CHAPTER 7: CONCLUDING SUMMARY, INSIGHTS AND FUTURE PROSPECTS **7**

Reverse genetics systems have been developed for many viruses, leading to the generation of valuable information regarding their replication and pathogenesis of these viruses. Regrettably, extrapolating either plasmid-based or transcript-based reverse genetics systems to rotavirus proved to be much more difficult than expected. A reverse genetics system will undeniably lead to the generation of valuable information regarding replication, pathogenesis, the correlates of protection and innate immune responses to rotaviruses.

The specific objectives of this study were to: (1) to determine the whole genome consensus nucleotide sequence and evolutionary history of the prototype rotavirus Wa strain using sequence-independent genome amplification and 454® pyrosequencing to use as template for rotavirus expression plasmid system; (2) to optimise transfection conditions in various mammalian cell lines for the expression of rotavirus proteins from transcripts and plasmids; (3) to transfect *in vitro* DLP-derived rotavirus SA11 transcripts into cultured cells in an attempt to generate viable rotavirus; (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.

Despite the fact that no viable rotavirus could be rescued from the reverse genetics approaches investigated, valuable insights were nevertheless gained.

In this study, the benefits of using the sequence-independent cDNA synthesis and amplification method (Potgieter et al., 2009) together with next generation sequencing and a variety of *in silico* analyses for the determination of the consensus sequence and evolutionary history of a rotavirus Wa strain were demonstrated. It was determined that the WaCS strain originated from an early cell culture adapted Wa variant from the initial gnotobiotic pig passaged Wa isolate. A total of 4 novel nucleotide changes, which also resulted in amino acid changes, were detected in genome segment 7 (NSP3), genome segment 9 (VP7) and genome segment 10 (NSP4)

.

In silico analysis indicated that none of the detected nucleotide changes, and consequent amino acid variations, had any significant effect on the viral structure. Despite serial passaging in animals, as well as cultured cells, the rotavirus Wa genome seems to be stable. The WaCS sequence was derived from a single rotavirus Wa population using next generation sequencing. Bearing in mind that the current rotavirus Wa reference strain is a composite sequence of various Wa variants, the rotavirus WaCS generated during this study may be a more appropriate reference sequence. The WaCS was used to synthesise cDNA for one of the expression plasmid sets.

In an effort to determine the best possible transfection conditions for a possible reverse genetics system, a variety of transfection reagents were tested on four different mammalian cell lines (MA104, BSR, COS-7 and HEK 293H). After extensive transfection condition optimisation, the X-tremeGENE HP transfection regent proved to provide the best expression of eGFP in MA104, COS-7, BSR and HEK 293H cultured cells. Bearing in mind all the other transfection reagents had poor transfection efficacy in MA104 cells and that rotavirus replicates well in these cells, X-tremeGENE HP transfection reagent may be the best choice for transfection of rotavirus transcripts and cDNA plasmids.

The rotavirus Wa and SA11 strains were selected for the plasmid-based expression of rotavirus proteins due to the fact that they propagate well in MA104 cells and the in-depth knowledge of their nucleotide sequences. After extensive transfection optimisation on a variety of mammalian cells, MA104 cells proved to be the best suited for the expression rotavirus proteins from plasmids. Due to a flaw in the T7 promoter sequence, these two plasmid-based sets could not be used for reverse genetics, but they still functioned as capable expression systems. The expression of VP1, VP6, NSP2 and NSP5 could be confirmed for the rotavirus Wa and SA11 expression constructs as well as the codon-optimised plasmid system. These systems were subsequently used to evaluate the effect of different rotavirus proteins on the innate immune response of the host cell.

No viable virus could be generated from the transcript-only based system. Nevertheless, the transcript derived expression of four different rotavirus proteins (VP1, VP6, NSP2 and NSP5) could be shown by immunostaining with antibodies. This result proved, for the first time,

that transfected *in vitro* derived rotavirus transcripts are translatable in a suitable cell culture system. In an effort to combine the transcript- and plasmid systems, a codon-optimized plasmid system was designed in an attempt to produce a rotavirus replication complex scaffold (VP1, VP2, VP3 and VP6). The expression of transcript derived rotavirus VP1, VP6, NSP2 and NSP5 could again be confirmed. A second transfection of rotavirus SA11 *in vitro* derived transcripts was performed 24 hours after the transfection of the codon-optimized plasmids. This pre-expressed replication core in combination with transcripts was not able to deliver any viable rotavirus. An expressed replication core scaffold may, therefore, not be able to recruit/import rotavirus mRNA as proposed by the core-filling model (McDonald and Patton, 2011).

Several other basic aspects of the rotavirus replication cycle are not well understood. For instance, the exact mechanism of how the transcripts are localised in the viroplasms or the mechanisms that direct the synthesis of negative sense ssRNA, assortment and packaging of dsRNA segments into viral cores are still unclear. Interestingly, both the replication cycles of the bluetongue virus or African horsesickness virus are completely understood, but it was still possible to develop reverse genetics systems for these viruses. However, there are some important replication cycle aspects that are unique to rotavirus, for example the occurrence of viroplasms, which is not found in the bluetongue virus or African horsesickness virus. As mentioned before, the exact function of viroplasms is not completely understood along with several other key aspects of the rotavirus replication cycle, making the development of a comprehensive rotavirus reverse genetic system extremely challenging. It will be important to dedicate more effort into examining and clarifying these elusive aspects in the life cycle of rotaviruses in the quest of developing a rotavirus reverse genetics system.

Currently, studies in cultured cells involving rotavirus transcripts have been severely hindered by the destructive innate immune response they elicit (Mlera, 2013, Uzri and Greenberg, 2013). In contrast to normal rotavirus infections, the innate immune response to rotavirus transcripts is poorly understood. The effect of plasmid-derived expression of all 12 rotavirus proteins individually on selected aspects of the innate immune response was investigated. The expression of certain viral proteins from plasmids (VP3, VP7 and NSP5/6)

stimulated specific interferon (IFN) responses. Some viral proteins (VP1, VP2, VP4 and NSP1) seemed to be able to actively suppress the expression of certain cytokines. In the light of these suppression results, specific rotavirus proteins were expressed from transfected plasmids to investigate their potential in supressing the interferon response provoked by rotavirus transcripts. Cells transfected with the plasmid encoding NSP1 and NSP2 reduced the expression of type I IFN and CXCL10. The transfection of plasmids encoding the viroplasm proteins, NSP2 and NSP5/6, significantly suppressed the type I and III interferon responses elicited by transfected rotavirus transcripts. These findings point to other possible viral innate suppression mechanisms in addition to the known degradation of IRFs by NSP1 (Graff et al., 2007). For the development of a rotavirus transcript-based reverse genetics system in the future, a few key elements will have to be taken into consideration. Preferably, the expression of all 12 rotavirus proteins should be confirmed. At present, there are only a few primary monoclonal antibodies to rotavirus proteins available commercially and more effort should be put into generating a full range of antibodies against all 12 rotavirus proteins. The lack of expression of only a single genome segment may have a grave effect on the viral replication cycle, thwarting an otherwise working reverse genetics system. Additionally, the influence of the innate response to rotavirus transcripts is not completely understood and should be examined more closely. The aggressive innate immune response to rotavirus transcripts may be the biggest hurdle for the development of a transcript-based reverse genetics system. Better knowledge of the exact innate pathways involved may enable us to suppress, or even prevent, this violent reaction which will be a large step towards a working transcript-based reverse genetics system.

In conclusion, this study investigated the importance of co-expressed rotavirus proteins in the development of a selection-free rotavirus reverse genetics system. Regardless of the fact that no viable rotavirus could be recovered from the reverse genetics approaches investigated, important progress was made. The consensus sequence of the prototype rotavirus Wa was determined by next generation sequencing and may be a more appropriate reference sequence than the composite sequence used today. When investigating the transcript-based approach, it could be shown that transfected *in vitro* derived transcripts are translatable in a suitable cell culture system. The transfection of plasmids encoding the viroplasm proteins, NSP2 and NSP5, significantly suppressed the type

I and III interferon responses elicited by transfected rotavirus transcripts. Based on these insights, it is possible that there could be additional viral innate suppression mechanisms or proteins. Sufficiently supressing the innate response to rotavirus transcripts might be central to the successful deployment of a transcript-based reverse genetics system.