

CHAPTER SEVEN

CLOSING REMARKS

In this final chapter, conclusions of the main findings and results of this study (presented in Chapters three to six) and their implications to the rotavirus field in general will be presented. Some aspects that were not completed in the current study that need to be addressed by future follow-up studies will also be highlighted.

7.1. Conclusion

In this study, a sequence-independent whole genome amplification method (Potgieter et al., 2009) coupled with an online rotavirus genotyping tool, RotaC (Maes et al., 2009), was used as a fast and effective approach to determine the genetic constellation, the possible evolutionary relationships and origin of rotavirus strains. All 11 dsRNA genome segments of the selected human and bovine African rotavirus strains were extracted directly from stool samples, converted to cDNA, amplified and 454[®] pyrosequenced. The same procedure was also used to characterise the tissue culture adapted RVA/Human-tc/USA/DS-1/1976/G2P1B[4] prototype strain, Appendix C. The human rotavirus strains characterised in this study were selected based on the following criteria: (i) emerging human rotavirus G genotypes (G9 and G12) (Santos and Hoshino, 2005, Le et al., 2008); (ii) commonly detected human rotavirus G genotypes in the sub-Saharan African region (G8 and G9) (Santos and Hoshino, 2005); (iii) human rotavirus G genotype that have been detected at higher frequencies in some regions where Rotarix[®] vaccine was introduced due to either vaccine pressure or natural rotavirus genotypes oscillation (Kirkwood et al., 2011, Gurgel et al., 2007, Carvalho-Costa et al., 2011), but detected frequently in Africa (G2) (Sanchez-Padilla et al., 2009); and (iv) P genotypes that are commonly associated with G2, G8, G9, and G12 during rotavirus infection (P[4], P[6], and P[8]) (Matthijnsens et al., 2011a). Bovine rotavirus strains frequently detected in cattle (G8P[1] and G6P[8]) (Martella et al., 2010) were also selected to obtain whole genome data of African bovine rotaviruses, which is not available, and determine their evolutionary relationship to rotavirus strains characterised from other bovine and mammalian species.

The findings of this study illustrate in numerous ways the usefulness, robustness and the application of this experimental approach. This was demonstrated by the fast complete characterisation of the first whole genomes of African human G9 (RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) and G12 (RVA/Human-wt/ZAF/3133WC/2009/G12P[4] and RVA/Human-wt/ZAF/3176WC/2009/G12P[6]) rotavirus strains, and bovine rotavirus strains (RVA/Cow-wt/ZAF/1603/2007/G6P[5], RVA/Cow-wt/ZAF/1604/2007/G8P[1] and RVA/Cow-wt/ZAF/1605/2007/G6P[5]). Classification and nomenclature of these strains were performed by following the guidelines stipulated by the RCWG (Matthijnsens et al., 2011a).

The genotypes of all the genome segments of the rotavirus strains characterised in this study were assigned using a single set of universal primers. This is contrary to the widely used multiplex RT-PCR which is labour-intensive and time consuming as several steps, more reagents and several genotype-specific primers, designed specifically for all the known genotypes for each of the 11 genome segments, are used (Gouvea et al., 1990, Gentsch et al., 1992, Iturriza-Gomara et al., 2004). One of the problems associated with the exclusive use of the RT-PCR assays that employ genotype-specific primers in epidemiological studies is its inability to detect emerging novel rotavirus strains that have mutations in the genotype-specific primer binding regions. This has bearing on the accuracy of the epidemiological data which is used for numerous purposes. The sequence-independent approach that employs a single set of primers that are ligated to the 5'- end of each of the 11 rotavirus genome segments can be used to characterise such mutant strains as these primers do not rely on conserved regions of any rotavirus genome segment. Similarly, the sequence-independent approach can solve the problem of genotyping numerous rotavirus-positive samples that are detected by ELISA, but are often reported as untypables. Furthermore, the approach used in this study to characterise rotaviruses could also be useful as a quality control tool for the sequence-dependent based dual genotyping system commonly used in assigning rotavirus genotypes. For instance, sequence-dependent RT-PCR equivocally assigned only G9 and P[8] genotypes to genome segments 9 (VP7) and 4 (VP4) of strain RVA/Human-wt/ZAF/2371WC/2008/G9P[8], respectively, Chapter four. However, analysing the 454[®] pyrosequence-generated data that was generated for RVA strain 2371WC also revealed the presence of rotaviruses with G2, G8, G12, P[4] and P[6] genotypes in the same sample.

Bioinformatic analysis revealed that most of the human rotavirus strains characterised in this study emerged through multiple reassortment processes between human and animal strains, whereas the bovine rotavirus strains might have emerged through genome reassortment

between *artiodactyls* strains. For instance, strain RVA/Human-wt/MWI/ MW1479/2001/ G8P[4] possibly emerged through interspecies reassortment between human and *artiodactyls* rotaviruses. Strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] which represents the first human rotavirus strain to be completely characterised with G9 (VP7) and P[6] (VP4) genotypes on a DS-1-like genetic backbone might have emerged through multiple genome reassortment events between Wa-like, DS-1-like, and human P[6] rotaviruses. Strain RVA/Human-wt/ZAF/3133WC/2009/G12P[4] which contained a DS-1-like P[4] encoding genome segment 4 might have emerged through intergenogroup genome reassortment events between human Wa-like and human DS-1-like rotavirus strains. Human strain RVA/Human-wt/ZAF/3176WC/2009/G12P[6] which contained a P[6] VP4 in a human Wa-like genetic backbone seems to share a common origin with porcine strains due to the genotype assigned to its genome segment 4. RVA strains 3133WC and 3176WC represent the first completely molecularly characterised G12 rotavirus strains from Africa. Furthermore, strain RVA/Human-wt/ZAF/3203WC/2009/G2P[4] is the first human African rotavirus strain of which all 11 genome segments have DS-1-like genotypes. In contrast to the human strains, comprehensive phylogenetic analyses of the three bovine rotavirus strains (RVA/Cow-wt/ZAF/1603/2007/G6P[5], RVA/Cow-wt/ZAF/1604/2007/G8P[1] and RVA/Cow-wt/ZAF/1605/2007/G6P[5]) showed that their nine non-G, non-P genome segments had identical genotypes but that the genotype of genome segment 4 (VP4) and genome segment 9 (VP7) of these three viruses differed, being G6P[5] and G8P[1]. This suggested that multiple reassortment events between rotaviruses of different animal species occurred in the genesis of the bovine rotavirus strains investigated. The evolutionary analyses suggest that the three bovine rotavirus strains emerged through multiple reassortment events between bovine, giraffe and antelope rotaviruses. The two G6P[5] bovine rotavirus isolates were very closely related, suggesting a clonal origin as they were isolated from calves with diarrhoea on the same farm in South Africa. These findings highlight the role of rotavirus intergenogroup and interspecies genome reassortment in generating rotavirus strain diversity. In addition to several full genomes of bovine rotavirus strains that have been reported from different countries, the three cattle rotavirus strains reported in this study are the first and thus far only bovine strains sequenced using a mass parallel sequencing platform. With the exception of strains RVA/Cat-wt/ITA/BA222/2005/G3P[9] (Martella et al., 2011), RVA/Antelope-wt/ZAF/RC-18-08/G6P[14] (Matthijnsens et al., 2009) and RVA/Horse-wt/ZAF/EqRV-SA1/2006/G14P[12] (Matthijnsens et al., 2011b), the three bovine rotavirus strains reported in this study represent the only animal rotavirus strains to be analysed by making use of the

next generation sequencing technology. Furthermore, the three bovine rotavirus strains reported here represent the only bovine rotaviruses whose genomes have been fully sequenced and characterised from Africa. The availability of more such data will be useful in epidemiology and evolutionary studies to decipher the origin of some of the zoonotic strains of bovine origin that are currently circulating in humans in this region.

The study also reported the first ever characterisation of the whole genomes of multiple rotavirus strains present in a single stool specimen. The evolutionary mechanisms such as potential animal-to-human interspecies transmission, human-to-animal reassortment and genome recombination followed by the co-infecting rotavirus strains were determined through analyses of the generated consensus nucleotide sequences of the co-infecting rotavirus strains. The study has illustrated how infection with multiple rotavirus strains of different genotypes may result in novel progeny rotaviruses through genome recombination in the same patient for the first time. Evidence of intergenotype genome recombination was detected in genome segments 6 (VP6), 8 (NSP2) and 10 (NSP4) that resulted in progenies with chimaeric sequences. Analysis of the nucleotide and amino acid sequence suggested that recombination between Wa-like and DS-1-like genome segments occurred in these three genome segments. Although intergenotype genome recombination has been reported in genome segment 8 (Donker et al., 2011), this study is the first to report rotavirus genome recombination in genome segments 6 and 10. This may suggest that genome segment recombination could be a more frequent evolutionary event in rotaviruses than originally thought. Tissue culture adaptation followed by plaque isolation might be necessary to determine the viability of the viral isolates bearing recombinant intergenotype sequences. The findings shed some new light on the complex mechanisms of the emergence of rotavirus genomic diversity. The advanced methodology used in generating the whole genome sequences employed in this study has not yet been widely applied to full genome-based studies on group A rotaviruses. The numerous advantages of this approach as demonstrated in the preceding chapters illustrate how valuable it will be towards whole genome analysis of rotaviruses to improve detection and epidemiological data.

Consensus nucleotide sequences of the genome segments generated from dsRNA isolated directly from stool samples containing human G2, G8, G9 and G12 rotavirus strains associated with P[4], P[6] and P[8] generated in this study (Chapters three and four) were used to prepare RV-VLPs. The pFBq plasmid, that allows cloning and expression of up to four foreign genes, was used as a donor for insect optimised ORFs coding for rotavirus

structural proteins to prepare recombinant bacmids which were used to generate recombinant baculoviruses. The size of the recombinant rotavirus structural proteins that were expressed by the recombinant baculoviruses in this study correlated with the theoretical polypeptides derived from the deduced amino acid sequences that were generated from the consensus nucleotide sequences (see Chapters three and four), which is also similar to other rotavirus protein expression studies (Crawford et al., 1994, Yao et al., 2012). By co-expressing recombinant baculovirus rotavirus proteins, RV-VLPs could be prepared to closely mimic the wild-type rotavirus particle conformation. Previously, cell culture-adapted rotavirus strains have been used to prepare RV-VLPs. However, “isolation and cultivation of human rotaviruses from clinical faecal specimens is difficult and adaptation to growth *in vitro* requires multiple rounds of passage in primary cells” (Arnold et al., 2009). In addition to these problems, rotavirus strains may also undergo genetic changes in order for them to adapt to the artificial *in vitro* environment. Therefore, generating RV-VLPs from tissue culture-adapted strains may result in RV-VLPs with some phenotypic differences to wild-type strains. This study has introduced a novel approach of producing RV-VLPs, namely by using dsRNA extracted directly from clinical specimens. This bypasses the need to adapt the viruses to tissue culture which saves time and circumvents the problems associated with cell culture adaptation. Thus, using this approach, it is now possible to generate RV-VLPs for evaluation as non-live vaccine candidates for any human or animal field rotavirus strain.

Based on the morphology and size of the RV-VLPs that were produced, the recombinant rotavirus proteins expressed in both Sf9 and High Five[®] cells assembled into both dRV-VLPs and tRV-VLPs. However, the number of tRV-VLPs formed were less than previously reported by others (Vieira et al., 2005), and most tRV-VLPs seemed to contain only a partial VP7 and VP4 outer capsid. In addition, the VP5* subunit of VP4 was associated with the tRV-VLPs in most cases, except when a step-wise infection strategy was used. This suggests that the step-wise strategy is a better option for producing cRV-VLPs. As expected, the yield of RV-VLP produced in High Five[®] cells was approximately three-fold higher than the yield obtained in Sf9 cells. Overall, the yield of RV-VLPs that was produced in this study correlates with the values reported by others. The immunogenicity of the tRV-VLPs produced in this study will be tested in follow-up studies.

The recombinant proteins and RV-VLPs generated in this study can be used for several purposes including basic research which involves the understanding of the function

relationships of rotavirus structural proteins, formulation of diagnostic assays and therapeutic products.

7.2. Recommendations and future perspectives

This study has shown some of the advantages of the use of a sequence-independent amplification procedure coupled with 454[®] pyrosequencing and RotaC analyses for the detection, surveillance, and epidemiological investigations of rotaviruses. In future, it would thus be prudent to use this approach in parallel with sequence-independent genotyping assays on selected specimens, especially in reference laboratories. Since prior knowledge of the sequence of the rotavirus strain is not required, the proposed approach used to characterise whole genomes of rotaviruses in this study can be useful for quality control purposes to check the accuracy or reliability of the genotype-specific sequence-dependent RT-PCR methods that are currently in use. This may enable identification of novel rotavirus strains that are circulating in communities. In addition, the approach should be employed in assigning genotypes to the ever increasing number of untypable rotavirus-ELISA positive strains that mostly result from genotype-specific primer inefficiencies or/and mismatches. Similarly, this approach is bound to uncover a wealth of new knowledge about the mechanisms driving strain evolution and transform rotavirus epidemiology if it were to be used to characterise the large number of mixed rotavirus infections that is frequently reported worldwide. Furthermore, the approach should be used in post-vaccine surveillance studies in areas where rotavirus vaccines have been introduced to monitor the impact of vaccination, for instance, in the generation of reassortants between the vaccine strains and the circulating wild-type strains, as has been recently reported from Nicaragua (Bucardo et al., 2012), and also to determine the ability of these vaccine-wild-type reassortant strains to persist in communities.

As evidence is now emerging that suggests a correlation between rotavirus vaccine efficacy and specific rotavirus genotypes (Gurgel et al., 2007, World Health Organization, 2007, Ruiz-Palacios et al., 2006), further full genome characterisation studies should be done to understand the complete genome constellations of regionally prevalent strains, such as G8 rotaviruses (Santos and Hoshino, 2005), and emerging genotypes, such as G12 (Le et al., 2008). This information can eventually be used to formulate regional rotavirus vaccines. Since most rotavirus mortalities are caused by rotaviruses that belong to either Wa- or DS-1-

like genogroups, combining genotypes from these two genogroups in the formulation of the next generation rotavirus vaccines may improve vaccine efficacy and induce broader protection.

Since the current licensed rotavirus vaccines are not as effective in developing countries as in developed countries and are quite expensive, further vaccine development efforts using new biotechnological approaches that involves genetic engineering are being encouraged world wide. Non-live RV-VLPs have been shown to be safe and can protect against rotavirus challenge in mice models (Zhou et al., 2011, Istrate et al., 2008, Bertolotti-Ciarlet et al., 2003, Ciarlet et al., 1998). The results of this study that show that it is possible to generate RV-VLPs by using dsRNA directly from human stool samples may lead to rational formulation of multivalent vaccine candidates that might give broader protection against several strains. This line of investigation should be continued.

Finally, although RV-VLPs are potentially attractive vaccine candidates, major challenges still exists with regard to the recovery of RV-VLPs and downstream processes such as purification. Since high expression of recombinant proteins is not directly proportional to the RV-VLPs yield, it is important to optimise the expression systems in order to improve the amount of recombinant rotavirus protein expression as well as the efficiency of RV-VLP assembly. Apart from parameters like MOI, TOI and stoichiometry that can be used to maximise RV-VLP production, the following approaches may also be considered.

- Recently Trask et al. (2012) speculated that by mimicking the natural rotavirus packaging of rotavirus, where VP4 associates with the DLP prior to the assembly of the VP7 layer, the possibility of obtaining cRV-VLP could be enhanced. This might be achieved by the use of a step-wise co-infection strategy whereby insect cells are infected with recombinant tricistronic baculoviruses containing ORFs expressing VP2/6/4. This can then be followed by infection with recombinant baculoviruses containing ORFs encoding VP7 12 – 24 hpi.
- Istrate and co-workers (2008) fused the VP8* subunit of VP4 to a truncated VP2 and were able to induce protective immunity in mice through parentally administration of the VP8*-VP2/6/7 RV-VLPs. Since dRV-VLPs can be produced much easier than RV-VLPs or cRV-VLPs, studies that can investigate the possibility of producing chimaeric dRV-VLPs containing antigenic regions of VP4s and/or VP7s conjugated to VP2 and/or VP6, respectively, should be considered.

- Another challenge associated with the production of RV-VLPs is the purification and differentiation between dRV-VLPs, tRV-VLPs and cRV-VLPs. The common methods currently in use for purifying RV-VLPs include ion exchange, affinity and size exclusion chromatography. Although size-exclusion chromatography is attractive as it can remove unassembled RV-VLPs and other particles that may be formed by baculoviruses, it generally leads to a lower RV-VLP yield (Mena et al., 2005). Furthermore, large scale production may be hampered due to its demand for specialised personnel and it being labour intensive. Therefore, studies that explore simpler, alternative methods to improve the recovery of purified RV-VLPs, and also facilitate purification of tRV-VLPs and dRV-VLPs without decreasing the RV-VLPs yield, should be encouraged.
- Finally, follow-up immunogenicity studies of the RV-VLPs prepared in this study should be conducted in animal models to determine their antigenic and protective abilities. The finding that immunising mice with rotavirus VP2/6 followed by VP6 expressed by an adenovirus as a booster regime elicits strong humoral, mucosal and cellular immune responses (Zhou et al., 2011) suggests the potential of using the dRV-VLPs prepared in this study as immunogens in our future rotavirus vaccine development efforts. In addition to using the prime-boost strategy, it would be interesting to assess the ability of the tRV-VLPs produced in this study in association with adjuvants, to induce homotypic or heterotypic immune response in animal models.

7.3. References

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