses by suppressing viral replication mechanisms and precedes the adaptive immune response (Samuel, 2001). The secretion of cytokines belonging to the interferon (IFN) family (IFN type I and III in particular) play an important part in this innate immune response by activating the expression of IFN-stimulated genes (ISGs) (Randall and Goodbourn, 2008, Takeuchi and Akira, 2009). ISGs are able to directly inhibit viral replication, to induce an antiviral state, or annihilating the virus-infected cells before the infectious agent can complete its replication cycle (Edinger and Thompson, 2004, Levy et al., 2011, Stetson and Medzhitov, 2006). Viruses have adapted specific strategies to neutralize and exploit these immune responses to aid their own replication cycle. Rotavirus, in particular, nullifies the type I IFN response by utilizing its non-structural protein, NSP1, to degrade various IFN transcription factors and by down regulating the RNA sensitive retinoic acid-induced gene (RIG-I) (Barro and Patton, 2007, Qin et al., 2011).

After the initial viral infection, the IFN response can be triggered by the recognition of viral gene segments (this includes genomic dsRNA/DNA, mRNA and replication intermediates) or proteins by pattern recognition receptors (PRR) (see ***Figure 2.5*** for summary of the innate response to a rotavirus infection). PRRs are divided into two families: 1) the cytoplasmic pathogen detectors which include the RNA sensitive retinoic acid-induced gene (RIG-I) and melanoma differentiation associated gene 5 (MDA5) (***Figure 2.5*****A**), and 2) the trans-membrane toll-like receptors (TLR) (***Figure 2.5*****B**) (Akira et al., 2006, Jensen and Thomsen, 2012, Loo et al.,

2008). The recognition of viral genomic material by these PRRs, triggers a complex cascade of cellular events which finally concludes with the deployment of immune regulatory proteins. Some of the first regulatory factors to be activated by the PRR are the IFN regulatory factors (IRFs) and nuclear factor-κB (NF- κB). After the detection of ssRNA/dsRNA in the cytoplasm by RIG-I and/or MDA5, a signal is sent through an antiviral sensor protein embedded in the mitochondria (MAVS). MAVS plays a role in the activation of IRF3/7 and NF- κB which can subsequently initiate the transcription of type I IFNs (***Figure 2.5*C**).

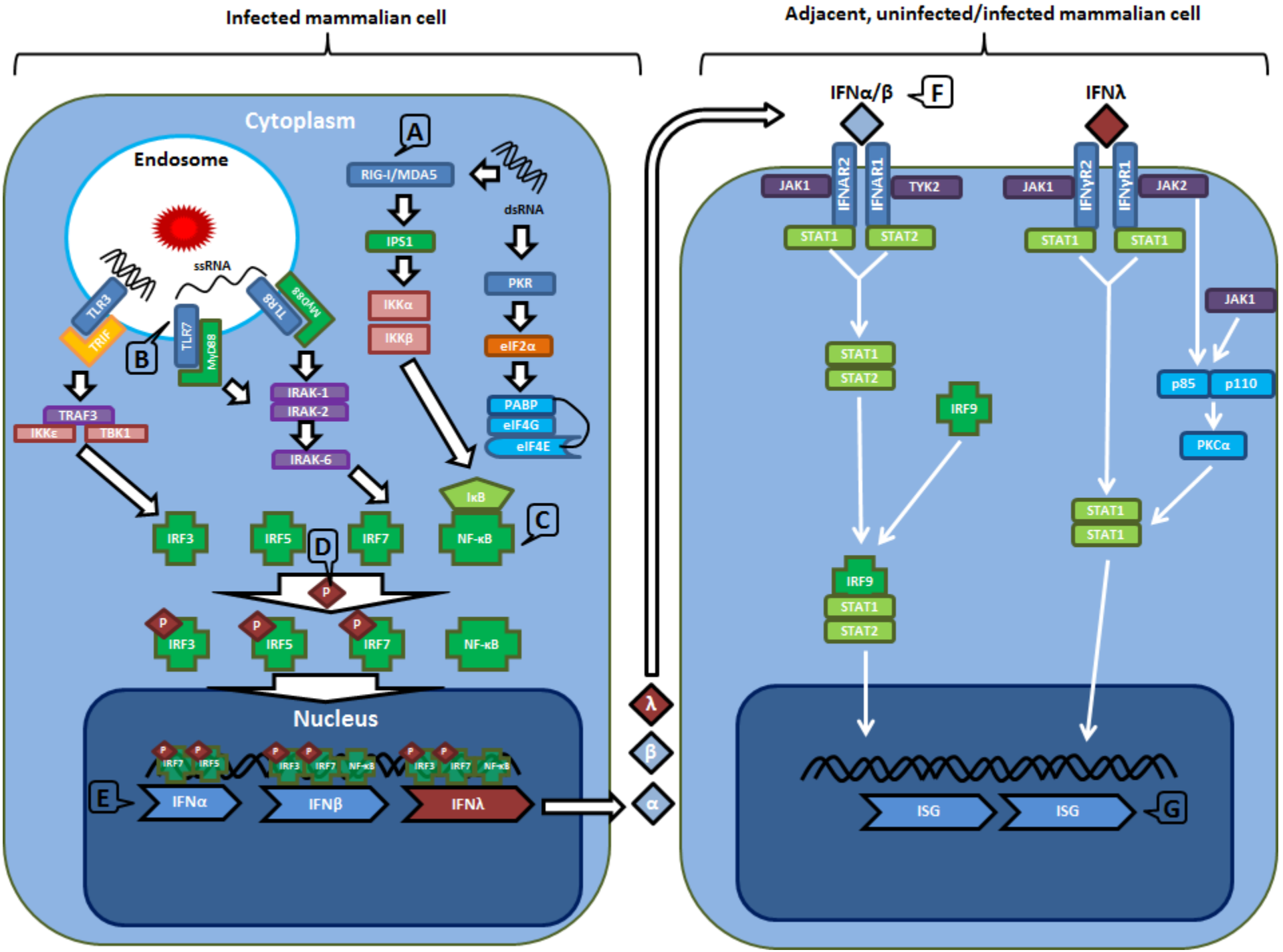
Infected mammalian cell
Adjacent, uninfected/infected mammalian cell
Cytoplasm
Endosome
ssRNA
TLR3
TRIF
TLR7
MyD88
TLR8
MyD88
TRAF3
IKKε
TBK1
IRAK-1
IRAK-2
IRAK-6
RIG-I/MDA5
IPS1
IKKα
IKKβ
dsRNA
PKR
eIF2α
PABP
eIF4G
eIF4E
IκB
NF-κB
IRF3
IRF5
IRF7
P
Nucleus
IFNα
IFNβ
IFNλ
A
B
C
D
E
F
G
IFNα/β
IFNλ
JAK1
TYK2
IFNAR2
IFNAR1
STAT1
STAT2
IRF9
IFNγR2
IFNγR1
JAK2
p85
p110
PKCα
ISG
λ
β
α

Figure 2.5: ***Schematic summary of the innate immune response of a mammalian host cell to a rotavirus infection.*** *After the initial viral infection, the IFN response can be triggered by the recognition of viral gene segments by (**A**) cytoplasmic pathogen detectors or (**B**) toll-like receptor (TLR)s. (**C**) After the detection of ssRNA/dsRNA in the cytoplasm by RIG-I and/or MDA5, IRF3/7 and NF- κB is activated which can subsequently initiate the transcription of type I IFNs. (**D**) TLRs can activate IRF3 and IRF7 by phosphorylation of the regulatory region C-terminal. (**E**) IRFs stimulate the expression of IFNs. The secretion of cytokines belonging to the IFN family, IFN type I and III in particular, play an important part in this innate immune response by activating the expression of IFN-stimulated genes. (**F**) Following the activation of IFNα/β/λ receptors, the creation of a heterotrimeric complex, IFN stimulated gene factor 3 (ISGF3) is prompt. (**G**) This complex is translocated to the nucleus and triggers the expression of ISGs. The expressed products of these ISGs are able to directly inhibit specific phases of the viral replication cycle. See text for details.*

The dsRNA genome segments of rotavirus range from 0.7 to 3.3 kb. RIG-I is best suited to recognize dsRNA between 21 bp to 1 kb, whereas MDA5 detects dsRNA segments longer than 1 kb (Kato et al., 2008). It seems feasible that both RIG-I and MDA5 will be able to recognize some rotavirus dsRNA genome segments. There are some controversy surrounding the presence of genomic rotavirus dsRNA in the cytoplasm due to the fact dsRNA is synthesized in double-layered particles situated inside viroplasms (Rojas et al., 2010, Trask et al., 2012). This means that dsRNA synthesis is supposed to be completely isolated from the host cell's PRR. The dsRNA detected in the cytoplasm may be ssRNA folding onto itself to form dsRNA-like secondary structures (Holloway and Coulson, 2013). Studies have reported that secondary structures of mRNA can also activate MDA5 (Li et al., 2010, Pichlmair et al., 2009). It has, however, been show that the delivery of dsRNA into embryonic mouse fibroblasts does indeed induce an IFN response (Sen et al., 2011). The rotavirus RNA-depentand RNA polymerase, VP1, produces dsRNA containing a 5' triphosphate moiety (Imai et al., 1983). This is common among viral polymerases and is a strong activator of RIG-I (Plumet et al., 2007). Rotavirus proteins are translated in the cytoplasm from plus-sense mRNA. These viral mRNAs are exposed to RIG-I and MDA5 in the cytoplasm. In order to shield the 5' triphosphate end from RIG-I, the methyl-guanylyl transferase protein, VP3, of rotavirus "caps" the plus-sense strand of dsRNA (Pizarro et al. 1991). The removal of the γ-phosphate is required for the formation of 5'-caps on negative sense ssRNA. γ-phosphate is universally absent in rotavirus negative sense ssRNA and NSP2 is the only rotavirus protein known to possess γ-phosphate removal activity (Hu et al., 2012). It is also interesting to note that NSP1 contains a RNA binding domain that recognizes the 5' end of rotavirus mRNA. This feature may also be used to mask the 5' triphosphate end of mRNA from RIG-I (Hua et al., 1994). In addition, it has been reported that NSP1 is able to bind to RIG-I in order to down regulate its activity (Qin et al., 2011).

On the other hand, foreign genomic material enclosed in endosomes can be detected by TLRs. After recognition of external genetic material, TLR3 signals by using the TIR-domain-containing adaptor-inducing IFNβ (TRIF), whereas TLR7 and TLR8 utilizes the myeloid differentiation primary response 88 (MyD88) (Honda and Taniguchi, 2006). IRF3 and IRF7 are two of the main regulators in the type I IFN expression and inactive forms of IRF3 and IRF7 are found in the cytoplasm under normal circumstances (Honda and Taniguchi, 2006).

IRF3 is constantly expressed in cells in relatively high concentrations while IRF7 is present in minute concentrations. IRF3 and IRF7 are activated by phosphorylation of the regulatory region C-terminal residues by the kinases IKKε or TBK1, forming homodimers or heterodimers which is transported to the nucleus (***Figure 2.5*D**) (Arnold and Patton, 2011). Both IRF3 and IRF7 activate IFN-α and IFN-β, but IRF7 has a wider ranging effect than IRF3 and promotes higher levels of IFN-α and IFN-β expression (***Figure 2.5*E**). Optimum type I IFN expression is only achieved with the combined effort of IRF3 and IRF7 (Arnold and Patton, 2011, Fitzgerald et al., 2003, Sato et al., 2000).

In contrast to IFN-α, IFN-β gene transcription is also dependant on the activation of NF-κB (Yeow et al., 2000). NF-κB is present in the cytoplasm and is dynamically held in an inactive form by κB inhibitors (IκB). In case of a viral infection, IκB is phosphorylated by IκB kinase with the substrate recognition protein β-transducin repeat containing protein (β-TrCP). IκB is degraded, thus lifting the inhibition of NF-κB and used to co-activate IFN-β transcription. NSP1 has also been shown to inhibit the expression of IFN-β by degrading β-TrCP, subsequently inhibiting the activation of NF-κB. This targeting of the NF-κB pathway by NSP1 has only been observed in the porcine rotavirus OSU (Graff et al., 2007).

Type I and III IFNs are released from the infected cell and binds to the surrounding uninfected cells using specific receptors (***Figure 2.5*F**). The IFNα and IFNβ receptors seem to be universally expressed on most cell surfaces, while IFNλ receptors are primarily found on epithelial cells (Donnelly and Kotenko, 2010). Following the activation of IFNα/β/λ receptors, the creation of a heterotrimeric complex, IFN stimulated gene factor 3 (ISGF3) is prompt. ISGF3 is comprised of the single transducer and activator of transcription 1 (STAT1), STAT2 and INF regulatory factor 9 (IRF9). This complex is translocated to the nucleus and triggers the expression of ISGs (***Figure 2.5*G**). The expressed products of these ISGs are able to directly inhibit specific phases of the viral replication cycle (Randall and Goodbourn, 2008). Apart from playing a crucial role in the innate immune response, ISGs also have a part in cell cycle regulation, initializing apoptosis and activating antigen-presenting dendritic cells in addition to T- and B-cells differentiation (Gonzalez-Navajas et al., 2012).

2.9 Rotavirus vaccines

The impact of viral diseases has been radically altered by the introduction of vaccines. The eradication of smallpox in the 1980s is just one example of the extremely positive impact vaccination programs can have against the burden of viral infections.

Before the introduction of vaccines, an estimated 1 billion diarrhoea cases were reported every year. Of these cases, approximately 2.4 – 5 million are fatal (Bryce et al., 2005, Lemaire et al., 2008). Rotaviruses are the leading aetiological agents of severe dehydrating diarrhoea globally. It is projected that every year rotavirus infections are linked to 111 million gastroenteritis episodes, of which approximately 25 million cases require medical attention and about 2 million people are hospitalised (Parashar et al., 2003). Rotavirus infections can have far reaching socio-economic impacts which can potentially be lightened by vaccination.

In the 1990s, RotaShield® was the first rotavirus vaccine (live-oral rotavirus vaccine) that was licensed worldwide (Hochwald and Kivela, 1999). The vaccine strain was a reassortant, engineered from the genetic rhesus rotavirus backbone and genome segment 9 (encoding for VP7) of DS-1, ST3 and D rotavirus strains. RotaShield® was removed from the vaccine market after the occurrence of a form of bowel obstruction, intussusception (1 out of 10 000 incident rate)(CDC, 1999, Abramson et al., 1999). It should be pointed out that no significant rotavirus-intussusception association have been identified and the pathogenic mechanism associated with intussusception is not known (Bines, 2005, Lynch et al., 2006).

At present, Rotarix™ (GlaxoSmithKline) and RotaTeq® (Merck) are the two licensed live-attenuated vaccines in use around the world. These two vaccines are now licensed in more than 100 countries globally. In 1998, the live-attenuated monovalent vaccine, Rotarix®, was developed by Ward and Bernstein (Bernstein et al., 1998). Rotarix® was previously known as *vaccine 89-12*, then RIX 4414 (De Vos et al., 2004), and is now marketed by the pharmaceutical giant GlaxoSmithKline (GSK). It was created by attenuating the human strain RVA/Human-wt/USA/89-12/1988/G1PA[8] through 33 passages in cultured primary African green monkey kidney cells and then several times in Vero cells (Bernstein et al., 1999). The

vaccine is administered to infants in 2 doses. The RotaTeq® vaccine is a bovine-human reassortant pentavalent vaccine. This vaccine contains five reassortant strains, each containing 10 genome segments of the bovine strain (RVA/Cow-tc/USA/WC3/1981/G6P[5]) and a single human genome segment that encodes for either G1, G2, G3, G4 or P1A[8] outer capsid protein (Heaton et al., 2005).

During large scale vaccine safety trials, high efficacies (>90%) in the prevention of severe dehydrating gastroenteritis were observed in most developed countries of North America and Europe and even in developing countries in South America for both vaccines (Ruiz-Palacios *et al*., 2006). Conversely, in most African countries the vaccine efficacies have been comparatively lower (Ruiz-Palacios et al., 2006, Vesikari et al., 2006). Despite the lower efficacies in African countries, the burden of disease is still significantly reduced by these vaccines (Madhi et al., 2010, Msimang et al., 2013). The need exits for vaccines that are specifically designed to contain region-specific strains. Rotavirus vaccine development is an on-going process with numerous clinical trials currently underway. Recently, one of the more unique therapies against rotavirus infection utilised transgenic rice that expressed the neutralizing domain of a rotavirus antibody fragment (Tokuhara et al., 2013). This transgenic product was shown to be stable at high temperatures and noticeably decreased the viral load in mice after oral administration (Mason et al., 2002). Taking into account the challenges facing the developing world, oral delivery of a rotavirus vaccine in a staple food, like rice or maize, may prove to be very useful in the development of future therapies against rotavirus infection.

2.10 Viral reverse genetic systems

Reverse genetics is a revolutionary molecular biology tool that enables the manipulation of specific viral genomes at the cDNA level in order to generate certain mutants or artificial viruses. This technology enables the generation of stable mutations in any viral genome. The effect of these induced mutations on the replication cycle or phenotype can then be studied in order to identify potential important molecular markers or to generate so-called rationally-designed vaccine strains. With the introduction of next generation sequencing, massive amounts of sequence data are generated. Reverse genetics endeavours to

associate a specific genetic sequence with a particular phenotype of an organism. The first successful reverse genetics system was developed by Goff and Berg for the rescue of a SV40 and λ-phage hybrid which was salvaged from cultured monkey kidney cells (Goff and Berg, 1976). Viable virus may be recovered from cDNA or infectious viral transcripts transfected directly into susceptible cultured cells. Since the development of the first reverse genetics systems, several important advances have been made. At present, reverse genetics systems exists for a variety mammalian RNA and DNA viruses, including influenza A viruses, flaviviruses, bornaviruses, paramyxoviruses and picornaviruses (Neumann et al., 1999, Perez et al., 2003, Racaniello and Baltimore, 1981, Yun et al., 2003). Developments in reverse genetics technology led to the generation of valuable information regarding the replication, pathogenesis and natural characterisation of these viruses.

The reverse genetics system for the influenza virus is arguably one of the best illustrations of the potential power of reverse genetics. In this system, ribonucleo-proteins and cDNA were transfected into cultured cells trailed by a helper influenza A virus infection (Luytjes et al., 1989). The function of the helper virus was to incorporate cDNA genome segments in order to create a recombinant virus. The reverse genetics system for influenza viruses used today has undergone multiple improvements and primarily makes use of recombinant cDNA plasmids (Neumann et al., 2012, Neumann et al., 1999). Originally, the influenza virus reverse genetics approach employed the transfection of 12 plasmids for the recovery of viable virus. Eventually the system was reduced to 8 plasmids and finally only 5 plasmids were needed to rescue infectious influenza virus (Hoffmann and Webster, 2000, Neumann et al., 2005). The influenza virus reverse genetics systems were cardinal in the development of influenza vaccines (Subbarao and Katz, 2004).

2.11 dsRNA and rotavirus reverse genetic systems

The first reverse genetics system for RNA viruses was developed for the positive-sense RNA virus, poliomyelitis virus (Racaniello and Baltimore, 1981). This system utilized the cDNA replica of whole RNA genome that was cloned into a vector. This recombinant vector was then transfected into mammalian cultured cells. After a few hours, infectious poliovirus

could be rescued from the cultured cells. Alternatively, transcripts generated from cDNA templates could also be used to generate infectious poliomyelitis virus (Kaplan et al., 1985).

Typically one of two main reverse genetic strategies is followed, namely a plasmid-based (cDNA) reverse genetics approach or a transcript-based (mRNA) reverse genetics strategy. In the plasmid-based reverse genetics approach the entire viral genome is synthesised as a cDNA copy and placed under the control of an upstream promoter sequence (cytomegalovirus IE or polymerase T7 promoters) and inserted into a suitable plasmid. This recombinant plasmid is then typically transfected into cultured mammalian cells that can support the replication of the specific virus. Viable viruses are then rescued from the cultured cells. Plasmid-based reverse genetic systems have been developed for many animal RNA viruses, including paramyxoviruses, bunyaviruses, coronaviruses, picornaviruses, bornaviruses, flaviviruses, orthoreoviruses, orthomyxoviruses, , and rhabdoviruses. A comprehensive plasmid-based reverse genetics system was described in 2007 for a member of the *Reoviridae* family, orthoreoviruses in 2007 (Kobayashi et al., 2007). Initially, cDNA copies of all 10 viral genome segments were placed in individual plasmids under the control of a T7 promoter and transfected into L929 cells. Viable orthoreoviruses could be rescued after 48 hours. An improved reverse genetic system was later introduced where multiple genome segments were placed into a single plasmid (four total plasmids) and transfected.

A transcript based approach to a reverse genetics system involves the generation of infectious virus particles in cultured cells by using transcriptively active plus sense ssRNA. Reovirus was the first orthoreovirus to be recovered from RNA by transfecting translated ssRNA of the ST3 strain (Roner et al., 1990). Other members of the *Reoviridae* family, including the bluetongue virus and the African horsesickness virus, were also successfully rescued from the complete set of viral mRNAs obtained from transcriptionally active viral cores or produced by *in vitro* transcription from cDNA (Boyce et al., 2008, Matsuo et al., 2010).

Unfortunately, extrapolating either the plasmid-based or transcript-based reverse genetics systems to rotavirus proved to be much more difficult. To date, no comprehensive reverse

genetic system exists for rotavirus. However, there are three selection dependant reverse genetics systems that have been developed for rotaviruses that are dependent on helper viruses. These systems permit the manipulation of only one of the eleven genome segments. The system developed by Komoto and colleagues were designed to manipulate genome segment 4 (VP4) (Komoto et al., 2006). This approach utilized plasmids containing the entire genome segment 4 sequence of rotavirus SA11 that were placed under control of a T7 promoter. This plasmid was transfected and 20 hours later the cells were infected with the "helper" rotavirus KU. After another 24 hours, the KU strain was suppressed and the recombinant virus containing VP4 of rotavirus SA11 were rescued.

In 2010, this reverse genetic system was modified by Troupin and co-workers to incorporate a recombinant rotavirus encoding for a rearranged genome segment 7 (NSP3) from *in vitro*-modified cDNA (Troupin et al., 2010). The plasmid was transfected into cultured cells followed by a helper bovine rotavirus RF strain infection. No selection pressures were applied resulting in the necessity of multiple serial passages in order to remove the helper bovine rotavirus (Kobayashi et al. 2010, Troupin et al., 2010).

As a creative alternative approach, Trask and co-workers made use of a temperature sensitive rotavirus mutant as a helper virus (Trask et al., 2010). A plasmid containing the entire cDNA genome segment 8 (NSP2) was placed under the control of a T7 promoter sequence and transfected into cultured cells. The transfection is followed by the infection of the temperature sensitive rotavirus mutant which is propagated at 30°C. After a specific time period, the incubation temperature is raised to 39°C to select for the recombinant viruses (Trask et al., 2010). The major drawback of this system is the lack of known temperature sensitive rotaviruses.

It is abundantly clear that the above discussed rotavirus reverse genetic systems are not comprehensive enough to allow for the manipulation of the entire rotavirus genome. There is a dire need of a comprehensive, flexible and helper-virus indepentant rotavirus reverse genetics system for manipulating of all eleven rotavirus genome segments. It is of great importance to select a suitable cell culture system for the standardisation of potential reverse genetic systems. The *in vitro* system must support good rotavirus replication and

expression as well as be able to be efficiently transfected by an effective transfection reagent. The successful co-expression influenza virus reverse genetics system was based on the successful co-expression of certain protein complexes from viral genomic RNA in the presence of other influenza viral proteins. Additionally, better knowledge of how specific cells react to rotaviruses (and rotavirus products such as transcripts and viral proteins) and how to manipulate these mechanisms may be of cardinal importance in implementing a comprehensive rotavirus reverse genetics system. A reverse genetics system will undeniably lead to the generation of valuable information regarding the replication, pathogenesis and natural characterisation of rotaviruses, paving the way to more effective vaccine development strategies.