# Chapter 1

# Introduction

## 1.0 Background

Classical virology techniques include virus culture, electron microscopy and various forms of immunoassays. These techniques are significantly complemented with recombinant DNA technology which was developed three decades ago. Recombinant DNA technology led to the development of a branch of genetics known as reverse genetics. This technology is a powerful cutting-edge technology in modern molecular virology. Classical genetics involves the identification of mutant phenotypes followed by mapping and sequencing of the responsible gene(s). On the other hand, reverse genetics involves the inference of function or phenotype from known and/or manipulated gene sequences. Viral reverse genetics enables the recovery of viruses with defined genomes as well as the manipulation of the viral genomes at cDNA level to generate mutants and "designer" viruses (Wimmer and Paul, 2011, Pekosz *et al*., 1999). Reverse genetics systems have been developed for various mammalian DNA and RNA viruses such as bornaviruses, flaviviruses, paramyxoviruses and picornaviruses (Neumann *et al*., 1999, Yun *et al*., 2003, Perez *et al*., 2003, Racaniello and Baltimore, 1981a, Racaniello and Baltimore, 1981b, Fodor *et al*., 1999). For these and other viruses, reverse genetics technology has led to the generation of valuable information regarding their replication, the study of attenuation markers, biological characterisation and pathogenesis.

The power of reverse genetics is excellently exemplified with the influenza virus which is an important human and animal pathogen. Using reverse genetics, the aetiological agent of the 1918–1919 Spanish influenza pandemic, which killed an estimated 50 million people, was resurrected from sequence fragments (Taubenberger *et al*., 2001, Tumpey *et al*., 2005a, Tumpey *et al*., 2005b, Tumpey *et al*., 2004). The sequences were generated by PCR using overlapping deoxyoligonucleotides corresponding to the published sequence of the 1918 influenza strain (Tumpey *et al*., 2005b). Initially, a recombinant influenza virus containing only the haemagglutinin and neuraminidase-encoding genome segments

from the 1918–1919 influenza virus was generated to demonstrate lethal virulence of the virus in mice (Tumpey *et al*., 2004). The recovery of the whole virus under high-containment biosafety level 3 conditions enabled studies which showed that the virus caused death in mice and embryonated eggs (Tumpey *et al*., 2005a). The rescued virus was also shown to replicate in the absence of trypsin and displayed a high growth phenotype in human bronchial epithelial cells (Tumpey *et al*., 2005a). Furthermore, a recombinant influenza H5N1 vaccine which was generated through reverse genetics was licensed by the American Food and Drug Administration (Tambyah, 2008). However, a reverse genetics system which utilises the rotavirus whole genome has not been developed yet.

*Rotavirus*, a member of the *Reoviridae* family, has a triple-layered icosahedral structure and measures about 75 nm in diameter (Bishop *et al*., 1973). Deaths due to rotavirus infections are currently estimated at 453 000 per annum (Tate *et al*., 2012). More than 85% of these deaths occur in developing countries in Africa and Asia (Patel *et al*., 2009, Tate *et al.*, 2012). The rotavirus genome consists of 11 double-stranded RNA (dsRNA) segments which encode six structural proteins (VP: VP1–4, VP6 and VP7) and five or six non-structural (NS) proteins (NSP: NSP1–NSP5/6) (Estes and Cohen, 1989). In some strains genome segment 11 is bicistronic and encode NSP6 in addition to NSP5 (Mitchell and Both, 1988). The inner capsid particle is made up of 120 copies of VP2 which enclose the dsRNA genome segments. The dsRNA genome associates with the enzymes VP1 (RNA-dependent RNA polymerase) and VP3 (guanylyl-methyl transferase) to form polymerase complexes in conjunction with VP2. The middle layer is composed of 780 molecules of VP6. Externally, 60 trimers of VP4 spikes and 260 trimers of VP7 form the outer capsid (Mathieu *et al*., 2001, McClain *et al*., 2010).

## 1.1 Problem identification

Several aspects of rotavirus biology are not well understood. Therefore, there are critical knowledge gaps and several contradictions in the literature. For instance, the exact order of events in genome replication and packaging is currently not known (Trask *et al*., 2012b, Jayaram *et al*., 2004, McDonald and Patton, 2011, Silvestri *et al.*, 2004). Intra-cytoplasmic electron dense inclusion bodies also referred to as

viroplasms are formed by NSP2 and NSP5 (Fabbretti *et al*., 1999). The co-localisation of viroplasms, lipid droplet (LD) lipids and LD-associated proteins appears to be required for the formation of functional viroplams (Cheung *et al*., 2010). Viroplasms contain VP1, VP2, VP3, VP6, NSP4 and mRNA but the precise molecular interactions and control mechanisms that direct minus-strand synthesis, assortment and packaging of dsRNA segments into viral cores are not known (Silvestri *et al*., 2004, Desselberger *et al*., 2009). Since rotavirus mRNA serves a dual role as templates for protein synthesis and minus-strand synthesis, no mechanism of how the transcripts localise in the viroplasm has been identified (Silvestri *et al*., 2004, Patton *et al*., 2006, Mitzel *et al*., 2003). Previously, it was thought that double-layered particles (DLPs) localise to viroplasms to generate transcripts that are packaged into newly made particles. However, a recent report shows that *in vitro* labelled virus particles do not co-localise with newly formed viroplasms, suggesting that they are not involved in viroplasm nucleation (Carreno-Torres *et al*., 2010). Understanding viral replication mechanisms can help in the discovery of inhibitors of the replication cycle which can be used for therapeutic purposes. There is currently no drug therapy for rotavirus infection. The only therapy is oral, subcutaneous or intravenous rehydration in severe cases of dehydration (Desselberger, 1999). However, recent evidence has shown that probiotics reduce the duration of rotavirus diarrhoea (Grandy *et al.*, 2010).

The mechanisms of VP4 cleavage and rearrangement during cell entry have been described recently (Yoder *et al*., 2009, Kim *et al*., 2010a, Trask *et al*., 2010a). However, pathogenesis and mechanisms of infectivity are not precisely clear. The role of VP7 in early interactions with the host cell is not well characterised, although it was postulated that it may complement VP4 in interacting with the cell membrane (Zarate *et al*., 2004). The innate immune response, which is characterised by the induction of interferon through several sensors of pathogen associated molecular patterns, is the first line of defence against rotaviruses (Lopez and Arias, 2012, Bowie and Unterholzner, 2008, Koyama *et al*., 2008). VP4 and VP7 elicit neutralising antibodies that correlate with protection from rotavirus disease (Desselberger and Huppertz, 2011). However, the exact determinants of immunological protection against rotavirus are also not well known. It is currently believed that intestinal IgA levels correlate best with protection against dehydrating rotavirus diarrhoea

(Molyneaux, 1995, Desselberger *et al*., 2009, Ward, 2009, Angel *et al*., 2012, Desselberger and Huppertz, 2011). The role of low level $CD4^+$ and $CD8^+$ T-cell responses is also not clearly understood (Angel et al., 2007, Angel et al., 2012, Desselberger and Huppertz, 2011). Immunization of mice with an NSP4-based vaccine and subsequent development of NSP4-specific antibodies attenuated symptoms of rotavirus induced diarrhoea (Hou *et al*., 2008). In humans, it is yet to be established whether NSP4 antibodies can exert a protective function. The precise role of the rotavirus specific cytotoxic T-cell response is not well understood (Desselberger and Huppertz, 2011). In humans, primary rotavirus infection induces systemic and intestinal antibodies that confer protection against severe diarrhoea upon re-infection (Davidson *et al*., 1983, Velázquez *et al*., 1996).

RotaShield®, the first licensed rotavirus vaccine, was withdrawn in 1999 following findings that it was associated with a higher than acceptable incidence of intussusception i.e. the bowel folding in on itself and causing obstruction (Abramson *et al*., 1999, Murphy *et al*., 2001). Currently, two rotavirus vaccines RotaTeq® (manufactured by Merck), and Rotarix™ (manufactured by GlaxoSmithKline) are licensed in many countries worldwide (WHO, 2007, Angel et al., 2007, SAGE, 2009b, SAGE, 2009c). However, global roll out has not yet been achieved. These vaccines prevent severity of subsequent rotavirus infection, but do not prevent follow-up infections. In the developed countries of Europe and the Americas these vaccines have a demonstrated efficacy of 85–98% (Vesikari *et al*., 2006b, Ruiz-Palacios *et al*., 2006, Patel *et al*., 2009). However, in trials conducted in developing countries such as South Africa and Malawi the combined efficacy of Rotarix™ was 61% (Madhi *et al*., 2010). In Malawi the efficacy was 49% and in South Africa it was 77% (Madhi *et al*., 2010). While this may save up to 1.5 million deaths from 2010 to 2025 (SAGE, 2009a), there is still a significant 40% of cases that would not be adequately protected by these two vaccines. The lower efficacy in African countries could be due to several factors including co-infections with pathogens such as infection with HIV, malaria parasites, mycobacteria, poor nutrition or that the vaccine strains do not adequately match the currently circulating strains in these geographical areas. A description of high vaccine failure against G1P[8] or G3[P8] strains in Nicaragua in partially or fully immunised (with RotaTeq®) children has been reported, suggesting that factors other than vaccine composition might impact

vaccine efficacy (Bucardo *et al*., 2012). In addition, vaccine manufacturing companies do not have the capacity to adequately fulfil global vaccine needs especially for developing countries and at affordable prices. Rationally designed vaccines, which target strains circulating in specific geographic areas, are required to complement the currently available vaccines.

For the reasons mentioned above, alternative technologies are required for understanding and unravelling the remaining questions in rotavirus replication. One such technology includes the development of a reverse genetics system. It would be possible to use reverse genetics technology to generate rotaviruses with stably introduced mutation(s) in any genome segment. The introduced mutations could be studied by investigating their effect on the replication cycle of the virus. The analysis of the replication cycle of mutants can be used to identify molecular targets that could be inhibited for the discovery and development of candidate anti-rotavirus therapies. Other biological studies that could be performed using a rotavirus reverse genetics system-generated mutant viruses include infecting animal models for further understanding of the host immune response as well as a complete elucidation of the immunological correlates of protection against rotaviruses. For instance, specific mutations could be introduced into VP4 or VP7, followed by analyses of the effect of the mutations on antigen processing and generation of neutralising antibodies. Such a system can also aid in the design and engineering of rationally designed vaccines for specific geographic locations. In addition, it will also be possible to rapidly generate rotaviruses expressing epitopes of emerging and potentially pathogenic rotavirus strains. Furthermore, a rotavirus reverse genetics system could be used to develop non-infectious vectors for the delivery of immunogenic or therapeutic molecules into cells. Therefore, this study sought to identify and investigate the factors that could hinder the establishment of a whole genome transcript-based reverse genetics system for rotaviruses.

## 1.2 Study motivation and rationale

Reverse genetics systems have been established for three mammalian segmented dsRNA members of the *Reoviridae* family, namely bluetongue virus (BTV), orthoreovirus and African horsesickness virus (AHSV). For BTV, *in vitro* synthesised

mRNA obtained from virus cores as well as T7 polymerase transcription of cDNA clones have been utilized to recover viable bluetongue virus (Boyce and Roy, 2007, Boyce *et al*., 2008). Furthermore, BTV has been reconstituted in a cell-free system consisting of core proteins and single-stranded RNA (Lourenco and Roy, 2011). Two recent field strains of BTV isolates (virulent BTV-8 and avirulent BTV-6) were recovered using reverse genetics based on transfecting *in vitro* synthesised transcripts from consensus sequences of the viruses (van Gennip *et al*., 2012). A plasmid-based reverse genetics system, requiring no helper virus or any selection steps, was also described for orthoreoviruses, another member of the *Reoviridae* family (Kobayashi *et al*., 2007). Efficiency of the orthoreovirus reverse genetics system was improved by cloning more than one genome segment into a single plasmid (Kobayashi *et al*., 2010). At 24 hours post infection, the titre of the virus recovered using four plasmids was 100-fold higher than that obtained for the virus recovered with 10 plasmids (Kobayashi *et al*., 2010). However, the orthoreovirus reverse genetics system is technically difficult. For instance, orthoreovirus may not be recovered due to lethal mutations, the quality of cDNA or inefficiency of the T7 polymerase (Boehme *et al.*, 2011). Lethal mutations are nucleotide changes that inhibit virus replication due to non-functional proteins or nucleotides (Boehme *et al*., 2011). Core-derived transcripts have also been used to recover AHSV in BHK-21 cells (Matsuo *et al*., 2010). In the AHSV reverse genetics system, it was possible to rescue reassortant AHSV by transfecting core-derived transcripts from two different AHSV serotypes (Matsuo *et al*., 2010). Furthermore, Matsuo and co-workers demonstrated that AHSV replicated in primary and secondary replication cycles of which the secondary replication cycle is the basis for genome packaging (Matsuo *et al*., 2010). However, a true plasmid-only reverse genetics system for AHSV is not available yet.

Despite the optimistic expectation that reverse genetics systems developed for orthoreoviruses and BTV could be quickly extrapolated to rotaviruses (Baric and Sims, 2007, Attoui *et al*., 2009), to date, attempts to develop a transcript- or plasmid-based reverse genetics system that does not require section or a helper virus have failed. The lack of a universal reverse genetics system for rotaviruses is thought to be a result of technical problems and a lack of proper understanding of the rotavirus replication cycle. Some of the apparent factors include the relatively low-titre

replication in cell culture characteristic of rotaviruses as well as the significant cytopathic effect of recombinant vaccinia virus which may be aggravated by trypsin in virus propagation medium (Trask *et al*., 2012a). However, strains such as rotavirus SA11 and OSU propagate to high titre in cell culture (Bohl *et al*., 1984, Londrigan *et al*., 2000). Infection with T7 polymerase-encoding vaccinia virus is used to provide the T7 polymerase which transcribes the cDNA genome segments from a T7 promoter as well as capping of the transcripts (Fuerst *et al*., 1987, Nielsen and Shapiro, 1986). Nevertheless, the first single-genome segment reverse genetics system has been described for rotavirus (Komoto *et al*., 2006) making use of a T7 polymerase-expressing vaccinia virus. With this system, a recombinant rotavirus was generated that contains a genome segment 4 (VP4) from the rotavirus SA11 strain on a rotavirus KU strain genetic background. The rotavirus SA11 genome segment was delivered in a plasmid, under the control of a T7 promoter, in combination with a recombinant vaccinia virus infection to provide the T7 polymerase. The wild-type, helper rotavirus KU strain was inhibited with specific anti-VP4 antibodies. Although this method allowed the engineering of the first rotavirus reassortant and the rescue of a chimeric virus containing cross-reactive neutralizing epitopes of VP4 (Komoto *et al*., 2006, Komoto *et al*., 2008), the system is limited to the manipulation of only genome segment 4 (VP4). The need for a helper virus and antibody selection technically limits the system to rotavirus strains for which specific antibodies are available. Two other single-genome segment rotavirus reverse genetics systems have also been reported. One utilises temperature sensitivity in combination with RNA interference to manipulate genome segment 8 (NSP2) (Trask *et al*., 2010b). For manipulating genome segment 7 (NSP3), a system dependent on the preferential packaging of rotavirus genome segments with a rearranged untranslated region (UTR) after the open reading frame has been reported (Troupin *et al.*, 2010). Both systems are limited by the use of a helper virus and, subsequently, the need for the selection for the rotavirus containing the rescued genome segment. Although Troupin and co-workers have used the reverse genetics system they developed to show that genome segment rearrangement does not confer growth advantage, the usefulness of their system may be very limited (Troupin *et al.*, 2011, Trask *et al*., 2010b).

The successful incorporation and expression of genome segments provided in cDNA form into helper rotaviruses, as indicated above suggests that it should be possible to recover rotavirus with a true plasmid-only or transcript-based reverse genetics system. However, there are fundamental problems that need to be overcome for the development of a true, robust and generally useful rotavirus reverse genetics system (Trask *et al*., 2012a). Some of the problems include the lack of knowledge about rotavirus replication and how cells respond to rotavirus genetic material. First attempts to develop a reverse genetics system for the bluetongue virus serotype 8, based on published sequences, failed until the consensus sequence was used (van Gennip *et al*., 2012). Due to the occurrence of minor viral population variants, (Domingo *et al*., 2006), some of the variants could be genetically unfit and interfere with the recovery of viable virus by reverse genetics.

Therefore, for this PhD project, it was decided to investigate the application of rotavirus transcripts obtained from *in vitro* transcription and 5'-capping of the consensus sequence of the well-characterised and cell culture-adapted rotavirus DS-1 strain in a transcript-based selection-free reverse genetics system approach. The consensus sequence represents the most viable genome of the viral population variants (Domingo *et al*., 2006). Due to the ability of the rotavirus SA11 strain to replicate well in cell culture, transcripts obtained by *in vitro* transcription using rotavirus SA11 double-layered particles were also used. In addition, the innate immune response to rotavirus transcripts was investigated based on the rationale that potent immune responses could contribute towards the failure to recover viable rotavirus by reverse genetics. The characterisation of the cellular innate immune response included the determination of the rotavirus transcript sensor(s) as well as establishing the cytokines that are significantly induced in response to the transfected transcripts.

## 1.3 Hypothesis

Studying the effect of transfecting cells with rotavirus transcripts should make it possible to identify factors that are important for the development of a transcript-based, helper-free reverse genetics system for rotaviruses.

## 1.4 Aims

The main objective of the study was to investigate and characterise the factors that need to be overcome for the development of a rotavirus reverse genetics system using transcripts derived from the *in vitro* transcription of a consensus nucleotide sequence as well as from double-layered particles.

### 1.4.1 Specific objectives

**1.4.1.1** To determine and analyse the whole genome consensus nucleotide sequence of the prototype rotavirus DS-1 and SA11 strains using sequence-independent genome amplification and 454® pyrosequencing.

**1.4.1.2** To evaluate the effect of transfecting rotavirus transcripts which were obtained from *in vitro* transcription of the consensus whole genome cDNA of rotavirus DS-1 and double-layered particles of rotavirus SA11, in appropriate cells.

1**.4.1.3** To determine the cellular response to transfected rotavirus transcripts and characterise the innate immune responses against rotavirus transcripts in cell culture.

## 1.5 Scientific approach and methodology

### 1.5.1 Viruses and propagation in cell culture

The well characterised and cell culture-adapted prototype rotavirus DS-1 strain was selected for the determination of the whole genome consensus sequence. The rotavirus DS-1 strain was a kind gift from Dr. C. Kirkwood (Murdoch Children's Research Institute, Melbourne, Australia). The rotavirus SA11 strain, which replicates rapidly and to high titre in cell culture, was also selected for the generation of *in vitro* transcribed transcripts using double-layered particles. The rotavirus SA11 sample was kindly provided by Mrs I. Peenze (Diarrhoeal Pathogens Research Unit, University of Limpopo, Pretoria, South Africa). The two rotaviruses were propagated in rhesus monkey kidney cells (MA104) in Dulbecco's modified Eagle's medium (Hyclone) containing 1 µg/ml porcine trypsin IX (Sigma), 1%

penicillin/streptomycin/amphotericin (Lonza) and supplemented with 1% non-essential amino acids (Lonza) (Ward *et al.*, 1984, Londrigan *et al*., 2000, Benureau *et al*., 2005).

### 1.5.2 Whole genome amplification

dsRNA was extracted from cell culture-propagated rotavirus DS-1 and rotavirus SA11. The extracted genomes were ligated to an anchor primer (Potgieter *et al*., 2009) to facilitate sequence-independent whole genome amplification. Sequence-independent genome amplification allowed the determination of correct 5'- and 3'-terminal end sequences. The whole genome cDNA was amplified using a sequence-independent method (Potgieter *et al*., 2009, Maan *et al.*, 2007).

### 1.5.3 Determination of whole genome consensus sequence

Attempts to recover rotavirus by reverse genetics were based on the observation by Dr. P. van Rijn (Department of Virology, Central Veterinary Institute of Wageningen UR, Lelystad, The Netherlands), that a consensus nucleotide sequence was needed for the efficient rescue of viable RNA viruses using T7 polymerase-derived transcripts. Therefore, it was decided that a consensus sequence of the virus was needed. The consensus sequence of the DS-1 virus strain was determined using 454® pyrosequencing technology (Roche; Margulies *et al*., 2005) at Inqaba Biotec™ (Pretoria, South Africa). For rotavirus SA11, the consensus whole genome sequence was also determined with 454® pyrosequencing, to characterise the virus and to determine a baseline for sequence comparison should the virus be recovered using reverse genetics. Whole genome consensus sequence analyses were performed using Lasergene™ (DNASTAR®) and the consensus sequences were compared to rotavirus DS-1 and SA11 nucleotide sequences in GenBank.

### 1.5.4 *In vitro* transcription and transfection of cells

To facilitate *in vitro* transcription, rotavirus DS-1 genome segments were engineered to contain the T7 polymerase promoter sequence at the 5'-end, and an enzyme restriction site at the 3'-end. The transcription cassettes containing the synthetic genome segments were purchased from GenScript (USA). Restriction enzymes were used to digest the plasmids containing the DS-1 genome segment resulting in a

linear, *in vitro* transcription template. Transcription was performed, in combination with capping, using a mMESSAGE mMACHINE T7 Ultra kit (Ambion®). The capped transcripts representing the whole rotavirus DS-1 genome were purified and transfected into appropriate cells after inhibiting the dsRNA-dependent protein kinase system (PKR) with PKR inhibitors. To obtain rotavirus SA11 transcripts, the outer capsid was removed and DLPs were purified with caesium chloride gradient ultracentrifugation. Transcription with DLPs was performed in a transcription cocktail as described before (Patton *et al.*, 2002). Transfections were performed using Lipofectamine® 2000 (Invitrogen) according to the manufacturer's recommendations.

### 1.5.5 Characterisation of the innate immune response to rotavirus transcripts

Results obtained during this study suggested that the failure to recover rotavirus could be attributed to an innate immune response against the rotavirus transcripts. The innate immune response was thought to induce an antiviral state which could lead to the prevention of transcript translation by degradation and possible editing (Samuel, 2001). To study the innate immune response to rotavirus transcripts, human embryonic kidney cells (HEK 293H) were transfected with rotavirus transcripts. The stimulation of type I interferon (IFN-α; IFN-1β), type III interferon (IFN-λ1), inflammatory and anti-inflammatory cytokines were subsequently analysed with quantitative real time PCR. The HEK 293H cells were selected based on the commercial availability of probes/primers against human innate immune response genes. The induction of the DExD/H box helicases RIG-I and MDA5 was evaluated using western blot analyses.

## 1.6 Structure of thesis

This thesis is presented in seven chapters which are briefly described below. For the experimental chapters (chapters 3–6), each chapter contains an Introduction, Materials and Methods section, Results section, and a Discussion section.

**Chapter 1**: Presents an introduction and overview of the scientific approach and methodology.

**Chapter 2**: Describes a current and relevant review of rotavirus literature applicable to the study.

**Chapter 3**: Contains details of 454® pyrosequencing of the prototype rotavirus DS-1 strain. Based on the results in chapter 3, the following original article was published:

**Luwanika Mlera**, Khuzwayo C. Jere, Alberdina A. van Dijk and Hester G. O'Neill (2011). Determination of the whole genome consensus sequence of the prototype DS-1 rotavirus using sequence-independent genome amplification and 454® pyrosequencing. *Journal of Virological Methods,* 175(2): 266–271 (Appendix 3).

**Chapter 4**: Describes the characterisation of a rotavirus SA11 stored in South Africa using 454® pyrosequencing and molecular clock analyses. Results from this chapter were accepted for publication in the journal *Archives of Virology* with the following details:

**Luwanika Mlera**, Hester G. O'Neill, Khuzwayo C. Jere, and Alberdina A. van Dijk. Whole genome consensus sequence analyses of a South African rotavirus SA11 sample reveals a mixed infection with two close derivatives of the SA11-H96 strain (Accepted 18 October, 2012; Appendix 4).

**Chapter 5**: The attempts to recover viable rotavirus using transcript-based reverse genetics, and the observations that rotavirus transcripts induce a characteristic cell death pattern, are presented in this chapter.

**Chapter 6**: Presents details of the characterisation of the innate immune responses induced by rotavirus transcripts.

A manuscript describing data from chapters 5 and 6 is currently in preparation for publication in the *PLoS Pathogens* journal and will be submitted shortly.

**Chapter 7**: Conclusions drawn from this study and recommendations for future work are presented.

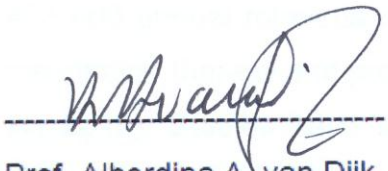
## 1.7 Publications authorship

For the article entitled *Determination of the whole genome consensus sequence of the prototype DS-1 rotavirus using sequence-independent genome amplification and 454® pyrosequencing*: Luwanika Mlera was involved in the study design, performed the laboratory experiments, data analysis and writing of the manuscript. Khuzwayo C. Jere was involved in data analysis and manuscript writing. Hester G. O'Neill and Alberdina A. van Dijk were involved in the study design, supervision and manuscript preparation.

Contributions for the manuscript entitled *Whole genome consensus sequence analyses of a South African rotavirus SA11 sample reveals a mixed infection with two close derivatives of the SA11-H96 strain* were as follows: Luwanika Mlera was involved in the study design, performing laboratory experiments, data analyses and writing of the manuscript. Khuzwayo C. Jere was involved in manuscript preparation. Hester G. O'Neill and Alberdina A. van Dijk were involved in the study design, supervision and manuscript writing.

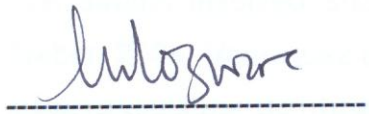
The articles in appendices 5–7 (Jere *et al*.) were not part of this PhD study. However, during the PhD studies Luwanika Mlera was involved in data analyses and writing of the manuscripts.

All co-authors in the articles and manuscript approve, indicated by their signing below, the use of the articles and manuscript in this thesis. They further declare that their contributions are as described herein.

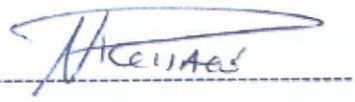


Prof. Alberdina A. van Dijk

Dr. Hester G. O'Neill



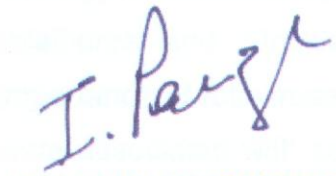
Dr. Khuzwayo C. Jere



Dr. Nicola A. Page

Dr. Abraham C. Potgieter

Dr. Mapaseka L. Seheri



Mrs Ina Peenze