Chapter 7

Concluding remarks and future prospects

The development of a whole genome rotavirus reverse genetics is critical for the understanding of several aspects of rotavirus biology that are currently not known. In addition, such a system will be instrumental in the efficient generation of rationally designed vaccines. This study sought to identify and investigate potential bottlenecks that need to be overcome to develop a transcript-based reverse genetics system for rotaviruses. The specific objectives were: (i) to determine the consensus whole genome sequence of the prototype rotavirus DS-1 strain; (ii) to characterise a rotavirus SA11 sample stored in South Africa; (iii) to attempt the recovery of rotavirus by transfecting *in vitro* transcribed transcripts generated from the consensus rotavirus DS-1 cDNA and rotavirus SA11 double-layered particles (DLPs); and (iv) characterise the innate immune response to rotavirus transcripts. Empirical insights were gained at each stage, despite the inability to recover rotavirus from transfecting rotavirus transcripts into cells in culture.

One of the important milestones of this study includes the first determination of the whole genome consensus sequences of the rotavirus DS-1 and SA11 strains. As expected, pyrosequence data analyses showed some sequence variations which suggested the potential occurrence of minor population variants (Figures 3.4 and 4.3). However, in the absence of a reverse genetics system, it is difficult to test the biological effect of the observed sequence variations. The importance of a consensus sequence was demonstrated for BTV-8\net06 which could not be recovered by reverse genetics using published sequences until a consensus sequence was used (van Gennip *et al*., 2012).

Another key finding was that rotavirus transcripts induced a characteristic cell death pattern in BSR and COS-7 cells (Tables 5.3 and 5.4). The cell death was similar irrespective of whether wild type rotavirus SA11 transcripts or consensus sequence-based single genome segment synthetic transcripts were used. It was interesting to find that BTV, which has a functional reverse genetics system (Boyce et al., 2008, Boyce and Roy, 2007), also induced cell death in COS-7 cells following transfection

of transcripts (Table 5.5). However, the BTV transcript-induced cell death was less than that induced by rotavirus transcripts (Table 5.5). This suggests a unique difference between BTV and rotavirus despite the viral family similarities. The precise contribution of the use of a consensus sequence could not be evaluated due to the occurrence of cell death without being able to recover rotavirus. Also noteworthy was the observation that rotavirus transcript-induced cell death is slowed but cannot be prevented with PKR inhibition (Table 5.4; Figure 5.7). This indicates that the cell death mechanism(s) include PKR-independent pathway(s). All indications are that the cell death was the end result of an antiviral state induced in response to the transcripts.

The results of investigations described in this study into the innate immune response to rotavirus transcripts also revealed the induction of interferon (IFN) which has not been described before. This important finding showed that interferon 1β (a type I IFN), and IFN-λ1 (a type III IFN) were strongly stimulated by rotavirus transcripts. Since the induction of IFN induces apoptosis (Chawla-Sarkar *et al*., 2003, Frias *et al*., 2011) it was, therefore, concluded that the basis of the cell death observed was a direct result of transfecting rotavirus transcripts. Furthermore, the induction of the cytokines by rotavirus transcripts implied that an antiviral state was established in transcript-transfected cells. The cytokine response is likely to be a very early event which is so potent that minus-strand synthesis, and other critical rotavirus replication steps, may be grossly inhibited. Therefore, the antiviral state could be the critical event that is preventing rotavirus recovery.

Despite the inability to recover rotavirus during this study, the demonstration that the transcripts were biologically active in the sense that they were translated was also important. However, antibodies against all the rotavirus DS-1 proteins should also be generated for the specific detection of rotavirus DS-1 proteins after transfections. The apparent higher expression in BSR cells than COS-7 cells suggested that the defective RIG-I system in BSR cells (Habjan *et al*., 2008) provides a translation advantage. The sensing of rotavirus transcripts by RIG-I was confirmed by western blot analyses and this was an important confirmation of the association between rotavirus and the RIG-I system. This further supports the idea that the cell line to be

used for developing a reverse genetics system for rotaviruses might be a crucial factor for success.

The inability to recover virus after transfection of rotavirus transcripts might be due to defective replication, particle assembly or both. Viral transcripts can be used for recovery of other closely related dsRNA viruses to rotaviruses i.e., AHSV and BTV (Boyce and Roy, 2007, Boyce *et al*., 2008, Matsuo *et al*., 2010). The basic steps of rotavirus replication are quite similar to those of AHSV, BTV and reovirus but the finer replication details are different. For instance, AHSV and BTV are able to replicate in arthropod vectors (Mertens, 2004, Roy, 2008). There are also distinct proteins and structural organisation differences between these *Reoviridae* family members. It could be reasonable to speculate that rotavirus transcripts need to be complexed with inner capsid proteins to form an “infectious nucleocapsid unit” similar to coated nucleocapsids described for the Φ6 bacteriophage (Olkkonen *et al*., 1990, Mindich, 1999). The dsRNA-filled rotavirus inner capsid particle has replicase activity (Patton *et al*., 2004, Patton and Gallegos, 1990) and could be used as a replication link if filled with synthetic dsRNA. However, filling DLPs with dsRNA seems to be extremely difficult. If the concerted model of assortment and packaging (McDonald and Patton, 2011) is valid, experiments should be performed to transfect rotavirus transcript-protein complexes into permissible cells. The recently described approach for the recovery of BTV by *in vitro* reconstitution of cores (Lourenco and Roy, 2011) should also be investigated for rotaviruses.

In future work, viroplasm formation should be confirmed with NSP2 and NSP5-specific antibodies as described in the literature (Carreno-Torres *et al*., 2010, Contin *et al*., 2010). In addition to viroplasm formation, a critical step in the reverse genetics system is the synthesis of the dsRNA from the transcripts. Therefore, it might be useful to determine whether genomic dsRNA is formed following transfection. The approach to determine the presence of rotavirus dsRNA should involve the use of the dsRNA-binding J2 antibody in combination with RNase A which cleaves single-stranded RNA and RNase III which specifically cleaves dsRNA (Lamontagne *et al*., 2001, Rojas *et al*., 2010) could be used to verify the presence or absence of dsRNA. If the presence of genomic dsRNA is detected, there might be problems with its quantity, packaging into particles or location in the cytoplasm. An alternative method

could be to transfect cells, incubate the cells with culture medium containing [α-^{32}P]dATP (Huang and Szostak, 1996), followed by RNA extraction and gel electrophoresis to visualise the rotavirus dsRNA migration pattern.

Transfections should be performed in cells that are RIG-I deficient. This is expected to bypass the RIG-I mediated interferon (IFN). However, RIG-I deficiency does not completely prevent the production of IFN (Kato *et al*., 2005). Therefore, BSR cells could be used, but it may be useful to knock-out IFN and engineer the cells so that they can express receptors which would allow re-infection of the cells. A possible approach to reduce or eliminate the innate immune response might be the permanent knock-out of appropriate gene in cells in culture using lentiviral hairpin-mediated RNAi (Sliva and Schnierle, 2010). The inhibition of IFN might allow sufficient amount of translation and possibly minimal transcript degradation. While NSP1 appears to be a broad spectrum IFN antagonist (Arnold and Patton, 2011, Sherry, 2009, Barro and Patton, 2007, Qin et al., 2011), it is currently not known whether the NSP1 inhibition of IFN extends to type III IFN and other molecules that may contribute to the establishment of an antiviral state in cells. This needs to be investigated further when rotavirus transcripts are transfected with the aim of recovering virus.

In summary, aspects deemed important for the development of a transcript-based rotavirus reverse genetics system were investigated. Based on insights gained in this study, it could be possible to develop a transcript-based rotavirus reverse genetics system. Since rotavirus cannot be recovered from cells that are rapidly progressing towards death, it is anticipated that the combination of a consensus sequence, appropriate cells and the targeted inhibition of an innate immune system-induced antiviral state could result in the recovery of rotavirus in a transcript-based reverse genetics system.