CHAPTER 1: INTRODUCTION **1**

1.1 Background and problem identification

The fact that humanity can improve human society and natural reality by the vigilant application of scientific knowledge is at the core of human progress and a prosperous future. Today, the future has become something that can be positively directed through scientific principles and reason. This refreshing viewpoint was an extremely commanding notion that transformed the Western world. Scientific discoveries/developments, like vaccines, and the application thereof have improved the lives of millions of people around the world. Basic research is of cardinal importance so that we may be able to better understand the world around us and, ultimately, to use that knowledge to better society.

Rotavirus infections are common among animals and humans (Estes and Kapikian, 2007). Since a link between rotavirus and gastroenteritis was established 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 (Sanchez-Padilla et al., 2009). 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.

Rotaviruses are classified under the *Reoviridae* family and are further classified into several serogroups (A – H). The modern classification system used for rotaviruses is based on the whole genome sequence of all 11 rotavirus genome segments in order to obtain a more complete picture of rotavirus strain constellations which, in turn, inform on strain diversity and evolutionary mechanisms employed (Matthijnssens et al., 2008, Matthijnssens et al., 2012). One of the prototype rotavirus strains, rotavirus Wa, was 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 current Wa reference strain sequence is a composite sequence of various Wa variants 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 predicted amino acid sequences. The need exists to determine the consensus sequence of rotavirus Wa strain derived from a single rotavirus Wa population. Today, whole genome characterization has become the gold standard for viral strain characterization as next generation sequencing technology has become more widely available and affordable.

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 (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. The reverse genetics system for the influenza virus is arguably one of the best illustrations of the potential power of reverse genetics. The system was based on the co-expression of ribonucleoprotein complexes from viral genomic RNA in the presence of other influenza viral proteins (Luytjes et al., 1989). This reverse genetics system was central in the development of influenza vaccines (Neumann et al., 2012, Subbarao and Katz, 2004). A selection-free plasmid-based reverse genetics system was described in 2007 for a member of the *Reoviridae* family, orthoreovirus (Kobayashi et al., 2007). Viable viruses were also successfully recovered using double-layered particle (DLP) derived transcripts in the bluetongue virus and African horsesickness virus reverse genetics systems (Boyce et al., 2008, Boyce and Roy, 2007, Matsuo et al., 2010). Unfortunately, extrapolating either the plasmid-based or transcript-based reverse genetics systems to rotavirus proved to be much more difficult than expected. No reverse genetics system (comprehensive reverse genetics system) exists to manipulate all eleven genome segments of rotavirus. There is a profound need for such a reverse genetics system for rotavirus to allow the manipulation of all eleven rotavirus genome segments.

In the quest for a rotavirus reverse genetics system it will be of great importance to select a suitable cell culture system. Practical problems include that tissue cultures in which rotavirus grows well (MA104 cells) are very difficult to efficiently transfect. On the other hand, cells that are easily transfected do not necessarily support rotavirus replication. The *in vitro* system must support good rotavirus replication which is based on rotavirus protein

expression. Although MA104 cells support good propagation of most group A rotaviruses, this cell line is notoriously difficult to transfect with either plasmids or transcripts. Additionally, it has been observed that rotavirus transcripts are neither translated nor infectious when transfected on their own (Richards et al., 2013). As an alternative reverse genetics approach, the rotavirus core-filling model proposes that rotavirus transcripts are recruited into the existing cores replication complexes consisting of VP1, VP2, VP3 and VP6 (McDonald and Patton, 2011). Little experimental evidence exists to support the core-filling model and should be investigated as a possible reverse genetics approach.

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 critical in the development of a comprehensive rotavirus reverse genetics system. To date, studies in cell culture involving rotavirus transcripts have been hampered by the aggressive innate immune response they elicit (Mlera, 2013, Uzri and Greenberg, 2013). In contrast to rotavirus infections, the innate immune response to rotavirus transcripts is poorly understood. A reverse genetics system will undeniably lead to the generation of valuable information regarding the natural characterisation, replication, pathogenesis and innate immune responses of rotaviruses, amongst many other applications of a rotavirus reverse genetics system.

1.2 Hypothesis

The following hypothesis was the basis for the work presented in this study:
It will be possible to develop a rotavirus reverse genetics system if a cell line, which the progeny rotavirus grows well in, can be efficiently transfected under conditions where the innate immune response can be shut-off or sufficiently suppressed.

1.3 Aims and objectives

1.3.1 Aim of this study

1 The main aim of the study was to investigate the importance of co-expressed rotavirus proteins in the development of a selection-free rotavirus reverse genetics system and to

investigate the potential of expressed rotavirus proteins to suppress the innate immune response induced by rotavirus transcripts.

1.3.2 Specific objectives of this study

1 To determine the whole consensus genome nucleotide sequence of the prototype rotavirus Wa strain using sequence-independent genome amplification and 454® pyrosequencing for use as template for the development of a rotavirus expression plasmid system (***Chapter 3***).

2 To optimise transfection conditions in various mammalian cell lines for the expression of rotavirus proteins from RNA transcripts and cDNA plasmids (***Chapter 4***).

3 To transfect *in vitro* DLP-derived rotavirus SA11 transcripts into cultured cells in an attempt to generate viable rotavirus (***Chapter 5***).

4 To transfect rotavirus transcripts in the presence of various expressed rotavirus proteins, including the core replication complex, in an attempt to generate viable rotavirus (***Chapter 5***).

5 To study the effect of rotavirus transcripts and expressed rotavirus proteins on specific interferon pathways of the host cell (***Chapter 6***).

6 To investigate the potential of expressed rotavirus proteins to suppress the innate immune response induced by rotavirus transcripts (***Chapter 6***).

1.4 Structure of thesis

The seven chapters presented in this thesis are summarised below. The four experimental chapters (chapters 3–6) have the same outline and contain a short introduction, materials and methods section, results and discussion section and a summary.

Chapter 1: **INTRODUCTION**

- Provides background, problem statement, scientific approach and methodology followed during this study.

Chapter 2: **LITERATURE OVERVIEW**

- An in depth review of the relevant literature on rotavirus and other aspects applicable to the study.

Chapter 3: **CONSENSUS SEQUENCE DETERMINATION AND EVOLUTIONARY ELUCIDATION OF A ROTAVIRUS Wa STRAIN**

- The consensus sequence determination and a detailed phylogenetic analysis of the prototype rotavirus Wa strain obtained by 454® pyrosequencing.

Chapter 4: OPTIMISATION OF TRANSFECTION CONDITIONS FOR MA104, COS-7, BSR and HEK293H CULTURED CELLS USING eGFP

- The transfection efficiency of four different transfection reagents in a variety of mammalian cell lines was investigated in order to identify a suitable cell type and the most effective transfection reagent for use in the development of a rotavirus reverse genetics system.

Chapter 5: INVESTIGATING THE ROLE OF EXPRESSED ROTAVIRUS PROTEINS IN THE DEVELOPMENT OF A ROTAVIRUS TRANSCRIPT-BASED REVERSE GENETIC SYSTEM

- Optimisation of plasmid derived rotavirus protein expression
- Designing of a codon-optimised reverse genetics plasmid system which can produce a rotavirus replication complex scaffold.
- The effect of co-expressed proteins on the recovery of viable virus from DLP-derived rotavirus transcripts as a possible reverse genetics system is investigated.

Chapter 6: INVESTIGATING APPROACHES TO SUPPRESS THE INNATE RESPONSE TO ROTAVIRUS TRANSCRIPTS USING PLASMID DERIVED ROTAVIRUS Wa AND SA11 PROTEINS

- The innate immune reaction to rotavirus transcripts and proteins is investigated with a specific focus on the potential of specific rotavirus proteins to supress the interferon response provoked by rotavirus mRNA.

Chapter 7: CONCLUSION AND FUTURE PROSPECTS

- Description of the conclusions drawn from this thesis and recommendations are made for future studies.

1.5 Methodology and experimental procedures

A variety of methods was utilized in this study and are summarised in Table 1.1.

Table 1.1: Specific methods used in this study. A detailed description of each method can be found in the specified chapters

Methods or Techniques utilized in this study	Applied in Chapter (•)				
	3	***4***	***5***	***6***	***7***
454® pyrosequencing FLX Titanium technology	•				
Adaption of rotaviruses to cultured cells		•	•	•	
Analysis of 454® pyrosequencing data and consensus sequence determination	•				
Bacterial cell transformation		•	•	•	•
cDNA synthesis using random primers	•				•
Detection of the expression of RIG-I and MDA5 with flow cytometry					•
dsRNA isolation and purification	•	•	•	•	•
Evolutionary pressure analyses	•				
eGFP plasmid expression in mammalian cell cultures		•	•	•	•
Immunofluorescence detection of antibodies and eGFP using a fluorescent light microscope		•	•	•	•
In vitro transcription of core derived rotavirus SA11 genome segments		•	•	•	•
In vitro transcription of GLYAT and RVFV mRNA					•
Mammalian cell culturing	•	•	•	•	•
Molecular clock analyses	•				
Nucleotide and amino acid alignments	•				
Nucleotide substitution rate analysis	•				
LiCl precipitation and purification of ssRNA	•	•	•	•	•
Online genotyping with RotaC	•				

Methods or Techniques utilized in this study	*Applied in Chapter (•)*				
	3	*4*	*5*	*6*	*7*
Phylogenetic analysis	•				
Plasmid purification		•	•	•	
Propagation of rotavirus Wa and SA11 in cultured cells	•	•	•	•	•
PCR and RT-PCR	•	•	•	•	•
Restriction enzyme analysis		•	•	•	•
CsCl gradient centrifugation				•	•
In silico secondary protein structures predictions	•				
qRT-PCR to detect the expression of innate immune response factors					•
Transfection of dsRNA, ssRNA and DNA in cultured cells		•	•	•	•
Ultracentrifugation		•	•	•	
Western blot techniques				•	•
Whole Genome amplification	•				•