CHAPTER SIX

GENERATION OF CHIMAERIC ROTAVIRUS VIRUS-LIKE PARTICLES DERIVED FROM THE CONSENSUS SEQUENCE OF AFRICAN ROTAVIRUS STRAINS CHARACTERIZED DIRECTLY FROM STOOL SAMPLES

6.1. Introduction

To date, developing countries are still faced with a high rotavirus burden of disease as reflected by high childhood morbidity and mortality (Parashar et al., 2009), whereas in developed countries rotavirus disease causes high health care cost (LeBaron et al., 1990). In view of the lack of antiviral therapy against rotavirus diarrhoea, the use of rotavirus vaccines at an early age is currently the only first line of defence against rotavirus disease. In addition to the Lanzhou lamb rotavirus vaccine licensed only in China (World Health Organization., 2001), RotaTeq® and Rotarix® were licensed by the WHO for routine use in children across the globe (World Health Organization, 2009). However, the use of these vaccines has revealed several short comings. Their high cost is beyond reach of most developing nations. Their safety still requires careful investigations (see section 1.1) and the lower efficacy of these vaccines in developing countries (Madhi et al., 2010) compared to their efficacy in developed countries (Vesikari et al., 2004) is another cause for concern (see section 2.8.1). Amongst several reasons, the difference in rotavirus strains circulating in developed and developing countries is thought to be one of the attributes to the lower efficacy of the vaccines in developing countries (Patel et al., 2009). However, this hypothesis has not been proven. Maternal antibodies could also interfere with the effectiveness of both Rotarix® and RotaTeq® as both vaccines are administered orally to infants in whom they need to replicate to elicit immunity (Glass et al., 2006b). Furthermore, there are currently only three commercial producers of rotavirus vaccines in the world and they cannot meet the global demand if all countries were to introduce mandatory vaccination of all infants. It is estimated that they can only produce around 20% of the anticipated global need (see section 2.8.1).

There is thus a need for further development of alternative rotavirus candidates. Amongst others, RV-VLP vaccines are some of the promising potential candidates that are currently considered as a viable alternative option (Glass et al., 2006a, Crawford et al., 1994). This is

based on the fact that RV-VLPs are (i) non-infectious as they do not contain the genomic material and can thus not replicate (Crawford et al., 1994); (ii) are highly immunogenic (Crawford et al., 1994) as the viral proteins are in their natural conformation (Brussow et al., 1990); (iii) can be genetically manipulated to provide broader protection by incorporating several rotavirus serotypes (Kim et al., 2002, Crawford et al., 1994); and (iv) are amenable to large-scale production (Vieira et al., 2005).

The three capsid layers of the rotavirus particle that enclose the 11 segments of the dsRNA genome are composed of 60 dimers of the 102 kDa VP2 (inner capsid; Labbe et al., 1991); 260 trimers of the 45 kDa VP6 (middle shell capsid; Prasad et al., 1988); and 780 monomers (260 trimers) of the glycosylated 37 kDa VP7 and spikes formed by 60 trimers of the protease-sensitive 87 kDa VP4 (outer capsid; Prasad et al., 1988, Settembre et al., 2011, Dormitzer et al., 2004). The genomic material and the inner layer comprise SLP. The DLP is composed of the genomic material, inner and the middle layer, whereas the complete particle or TLP contains all three capsid layers (see section 2.2.1).

Single-layered rotavirus-like particles (sRV-VLPs), double-layered RV-VLPs (dRV-VLPs) and triple-layered RV-VLPs (tRV-VLPs) have been produced successfully in BVES previously by using either monocistronic (Jiang et al., 1998, Kim et al., 2002) or multicistronic (Dee and Shuler, 1997, Peixoto et al., 2007) baculovirus recombinants expression strategies. Co-infection strategies make use of either monocistronic or dualcistronic baculoviruses expressing either a single or two rotavirus proteins, respectively. Often the yield of VP7 expression is lower in co-infection strategies compared to tricistronic baculovirus systems (Roldao et al., 2007, Vieira et al., 2005). Tricistronic baculovirus systems expressing VP2/6/7 have the advantage of producing a higher yield of VP7 (Roldao et al., 2007, Vieira et al., 2005). However, RV-VLP kinetic studies showed that this system can be wasteful, as excess VP7 proteins assemble into defective particles, thereby limiting the production of tRV-VLPs (Roldao et al., 2007). However, controlled optimised MOI, TOI and taking the stoichiometry of rotavirus proteins that makes the particles into account can be used to increase the yield tRV-VLPs (Roldao et al., 2007).

Applications of RV-VLPs other than as vaccine candidates include their use in basic research to understand the structural conformation of rotavirus particles, functions of the structural proteins and understanding the interaction between rotaviruses and their hosts (Mathieu et al.,

2001, Crawford et al., 1994, Labbe et al., 1991). In nanotechnology, RV-VLPs have been demonstrated to be useful as possible drug delivery systems to the gut as novel nanocarriers due to their natural epithelial cell tropism that can efficiently transfer bioactive molecules to other specific target tissues (Cortes-Perez et al., 2010).

Most RV-VLPs have been prepared for the purpose of developing rotavirus vaccine candidates because the multimeric RV-VLP protein structures have the same conformation as the native rotaviruses (Istrate et al., 2008, Crawford et al., 1994). Several experimental RV-VLPs generated from non-human rotavirus strains have been shown to induce protective immunity against rotavirus challenge in mice (Zhou et al., 2011). Although dRV-VLPs prepared from strain SA11 could not induce serum neutralising antibodies in mice, immunogenic protection was generated as demonstrated by results of rotavirus challenge experiments of these mice (Bertolotti-Ciarlet et al., 2003). Serum neutralising antibodies were elicited in mice that were immunised with tRV-VLP experimental vaccines containing VP2/4/6/7 proteins that were generated from strain SA11 (Crawford et al., 1999). Similarly, several RV-VLPs have been prepared from bovine rotaviruses that also induced immune responses in animal models (El-Attar et al., 2009, Parez et al., 2006, Vieira et al., 2005, Madore et al., 1999, Kim et al., 2002). To date, there is no data reporting on RV-VLPs formulated from human rotavirus strains that emerged in the past two decades. Most RV-VLPs have been generated from tissue culture adapted rotavirus strains. However, it is very difficult to adapt wild-type human rotaviruses to tissue culture. In addition, the need for plaque picking to select and characterise the adapted rotaviruses is extremely time-consuming (Ward et al., 1991, Ward et al., 1988, Ward et al., 1984). In this study, the possibility of generating chimaeric RV-VLPs using the outer capsid proteins of selected G2, G8, G9, and G12 wild-type African rotavirus strains associated with P[4], P[6] and P[8] genotypes of which their dsRNA was isolated directly from human stool samples was investigated. Adapting rotavirus strains to tissue culture may result in changes especially in the epitopes of receptor binding regions of VP4 and VP7 that are involved in viral attachment to host cells (Ciarlet and Estes, 1999). Similarly, tissue culture adapted strains may undergo as yet unknown biological changes that enable them to adapt to the artificial *in vitro* environment which may cause them to have phenotypic differences to wild-type strains. Therefore, preparing RV-VLPs directly from field strains using the consensus sequences derived from their dsRNA may represent a novel strategy of producing non-live RV-VLP vaccines from

any human or animal field strains without the prior intermediate step of cell culture adaptation. RV-VLPs produced in this manner may not only shorten the time required to prepare RV-VLPs from wild-type strains, but also may permit production of RV-VLPs with antigenic characteristics that closely mimic the wild-type strains. In the current study, RV-VLPs were prepared directly from the consensus sequences characterised from the dsRNA of rotaviruses that was extracted from human stool samples. Chimaeric RV-VLPs were generated by assembling outer capsid layers containing various combinations of VP4 and VP7 proteins derived from different rotavirus strains onto dRV-VLPs generated from VP2 and VP6 proteins of strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6]. These RV-VLPs may in future be used to formulate potential alternative next generation polyvalent non-live rotavirus vaccine candidates. Unlike reassortment observed between the live-attenuated vaccine administered to children and the wild-type strains circulating in communities (Bucardo et al., 2012), RV-VLP vaccines would not present such safety concerns as they can not replicate.

6.2. Materials and Methods

6.2.1. Rotavirus strains, plasmids, bacteria, insect cell lines and baculoviruses

Human African rotavirus strains RVA/Human-wt/MWI/1473/2001/G8P[4], RVA/Human-wt/ZAF/2371WC/2009/G9P[8], RVA/Human-wt/ZAF/3203WC/2009/G2P[4], RVA/Human-wt/ZAF/3176WC/2009/G12P[6], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] present in stool samples (characterised in Chapters three and four: Jere et al., 2011a, Jere et al., 2011b) were used to prepare chimaeric RV-VLPs. pFastBACquad (pFBq), kindly provided by Dr. A.C. Potgieter (Deltamune, South Africa; Fig. 1), was used as a donor plasmid to prepare expression cassettes in which various combinations of open reading frames (ORFs) coding for VP4 and VP7 were cloned as indicated in the text (see section 6.2.4). pFBq was generated by incorporating the multiple cloning site (MCS) of pBacgus4x-1 (Novagen, Merck Chemicals Ltd., Nottingham, UK), which allows cloning and co-expression of up to four target genes under the control of two polyhedron (polh) or two p10 *Autographa californica* multi-capsid nucleopolyhedrosis virus (AcMNPV) promoters into the pFastBac™ (Invitrogen, Life Technologies, Grand Island, NY). This manipulation was performed by Dr. A.C. Potgieter. The *E. coli* strain JM109 was used as host for propagation of pUC57 plasmids

containing the synthetic rotavirus genome segments and pFBq expression donor plasmids as well as cloning experiments. The *E. coli* strain AcBACΔCC (Kaba et al., 2004) was used to construct and propagate recombinant baculovirus expression shuttle vectors (bacmids). The AcMNPV-Sf9 cell lines, referred to here as Sf9 cells, were used to prepare, amplify, purify and titrate recombinant baculoviruses and to produce RV-VLPs. The *Trichoplusia ni* cells, referred to here as High Five® cells, were also used to produce RV-VLPs as indicated in the text (see section 6.2.8). Baculoviruses expressing recombinant VP2 and VP6 prepared in a previous study by Dr. H.G. O'Neill were used in the current study to generate the DS-1-like

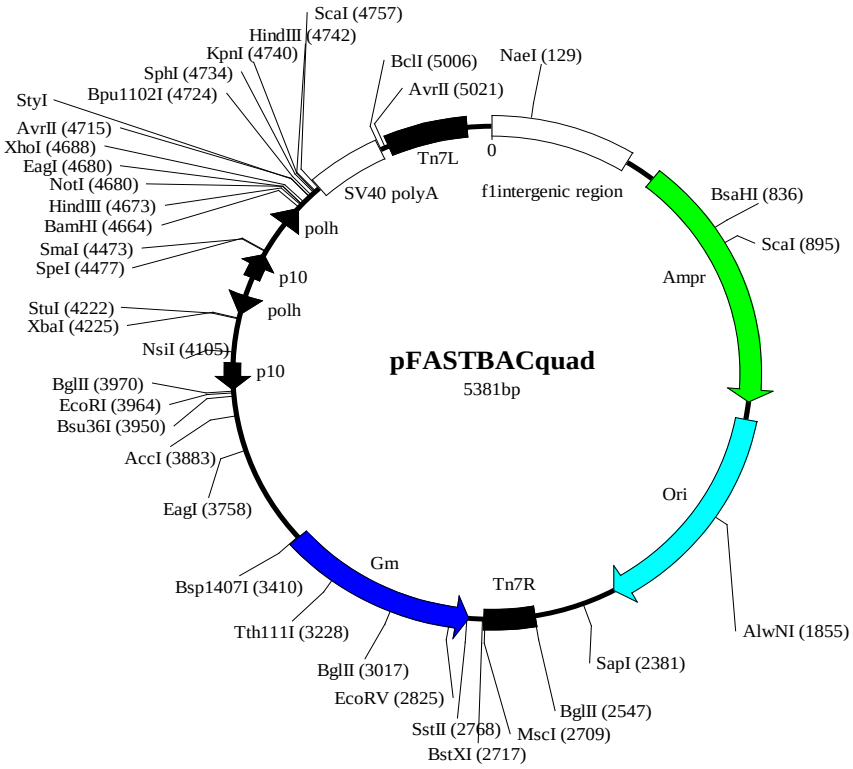


Fig. 6.1. Map of the pFastBACquad (pFBq) baculovirus transfer plasmid used for cloning and co-expression of rotavirus proteins in insect cells in the current study. The elements of pFBq were derived from the pFastBac™ (Invitrogen, Life Technologies, Grand Island, NY) and pBACgus4x-1 (Novagen, Merck Chemicals Ltd., Nottingham, UK) transfer plasmids. The two polyhedron (polh) and two p10 promoters are located upstream of the MCS (flanked by Tn7R and Tn7L transposition elements) that allow restriction enzyme-mediated cloning of foreign genes of interest. The homologous promoters allows efficient, high-level expression of recombinant protein in insect cells and are in opposite orientation to minimise recombination (O'Reilly et al., 1992). *Ori* origin permits high-copy replication, allows rescue of single-stranded DNA virion with the assistance of helper phage and maintenance of pFBq plasmid in *E. coli*. SV40 polyA (polyadenylation signal) permits efficient transcription termination and polyadenylation of mRNA (Westwood et al., 1993). The ampr (ampicillin resistance gene) allows for selection of the plasmid in *E. coli*, whereas the Gm (gentamicin resistance gene) permits selection of the recombinant bacmid in AcBACΔCC *E. coli*. Mini Tn7 elements (Tn7R and Tn7L) permits site-specific transposition of the gene of interest into the baculovirus genome (Luckow et al., 1993). (The pFBq transfer expression plasmid was constructed by Dr. A.C. Potgieter, personal communication).

VP2/6 backbone (dRV-VLP) of the chimaeric tRV-VLPs. Dr. H.G. O'Neill cloned the coding regions for VP2 and VP6 from strain RVA/Human-wt/GR10924/1999/G9P[6] into pFBq under the control of p10 and polh promoters, respectively.

6.2.2. Selection of rotavirus strains

Several human African rotavirus strains present in stool samples having G2, G8, G9 or G12 VP7 encoding genome segments associated with either P[4], P[6] or P[8] VP4 encoding genome segments that have been either linked with lower vaccine efficacy, frequently detected in Africa or have emerged in the past two decades, were solicited from NICD and UL, Medunsa, Table 6.1. The genotypes for the VP4 and VP7 encoding segments of these rotavirus strains were assigned at NICD and UL, Medunsa at the time when these samples were collected. Since these samples were stored for long periods at different conditions (some at -20 ^{o}C and others at +4 ^{o}C), the capsids of the rotavirus particles or/and the genomic material might have degenerated. Therefore, the stool specimens were screened using TEM at OVI and UL-Medunsa to select only samples that had complete rotavirus particles.

Table 6.1. List of all rotavirus strains in stool samples screened initially prior to selection of the strains used for dsRNA extraction for whole genome characterisation and preparation of chimaeric RV-VLPs.

		Genome segment 4 (VP4) genotypes		
		P[4]	**P[6]**	**P[8]**
Genome segment 9 (VP7) genotypes	**G2**	480 ¥ 518 ¥ 2329 † **3203** †, *	510 ¥ 2328 †	
	G8	842 ¥ **1473** ¥,	1241 ¥ 1396 ¥ 2070 ¥	781 ¥
	G9		2192 ¥	**2371** †, *
	G12	**3133** †, *	**3176** †, *	3152 †

The samples in the Table are designated by common laboratory names assigned by NICD and UL, Medunsa. The numbers in the table refer to sample numbers designated to the samples as laboratory sample names {referred to as common name in the rotavirus nomenclature (Matthijnssens et al., 2011)}.
† Samples solicited from NICD.
¥ Samples solicited from UL, Medunsa.
* Samples indicated in bold were used in this study.

6.2.3. Codon optimisation and design of the synthetic rotavirus genome segments coding for VP4 and VP7 for expression in insect cells

Synthetic genome segments coding for VP4 and VP7 proteins derived from consensus nucleotide sequences of the selected rotavirus strains, characterised in Chapters three and four, were codon optimised for insect cell expression and purchased from GenScript (GenScript USA Inc. New Jersey, NJ). The codon optimised genome segments were each sub-cloned into pUC57 using *EcoR* V blunt ligation at GenScript (see Fig. 6.2 for the ORF coding for VP7 (G8) protein as an example. GenScript used the OptimumGene™ algorithm (GenScript USA Inc. New Jersey, NJ) that considers many factors that regulate and influence gene expression levels in producing single genes that can give the highest possible level of expression in insect cells. In all the selected consensus nucleotide sequences of this study, GenScript increased the codon usage bias in insect cells by upgrading the codon adaptation index (CAI), increasing the GC content, prolonging the half-life of the mRNA by optimising unfavourable peaks, breaking the stem-loop structures which impact ribosomal binding and stability of mRNA, screening and modifying the negative cis-acting sites.

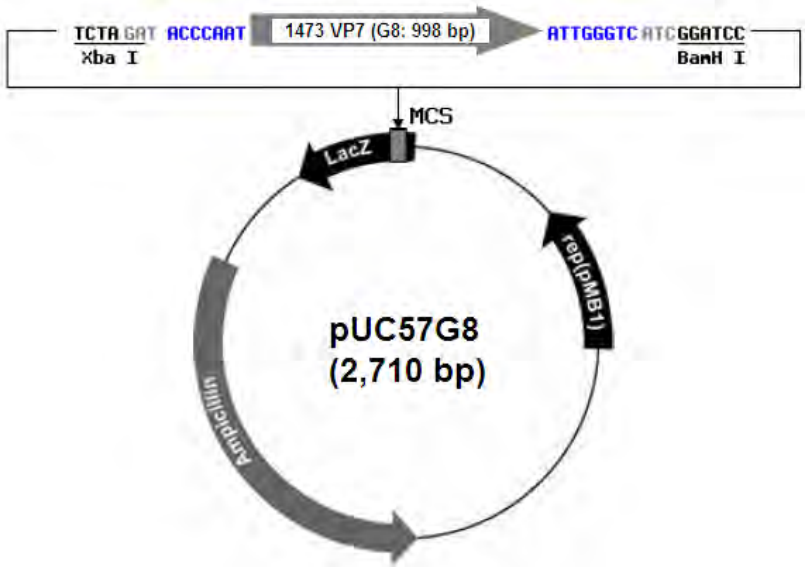


Fig. 6.2. Plasmid map of the genome segment encoding VP7 (G8) cloned in pUC57 plasmid prepared at Genscript. The 998 bp codon optimised consensus nucleotide sequence generated from strain RVA/Human-Wt/MWI/1473/G8P[4], accession number HQ657143, was designed to include *Bam* HI at 5′- end and *Xba* I/*Not* 1 at -3′ ends to facilitate directional sub-cloning into pFBq plasmid. Selection of the JM109 bacteria cells that acquired pUC57 plasmid DNA were selected through resistance to ampicillin conferred by the ampicillin gene (selectable marker).

Table 6.2. Rotavirus strains and restriction endonuclease enzymes used to clone the selected VP4 and VP7 encoding ORFs into the pFBq donor plasmid.

Strain	Accession number	Encoded protein	Restriction enzyme	Incubation period and temperature	Heat inactivation temperature [C]
RVA/Human-wt/ZAF/3203WC/2009/G2P[4][a, ¥]	HQ657176	VP7 (G2)	*Bam* HI and *Not* I	1 h at 37 °C	80 °C
RVA/Human-wt/MWI/1473/2001/G8P[4][a]	HQ657143	VP7 (G8)	*Bam* HI and *Not* I	1 h at 37 °C	80 °C
RVA/Human-wt/ZAF/GR10924/1999/G9P[6][a]	FJ183360	VP7 (G9)	*Bam* HI and *Not* I	1 h at 37 °C	80 °C
RVA/Human-wt/ZAF/3176WC/2009/G12P[6][a]	HQ657165	VP7 (G12)	*Bam* HI and *Not* I	1 h at 37 °C	80 °C
RVA/Human-wt/ZAF/3133WC/2009/G12P[4][b, ¥]	HQ657174	VP4 (P[4])	*Spe* I and *Sma* I	1 h at 30 °C, then 1 h at 37 °C	Heat inactive 65 °C
RVA/Human-wt/ZAF/GR10924/1999/G9P[6][b]	HQ657152	VP4 (P[6])	*Spe* I and *Sma* I	1 h at 30 °C, then 1 h at 37 °C	Heat inactive 65 °C
RVA/Human-wt/ZAF/2371WC/2009/G9P[8][b]	JN013994	VP4 (P[8])	*Spe* I and *Sma* I	1 h at 30 °C, then 1 h at 37 °C	Heat inactive 65 °C

[a] Consensus nucleotide sequence of genome segment 9 was used (Jere et al., 2011a).
[¥] Consensus nucleotide sequence of genome segments 4 (P[4] genotype) and 7 (G2 genotype) were derived from the same strain (RVA/Human-wt/ZAF/3203WC/2009/G2P[4]) (Jere et al., 2011a).
[b] Consensus nucleotide sequence of genome segment 4 was used (Jere et al., 2011b).
[C] The temperatures were used to stop the RE reactions.

All synthetic genome segments were designed to contain restriction endonuclease (RE) sites at both 5'- and -3' ends, flanking the ORFs, and were used for sub-cloning. Two additional stop codons were inserted at -3' end. The REs used for cloning each of the selected genes are listed in Table 6.2. Verification of the presence of restriction sites at the 5'- and -3' end was done by aligning nucleotide sequences of the codon optimised and un-optimised genome segments using DNAMAN version 6 (Lynnon Corporation, St-Louis, Canada). The codon optimised genome segments were amplified by transforming the various pUC57 into chemical competent JM109 bacteria cells prepared using the rubidium chloride method (Hanahan, 1983). In brief, the lyophilised codon optimised ORFs cloned in pUC57 were suspended in 50 µl elution buffer (EB; Qiagen, Hilden, Germany). Precisely, 100 µg plasmid DNA suspended in 5 µl EB buffer was transformed into 200 µl JM109 with transformation efficiency of 1 x 10^7 colony forming units (cfu)/µg DNA using a heat-shock method (Sambrook and Russell, 2001). Approximately 800 µl of the super optimal broth with catabolite repression (SOC) (0.5% w/v yeast extract, 8.56 mM NaCl, 2% w/v tryptone, 2.5 mM KCl pH 7.0, 20 mM $MgCl_2$ and 10 mM glucose) (Hanahan, 1983) was added to the transformed JM109 cells, followed by incubation for 1 h at 37 °C. This was followed by

Table 6.3. Primers used for PCR bacmid screening and Sanger sequencing of the nucleotide sequences of VP4 and VP7 encoding genome segments.

Primer	Encoding ORF	Primer sequence	Tm [¥]
		Bacmid PCR Primers [α]	
M13F (-40)	4, 9 or both 4 and 9	5′-GTTTTCCCAGTCACGAC-3′	55 °C
M13R	4, 9 or both 4 and 9	5′-CAGGAAACAGCTATGAC-3′	55 °C
Sanger sequencing primers			
Flanking primers for VP4 and VP7 encoding ORF sub-cloned into pUC57 [β]			
pUC57 F	4 and 9	5′-GTAAAACGACGGCCAGTG-3′	54 °C
pUC57 R	4 and 9	5′-GGAAACAGCTATGACCATG-3′	50 °C
Flanking primers for VP4 and VP7 encoding ORF sub-cloned into pFBq [δ]			
MCS_F3	4 (VP4)	5'-CGACCTTTAATTCAACCCAACAC-3'	51.6 °C
MCS_R3	4 (VP4)	5'-CGATCCTCTAGGTTTTATTACTAG-3'	50.3 °C
MCS_F2	9 (VP7)	5'-CCGGATCTGATCATGGAG-3'	49.3 °C
MCS_R	9 (VP7)	5'-GTGAAATTTGTGATGCTATTGC-3'	47.5 °C
Internal primers for VP4 encoding ORF sub-cloned into pFBq and pUC57 [§]			
pFBqP4_F	4 (VP4: P[4])	5'-CCGATCCAAAACACTAAGAACG-3'	53.2 °C
pFBqP4_R	4 (VP4: P[4])	5'-GATAGTAGACTTGATTCCGCTGAAC-3'	55.2 °C
pFBqP8_F	4 (VP4: P[8])	5'-CAGGCTCGTGGGCATCTTG-3'	56.3 °C
pFBqP8_R	4 (VP4: P[8])	5'-GGAGAATGTCGAGATGTTTGAGC-3'	54.5 °C

[α] Primer sequences as recommended in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY).
[β] Primer sequences designed at GenScript (GenScript USA Inc. New Jersey, NJ).
[¥] Hybridization melting temperature for bacmid (M13), pUC57 and pFBq (MCS) primers were provided by the manufactures/designer, whereas those of the internal primers was calculated using SeqMan, DNASTAR® Lasergene™ software package, version 8.1.2 (DNASTAR, Inc. Madison, WI).
[δ] Flanking primers were designed by Dr. H.G. O'Neill (Personal communication).
[§] Designed in the current study.

inoculation on Luria Bertani (LB) agar plates containing 50μg/ml ampicillin and incubated overnight at 37 °C. Separate colonies were inoculated in LB media containing 50 μg/ml ampicillin followed by overnight incubation at 37 °C. Plasmid DNA was isolated from the culture using the PureYield™ Plasmid Midiprep System kit (Promega Corporation, Madison, WI) by following the manufacturer's instructions. Stocks were prepared by freezing 500 μl of the culture in 50% glycerol at -80 °C. The correct sizes of the cloned segments (inserts) of each construct were verified with RE digestion reactions using the REs listed in Table 6.1. The fidelity of the nucleotide sequences for each clone was verified by sequencing the isolated plasmid DNA using Sanger sequencing at the Central Analytical DNA Sequencing Facility (Stellenbosch University, South Africa) by targeting the nucleotide sequences of the inserts using the pUC57 forward and reverse primers that flank the MCS, Table 6.3 (results not shown).

6.2.4. Sub-cloning of VP4 and VP7 coding region (s) into pFBq donor plasmid

The pFBq and pUC57 containing the synthetic rotavirus genome segments (pUC57 + codon optimised ORFs, Table 1.1) were double-digested with the REs listed in Table 6.2. pUC57 + codon optimised ORFs were digested to liberate the DNA fragments coding for VP4 and VP7 proteins, whereas pFBq (vector) was cleaved to prepare it for the insertion of the codon optimised ORFs. The nucleotide sequences of the inserts, plasmid DNA and REs were used to generate *in silico* plasmid maps using DNAMAN version 6 (Lynnon Corporation, St-Louis, Canada) to predict the sizes and pattern of the fragments resulting from RE digestion reactions. In brief, 1 µg pUC57 + insert or pFBq DNA was double-digested with 0.2 U REs. VP4 DNA fragments were generated with *Spe* I and *Sma* l, whereas *Bam* HI and *Not* I were used for VP7 DNA fragment, Table 6.2. The total volume of the reaction mixture was made up to 20 µl by adding 10 x reaction buffer and nuclease free water. Following the incubation times listed in Table 6.2, 1 U shrink alkaline phosphatase (SAP) was added to the digestion reaction for preparing vectors. This was followed by incubation at 37 ^{o}C for 30 min. SAP prevents re-circularisation of the vector by de-phosphorylating the 5'- phosphate group of DNA. The reactions were stopped by heat-inactivation at the temperatures indicated in Table 6.2. The RE stop solution at pH 8.0 containing 6 parts orange loading dye (Fermentas Inc, Glen Burnie, MA), 0.5 M ethylene-diamine-tetra-acetic acid (EDTA) and RNase was used to inactivate *Spe* I. The entire reactions were run on 0.8% agarose gels stained with 0.5 µg/ml ethidium bromide using 1x Tris-acetate-EDTA (TAE) buffer at 80 volts (Volts: Sambrook and Russell, 2001). The restricted DNA fragments of interest were excised from agarose gels on a Dark Reader® Transilluminator (Clare Chemical Research, Inc, Dolores, CO), imaged using the Syngene ChemiGenius Bio-imaging system and analysed with the GenSnap version 6.05 software (Syngene, Cambridge, England). The DNA was purified with the Qiagen gel purification kit (Qiagen, Hilden, German) by following the manufacturer's instructions.

The purified insert and vector DNA was quantified using a ND-1000 Spectrophotometer (NanoDrop Products, Wilmington, DE). Ligation reactions were carried out to prepare the recombinant plasmid DNA by covalently joining the vector and insert DNA. The DNA fragments of the ORFs coding for the rotavirus proteins were inserted downstream of the polh (VP7) and p10 (VP4) promoters that regulated their expression. In brief, a 3:1 of insert to vector molar ratio was used in a 20 µl ligation reaction containing 1x T4 DNA ligase buffer, 0.6 U T4 DNA ligase (Takara Bio Inc., Shiga, Japan) and nuclease free water. The mixture

was incubated at 4 ^{o}C for 48 h. The required amounts of insert and vector DNA were calculated using the formula below (from the subcloning notebook, Promega Corporation, Madison, WI):

$$\frac{\text{X ng vector x kb size of insert}}{\text{size of vector (kb)}} \times \text{molar ratio} \left\{\frac{\text{insert}}{\text{vector}}\right\} = \text{ng of insert}$$

The ligation reaction was then transformed into chemically competent JM109 cells as described in section 6.2.3. The colonies were inoculated in 50 ml LB media containing 50 μg/ml ampicillin. The plasmid DNA was isolated from the culture with the Pure Yield™ plasmid Midiprep System kit (Promega Corporation, Madison, WI) by following the manufacturer's instructions. Stocks were prepared as described in section 6.2.3. Plasmid DNA was quantified using a ND-1000 Spectrophotometer (NanoDrop Products, Wilmington, DE).

Following preparation of *in silico* plasmid maps (see Appendix B), RE digestion reactions were performed to determine if cloning was successful. The plasmid DNA was then double digested with two different REs. The correct sizes of the inserts were verified by digesting with REs that were used to construct the recombinant pFBq expression construct. The proper ligation of the pFBq + insert (s) and their correct sizes was checked by digesting with RE that cleaves inside the nucleotide sequences of both insert and vector DNA. The fidelity of the nucleotide sequences of the inserts were verified through Sanger sequencing at the Central Analytical DNA Sequencing Facility (Stellenbosch University, South Africa). The flanking primers listed in Table 6.2 were used to sequence the 5′- and -3′ ends of the insert DNA (coding regions of VP4 and VP7). Since Sanger sequencing only produces useful data for around 700 bp, internal primers were designed using DNASTAR® Lasergene™ software, version 8.1.2 (DNASTAR, Inc. Madison, WI) to verify the sequence of the whole ORFs encoding the VP4 (2343 bp), Fig. 6.3 and Table 6.2.

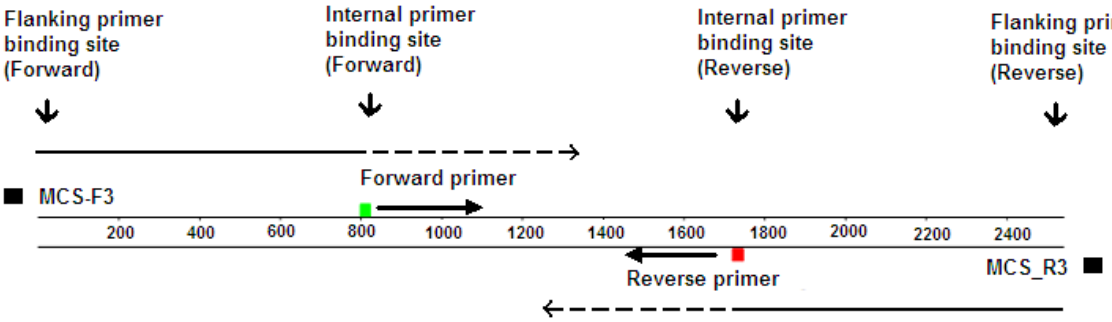


Fig. 6.3. Schematic diagram of the sequence strategy employed to verify the VP4 protein ORF coding region. The regions indicated with an unbroken straight line represent the regions determined with MCS_F3 and MCS_R3. The broken lines represent regions determined with forward primer (green) and reverse primer (red). Black rectangles represents binding site for flanking primers used for sequencing the outer region. The flanking primers targets the nucleotide sequences of the pFBq flanking the nucleotide sequence of the cloned insert. The arrows indicate direction of sequencing. The names and nucleotide sequences of the primers are provided in Table 6.3.

6.2.5. Transposition of the ORFs encoding VP4 and VP7 cloned in pFBq donor plasmids into DNA of the baculovirus shuttle vector (bacmid)

Various pFBq expression cassettes were transformed into AcBAC∆CC *E.coli* cells (Kaba et al., 2004) to produce recombinant bacmids. The transposition sites Tn7R and the Tn7L on the pFBq expression plasmid and the attachment site, mini-*att*Tn7, on the bacmid facilitated site-specific transposition (Luckow et al., 1993). With minor variations to the procedure described in section 6.2.3, chemically competent AcBAC∆CC *E. coli* cells were transformed as described in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY) to generate recombinant bacmids, Fig. 6.4. For example: (i) LB-agar plated contained three antibiotics; 50 µg/ml kanamycin, 10 µg/ml tetracycline and 7 µg/ml gentamicin were used; (ii) LB media plates contained 40 µg/ml Isopropyl β-D-1-thiogalactopyranoside (IPTG) inducer to trigger transcription of the *LacZ* operon; (iii) LB media plates contained 40 µg/ml X-gal substrate to enable determination of colonies transformed with the recombinant DNA (Hansen et al., 1998); and (iv) the transformation reaction plated on LB agar plates was incubated for 48 h.

Blue/white colony selection was used to identify AcBAC∆CC *E. coli* cells that acquired the pFBq expression plasmids as instructed in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY). Since restriction analysis is difficult to perform on large DNA, colonies containing recombinant bacmid DNA were screened using PCR as follows: a colony was suspended in 25 µl PCR reaction mixture containing 1x Ex-Taq buffer, 1.2 µM

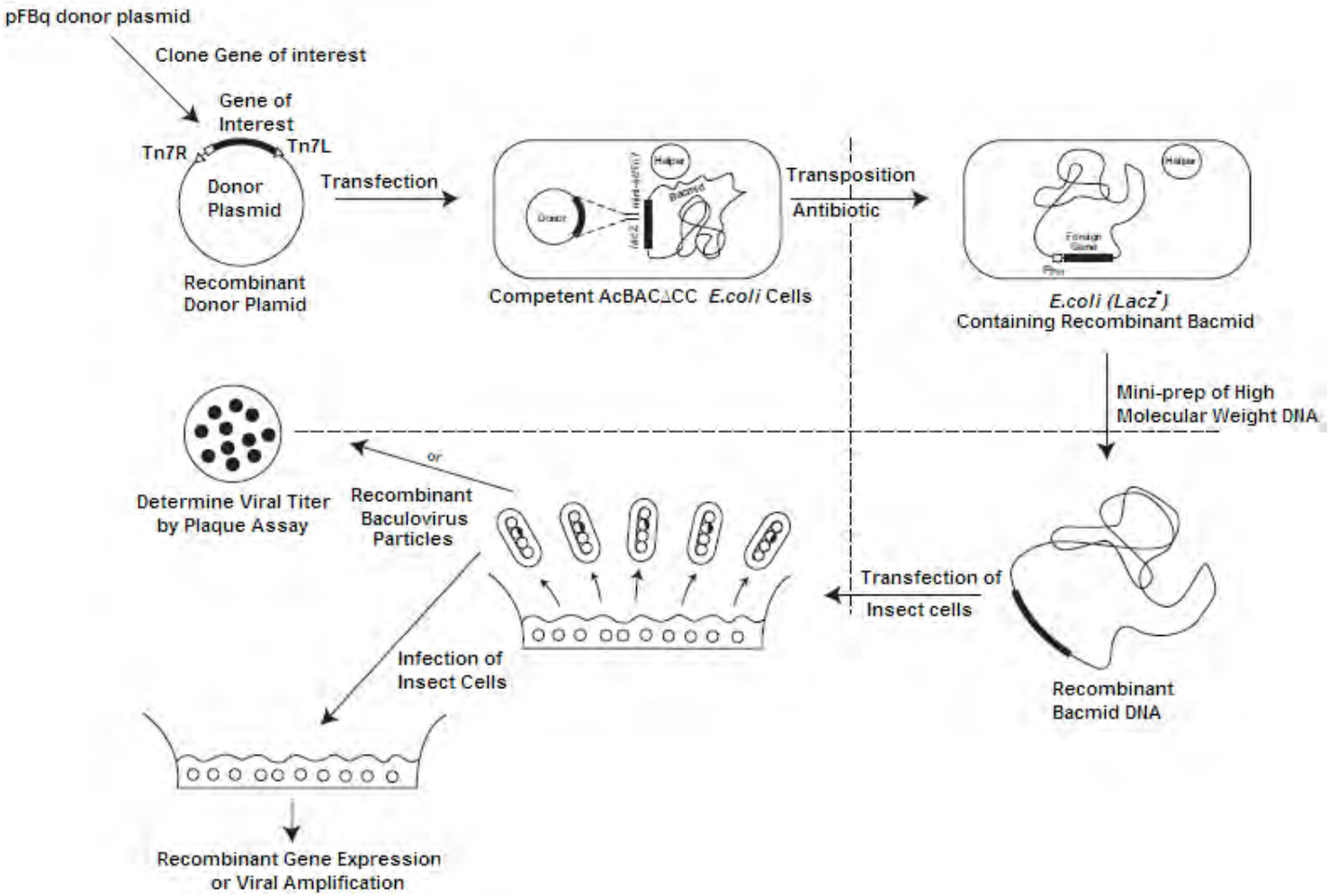
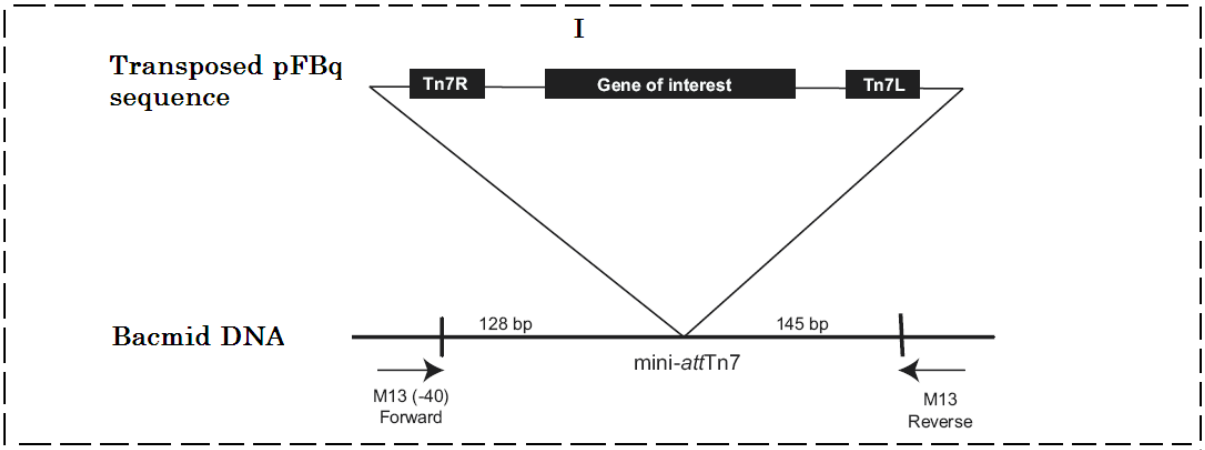


Fig. 6.4. Diagram of the Bac-to-Bac® System. The figure above depicts the generation of recombinant baculovirus and the expression of the gene of interest using the Bac-to-Bac® Baculovirus Expression System. [Bac-to-Bac® Baculovirus Expression System manual: an efficient site-specific transposition system to generate baculovirus for high-level expression of recombinant proteins. Catalog nos. 10359-016 (Life Technologies, Grand Island, NY)].

M13F primer, 1.2 µM M13R primer, 2.5 U Taq polymerase (Takara Bio Inc., Shiga, Japan) and nuclease free water. The PCR steps recommended in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY) were modified as follows: an initial boiling step at 94 °C for 10 min was included to liberate plasmid DNA by lysing the bacterial cell wall. This was followed by 25 cycles of denaturation at 94 °C for 45 sec, annealing at 55 °C for 45 sec and elongation at 72 °C for 5 min. A final extension step of 72 °C for 10 min was included. The expected sizes were calculated as described in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY), Fig. 6.5. Five positive colonies per construct were each inoculated in 4 ml LB media containing 50 µg/ml kanamycin, 10 µg/ml tetracycline and 7 µg/ml gentamicin. The culture was incubated for 12 – 16 h at 37 °C shaking at 200 revolution per minute (rpm). The high molecular weight bacmid DNA was isolated using the BACMAX™ DNA purification kit (Epicentre® Biotechnologies, Madison, WI) by following the manufacturer's instructions. Bacmid DNA was quantified using a ND-1000 Spectrophotometer (NanoDrop Products, Wilmington, DE).



II

Sample	Size of PCR Product
Bacmid alone	~300 bp
Bacmid + pFBq + gene of interest	~2500 bp + 300 bp + size of insert
Bacmid + pFBq + VP4 encoding gene segment	~5100 bp
Bacmid + pFBq + VP7 encoding gene segment	~3800 bp
Bacmid + pFBq + VP4 +VP7 encoding gene segments	~6100 bp

Fig. 6.5. **Schematic representation of the expected sizes of transposed bacmid DNA**. **I**) The M13 forward (-40) and M13 reverse priming sites flanking the mini-*att*Tn7 site within the *lacZα*-complementation in the bacmid that facilitates PCR analysis. **II**) The expected PCR product of the bacmid DNA transposed with pFBq expression cassettes, Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY).

6.2.6. Transfection of insect cells

With minor variations, the procedures described in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY), Fig. 6.4, were followed to transfect Sf9 cells with each of the five bacmid DNA isolated for each pFBq construct. Instead of the recommended Cellfectin® transfection reagent, FuGENE 6 or X-tremeGENE (Roche Diagnostics, Mannheim, Germany) was used per manufacturer's instructions. In brief, the transfection complex was prepared by combining 3 µl transfection reagents with 97 µl TC-100 serum-free media (Sigma-Aldrich, St. Louis, MO). Following incubation for 5 min, 1µg of purified recombinant bacmid DNA was added to form the transfection complex. This was followed by incubation at room temperature for 45 min. The complex was then added to Sf9 cells seeded 1 h earlier in 6-well plates (NUNC, Roskilde, Denmark) at density of 1 x 10^6 cells/ml. The 6-well plates were incubated at 28 ^oC until the cytopathic effect (CPE) was complete. The transfection supernatant was harvested from each well, centrifuged at 500 x *g* for 2 min, and the supernatant (P1 viral stock) was stored at 4 ^oC for infection experiments. Sf9 cells were maintained in complete TC-100 medium (Sigma-Aldrich, St. Louis, MO) containing 10% foetal bovine serum (FBS), 1% antibiotic (streptomycin/penicillin, Lonza, Wakersville, MD) and 1% fungizone (amphothericin B, Lonza, Wakersville, MD). The Sf9 shaker cultures were maintained in the presence of 0.1% Pluronic® F-68 solution (Sigma-Aldrich, Ayrshire, KA), which is a surfactant, to minimise shearing of the cell membranes.

6.2.7. Infection of insect cells with baculoviruses to verify expression of recombinant rotavirus proteins

Sf9 cells were seeded at density of 1 x 10^6 cells/ml in 6-well plates and infected with 400 µl P1 viral stocks followed by incubation at 28 ^oC until CPE was complete. The supernatant, P2 viral stock, collected from the harvested culture after centrifugation at 4 ^oC for 2 min at 500 x *g*, was used to purify and amplify the viral stocks. The cell pellet was lysed for 1 h with 200 µl of cell lysis buffer 1 [0.5% NP40 in 1x phosphate-buffered saline (PBS) containing complete mini-EDTA free protease inhibitors, (Roche Diagnostics, Mannheim, Germany)]. The expressed proteins were visualised by using SDS-PAGE or western blot analysis as indicated in the text, see section 6.2.7.1 and 6.2.7.2 below.

Agarose overlay plaque assays were used to purify and determine the titre of the virus stocks as described in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island,

NY). A total of ten plaques per recombinant baculovirus were each diluted in 1 ml complete TC-100 media (Sigma-Aldrich, St. Louis, MO) which was used to infect Sf9 cells at density of 1 x 10^6 cells/ml. The cultures were harvested after CPE was complete as described above for preparing P1 stocks. The pellet was used to screen for plaque-purified viral stocks capable of expressing the expected recombinant rotavirus proteins using SDS-PAGE or western blot analysis. The supernatant collected from plaque-purified cultures were used to amplify the viral stocks as described for producing P2 viral stocks above. Higher volumes of plaque-purified viral stocks that were used to produce RV-VLPs were prepared by infecting Sf9 cells at 1 x 10^6 cells/ml in 50 ml shaker cultures with 600 µl purified baculovirus stock followed by incubation at 28 ^oC under shake at 98 rpm until CPE was complete. The titres of the plaque-purified stocks were also determined using agarose overlay plaques assays as described above. Expression of recombinant rotavirus proteins by the purified baculoviruses was also verified by harvesting cell pellets from each virus stock followed by performing SDS-PAGE and western blot analysis as described above.

6.2.7.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

The method of Laemmli (1970) described in Sambrook and Russell (2001) was used to separate 14 µl of the cell lysate on SDS-PAGE gels which were made of resolving and separating gels with a final acrylamide concentration of 10% and 3.9%, respectively. Detection of the recombinant proteins was made possible after staining of the SDS-PAGE gels with Coomassie blue.

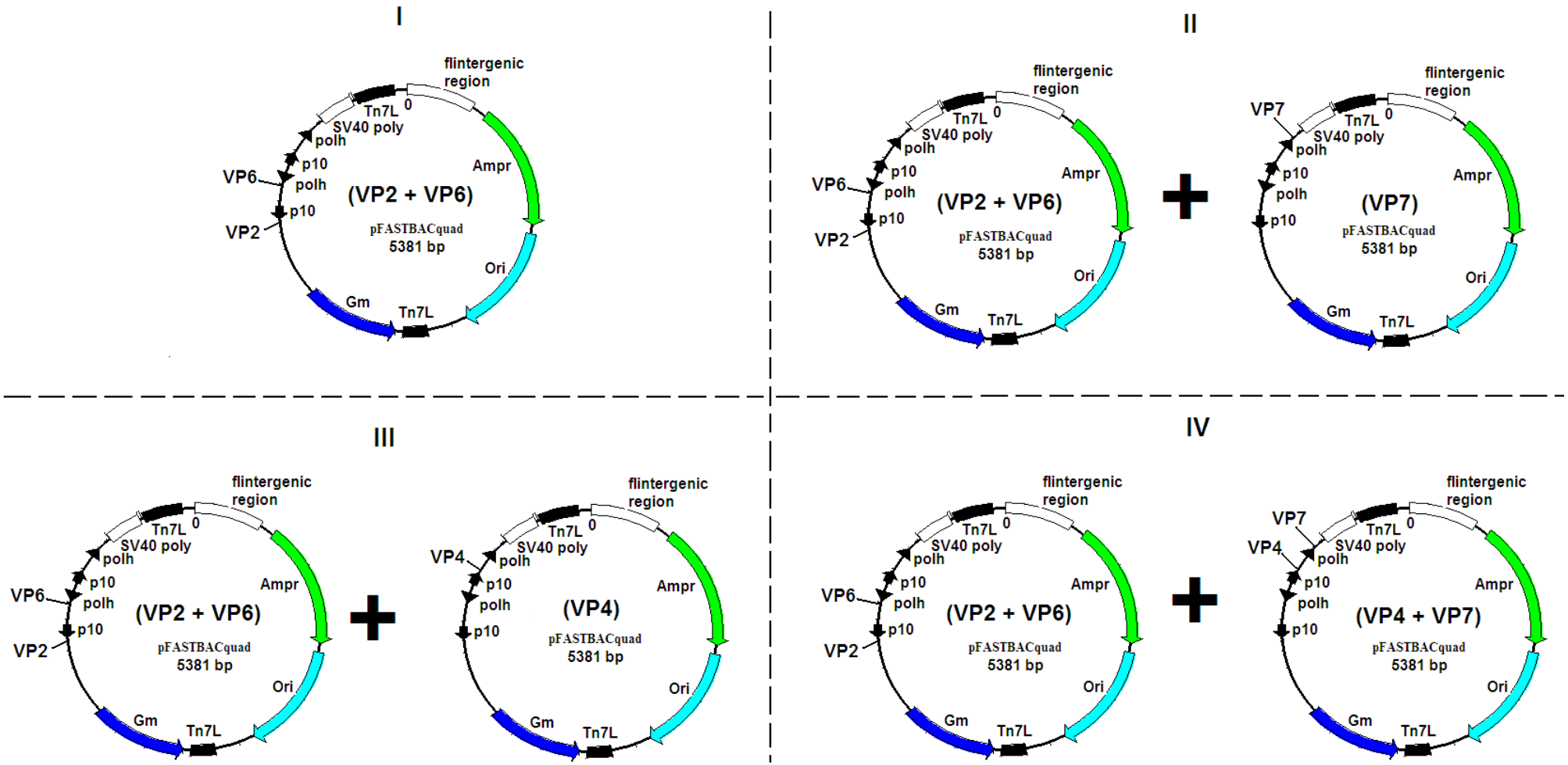
6.2.7.2. Western blot analysis

Western blot analysis was used to verify expression of the recombinant proteins, especially VP7. In brief, before staining the SDS-PAGE gels with Coomassie blue (see section 6.2.7.1), the proteins were electro-blotted onto the nitrocellulose membrane (Whatman® GmbH. Dassel, Germany) submerged in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100 V for 1 h. Transfer of proteins was verified by staining the membranes with Ponceau S solution (Sigma-Aldrich, St. Louis, MO). The membranes were blocked with 5% non-fat skim milk [Nestle (Pty) Ltd, Randburg, South Africa] in TNT buffer [0.05% Tween, 0.2 M NaCl and 0.05 M Tris-HCl (pH 7.4)] for 3.5 h at 4 ^oC. Then the membranes were washed three times with TNT buffer, and incubated for 8 h at 4 ^oC with goat polyclonal anti-rotavirus antibody prepared against rotavirus Nebraska calf diarrhoea virus (NCDV) antibody

(Biotin) (ab69560) (Abcam, San Francisco, CA) which was diluted to 1:1000 ratio in TNT buffer. The membranes were washed three times with TNT buffer followed by incubation in secondary antibody, donkey horseradish peroxidase conjugated anti-goat IgG (Abcam San Francisco, CA) diluted 1:500 in TNT buffer for 1 h. The membranes were washed three times with TNT buffer, followed by incubation until bands were visible in 4-chloro-1-naphthol which is a peroxidase substrate that produces an insoluble dark blue end product that can be observed visually. This substrate was prepared by dissolving 1 tablet of 4-chloro-1-naphthol (Sigma-Aldrich, St. Louis, MO) in 10 ml of methanol (Merck, Darmstadt, Germany) as stock solution. Then 2 ml of the methanol stock solution was added to 10 ml PBS, pH 7.5. This was followed by adding 5 µl of fresh 30% hydrogen peroxide (Sigma-Aldrich, St. Louis, MO) immediately prior to use.

6.2.8. Co-infection of insect cells with viral stocks to produce chimaeric RV-VLPs and gradient fraction purification

RV-VLPs were produced in Sf9 cells maintained in Ex-Cell™ TitreHigh Medium (SAFC Biosciences™, Lenexa, KS) containing 10% FBS (Gibco®, Life Technologies, Grand Island, NY), 1% Penicillin/Streptomycin/Amphotericin B (Lonza, Wakersville, MD), 0.1% Pluronic F-68 solution (Sigma-Aldrich, Ayrshire, KA) and 0.5 – 1 µg/ml Leupeptin or Aprotinin protease inhibitors (Roche Diagnostics, Mannheim, Germany). High Five® cells were maintained in Express 5 serum-free medium (Gibco®, Life Technologies, Grand Island, NY) containing 0.6 mg/L L-glutamine (Sigma-Aldrich, Ayrshire, KA), 10 µg/ml gentamycin (Sigma-Aldrich, Ayrshire, KA) and 0.5 – 1 µg/ml Leupeptin or Aprotinin protease inhibitors (Roche Diagnostics, Mannheim, Germany). The infected cultures were incubated at 28 °C at 96 rpm until CPE reached approximately 95% (between 5 – 6 days). A MOI of 2 was used to infect Sf9 cell with each of the plaque-purified viral stocks confirmed to express rotavirus proteins. To produce the G9P[6] dRV-VLPs (VP2/6) backbone to be used in this investigation, insect cells at density of 1 x 10^6 cells/ml in 100 ml were infected with viral stocks (recombinant baculoviruses prepared by Dr H.G. O'Neill, see section 6.2.1) confirmed in the current study to simultaneously express VP2 and VP6 proteins, Fig. 6.6.I. Different strategies were used to produce chimaeric tRV-VLPs. tRV-VLPs consisting of VP2/6/7 protein layers were produced by simultaneously co-infecting Sf9 or High Five® cells with dualcistronic recombinant baculoviruses that were verified to express VP2 and VP6 and monocistronic recombinant baculoviruses confirmed to express VP7, 6.6.II. To produce RV-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flintergenic region
Tn7L
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SV40 poly
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p10
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p10
VP6
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(VP2 + VP6)
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VP6
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(VP2 + VP6)
pFASTBACquad
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Tn7L
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SV40 poly
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p10
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(VP2 + VP6)
pFASTBACquad
5381 bp
Ampr
Ori
Gm
Tn7L
+
flintergenic region
VP4
Tn7L
0
SV40 poly
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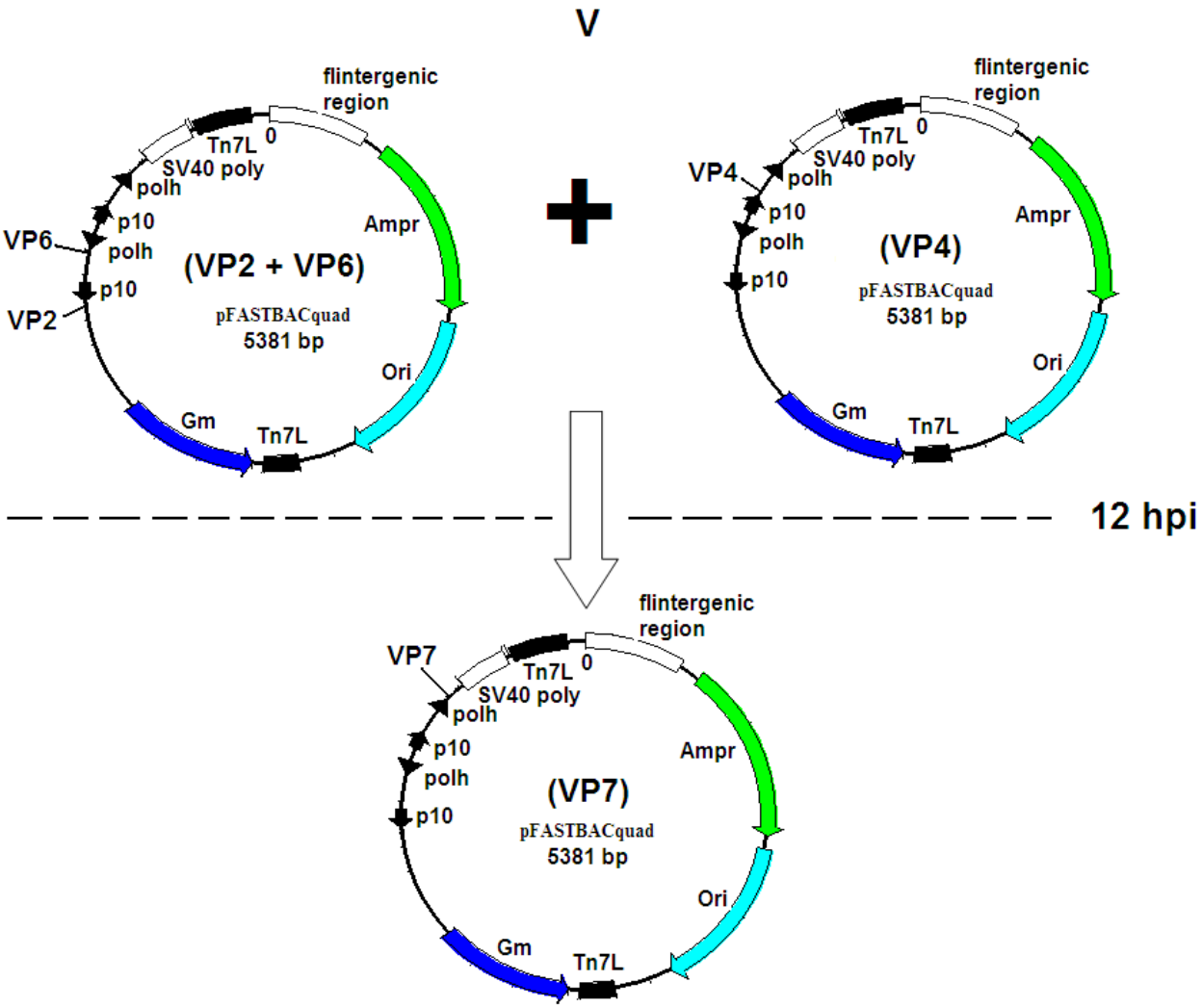


Fig. 6.6. Schematic presentation of the baculovirus expression strategies used to generate RV-VLPs. The transfer plasmids contains ORFs coding for specific rotavirus proteins (labelled downstream to the promoters regulating their expression as described in the text) that were transposed into bacmids which were subsequently used to generate baculoviruses. The restriction sites are not indicated for REs on the pFBq plasmids, see Fig 6.1 for more details. (**I**) pFBq plasmid construct used to generate recombinant dualcistronic baculoviruses that was used to prepare dRV-VLPs (VP2/6) through single infection of insect cells. (**II**) pFBq plasmid constructs used to generate recombinant dualcistronic and monocistronic baculoviruses that were used to prepare tRV-VLPs **(VP2/6/7)** through simultaneous infection of insect cells. (**III**) pFBq plasmid constructs used to generate recombinant dualcistronic and monocistronic baculoviruses that were used to prepare tRV-VLPs **(VP2/6/4)** through simultaneous infection of insect cells. (**IV**) pFBq plasmid constructs used to generate recombinant dualcistronic baculoviruses that were used to prepare tRV-VLPs **(VP2/6/7/4)** through simultaneous infection of insect cells. (**V**) pFBq plasmid constructs used to generate recombinant dualcistronic and monocistronic baculoviruses that were used to prepare tRV-VLPs **(VP2/6/7/4)** through step-wise co-infection strategy. Insect cells were initially infected with dualcistronic baculoviruses confirmed to express VP2/6 and recombinant monocistronic baculoviruses confirmed to express VP4. This was followed by infection with recombinant monocistronic baculoviruses confirmed to express VP7 12 hpi.

VLPs comprising of VP2/6/4, Sf9 or High Five® cells were simultaneously co-infected with recombinant baculoviruses confirmed to express VP2 and VP6 simultaneously with monocistronic recombinant baculoviruses confirmed to express VP4, 6.6.III. To produce RV-VLPs consisting of VP2/6/4/7, cells were either simultaneously co-infected with dualcistronic recombinant baculoviruses confirmed to express VP2 and VP6 proteins concurrently with recombinant dualcistronic baculoviruses confirmed to express VP4 and VP7, 6.6.IV; or co-infected simultaneously with dualcistronic recombinant baculoviruses confirmed to express VP2 and VP6 with monocistronic recombinant baculoviruses expressing VP4 as indicated in the text. This was followed by infecting the culture 12 hpi with monocistronic recombinant baculoviruses confirmed to express VP7 (step-wise infection), Fig. 6.6.V. Stepwise infection was opted to allow proper mounting of VP4 on the DLP prior to VP7 assembly as recommended by Trask and Dormitzer (2006).

The cells and medium were harvested 5 – 6 days post-infection (dpi) followed by centrifugation at 3,000 x *g* for 15 min at 4 °C. The cell pellet was resuspended in 10 ml lysis buffer 2 (10 mM Tris, 0.1 mM EDTA, 1% DOC, 0.1% SDS and protease inhibitors) and centrifuged at 3,000 × *g* for 15 min at 4 °C to remove cell debris. The supernatant recovered from culture and cells was layered on a 40% sucrose cushion prepared in Tris-calcium buffer (10 mM Tris-HCl [pH 7.4], 10 mM $CaCl_2$) followed by ultracentrifugation in a Sorvall WX Ultra Series centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) using a Surespin 630 rotor (36 ml) at 135,172 × *g* for 2 h at 4 °C. The supernatant was discarded and the pellet was resuspended in 400 µl Tris-calcium buffer of which 100 µl was applied to the top of either 10% – 60% sucrose or iodixanol gradients prepared in Tris-calcium buffer (Patient et al., 2009, Nielsen et al., 2006). The samples were centrifuged for 1 h at 106401 × *g* in a Sorvall TH-660 rotor (Thermo Fisher Scientific Inc., Waltham, MA) at 4 °C. Another 100 ul of the pellet suspension was mixed with cesium chloride (CsCl) dissolved in Tris-calcium buffer of which its density was adjusted to a refractive index of 1.368 followed by ultracentrifugation at 166251 × *g* at 15 ^{o}C for 16 h. The bands containing RV-VLPs were visualised with the Gradient Station *ip* fractionator (BioComp Instruments Inc, New Brunswick, Canada) which was also used to collect 18 fractions of 220 µl each from iodixanol and sucrose gradients by following manufacturer's instructions. The bands from CsCl gradients were collected with a syringe and dialysed with Slide-A-Lyzer G2 dialysis cassettes (Pierce Biotechnology, Inc, Rockford, IL) submerged in Tris-calcium buffer by following the manufacturer's instructions.

The protein concentration that constituted the RV-VLPs was determined using a BCA Protein Assay Reagent (Pierce, Rockford, IL) by following the manufacturer's instructions.

6.2.9. Verification of the production of chimaeric rotavirus-like particles using transmission electron microscopy (TEM)

To verify the production of RV-VLPs, the presence of the recombinant rotavirus structural proteins was determined by screening the gradient fractions using SDS-PAGE or western blot analysis as described in section 6.2.7. The fractions confirmed to contain RV-VLPs were pooled and washed by using Tris-calcium buffer followed by ultracentrifugation at 106401 × *g* for 1 h at 4 ^{o}C. The pellet was suspended in 100 µl Tris-calcium buffer. The structural integrity of the RV-VLPs was verified by negative staining with a TEM using 1% uranyl acetate stain (Kim et al., 2002). In brief, a 400-mesh, 0.5% Formvar-coated copper grid was placed on a 5 µl drop of RV-VLP sample in Tris-calcium buffer for 15 – 20 sec on a dental-wax sheet. The grid was drained of excess fluid by gently applying the torn edge of a piece of filter paper and immediately placed on a drop of 1% aqueous uranyl acetate dihydrate [$(CH_3COO)_2UO_2.2H_2O$; molecular weight = 424.15g/mol] (E. Merck, Darmstadt, Germany) for a further 15 – 20 sec. After draining excess stain from the grid, it was allowed to air dry before viewing using a Jeol JEM-1200 Mk-I (JEOL Ltd, Tokyo, Japan) operated at 80 kV. Micrographs were recorded at 60 kX magnification. This was followed by examination using a Carl Zeiss TEM (Carl Zeiss NTS GmbH, Oberkochen, Germany).

6.3. Results

6.3.1. Selection of rotavirus strains for preparing chimaeric rotavirus virus-like particles

To select rotavirus strains that were used for whole genome characterisation and preparation of chimaeric RV-VLPs, strains with specific VP4 and VP7 genotypes that were assigned to rotavirus strains present in human stool specimens at NICD and UL, Medunsa were considered. Rotavirus strains with VP4 genotypes (P[4, P[6] or P[8]) associated with VP7 genotypes that have been linked with lower vaccine efficacy (G2), or frequently detected in Africa (G8) or have emerged in the past two decades (G9 or G12) were selected (Table 6.1). TEM was used to screen the integrity of the rotavirus particles present in the human stool

specimens. Both DLPs and TLPs were visualised in most of the faecal samples. Only samples containing TLPs were selected. TLPs were identified based on the size (more than 75 nm in diameter) and morphology (smooth outer rims and spikes) of the rotavirus particles visualised in the stool samples. Five rotavirus strains (Table 6.1 and 6.2) were selected for 454® pyrosequencing to generate consensus nucleotide sequences of their genome segments as described in Chapters three and four. The electron micrographs of the selected human rotavirus strains viewed directly from human faecal samples are shown in Fig. 6.7. The codon optimised ORFs for insect cell expression coding for VP7 proteins with G2, G8, G9 and G12 genotypes were derived from strains RVA/Human-wt/ZAF/3203WC/2009/G2P[4], RVA/Human-wt/MWI/1473/2001/G8P[4], and RVA/Human-wt/ZAF/GR10924/1999/G9P[6] and RVA/Human-wt/ ZAF/3176WC/2009/ G12P[6], respectively, whereas ORFs coding for VP4 with P[4], [6] and [8] genotypes were derived from strains RVA/Human-wt/ZAF/3203WC/2009/G2P[4], RVA/Human-wt/ZAF/GR10924/1999/G9P[6] and RVA/ Human-wt/ZAF/2371WC/2009/G9P[8], respectively (Tables 6.2).

6.3.2. Codon optimisation for insect cell expression and design of pUC57 constructs containing rotavirus nucleotide sequences coding for the selected VP4 and VP7 proteins

Since ORFs coding for rotavirus proteins used in this study were derived from human rotavirus strains of which dsRNA was directly extracted from stool samples, the selected genome segments encoding VP4 and VP7, Table 6.2, were codon optimised for insect cell expression at GenScript to increase the yield of the mammalian rotavirus viral proteins in insect cells. To facilitate sub-cloning into the pFBq donor plasmid, the 5′- and -3′ ends of all codon optimised ORFs of the selected rotavirus proteins contained RE sites (see section 6.2.3). The amino acid sequence alignments derived from nucleotide sequences of the codon optimised and un-optimised (wild-type) coding regions for the selected VP4 and VP7 proteins were identical (data not shown). They were subsequently prepared for insertion into a series of expression pFBq donor plasmids as described below.

6.3.3. Preparation of recombinant pFastBACquad expression donor plasmids

As already mentioned, Dr. H.G. O'Neill cloned the coding regions for VP2 and VP6 from strain RVA/Human-wt/GR10924/1999/G9P[6] into pFBq under the control of p10 and polh promoters, respectively. This construct was used to express recombinant VP2 and VP6 that formed the DS-1-like VP2/6 backbone (dRV-VLP) of the chimaeric tRV-VLPs produced in

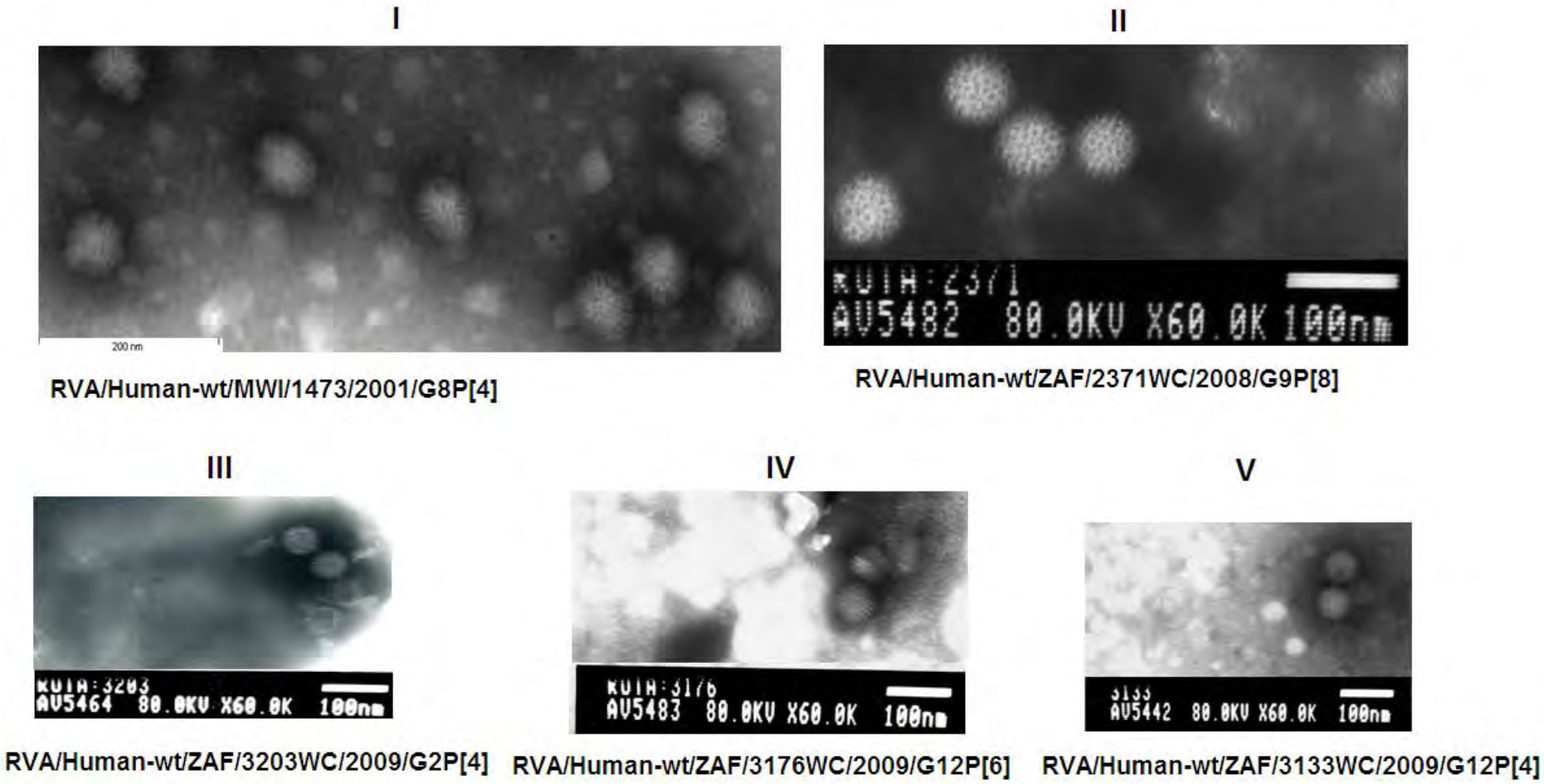


Fig. 6.7. Electron micrographs of rotavirus particles visualised from stool samples used for RNA extraction in this study.
Scale bar, 200 nm (I) and 100 nm (II – V).

this study. The ultimate goals of this project were to produce chimaeric tRV-VLPs and also evaluate the extent to which various combinations of VP4 and VP7 recombinant proteins would possibly assemble onto the G9P[6] (DS-1-like) dRV-VLPs backbone.

The pFBq expression cassettes were generated as donor plasmids (described in section 6.2.3) to facilitate transposition of ORFs coding for rotavirus outer proteins into recombinant baculovirus shuttle vectors that were subsequently used to generate baculoviruses for expression of rotavirus proteins in follow-up experiments. The ORFs coding for VP4 were sub-cloned into pFBq to be expressed under the control of p10 promoter, whereas ORFs coding for VP7 were under the control of the polh promoter. Sub-cloning of foreign DNA into the MCS of pFBq plasmid allowed their site specific-transposition into bacmid DNA.

To generate compatible inserts (ORFs encoding rotavirus proteins) and vectors (pFBq for cloning plasmid), the same REs were used in their preparation as described in Materials and Methods (section 6.2.4). *Spe* I and *Sma* I were used to sub-clone VP4 inserts, whereas *Bam* HI and *Not* I were used to sub-clone VP7 inserts (Table 6.2). The DNA fragments of the ORFs coding for the selected VP4 and VP7 proteins and the pFBq plasmid vectors were isolated, double-digested, purified and quantified as described in section 6.2.4. Fig. 6.8 shows that the expected DNA fragments were generated for both the vectors and inserts as predicted by *in silico* plasmid analysis, Appendix B. A total of 16 recombinant pFBq expression cassettes were generated by covalently ligating purified insert DNA to pFBq plasmid vector DNA as described in section 6.2.4, and listed in Table 6.4. Restriction enzyme analysis using *Bst* XI confirmed that the nucleotide sequences of the ORFs coding for VP4 and VP7 proteins were present in the plasmid as predicted by the *in silico* plasmid analysis. Fig. 6.9 is representative of the successful cloning indicating the expected sizes of the DNA fragments for four of the 16 expression constructs. *Bst* XI was used to screen the generated pFBq constructs because it cleaves at more than one RE site inside the nucleotide sequences of pFBq and the ORFs encoding G2, G8, G9, and G12 rotavirus proteins (see predicted DNA fragment sizes and RE cleavage position generated through *in silico* plasmid analysis in Appendix B). Not all the screened colonies exhibited the expected restriction DNA fragments. For example, restriction fragments in lanes not highlighted with asterisks in Fig. 6.9.III have non-specific DNA fragments. Only recombinant pFBq plasmids whose restriction patterns correlated with the predicted sizes generated by *in silico* plasmid analysis were transformed into and propagated in JM109 cells by culturing in LB media, isolated,

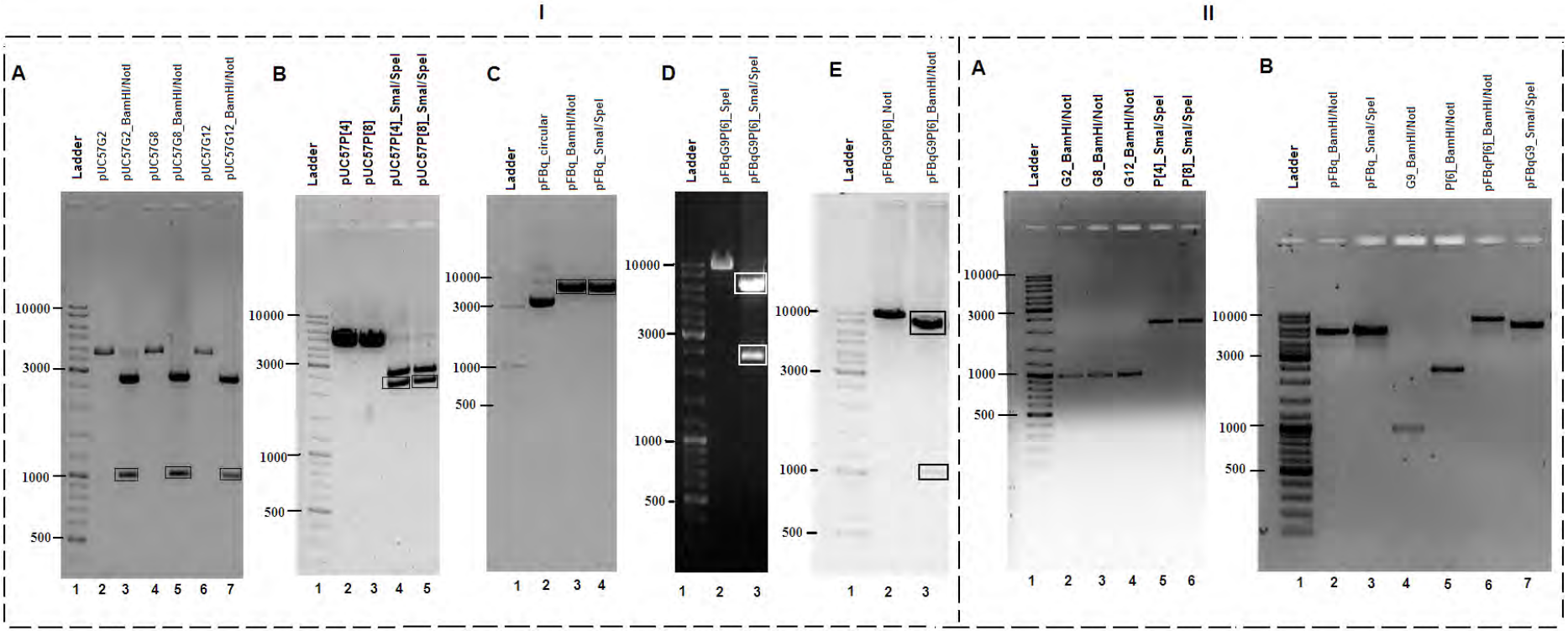


Fig. 6.8. Agarose gel analysis of prepared and purified DNA fragments from GenScript plasmids containing ORFs coding for VP4 (P[4] and P[8]) and VP7 (G2, G8 and G12) rotavirus proteins. (I) Double-digested DNA fragments of pUC57 and pFBq plasmids. **A**) Circular (lanes 2, 4 and 6) and restricted fragments of pUC57G2, pUC57G8 and pUC57G12 plasmid DNA double-digested with *Bam* HI and *Not* I (lanes 3, 5 and 7). **B**) Circular (lanes 2 and 3) and restricted fragments of pUC57P[4] and pUC57P[8] plasmid DNA double-digested with *Sma* I and *Spe* I (lanes 4 and 5). **C**) Circular (lane 2) and restricted fragments of pFBq plasmid DNA double-digested with *Bam* HI and *Not* I (lane 3), *Sma* I and *Spe* I (Lane 4). **D**) Linearised pFBqG9P[6] digested with *Spe* I (lane 2) and restricted fragments of pFBqG9P[P6] plasmid DNA double-digested with *Sma* I and *Spe* I (Lane 3). **E**) Linearised pFBqG9P[6] digested with *Not* I (lane 2) and restricted fragments of pFBqG9P[P6] plasmid DNA double-digested *Bam* HI and *Not* I (lane 3). **(II)** Gel-purified endonuclease restricted DNA fragments excised from boxed fragments in 6.7.I. **A)** Purified DNA fragments for nucleotide sequences coding for VP7 (991 bp) and VP4 proteins (2,335 bp). **B**) Purified pFBq and pFBqG9P[6] restricted DNA fragments: The DNA concentration of the purified fragments ranged from 30 – 90 µg/µl. The fragment sizes were 5,377 bp (lane 2), 5,377 bp (lane 3), 991 bp (lane 4), 2,335 bp (lane 5), 7,699 bp (lane 6) and 6,361 bp (lane 7). Lane 1 in all the gels; O'GeneRuler DNA Ladder Mix molecular marker (Fermentas Inc., Maryland, USA).

Table 6.4. A description of the pFastBACquad constructs prepared in this study from genomic data obtained from human faecal samples containing human African rotavirus strains.

Cloning strategy	pFBq constructs
Monocistronic: with one gene coding for VP7	**pFBqG2 [a], pFBqG8 [a], pFBqG12 [a,¥]**
Monocistronic: with one gene coding for VP4	**pFBqP[4] [b,¥], pFBqP[8] [b,¥]**
Dualcistronic: with two genes coding for VP4 and VP7	**pFBqG2P[6] [c,¥], pFBqG2P[4] [c], pFBqG2P[8] [c], pFBqG8P[4] [d], pFBqG8P[8] [d,¥], pFBqG12P[4] [e,¥], pFBqG12P[8] [e,¥], pFBqG8P[6] [f], pFBqG12P[6] [f,¥], pFBqG9P[4] [f,¥], pFBqG9P[8] [f,¥], pFBqG9P[6] [f,¥,†]**

[a] **pFBqG2, pFBqG8 and pFBqG12:** Generated by ligating coding regions for G2, G8 and G12 VP7 proteins excised from pUC57G2, pUC57G2 and pUC57G8 plasmids to pFBq vector DNA, respectively. Both insert and vector DNA were prepared by double-digestion with *Bam* HI and *Not* I.

[¥] VP4 and VP7 outer capsid proteins expressed by baculoviruses prepared from these expression cassettes were used to generate RV-VLPs in the current study. The VP2 and VP6 proteins that were prepared from strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6] formed the scaffolds on to which the outer capsid proteins were assembled.

[b] **pFBqP[4] and pFBqP[8]:** Generated by ligating coding regions for P[4] and P[8] VP4 proteins excised from pUC57P[4] and pUC57P[8] plasmids to pFBq vector DNA. The vector and insert DNA were prepared by digestion with *Sma* I and *Spe* I.

[c] **pFBqG2P[4], pFBqG2P[8]:** Generated by ligating the coding regions for P[4] and P[8] VP4 proteins excised from pUC57P[4] and pUC57P[8] plasmids to recombinant pFBqG2 expression cassettes, respectively. Both insert and vector DNA were prepared by double-digestion with *Sma* I and *Spe* I.

[d] **pFBqG8P[4], pFBqG8P[8]:** Generated by ligating coding regions for P[4] and P[8] VP4 proteins excised with *Sma* I and *Spe* I from pUC57P[4] and pUC57P[8] plasmids to the recombinant pFBqG8 expression cassettes, respectively. The vector was also prepared by restricting with *Sma* I and *Spe* I.

[e] **pFBqG12P[4],pFBqG12P[8]:** Generated by ligating the coding regions for P[4] and P[8] VP4 proteins excised from pUC57P[4] and pUC57P[8] plasmids to the recombinant pFBqG12 expression vector. Both the insert and vector were double-digested with *Sma* I and *Spe* I.

[f] **FBqG9P[4], pFBqG9P[8], pFBqG2P[6], pFBqG8P[6], pFBqG12P[6]:** Double-digestion of pFBqG9P[6] with *Sma* I and *Spe* I resulted in pFBqG9_*Sma*I/*Spe*I and P[6]_*Sma*I/*Spe*I DNA fragments. Double digesting pFBqG9P[6] with *Bam* HI and *Not* I resulted in pFBqP[6]_*Bam*HI/*Not*I and G9_*Bam*HI/*Not*I fragments, Fig. 6.7.IIB lanes 2 – 7. Recombinant pFBqG9P[4], pFBqG9P[8], pFBqG2P[6] expression cassettes were engineered by ligating coding regions for P[4] and P[8] VP4 proteins to the recombinant pFBqG9_*Sma*I/*Spe*I vector. Ligating G2_*Bam*HI/*Not*I, G8_*Bam*HI/*Not*I and G12_*Bam*HI/*Not*I fragments to recombinant pFBqP[6]_*Bam*HI/*Not*I vector resulted in pFBqG2P[6], pFBqG8P[6], pFBqG12P[6] expression cassettes, respectively.

[†] Expression construct prepared previously by Dr HG O'Neill.

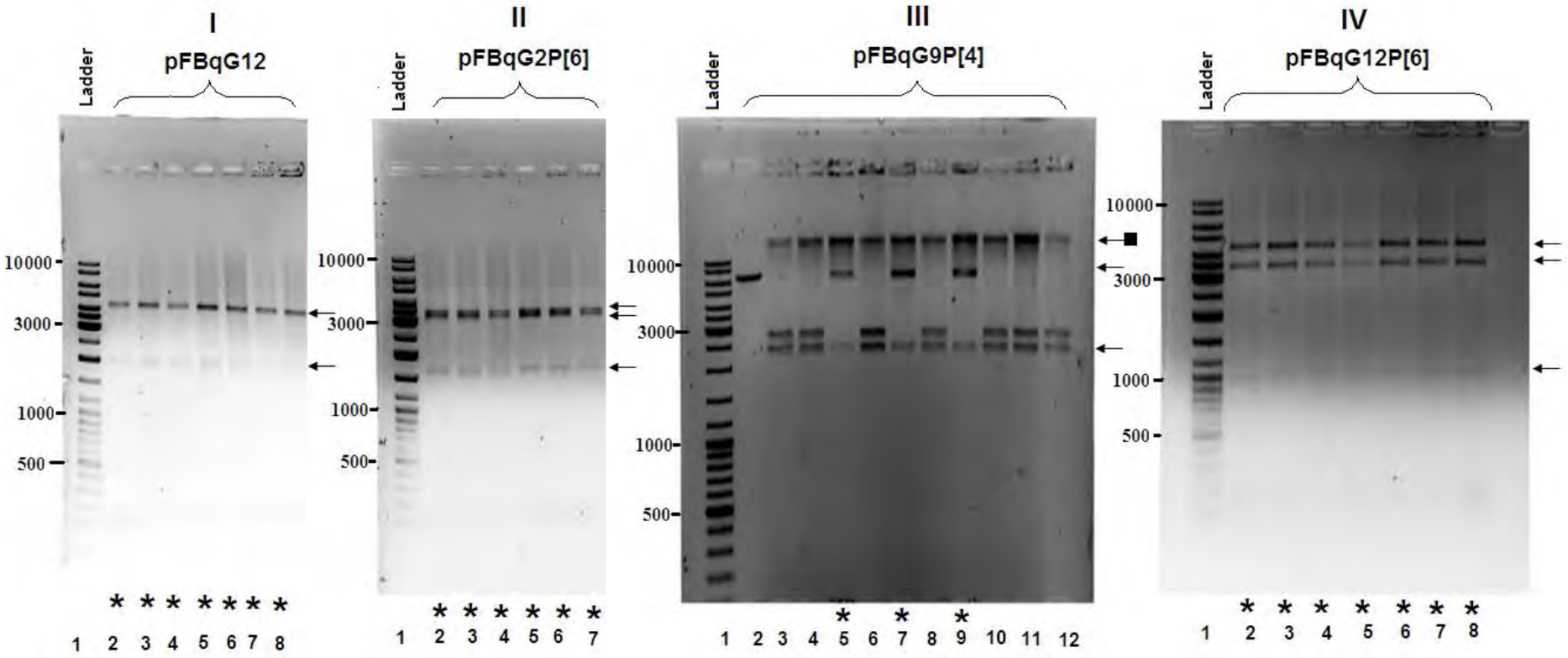


Fig.6.9. **Verification cloning of ORF (s) encoding selected VP4 and VP7 into the pFBq expression cassettes on 1% agarose gels**. The purified plasmid DNA was digested with *Bst* XI. (**I**) DNA fragments generated after digesting pFBqG12 expression construct. (**II**) DNA fragments generated after digesting pFBqG2P[6] expression construct. (**III**) DNA fragments generated after digesting pFBqG9P[4] expression construct. (**IV**) DNA fragments generated after digesting pFBqG12P[6] expression construct. Arrows point at the expected restriction DNA fragments. The arrow conjugated to a black square, points at linearised plasmid DNA. The lanes indicated with asterisks (*) represent colonies that contains pFBq + selected ORF (s) encoding VP4 and VP7 which were used to generate bacmids. The other lanes represent colonies that contained pFBq expression plasmids with incorrect pieces of DNA which were not used. Lane 1; O'GeneRuler DNA Ladder Mix molecular marker (Fermentas Inc., Maryland, MD).

purified and quantified as described in section 6.2.4. Between 200 – 1,200 µg/µl plasmid DNA was obtained for all the generated pFBq expression donor plasmid constructs. To confirm further the fidelity of the nucleotide sequences of the ORFs sub-cloned into pFBq plasmids, the purified recombinant pFBq plasmid DNA was sequenced using Sanger technology. MCS_F2 (forward) and MCS_R (reverse) flanking primers were used to sequence both ends of the ORFs coding for VP7 proteins. MCS_F3 (forward) and MCS_R3 (reverse) flanking primers were used to sequence the ends of the ORFs coding for VP4 proteins (Table 6.3). The flanking primers could only generate data of approximately 800 bp. Since nucleotide sequences of the ORFs coding for VP4 were 2343 bp, internal primers listed in Table 6.3 were designed for inner regions of the VP4 encoding ORFs, as described in section 6.2.4, were used to sequence the inner regions of the VP4 inserts. DNA sequencing of the cloned VP4 and VP7 inserts did not show nucleotide mismatches when compared to the codon optimised consensus nucleotide sequences (data not shown). This confirmed the presence of ORFs coding VP4 and VP7 proteins in the generated pFBq expression donor plasmids.

6.3.4. Generation and analysis of the recombinant bacmid DNA of 16 expression cassettes

To generate recombinant bacmid DNA containing ORFs coding for rotavirus proteins, *E. coli* host strain, AcBACΔCC, that contains a baculovirus shuttle vector and a helper plasmid, was transformed with 100 ng recombinant pFBq expression construct DNA as described in section 6.2.5. Blue/white selection was used to identify colonies containing recombinant bacmids. Since insertions of the mini-Tn7 of the pFBq expression plasmid into the mini-*att*Tn7 attachment site on the bacmid disrupt expression of the *LacZα* peptide (Luckow et al., 1993), colonies containing recombinant bacmid were white in the background of blue colonies that harboured unaltered bacmid. Therefore, the recombinant bacmid plasmid DNA generated by using the 16 recombinant expression pFBq constructs listed in Table 6.4 was isolated from white colonies. Plasmid DNA isolated from colonies in which transposition was unsuccessful (blue) were used as positive controls, designated here as wild-type.

PCR analysis of the bacmid DNA using M13 forward (-40) and M13 reverse primers as described in section 6.2.5 resulted in the following DNA fragments on agarose gels: the bacmid DNA transposed with pFBq containing ORFs coding for VP4s were approximately 5, 100 bp; bacmid DNA transposed with pFBq containing ORFs coding for VP7 proteins were

approximately 3, 800 bp; bacmid DNA transposed with pFBq containing ORFs coding for both VP4 and VP7 were approximately 6, 100 bp; whereas a 300 bp DNA fragment was obtained for bacmid DNA isolated from blue colonies [Fig. 6.10, the fragments generated from bacmid DNA containing rotavirus ORFs are shown with asterisks (*), whereas the bacmids that did not transpose are in the last lanes on each gel]. The bacmids transposed with pFBq without inserts (empty) were approximately 2, 500 bp (data not shown). These sizes correlated with the expected PCR fragments predicted in the Invitrogen® Bac-to-Bac® BEVS manual (Life Technologies, Grand Island, NY) as described in section 6.2.5. Therefore, for each bacmid construct, five white colonies confirmed to contain recombinant bacmid DNA and one wild-type blue colony (non-recombinant bacmid) were selected and used to inoculate LB media containing the appropriate antibiotics as described in section 6.2.5. The high molecular weight bacmid DNA for each of the 16 recombinant baculovirus shuttle vectors were isolated and the concentrations ranged from 600 – 1,300 µg/µl. DNA from these bacmids were used to transfect insect cells.

6.3.5. Generation of recombinant baculoviruses expressing rotavirus structural proteins (VP2, VP4, VP6 and VP7)

Recombinant baculoviruses were generated by transfecting Sf9 cells with purified bacmid DNA as described in section 6.2.6. Complete CPE was observed six to seven dpi (~150 hpi). Altogether 16 recombinant baculoviruses were generated and were confirmed to express recombinant rotavirus. Fig. 6.11 and 6.12 shows selected examples for the expression of various rotavirus proteins by these recombinant baculoviruses using SDS-PAGE and western blot as described in section 6.2.7 : VP4: Fig. 6.11.I, lanes 2 – 6; Fig. 6.11.II, lanes 5 and 6; VP7: Fig. 6.12.IB, lanes 2 – 6; both VP4 and VP7: Fig.6.11.III, lanes 2 – 6; Fig. 6.11.IV, lanes 2 – 5; Fig. 6.11.V, lanes 2 – 6; Fig. 6.12.IIA and B, lanes 2 – 6; Fig. 6.12.IIIA and B, lane 4. The various combinations of the generated baculoviruses are summarised in Table 6.4. Baculovirus titres ranging from 1 x 10^7 to 1 x 10^9 pfu/ml were determined using agarose-plaque assays. The ability of the individual purified, titred and amplified recombinant baculoviruses to produce VP4 and VP7 was also confirmed as depicted in Fig. 6.12.IIIA and 6.12.IIIB, lanes 2 – 4, for example. The ability of the recombinant VP2/6 baculoviruses to express VP2 and VP6 was also demonstrated as shown in Fig. 6.12.IIIA and 6.12.IIIB, lanes 5. In summary, the recombinant rotavirus proteins were expressed in Sf9 cells with recombinant baculovirus stocks between five to six dpi (~140 hpi). Recombinant VP2, VP4 and VP6 of approximately 100 kDa, 87 kDa and 45 kDa, respectively, were visualised on

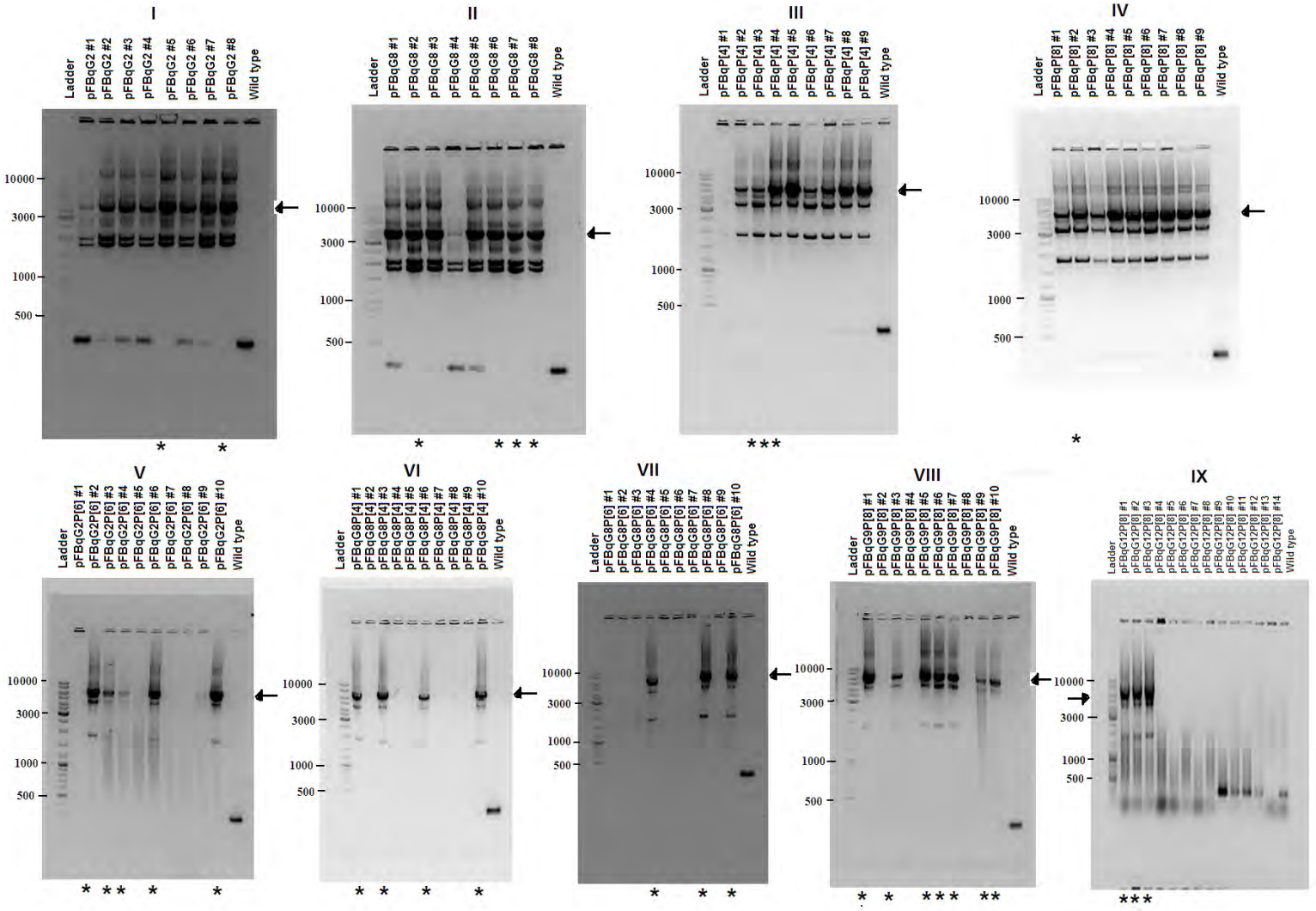


Fig. 6.10. Agarose gel electrophoresis confirming transposition of expression cassettes from donor pFBq plasmids into the respective bacmid DNA. I – IV represents bacmid expression cassettes engineered for preparing monocistronic baculoviruses for expression of either VP4 or VP7 proteins, whereas **V – IX** are representing dualcistronic baculoviruses for expression of both VP4 and VP7. Genotypes of the nucleotide sequences of genome segments 4 and 7 used to prepare the expression pFBq constructs are included in the name of each expression cassette. The arrows point at the expected bands. Stars below the gels indicate colonies that contained recombinant bacmid DNA. The last lane on each gel represents the empty bacmid (wild-type). The first lane on each gel contains an O'GeneRuler DNA Ladder Mix molecular marker (Fermentas Inc., Maryland, MD).

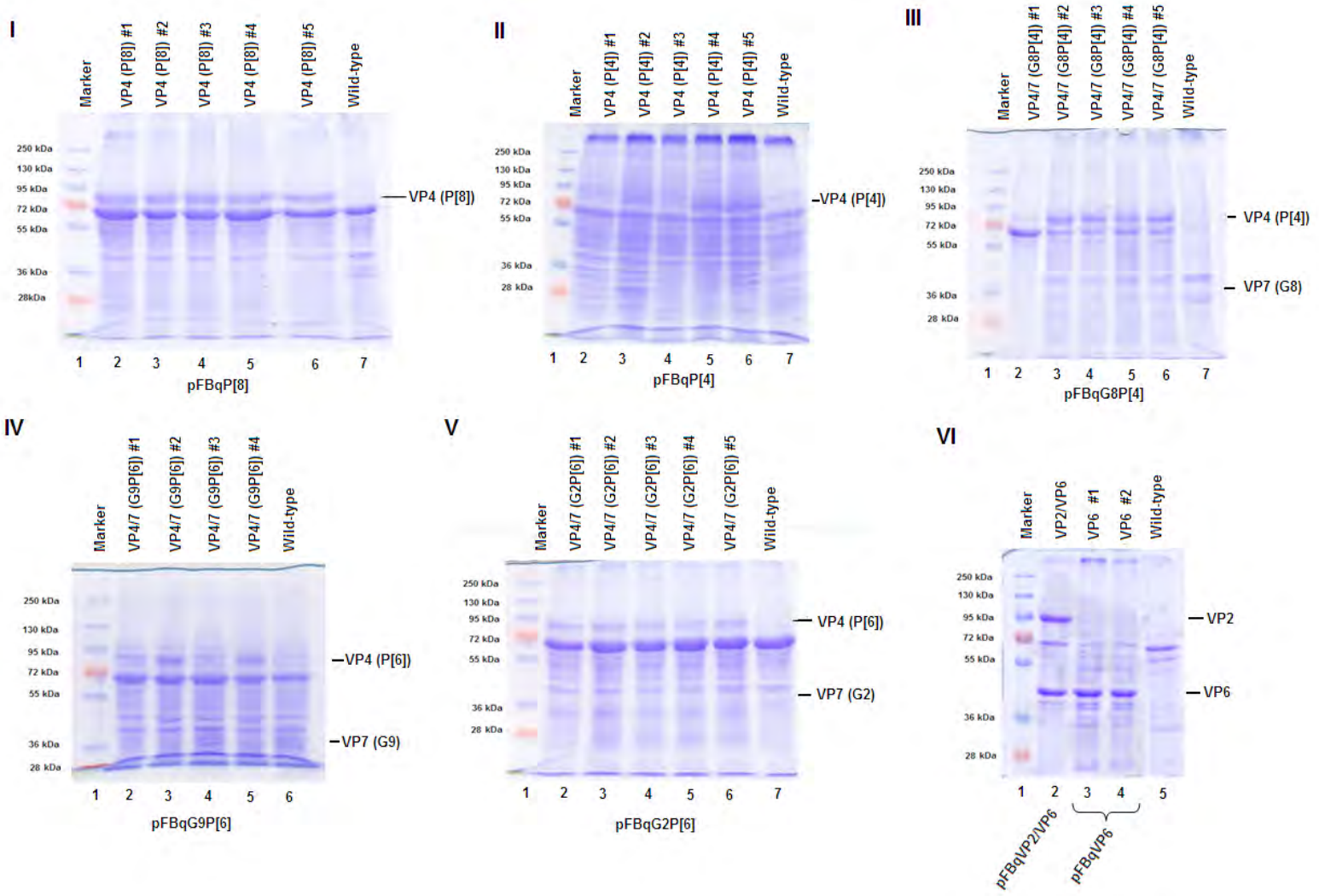


Fig 6.11. SDS-PAGE gels of recombinant rotavirus proteins expressed by recombinant baculoviruses. (**I** and **II**) Expressed recombinant VP4 with P[4] and P[8] genotypes, respectively. (**III, IV** and **V**) VP4 with P[6] genotypes expressed by recombinant dualcistronic baculoviruses containing genes coding for VP4 and VP7. Expressed VP7 was not visible. (**VI**) Recombinant VP2 and VP6 (lane 2) expressed by dualcistronic baculoviruses containing VP2 and VP6 encoding ORFs, and recombinant VP6 (lanes 3 and 4) expressed by monocistronic baculoviruses containing the VP6 encoding ORF. #1, #2, #3, #4 and #5 designate plaques purified from the same construct on each gel. Lane 1. Ladder, PageRuler™ Plus Prestain Protein Ladder (Fermentas UAB**,** Vilnius, Lithuania). Wild-type, empty baculovirus used as a positive control.

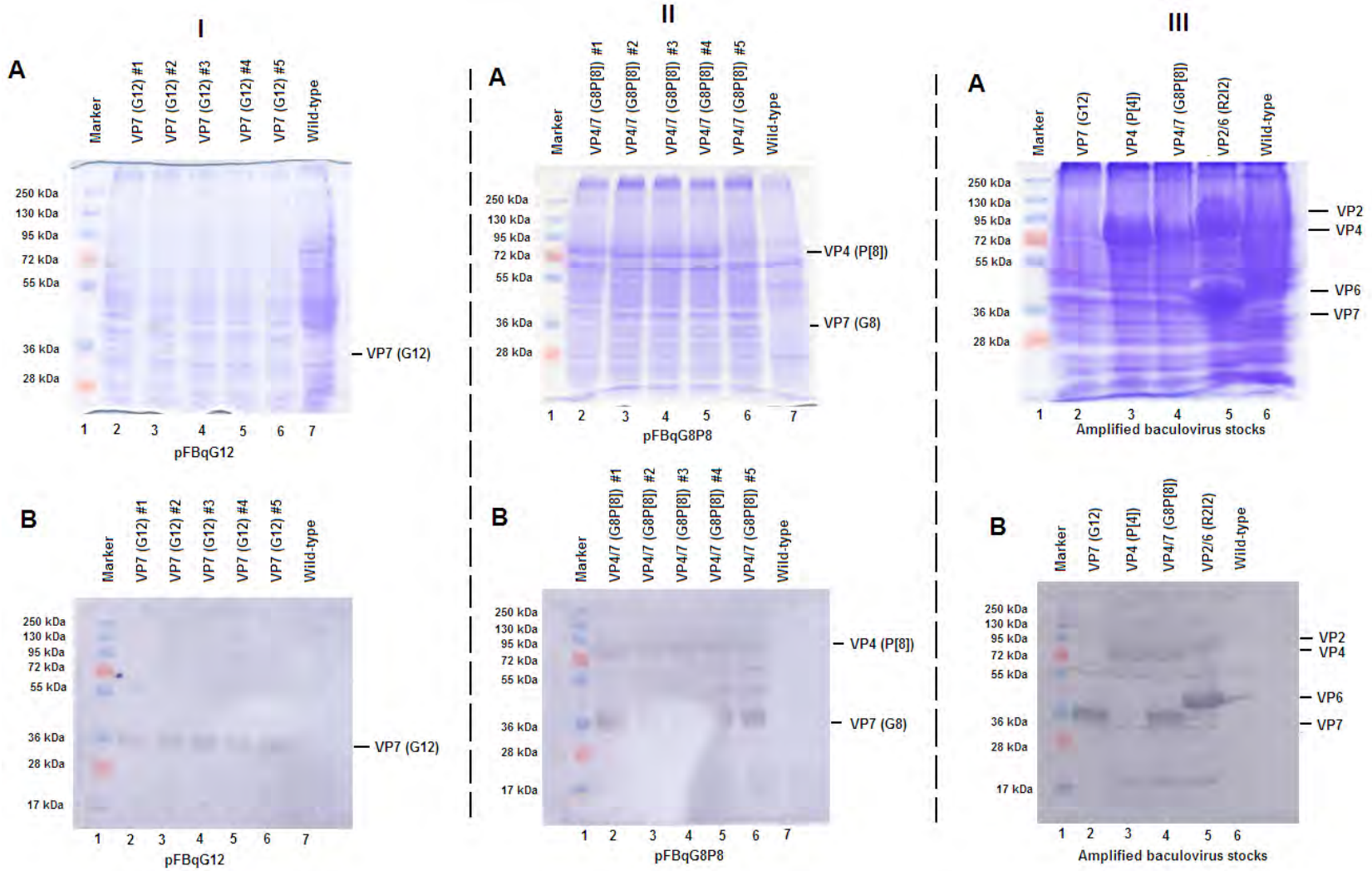


Fig. 6.12. Evaluation of expression of VP7 by recombinant baculoviruses as indicated by SDS-PAGE (A) and western blot analysis (B) in duplicates. (**I**) Recombinant VP7 expressed by five plaque-purified baculoviruses that contain VP7 encoding ORFs. (**II**) Recombinant VP4 and VP7 expressed by five plaque-purified baculoviruses that contain VP4 and VP7 encoding ORFs. (**III**) Recombinant VP7 (lane 2), VP4 (lane 3), VP4 and VP7 (lane 4), VP2 and VP6 (lane 5) expressed by amplified recombinant baculoviruses containing rotavirus ORFs encoding these proteins, respectively. Lanes 1. Ladder, PageRuler™ Plus Prestain Protein Ladder (Fermentas UAB**,** Vilnius, Lithuania). Wild-type, empty baculovirus used as a positive control.

SDS-PAGE gels, Fig 6.11 and 6.12. Expression of recombinant VP7, which was approximately 36 kDa, could only be confirmed with western blot analysis, Fig 6.12.

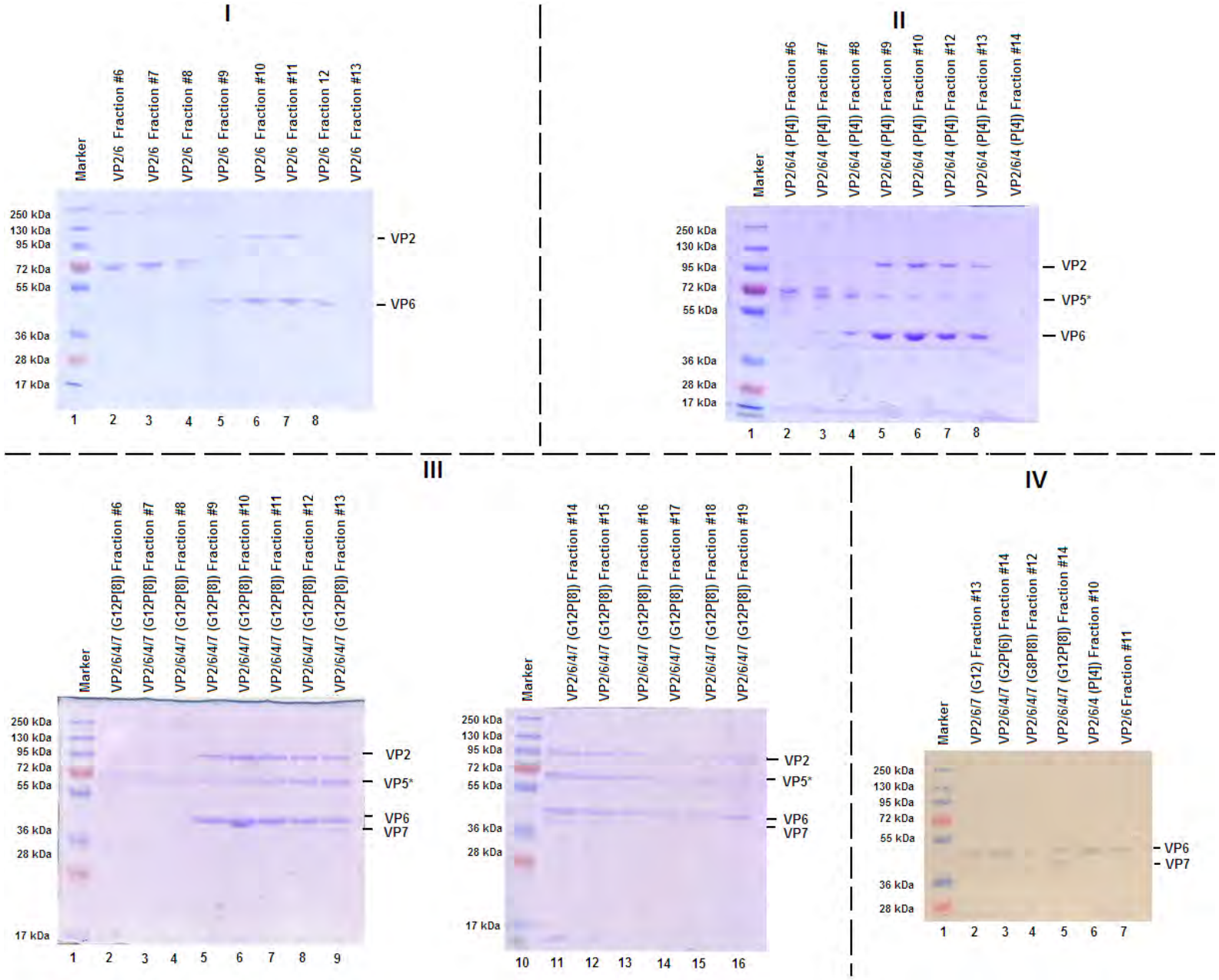
6.3.6. Evaluation of the assembly of baculovirus expressed rotavirus proteins into chimaeric rotavirus virus-like particles

To generate chimaeric RV-VLPs, the G9P[6] dRV-VLPs were co-expressed with outer capsid protein layers that comprised of various combinations of VP4 and VP7 having G2, G8, G9 or G12, associated with either P[4], P[6] or P[8] genotypes using recombinant baculoviruses (see Table 6.4 for the expression constructs used to prepare respective recombinant baculoviruses). The recombinant baculovirus stocks described in section 6.3.5 were used to produce RV-VLPs by co-infecting insect cells with various infection strategies as described in section 6.2.8 and illustrated in Fig. 6.5. Sucrose, iodixanol and CsCl gradient matrices were assessed to determine which purification procedure may result in better yield of RV-VLPs. Presence of specific recombinant structural proteins on RV-VLPs was determined using SDS-PAGE and western blot analysis, whereas RV-VLP assembly was confirmed with TEM.

RV-VLPs were isolated from the supernatant of cultures harvested from both Sf9 and High Five® insect cells co-infected with recombinant baculoviruses as described in section 6.2.8. Initially, dRV-VLPs and chimaeric tRV-VLPs were produced in Sf9 cells. RV-VLPs production in High Five® cells was also done to determine if the yield could be improved as observed previously (Jiang et al., 1998). On both sucrose and iodixanol gradients, two to three bands were usually visualised for dRV-VLPs and tRV-VLPs preparations, respectively.

6.3.6.1. Verification of the presence of VP2, VP4, VP6 and VP7 on the assembled RV-VLPs using SDS-PAGE and western blot analysis

Using SDS-PAGE, VP2, VP4, VP6 and VP7 proteins could be detected in fractions 9 – 15 in most cases, which correlated to the positions where the band was located on the gradients, Fig. 6.13.I.II and 6.13.I.III. Often, VP4 appeared to have been cleaved by endogenous proteases since an approximately 60 kDa product was detected using SDS-PAGE which may represent a VP5* subunit, Fig. 6.13 (II lanes 5 – 8: where VP2 and VP6 were also visible; III lanes 5 –15). A complete VP4 was only detected when insect cells were co-infected with baculoviruses expressing VP2/6 and VP4 first followed by step-wise infection 12 hpi with baculoviruses expressing VP7, Fig 6.13.VI.A, lane 2. Combining the total amount of RV-VLPs in the gradient fractions in which expected recombinant structural rotavirus protein were detected and


I
Marker
VP2/6 Fraction #6
VP2/6 Fraction #7
VP2/6 Fraction #8
VP2/6 Fraction #9
VP2/6 Fraction #10
VP2/6 Fraction #11
VP2/6 Fraction 12
VP2/6 Fraction #13
250 kDa
130 kDa
95 kDa
72 kDa
55 kDa
36 kDa
28 kDa
17 kDa
VP2
VP6
1 2 3 4 5 6 7 8
II
Marker
VP2/6/4 (P[4]) Fraction #6
VP2/6/4 (P[4]) Fraction #7
VP2/6/4 (P[4]) Fraction #8
VP2/6/4 (P[4]) Fraction #9
VP2/6/4 (P[4]) Fraction #10
VP2/6/4 (P[4]) Fraction #12
VP2/6/4 (P[4]) Fraction #13
VP2/6/4 (P[4]) Fraction #14
250 kDa
130 kDa
95 kDa
72 kDa
55 kDa
36 kDa
28 kDa
17 kDa
VP2
VP5*
VP6
1 2 3 4 5 6 7 8
III
Marker
VP2/6/4/7 (G12P[8]) Fraction #6
VP2/6/4/7 (G12P[8]) Fraction #7
VP2/6/4/7 (G12P[8]) Fraction #8
VP2/6/4/7 (G12P[8]) Fraction #9
VP2/6/4/7 (G12P[8]) Fraction #10
VP2/6/4/7 (G12P[8]) Fraction #11
VP2/6/4/7 (G12P[8]) Fraction #12
VP2/6/4/7 (G12P[8]) Fraction #13
250 kDa
130 kDa
95 kDa
72 kDa
55 kDa
36 kDa
28 kDa
17 kDa
VP2
VP5*
VP6
VP7
1 2 3 4 5 6 7 8 9
Marker
VP2/6/4/7 (G12P[8]) Fraction #14
VP2/6/4/7 (G12P[8]) Fraction #15
VP2/6/4/7 (G12P[8]) Fraction #16
VP2/6/4/7 (G12P[8]) Fraction #17
VP2/6/4/7 (G12P[8]) Fraction #18
VP2/6/4/7 (G12P[8]) Fraction #19
250 kDa
130 kDa
95 kDa
72 kDa
55 kDa
36 kDa
28 kDa
17 kDa
VP2
VP5*
VP6
VP7
10 11 12 13 14 15 16
IV
Marker
VP2/6/7 (G12) Fraction #13
VP2/6/4/7 (G2P[6]) Fraction #14
VP2/6/4/7 (G8P[8]) Fraction #12
VP2/6/4/7 (G12P[8]) Fraction #14
VP2/6/4 (P[4]) Fraction #10
VP2/6 Fraction #11
250 kDa
130 kDa
95 kDa
72 kDa
55 kDa
36 kDa
28 kDa
VP6
VP7
1 2 3 4 5 6 7

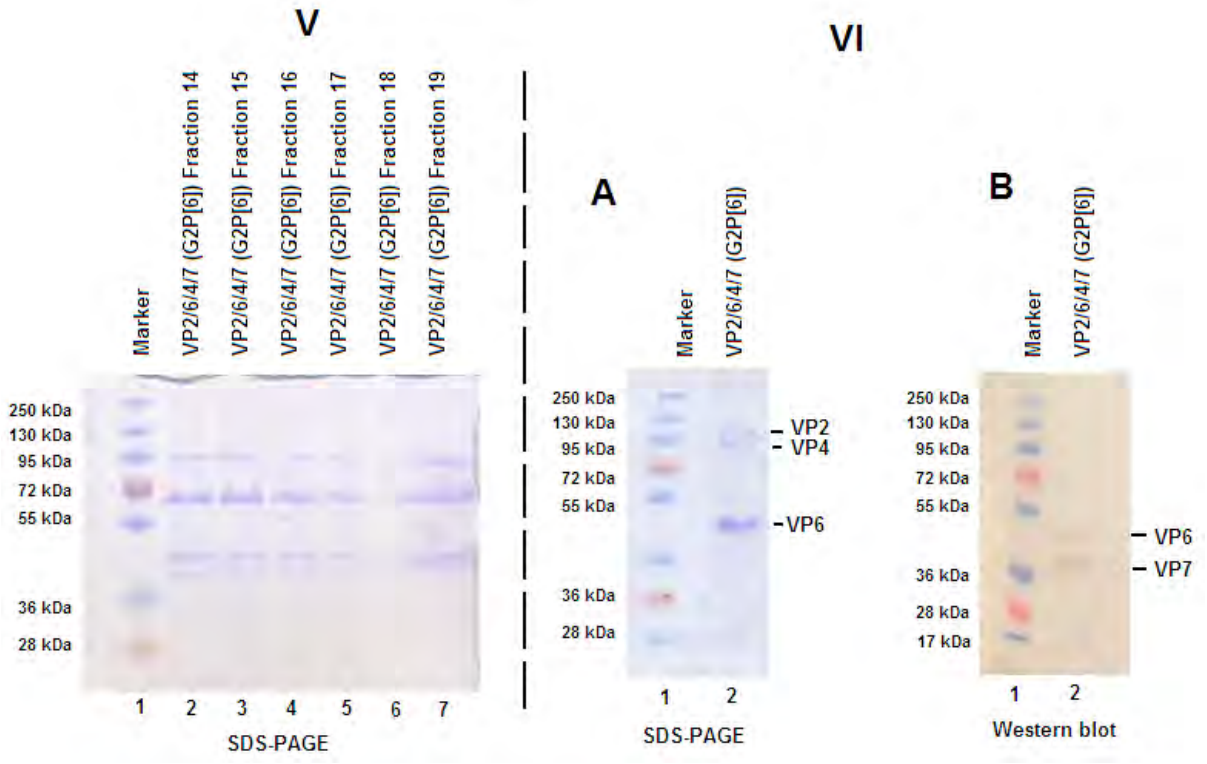


Fig 6.13. SDS-PAGE and western blot analysis of RV-VLP gradient fractions. Gradient fractions which contained structural proteins out of the 18 fractions of 220 ul collected for each sample are depicted. **(I)** Gradient fractions for RV-VLPs (dRV-VLP) prepared with baculoviruses confirmed to express VP2 (C2 genotype) and VP6 (I2 genotype). (**II**) Gradient fractions for RV-VLPs (tRV-VLPs) prepared with baculoviruses confirmed to express VP2 (C2 genotype), VP5* (P[4] genotype) and VP6 (I2 genotype). (**III**) Gradient fractions for RV-VLPs (tRV-VLP) prepared with baculoviruses confirmed to express VP2 (C2 genotype), VP5* (P[8] genotype), VP6 (I2 genotype) and VP7 (G2 genotype). (**IV**) Detection of VP6 (I2 genotype) and VP7 (G8 genotype) with anti-rotavirus IgG antibodies using western blot in selected gradient fractions in which other structural proteins (VP2, VP5* or VP6) were detected using SDS-PAGE. (**V**) Gradient fractions for RV-VLPs on SDS-PAGE prepared by step-wise co-infection with baculoviruses confirmed to express VP2 (C2 genotype), VP5* (P[6] genotype), VP6 (I2 genotype) and VP7 (G2 genotype). (**VI**) Gradient fractions for RV-VLPs on SDS-PAGE (A) and nitrocellulose membrane, western blot (B) prepared by step-wise co-infection with baculoviruses confirmed to express VP2 (C2 genotype), VP4 (P[6] genotype), VP6 (I2 genotype) and VP7 (G2 genotype). Lane 1, Ladder, PageRuler™ Plus Prestain Protein Ladder (Fermentas UAB**,** Vilnius, Lithuania).

concentrating them by centrifugation, the average RV-VLP yield in High Five® cells was almost three-fold higher than in Sf9 cells, Table 6.5. The yields achieved through sucrose gradient purification of the RV-VLPs were higher than when iodixanol was used as gradient matrices, Table 6.5. A clear band was visible on CsCl gradients. However, due to some technical problems, the amount of RV-VLPs determined with BCA assay and the visibility of the protein band on SDS-PAGE gels were too low and faint, respectively, when CsCl gradient was used (data not shown). This may suggest that a significant amount of RV-VLPs yield obtained from CsCl gradients. Therefore, only RV-VLPs purified with sucrose and

Table 6.5. Yield of RV-VLPs quantified with BCA assay.

Infection scheme	Yield after recovery of RV-VLP/1 x 10^6 cells by sucrose and iodixanol gradients			
	Sf9		High five®	
	Sucrose	Iodixanol	Sucrose	Iodixanol
D: VP2 + VP6	1.99 mg/L	Not done	6.55 mg/L	Not done
DC and MC: (VP2 + VP6) + VP7	1.34 mg/L	Not done	6.87 mg/L	Not done
DC and MC: (VP2 + VP6) + VP4	3.6 mg/L	Not done	12.56 mg/L	Not done
DC and MC + MC: (VP2 + VP6) + (VP4) + (VP7)	5.4 mg/L	Not done	9.88 mg/L	Not done
DC and DC: (VP2 + VP6) + (VP4 + VP7)	5.75 mg/L	1.34 µg/L	14.08 mg/L	4.49 mg/L

The concentration values determined with BCA assays for RV-VLPs indicated in the table are averages obtained from baculoviruses expressing rotavirus proteins with G9, G8, G12, P[4], P[6] and P[8] genotypes. The RV-VLPs were produced in 100 ml shaker cultures and the listed yields were converted from µg/100 ml to mg/L. D: dualcistronic, DC: dualcistronic co-infection, MC: monocistronic co-infection.

could have been lost during the dialysis steps. Thus no comparison was made with regard to iodixanol gradients were used for TEM and subsequent comparisons.

6.3.6.2. Verification of RV-VLP assembly using transmission electron microscopy

On electron micrographs, dRV-VLPs were visible from gradient purified RV-VLPs produced through infection of insect cells with baculoviruses expressing VP2 and VP6, Fig. 6.14.I. tRV-VLPs were visualised from gradient purified RV-VLPs prepared by co-infecting insect cells with baculoviruses expressing VP2, VP4, VP6 and VP7. There was evidence of the formation of chimaeric RV-VLPs that were produced by assembling outer capsid proteins made of various combinations of VP7 (G2, G8, G9 or G12) interchanged with VP4 (P[4], P[6] or P[8]) outer capsid onto G9P[6] dRV-VLPs (see Table 6.4 for the proteins expected to be expressed by various generated recombinant baculoviruses). Representatives of the RV-VLPs produced in the current study are shown in Fig. 6.14. Up to 30% of the assembled RV-VLPs produced by homologous VP2/6/7/4 recombinant proteins derived from the same strain (RVA/Human-wt/ZAF/GR10924/1999/G9P[6]) were chimaeric tRV-VLPs. The rest were dRV-VLPs without VP4 and VP7, Fig. 6.14.II. When recombinant baculoviruses containing ORFs coding for VP7 with G2, G8, G12 genotypes or VP4 with P[4], P[6] or P[8] genotypes that were derived from heterologous rotavirus strains, the chimaeric tRV-VLP yield varied from 10 – 20%, Fig. 6.14.III – VII. The distinction between the dRV-VLPs and tRV-VLPs

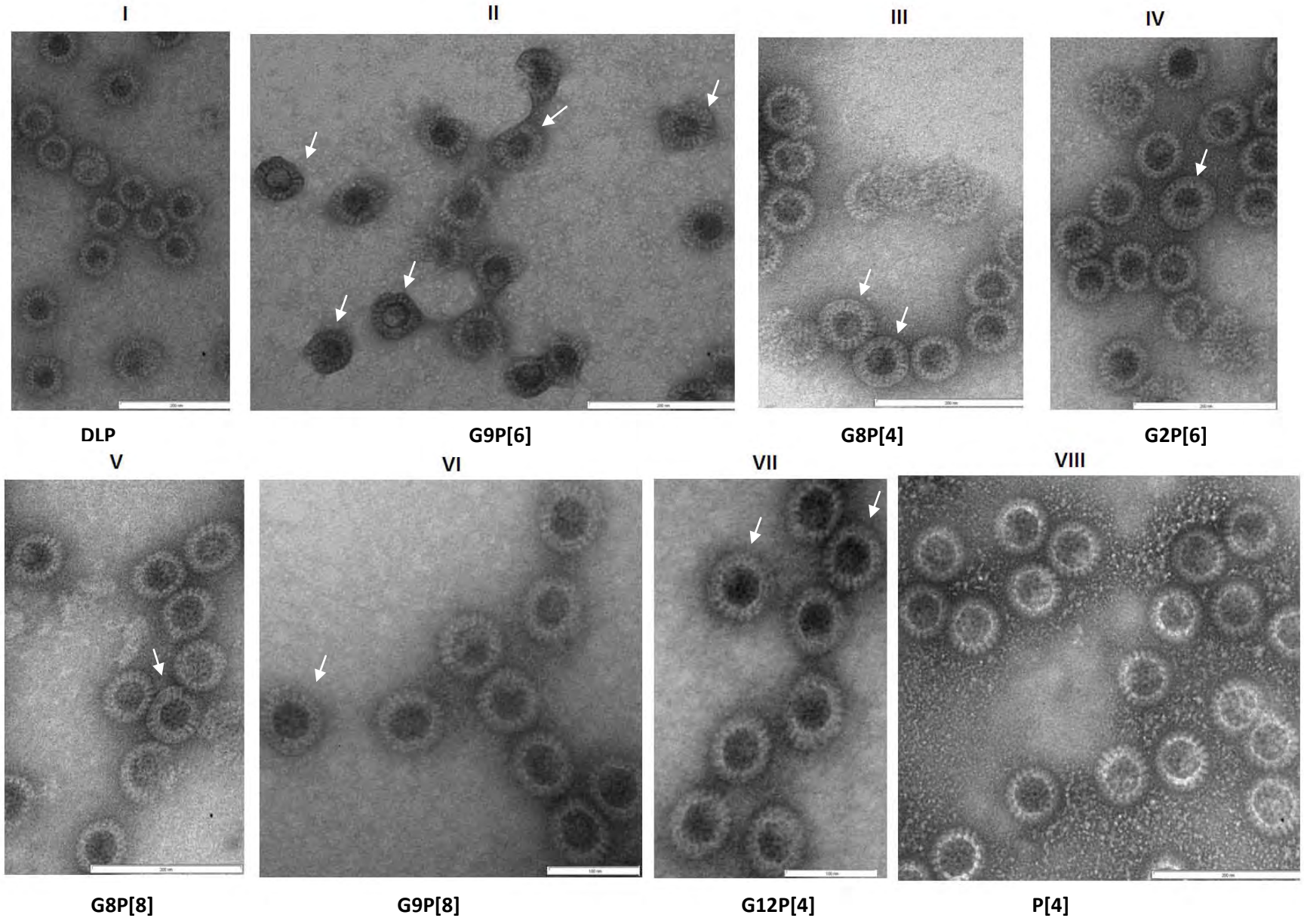

I
II
III
IV
DLP
G9P[6]
G8P[4]
G2P[6]
V
VI
VII
VIII
G8P[8]
G9P[8]
G12P[4]
P[4]

Fig. 6.14. Rotavirus virus-like particles produced in insect cells by using wild-type strains isolated directly from stool samples. (I) dRV-VLPs produced by infecting High Five® cells with recombinant baculoviruses containing ORFs coding for VP2 (C2) and VP6 (I2) proteins. Scale bar 200 nm. (**II**) tRV-VLPs produced by infecting Sf9 cells with recombinant baculoviruses containing ORFs coding for VP2 (C2), VP4 (P[6]), VP6 (I2) and VP7 (G9) proteins. Scale bar 200 nm. (**III**) Chimaeric tRV-VLPs produced by infecting High Five® cells with recombinant baculoviruses containing ORFs coding for VP2 (C2), VP4 (P[4]), VP6 (I1) and VP7 (G8) proteins. Scale bar 200 nm. (**IV**) Chimaeric tRV-VLPs produced by infecting Sf9 cells with recombinant baculoviruses containing ORFs coding for VP2 (C2), VP4 (P[6]), VP6 (I1) and VP7 (G2) proteins. Scale bar 200 nm. **V**) Chimaeric tRV-VLPs produced by infecting High Five® cells with recombinant baculoviruses containing ORFs coding for VP2 (C2), VP4 (P[8]), VP6 (I2) and VP7 (G8) proteins. Scale bar 200 nm. **VI**) Chimaeric tRV-VLPs produced by infecting High Five® cells with recombinant baculoviruses containing ORFs coding for VP2 (C2), VP4 (P[8]), VP6 (I2) and VP7 (G9) proteins. Scale bar 100 nm. **VII**) Chimaeric tRV-VLPs produced by infecting Sf9 cells with recombinant baculoviruses containing ORFs coding for VP2 (C2), VP4 (P[4]), VP6 (I2) and VP7 (G12) proteins. Scale bar 100 nm. **VIII**) Chimaeric tRV-VLPs produced by infecting High Five® cells with recombinant baculoviruses containing ORFs coding for VP2 (C2), VP4 (P[4]) and VP6 (I2) proteins. Scale bar 200 nm. tRV-VLPs with a smooth extra outer ring are shown with arrows.

was made based on the size and their morphology. dRV-VLPs were approximately 70 nm, whereas tRV-VLPs were approximately 75 – 80 nm. Furthermore, tRV-VLPs appeared to contain an extra smooth outer rim which may represent the VP7 layer, Fig.6.14. II – VII. However, this outer VP7 layer was partial in most of the tRV-VLPs that were obtained. Although the step-wise co-infection strategy resulted in the detection of complete VP4 when the RV-VLP sample were screened using SDS-PAGE, the amount and quality of the tRV-VLPs formed was not influenced by the strategy used. The efficiency of recombinant outer spike VP4 onto the dRV-VLPs appeared to be similar regardless of the co-infection strategy used to generate VP2/6/4/7 tRV-VLPs. This may suggest that either VP4 assembly was relatively poor hence could not be visualised clearly, or if VP4 assembled, it was relatively unstable on the particle as observed by others (Istrate et al., 2008), hence could not be seen on electron micrographs.

6.4. Discussion

In the current study, chimaeric RV-VLPs were derived from the consensus sequence of rotavirus strains characterised directly from stool samples without prior adaptation to cell

culture. dsRNA of rotavirus strains (G2, G8, G9 or G12 associated with either P[4], P[6] or P[8] genotypes) extracted directly from stool specimens were used. Recombinant rotavirus proteins, which have been used to prepare RV-VLPs, have been expressed before in insect cells, mammalian cells and larva using baculovirus expression systems (Matic et al., 2012, Bundy et al., 2008, Crawford et al., 1994, Gavilanes et al., 1982, Kost et al., 2010). The majority of the recombinant baculovirus expressed rotavirus proteins have been derived from tissue culture adapted animal and human rotavirus strains. However, adaptation of rotaviruses to tissue culture is difficult, inefficient, labour, intensive and time consuming as it requires multiple rounds of passage in primary cells (Arnold et al., 2009, Ward et al., 1984). No study has yet attempted to prepare RV-VLPs from dsRNA of rotaviruses extracted directly from faecal specimens. Furthermore, most of the recombinant rotavirus proteins have been prepared from strains that have genotypes commonly associated with animal infections (see section 2.8.2) and very few recombinant rotavirus proteins bearing genotypes commonly characterised in humans such as G1 and G3 serotypes have been produced (Yuan et al., 2000, Yuan et al., 2001). To date, expression of rotavirus proteins with G8, G9 and G12 serotypes/genotypes that emerged globally in the past two decades (Santos and Hoshino, 2005) which are also frequently characterised in African (Santos and Hoshino, 2005) and Asian countries (Nelson et al., 2009) have not been reported. In the current study, field rotavirus strains were utilised to generate recombinant baculoviruses that were used to express the four major recombinant structural rotavirus proteins, VP2, VP6, VP7 and VP4, and to prepare RV-VLPs using the BEVS.

Previously, the coding regions for VP2 and VP6 from strain RVA/Human-wt/GR10924/99/1999/G9P[6] were cloned into pFBq under the p10 and polh promoters, respectively (Dr H.G. O'Neill, personal communication). This construct was used to express recombinant VP2 and VP6 that formed the VP2/6 backbone (dRV-VLP) for the chimaeric RV-VLPs produced in this study. The ORFs coding for VP4 and VP7 proteins were cloned into pFBq plasmid under the p10 and polh promoters, respectively. Vieira, et al (2005) reported that promoters may affect the amount of the expressed proteins. Therefore, the recombinant rotavirus VP7 that we desired to be expressed in relatively higher amounts was positioned to be expressed under the control of polh to enhance its expression as it is a stronger promoter compared to p10. This was done due to reports in the literature (Mena et al., 2006, Vieira et al., 2005, Crawford et al., 1994) as well as in our own laboratory (unpublished data) that recombinant

glycosylated VP7 expresses poorly in insect cells. Furthermore, the cloning strategy of the ORFs coding structural rotavirus proteins was based on the number of copies required for each protein to form the viral capsid, see Table 2.2. ORFs of the proteins that requires more copies (VP6 = 260 trimers; VP7 = 260 trimers) were cloned downstream of the polh promoters, whereas those requiring lesser copies (VP2 = 120 dimers; VP4 = 120 trimers) were cloned downstream of the p10 promoters (Fig. 6.6).

Simultaneous recombinant expression of one or more baculovirus expressing more than one rotavirus protein is required to produce dRV-VLPs or tRV-VLPs that can be used for various purposes (refer to section 2.8.2 for more details). The efficiency and versatility of the BEVS makes it an ideal system for production of recombinant proteins (Ciccarone et al., 1998). Since RV-VLPs can mimic the structural conformation and size of the authentic native rotaviruses (Crawford et al., 1994), they are easily taken up by dendritic cells. The large number of structural epitopes on RV-VLPs, which is analogous to native rotaviruses, enables activation of B cells that subsequently leads to production of anti-rotavirus specific antibodies (Grgacic and Anderson, 2006). Such findings makes RV-VLPs potentially safe rotavirus vaccine candidates compared to live-attenuated candidates since RV-VLPs do not have any genomic material. Furthermore, it has been proposed that broader protection might be achieved by preparing chimaeric RV-VLPs containing outer capsid proteins with different serotypes (Crawford et al., 1994). In the current study, attempts were made to prepare chimaeric RV-VLPs by using G9P[6] VP2/6 dRV-VLPs containing various combinations of VP4 and VP7 outer capsids. Different strategies and insect cell lines were used to produce chimaeric RV-VLPs that will be tested in animal models as potential vaccine candidates for rotaviruses in future. Characterisation of the chimaeric tRV-VLPs that were prepared suggests that both VP4 and VP7 of all the various combinations that were selected in this study assembled on to the G9P[6] dRV-VLPs, although at low frequency (Fig. 6.14).

Successful assembly of outer capsid proteins derived from different strains onto a common dRV-VLP was expected considering that hybrid tRV-VLPs with structural proteins from group A and C have been produced previously (Kim et al., 2002). Various combinations of recombinant VP4 and VP7 derived from the selected African rotavirus strains were evaluated for compatibility as outer capsid proteins. The resultant chimaeric RV-VLPs were designed to contain aVP2/bVP6/cVP4/dVP7 components where a = C1, b = I2, c = either P[4], P[6] or P[8] and d = either G2, G8, G9 or G12 genotypes/serotypes of the proteins. The VP2 and

VP6 were derived from dsRNA of the same strain RVA/Human-wt/ZAF/GR10924/1999/G9P[6], whereas VP4 and VP7 were derived from dsRNA of several strains listed in Table 6.2. Of the RV-VLPs observed by TEM, about 30% were tRV-VLPs when VP2, VP4, VP6 and VP7 were derived from the same strain (GR10924). When the G9P[6] VP2/6 dRV-VLPs were co-expressed with outer capsid VP4 (with P[4], P[6] or P[8] genotypes) and VP7 (with G2, G8 or G12 genotypes) derived from heterologous strains (Table 6.2) were used, between 10 to 20% of the assembled particles were tRV-VLPs. The rest were dRV-VLPs. This suggests that neither outer capsids with various combinations of Wa- (G9, P[8]), DS-1-like (G2, P[4]) nor serotypes/genotypes that have not been associated to either Wa- or DS-1-like genogroups (G12, P[6]) (VP4 and VP7 combinations listed in Table 6.4) assembled readily on a common G9P[6] DS-1-like VP2/6 backbone compared to outer capsids consisting of VP4 (P[6]) and VP7 (G9) from the same GR10924 strain. This may suggest that assembly of the outer capsid on the DS-1-like dRV-VLPs was not serotype-dependent, but may be improved by assembling recombinant structural VP2/6/7/4 derived from a homologous strain. The current study further illustrated how the BEVS can be applied to generate tRV-VLPs containing different genotypes, albeit that the efficacy of their assembly will have to be improved in future investigations. The current study also demonstrates the benefit of codon optimisation in terms of increased yields to the extent that the constituents proteins (VP2/6/7/4) of the RV-VLPs were readily visible on Coomassie stained SDS-PAGE (Fig. 6.13).

Compared to other studies (Crawford et al., 1994, Zeng et al., 1994, Labbe et al., 1991), we did not detect other VP2-specific polypeptides that migrate either lower or higher than the authentic VP2. However, two forms of VP4 (intact VP4, 87 kDa when expressed under conditions where no assembly on to VP2/6 particles are possible (Figs. 6.11.I lanes 2 – 6; 6.11.II lanes 5 and 6; 6.11.III lanes 3 – 6; 6.11.IV lanes 2 – 5; 6.11.V lanes 2 – 6; 6.12.II A and B lanes 2 – 6; and 6.12.II A and B lanes 2 and 3): and VP5*; 60 kDa in cases where RV-VLPs were assembled (Fig. 6.13.II lanes 4 – 8 and 6.13.III lanes 5 – 16) that migrated slightly lower or equivalent to the authentic VP4 proteins were characterised. Mellado et al. (2008) confirmed with western blot that the baculovirus envelope glycoprotein gp64 is around 62 kDa. The fact that RV-VLP samples were purified by sedimentation through a 40% sucrose cushion prior to gradient fractionation may suggest that unassembled proteins like gp64 were removed (Jiang et al., 1998). Furthermore, the approximately 60 kDa product

was observed only when baculoviruses expressing VP4 were used and not when RV-VLPs were produced using recombinant baculoviruses expressing VP2 and VP6 only, Fig 6.13.I. Therefore, the 60 kDa band could be potentially a VP4 cleaved product, VP5*, as also observed previously by Crawford and co-workers (1994). Crawford et al. (1994) hypothesised that endogenous proteases from the serum in the media or cells could be responsible for the cleavage of both the VP4 associated with RV-VLPs that they produced and the VP4 of their native SA-11 rotavirus into VP5* and VP8* subunits. Although protease inhibitors were used during the production of the RV-VLPs in this study, VP4 appeared to have been cleaved regardless. This was unexpected considering that the recombinant baculoviruses were prepared using cathepsin negative bacmids (Kaba et al., 2004). The other VP4 subunit, VP8*, was not visualised when RV-VLPs were analysed by SDS-PAGE. This was expected as only proteins associated with the particles were expected to sieve through the 40% sucrose cushion prior to centrifuging the samples through sucrose or iodixanol gradients.

Despite detecting only a VP5* subunit portion of VP4 on most of the RV-VLPs that were prepared, an approximately 87 kDa VP4 protein was always expressed when Sf9 cells were infected with baculoviruses containing ORFs (s) coding for VP4 or both VP4 and VP7 in the absence of protease inhibitors (Figs. 6.11.I lanes 2 – 6; 6.11.II lanes 5 and 6; 6.11.III lanes 3 – 6; 6.11.IV lanes 2 – 5; 6.11.V lanes 2 – 6; 6.12.II A and B lanes 2 – 6; and 6.12.II A and B lanes 2 and 3). This may perhaps suggest that association of VP4 to the DLP facilitates VP4 cleavage by exposing the cleavage sites to endogenous protease which was perhaps inaccessible in VP4 that is not associated to other proteins. In order to produce cRV-VLP consisting of intact spike VP4 protein which could closely mimic rotaviruses in future, further studies are warranted to understand why VP4 was cleaved in the presence of protease inhibitors even when the concentration of Leupeptin or Aprotinin was doubled from 0.5 – 1 µg/ml. Nevertheless, the tRV-VLPs containing VP5* might still be useful as potential vaccine candidates due to the revelation from crystal structure studies that showed that the VP5* antigen domain on VP4 is responsible for heterotypic neutralisation as it contains the neutralising epitopes (Dormitzer et al., 2004).

Based on the complexity of the structure of rotaviruses, wasteful accumulation of unassembled proteins or formation of incomplete particles can occur during assembly of RV-VLPs due to inadequate thermodynamics because of incorrect stoichiometric ratios of the

recombinant structural proteins. Vieira and co-workers (2005) demonstrated that only 15% of the expressed proteins correctly assemble into RV-VLPs. This makes the process productivity of RV-VLPs somehow discouraging. Based on the morphology and diameter of the particles produced in this study, most of them were dRV-VLPs. Some studies suggest that when VP2 and VP6 are individually expressed they form nanotubes which are approximately 45 nm in diameter and compact insoluble ordered aggregates, respectively (Mena et al., 2006). This was not observed in the current study, suggesting efficient assembly of expressed VP2 and VP6 proteins. Almost all the baculoviruses with ORFs coding for VP2 and VP6 proteins formed clearly defined wheel-like spherical dRV-VLPs that had rough surfaces in both Sf9 and High Five® cells (Fig. 6.14.I) as observed by others (Crawford et al., 1994, Kim et al., 2002). Therefore, the findings of the current study correlates with kinetic studies conducted by Mena et al, (2006) that demonstrated that simultaneous expression of VP2 and VP6 result in well defined dRV-VLPs.

Despite detecting VP7 when the fractions of the gradients containing RV-VLPs were screened using SDS-PAGE and western blot, EM micrographs suggest that very few particles assembled into tRV-VLPs. Most of the tRV-VLPs were partial as seen by having partly smooth and mostly rough edges, suggesting that they partially assembled into tRV-VLPs or the outer layer on some tRV-VLPs was not that stable. This calls for further optimisation studies to improve the assembly efficiency of the outer capsid layer onto the dRV-VLPs that may lead to production of stable cRV-VLPs. The TOI and MOI are some of the parameters that need to be optimised as several studies have shown their effect on the amount and quality on the BEVS-produced RV-VLPs. Lower MOI leads to longer process time but minimises the production of defective interfering viral particles. Higher MOI achieves higher RV-VLP yield and increases the amount of RV-VLP production within a shorter time as all the cells are infected at the same time (Palomares et al., 2002, Licari and Bailey, 1992). Mellado and co-workers (2008) showed that lower MOI, for instance 0.1 pfu/cell, result in a higher VP7/gp64 ratio, which may simplify purification of RV-VLPs as the production of free-gp64 trimers by baculoviruses is greatly reduced. Furthermore, Mena et al. (2005) and Vieira et al. (2005) found that the highest yield of RV-VLPs is achieved when the culture is harvested at 4 dpi. However, most studies have used MOI ranging from 1 – 5 pfu/cell and have harvested the cultures approximately 4 – 5 dpi (Vieira et al., 2005, Ribes et al., 2011, Mena et al., 2005). During preliminary investigations in our laboratory, MOI of 0.1, 1, 2, 5 and 10 pfu/cell were

compared for dRV-VLP formation of the G9P[6] backbone (data not shown). A MOI of 2 pfu/cell was shown to give the best yield of dRV-VLP formation (M.J. van der Westhuizen, personal communication). Therefore, RV-VLPs were produced by infecting either Sf9 or High Five® insect cells with one or more baculoviruses containing the ORF (s) encoding one or more rotavirus structural proteins with MOI of 2 pfu/cell. Production of RV-VLPs in High Five® cells was almost three-fold higher than in Sf9 cells. This was expected based on findings of Jiang and co-workers (1998) who suggested that High Five® cells may offer a better environment for expression of recombinant protein and assembly into RV-VLPs. Therefore, High Five® cells will be used for production of RV-VLPs in our future optimisation studies.

Different co-infection strategies were used to produce RV-VLPs. The largest protein, VP2, accumulates at the lowest rate during expression of the four structural proteins (VP2, VP4, VP6 and VP7) in the BEVS system (Vieira et al., 2005, Roldao et al., 2007). VP6 is required for the release of the VP2 into the supernatant. Therefore, insect cells were initially infected with baculoviruses expressing these proteins before infecting them with recombinant baculoviruses expressing outer capsid VP7 proteins. Amongst all the co-infection strategies that were used, it appeared that co-infecting insect cells simultaneously with baculoviruses expressing VP2, VP6 and VP4 followed by baculoviruses expressing outer capsid proteins VP7 is a better strategy for producing cRV-VLPs. This correlates with Trask and Dormitzer (2006) findings. With this strategy, intact 87 kDa VP4 was detected and also the band of VP7 from the RV-VLPs was visible on most Coomasie blue stained SDS-PAGE gels, for example in Fig. 6.14.V and VI. Having yields of recombinant rotavirus proteins that can be seen by Coomasie blue staining of SDS-PAGE gels is quite good, since not many studies has reported this. Most other studies to date, relied on radioactive labelling of proteins and visualisation by autoradiography. The size and position on SDS-PAGE gels of the approximately 36 kDa VP7 correlated with Yao et al. (2012) findings who also managed to visualise the glycosylated VP7 expressed in silkworms on SDS-PAGE gels. This observation correlates with the proposed packaging strategy of rotavirus protein during the replication process where VP4 is associated first with the DLP prior to the addition of the VP7 layer (Trask et al., 2012). This fact that VP4 is anchored by VP6 and protrudes through the five-fold apices of VP7, was also found by X-ray crystallography studies reported by Settembre et al. (2011). This suggests that assembling VP7 first may prohibit assembly of VP4 onto the dRV-VLP that attaches to VP6

as the VP7 channels may be blocked. This may lead to formation of tRV-VLPs having a VP7 layer but devoid of VP4 proteins. CryoEM studies of the RV-VLPs generated in the current study could shed more light on the order of assembly in addition to findings of Settembre et al. (2011). Nevertheless, Ciarlet et al. (1988) concluded that the inclusion of VP7 and VP4 was not required to achieve protection from rotavirus infection in a rabbit model when Freund's adjuvant was used. Ciarlet and co-workers (1998) reported high levels of protection ranging from 87 to 100% against rotavirus infection in individual rabbits immunised parenterally with VP2/6/7- or VP2/6-VLPs in Freund's adjuvants. This has also recently been supported by Zhou and co-workers (2011) who achieved protection of up to 92% against rotavirus challenge in mice by administering dRV-VLPs with an adenovirus-expressed VP6 booster regime. Furthermore, the heterotypic immune response against strains like G2P[4] achieved by the monotypic Rotarix vaccine may suggest heterotypic component of rotavirus-specific immune responses (Desselberger and Huppertz, 2011, Ruiz-Palacios et al., 2006) Therefore, the dRV-VLPs prepared in this study from wild-type rotavirus strains generated from dsRNA extracted directly from stool specimens have the potential to be used as immunogens to formulate rotavirus vaccine candidates in future.

The potential of the RV-VLPs generated in this study to induce protection against broader spectrum of rotavirus strain commonly isolated from African and rotavirus strains that have emerged within the past two decade across the globe will be evaluated in animal models in follow-up studies. Several regimens that include combined parenteral-oral vaccination, combined dRV-VLP-adenovirus-associated, and use of adjuvants are some of the strategies that can be evaluated to improve the immunogenecity of these RV-VLPs. In conclusion, this study proves the concept that chimaeric RV-VLPs can be produced from rotavirus dsRNA extracted directly from clinical specimens. The outcomes of this current study will support initiatives of developing alternative rotavirus subunit vaccines especially in Africa where the burden of rotavirus is high. The various expression cassettes and recombinant baculoviruses prepared here can be used by several research groups interested in formulating mono- or polyvalent RV-VLP vaccines against strain (s) that have emerged in the past two decades and also strains detected frequently from Africa. Such endeavours may contribute towards efforts of producing alternative strain-specific rotavirus vaccines applicable to specific regions.

6.5. References

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