

Molecular characterization and expression of human Rotavirus recombinant protein VP2, VP6, and VP7 transfected in Vero cellLatri Rahmah^{1,2,*}, Ernawati G Rachman², Debbie S Retroningrum², Marselina I Tan²¹ Rotavirus Vaccine Production PT Biofarma, Bandung, Indonesia² Institut Teknologi Bandung, Bandung, Indonesia

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Abstract

Rotavirus is a non-enveloped viruses containing double-stranded RNA genetic material. The 11 ds-RNA encodes 6 structural and 6 nonstructural proteins. Three major structural capsid proteins of human rotavirus RV4 (VP2, VP6, and VP7) had been isolated, cloned and transfected into Vero cell. VP2 (VP2LRV4), VP6 (VP6LRV4), and VP7 (VP7LRV4) was isolated with the size of coding sequence (CDS) 2673 bp, 1194 bp, and 981 bp, respectively. In silico analysis showed that VP2LRV4, VP6LRV4, and VP7LRV4 protein sequences (prediction) comprised essential subdomain and residues to construct triple layer structural capsid protein. Gene transcription and protein expression of VP2LRV4, VP6LRV4 and VP7LRV4 in Vero cell were examined by RT-PCR and immunofluorescence assay (IF). Transcriptional analysis led to the detection of VP6 and VP7 cDNA on day 1 post transfection while VP2 cDNA was expressed on day 3 post transfection. VP6 and VP7 protein expression marked with fluorescence luminescence in Vero cell on day 1 post transfection.

Keywords: Rotavirus; Vero Cell; VP2; VP6; VP7.**INTRODUCTION**

Rotavirus has been recognized as a major cause of infantile diarrhea in young children. Classify as non-enveloped virus, the genomes of Rotavirus are 11 ds-RNA which encoding 6 structural proteins (VP1-VP4, VP6, and VP7) and 6 nonstructural protein (NSP1-NSP6) (Desselberger, 2014). Rotavirus is composed by three layers (outer, inner and core) capsid protein. In the core site, VP2 is a major protein with VP1 and VP3 as the minor protein. The major capsid protein VP6, which comprises >80% of the protein mass of the particle, is located on the inner capsid and contains the subgroup antigen. The rotavirus outer layer is composed of the glycoprotein VP7 and VP4 (Ruiz *et al.*, 2009).

A number of research reported that the recombinant structural protein of Rotavirus had been expressed in prokaryotic host cell, such as *Escherichia Coli*, and

eukaryotic host cell such as yeast, insect cell, and mammalian cell system. However the expression of recombinant structural protein found several limitations. Rotavirus recombinant protein produce in *Escherichia coli* failed to express and folded properly (Kato *et al.*, 2012). In yeast, it is produced in a lower quantity or inadequate aggregation condition related to glycosylation patterns (Rodriguez-Limas *et al.*, 2011). In Baculovirus system, recombinant Rotavirus protein is degraded and partial loss of ability to form trimmers (Da silva *et al.*, 2012).

Mammalian cells have been chosen as a system for the production of recombinant proteins for its excess on proper protein folding, assembly, and post-translational modification. Vero cells is a mammalian cells that has been widely used for expression recombinant virus protein, such as JEV, PPRV, SV40, rabies, Coronavirus (SARS), Ebola, and Influenza (Hua *et al.*, 2014; Mulherkar *et al.*, 2011; Chen *et al.*, 2011; Siu *et al.*, 2008; Balamurugan *et al.*, 2006; Hsieh *et al.*, 2005; Konishi *et al.*, 2001; Wagner *et al.*, 2000). In the current study, we used Vero cell as mammalian expression system to generate the three major structural recombinant Human Rotavirus RV4 capsid proteins.

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MATERIALS AND METHODS

Amplification and cloning of VP2, VP6, and VP7 rotavirus gene

Amplification of the complete open reading frame (ORF) of VP2, VP6, and VP7 gene of rotavirus RV4 strain was performed using RT-PCR. Amplification of VP2, VP6, and VP7 were using primer's pair VP2, VP6 and VP7. RT-PCR was performed with an aliquot of dsRNA (100 ng), 4 μ M each of specific primer, 2x Reaction Mix (a buffer containing 0,4mM of each dNTP, 2,4 mM MgSO₄) (Invitrogen) and 1U Super Script™ III RT/Platinum Taq High Fidelity Enzyme Mix (Invitrogen) in a final volume 50 μ L. The full-Length VP2 (2.6 kb) cDNA synthesis was obtained by one cycle at 45 °C for 45 min, followed by PCR amplification with 35 cycles at 94 °C for 1 min, 62 °C for 2 min, 72 °C for 2 min and 1 cycle at 72 °C for 7min. The full-Length VP6 (1.3 kb) cDNA synthesis was obtained by one cycle at 45 °C for 45 min, followed by PCR amplification with 35 cycles at 94 °C for 1 min, 47 °C for 2min, 72 °C for 2 min and 1 cycle at 72 °C for 7min. The full-Length VP7 (1 kb) cDNA synthesis was obtained by one cycle at 45 °C for 45 min, followed by PCR amplification with 35 cycles at 94 °C for 1 min, 42 °C for 2min, 72 °C for 2 min and 1 cycle at 72 °C for 7min. The amplified cDNA of VP2 gene was cloned into pCDNA 3.1/TOPO while VP6 and VP7 gene was cloned into pEF6/V5-His-TOPO. Both vectors are dual-host vectors (Invitrogen) for both bacteria and mammalian cells and protein expression in mammalian cells. The recombinant VP2-pCDNA, VP6-pEF, and VP7-pEF was transformed into competent *E.Coli* Top 10 and ampicillin-resistant colonies were screened by ampicillin medium selection. In addition the expected inserts were verified by colony PCR. Colonies PCR were performed using primer's pairs VP2, VP6, and VP7 as described above. Finally, nucleotide sequences analyses were carried out to obtain the full sequences of genes and to confirm the exact in-frame position and correct orientation for subsequent expression.

Protein sequence and structure analysis

Protein sequences (prediction) of VP2, VP6, and VP7 were performed using Bioinformatics tools ExPasy. Three dimensional structures of VP2, VP6, and VP7 were implemented via Phyre2 and Pymol Molecular Graphic System.

Transfection

Vero Cell were seeded at a concentration 1.5 x 10⁴ cells per well in 96–well plates and grown in Opti-MEM for 1 d prior to transfection. To achieve transfection, according to the manufacturer's instruction, 0,2 μ g of Recombinant Vector (pCDNA/VP2, pEF/VP6, pEF/VP7) were mixed with 0.5 μ L lipofectamine

3000™, 0.4 μ L P3000 reagent, and 10 μ L Opti-MEM (Invitrogen) according to the instruction supplied by the company. Transfection mixture was then added to the seeded cells and incubated at 37 °C from day 1 to 5 for transfection.

RT-PCR analysis

Vero cells transfected with Recombinant Vector (pCDNA/VP2, pEF/VP6, or pEF/VP7) washed two times with PBS and RNA total was extracted by SV Total RNA isolation System (Promega) according to the manufacturer's instruction. The expected fragment of VP2, VP6, and VP7 was amplified by RT-PCR using the primer's pair described above.

Immunofluorescence Assay

Vero cells transfected with Recombinant Vector (pCDNA/VP2, pEF/VP6, or pEF/VP7) fixed in methanol-acetone (1:1) and incubated for 10 m. After fixation, the cells were added by blocking solution (BSA 1%, in PBS-Tween 0.05%) followed with rabbit anti-RV polyclonal antibody (1:800 in PBS). Second-stage antibody was Alexa-488-conjugated secondary antibody diluted at 1:500 in PBS. Cells were examined with a Fluorescence Microscope.

RESULTS AND DISCUSSION

The complete VP2 cDNA sequences (VP2LRV4) with 2679 nucleotides in length contained a 2673 bp CDS that encoded 889 amino acids (Fig. 1A). The complete cDNA sequences (1356 bp) of VP6 (VP6LRV4) comprised an 1194 bp encoding a peptide of 397 amino acids (Fig. 1B). The VP7 sequence (VP7LRV4) was 1062 consecutive bases and contained CDS with 981 bp that encoded 326 amino acids (Fig. 1C). In silico study showed that protein structure (prediction) of VP2LRV4, VP6LRV4, and VP7LRV4 comprised essential subdomain and residues to construct the structural capsid protein (Fig. 2).

The three dimensional structure (prediction) at Figure 2.A showed the dimer domain of VP2LRV4. The dimer subdomain of VP2LRV4 was predicted located in residues 711 to 836 (Fig. 1A). The dimerization of VP2 performed on its dimer domain at residues ~700-826 (Boudreaux *et al*, 2013; McClain *et al*, 2010). Protein structure (prediction) of VP6LRV4 in Fig. 2B showed the domains and residue that was predicted essential to perform trimerization. The essential domain of VP6LRV6 to performed trimerization was predicted located in residues 1 to 150 and residues 151 to 331 (Fig. 1B). Mathieu, *et al* (2001) reported that the VP6 subunit performed trimerization at the domain B (residues 1-150) and domain H (residues 151-331) to construct inner layer Rotavirus capsid protein.

recombinant proteins (Jager *et al.* 2007). In this paper, the transient expression of recombinant VP2, VP6 and VP7 of RV4 was described for the first time in Vero cells.

The transcription of VP2, VP6, and VP7 gene in transfectant Vero cells was verified by the RT-PCR analysis. Agarose gel electrophoresis of the RT-PCR products obtained from total RNA extracted from Vero cells that was transfected with the pCMV/VP2, showed expected fragment of 2.6 kb at day 3 post transfection (pt) to day 5 pt (Figure 3). The RT-PCR confirmed that VP2 mRNA was expressed in transfectant Vero cells at day 3 and acquired the highest expression at day 5. These data may indicate that the production of rVP2 protein in Vero cell optimize at day 3 to 5. Several researches also suggest that rVP2 in transfectant cells expressed on day 3 pt (Labbe *et al.* 1991; Pera *et al.* 2015; Pourasgari *et al.* 2007). The RT-PCR assay also confirmed that the VP6 and VP7 mRNA was expressed in transfectant Vero Cell at day 1 to 5 pt. RT-PCR products of Vero cells transfected with the pEF/VP6 or pEF/VP7 were expressed with expected fragment of 1.3 kb and 1.1 kb, respectively (Fig. 3).

Transfected transient cell lines were further examined for the intracellular expression of VP6 and VP7 gene by indirect immunofluorescence assay. As expected, the specific cytoplasmic staining was clearly evident when the transfected cells were reacted with primary polyclonal rabbit anti-rotavirus antibody. The results showed that Vero cells expressed VP6 and VP7 proteins possess a serological specificity similar to that

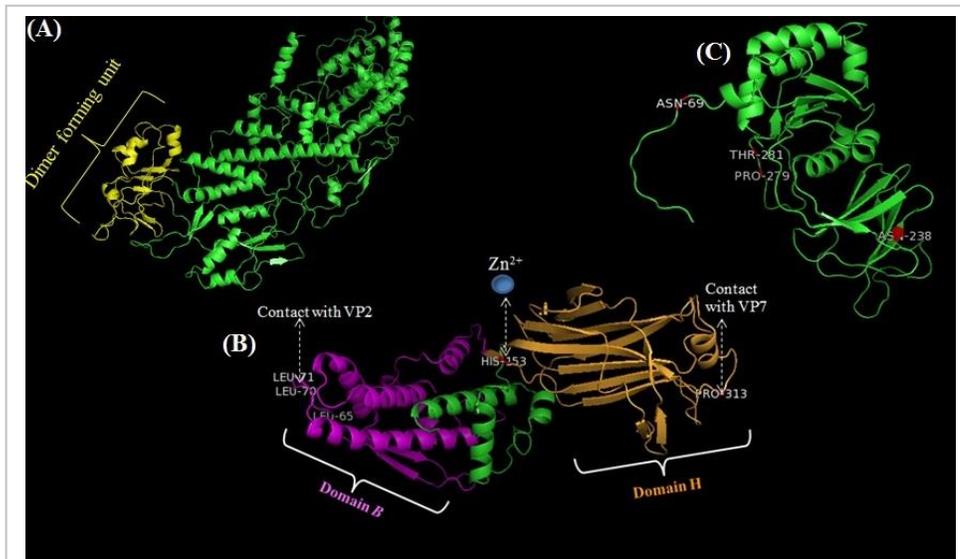


Figure 2: Protein structure (prediction) of VP2LRV4 (A), VP6LRV4 (B), and VP7LRV4 (C). Dimer forming subdomain of VP2LRV4 in yellow. In silico analysis of VP6LRV4 determined domain B in purple (residues 1-150), domain H (residues 151-331) in gold and residue His-153 that essential in the stabilizing the VP6 trimeric molecule. The VP6LRV4 also contained residues 65-LLGTTLL-71 and Pro313 that may contact with VP2 and VP7. VP7LRV4 contained the conserved N-Glycosylated (Asn-69 and 238) and residues Pro-279 and Thr-281 that may interact with VP6.

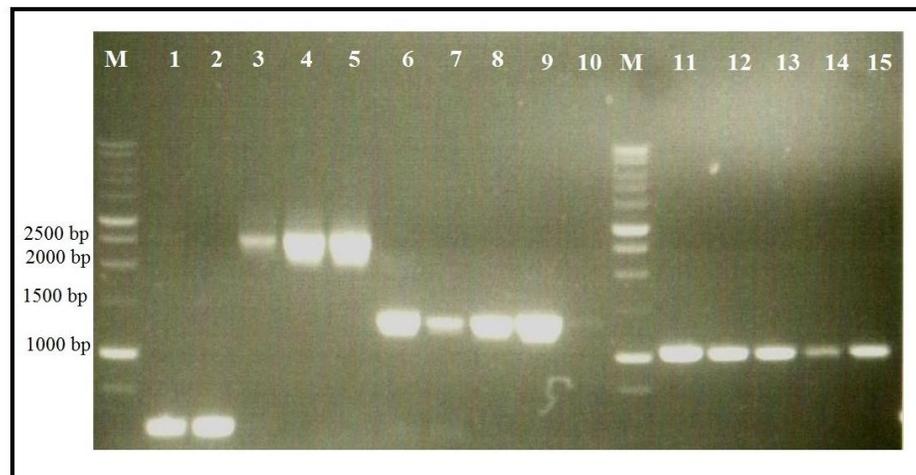


Figure 3: Agarose gel electrophoresis of the RT-PCR products obtained from total RNA extracted from Vero cells transfected with the pCMV/VP2, pEF/VP6, and pEF/VP7 genes. Lanes L: 1kb ladder; Lanes 1 to 5: RT-PCR product of VP2 at 1d to 5d post-transfection; Lanes 6 to 10: RT-PCR product of VP6 at 1d to 5d post-transfection; Lanes 11-15: RT-PCR product of VP7 at 1d to 5d post-transfection

of the viral VP6 and VP7. Our result shows that the transfected Vero cell expressed VP6 and VP7 protein at day 1 to 5 pt (Fig. 4). Interestingly, we observed that the Vero cells transfected with VP7 shows the fluorescence cell with a cell nucleus that more obvious than in the Vero cells transfected with VP6. VP7 is translated at the ribosomes which bind to the membranes of organelles ER while VP6 is translated in polyribosome (Desselberger, 2014). ER membrane lies

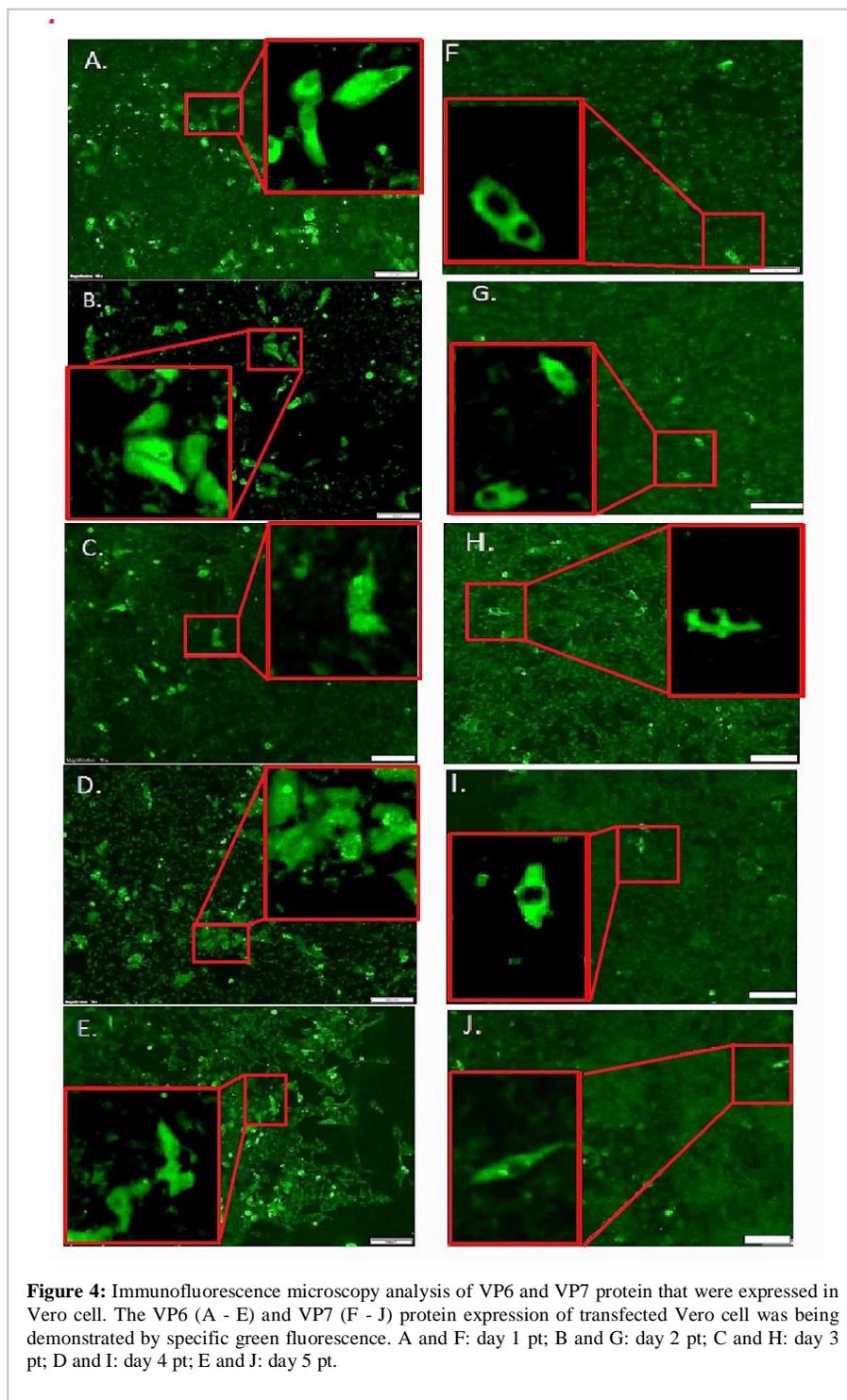


Figure 4: Immunofluorescence microscopy analysis of VP6 and VP7 protein that were expressed in Vero cell. The VP6 (A - E) and VP7 (F - J) protein expression of transfected Vero cell was being demonstrated by specific green fluorescence. A and F: day 1 pt; B and G: day 2 pt; C and H: day 3 pt; D and I: day 4 pt; E and J: day 5 pt.

adjacent to the nucleus while polyribosome scattered in the cytoplasm. Those differences may lead to differing form of fluorescence in transfected cell.

CONCLUSION

The three major structural capsid proteins VP2, VP6, and VP7 of human rotavirus RV4 had been isolated and expressed in Vero cell. In silico analysis showed that

the VP2LRV4, VP6LRV4, and VP7LRV4 protein sequences (prediction) comprised essential subdomain and residues to construct triple layer structural capsid protein. Transcriptional analysis confirmed that the VP2 gene was expressed at day 3 post transfection while VP6 and VP7 were expressed at day 1 post transfection. VP6 and VP7 protein expression in transfected Vero cell occurred on day 1 post single transfection. All of it suggested that this study can be developed as a reference on producing triple layer VLP consist of VP2, VP6, and VP7 in Vero cell.

Conflict of interest

The authors declare no conflict of interest with respect to the content and writing of the paper.

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References

- Balamurugan V, Sen A, *et al.* (2006) Development and characterization of a stable Vero cell line constitutively expressing peste des petits ruminants virus (PPRV) hemagglutinin protein and its potential use as antigen in enzyme-linked immunosorbent assay for sero surveillance of PPRV, *Clin. Vaccine Immunol.*, **13**(12): 8036-8048.
- Boudreaux CE, Vile DC, *et al.* (2013) Rotavirus core shell subdomains involved in polymerase encapsidation into virus-like particles. *J. Gen. Virol.*, **94**: 1818-1826.
- Chen Y, Guo W, *et al.* (2011) A novel recombinant pseudorabies virus expressing parvovirus VP2 gene: immunogenicity and protective efficacy in swine. *Virol J.*, **8**: 307-315.
- Chen JZ., Settembre EC, *et al.* (2009) Molecular interaction in rotavirus assembly and uncoating seen by high-resolution cryo-EM. *PNAS*, **103** (26): 10644-10648.
- Coulson BS, Kirkwood C., (1991) Relation of VP7 amino acid sequence to monoclonal antibody neutralization of rotavirus and rotavirus monotype. *J. Virol.*, 5968-5974.

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Da Silva Junior HC, Mouta Junior SS, *et al.* (2012) Comparison of two eukaryotic systems for the expression of VP6 protein of rotavirus specie A: transient gene expression in HEK293-T cells and insect cell-baculovirus system. *Biotechnol. Lett.*, **34**: 1623–1627.

Desselberger U (2014) Rotaviruses. *Virus Res.*, **190**: 75-96.

Hsieh PK, Chang SC, *et al.* (2005) Assembly of severe acute respiratory syndrome coronavirus RNA packaging signal into virus-like particles is nucleocapsid dependent. *J. Virol.*, **79**(22): 13848-13855.

Hua, RH, Li, YN, *et al.* (2014) Generation and characterization of new mammalian cell line continuously expressing virus-like particle of Javanese encephalitis virus for a subunit vaccine candidate. *BMC Biotechnol.*, **14**: 62-69.

Jager, V, Bussow K, *et al.* (2015) Transient recombinant protein expression in mammalian cells. *Animal Cell Culture, Cell Engineering 9*. Springer International Publishing, ISBN: 978-3-319-10319-8, **12**: 27-63.

Kato T, Deo VK, *et al.* (2012) Functional virus-like particles production using silkworm and their application in life science. *J. Biotechnol. Biomaterial*, S9:001, doi:10.4172/2155-952X.S9-001.

Konishi E, Fujii A, *et al.* (2001) Generation and characterization of a mammalian cell line continuously expressing japanese encephalitis virus subviral particles. *J. Virol.*, **75** (5): 2204-2212.

Labbe M, Charpilienne A, *et al.* (1991) Expression of rotavirus VP2 produces empty core like particles. *J. Virol.*, **65** (6): 2946-2952.

Mathieu M, Petitpas I, *et al.* (2001) Atomic structure of the major capsid protein of Rotavirus: implication for the architecture of the virion. *EMBO J.*, **20**(7): 1485-1497.

McClain B, Settembre E, *et al.* (2010) X-Ray Crystal structure of the Rotavirus inner capsid particle at 3.8 Å resolution. *J. Mol. Biol.*, **397**: 587-599.

Mulherkar N, Raaben, M, *et al.* (2011) The ebola virus glycoprotein mediates entry via a non-classical dynamin-dependent macropinosocytic pathway. *Virology*, **419**: 72–83.

Pourasgari, Ahmadian, S, *et al.* (2007) Expression and characterization of VP2 protein of human rotavirus a in a mammalian lung cell line. *Acta Virol*, **51**: 261 – 264.

Pera FFPG, Mutepfa DLR, *et al.* (2015) Engineering and expression of a human rotavirus candidate vaccine in *Nicotiana benthamiana*. *Virol J.*, **12**: 205-211.

Ruiz MC, Leon T, *et al.* (2009) Molecular biology of rotavirus entry and replication, *Sci. World J.*, **9**, 1476–1497.

Rodríguez-Limas WA, Tyo KE, *et al.* (2011) Molecular and process design for rotavirus-like particle production in *Saccharomyces cerevisiae*. *Microb Cell Fact.*, **10**: 33-43.

Siu YL, Teoh KT, *et al.* (2008) The M, E, and N structural proteins of the severe acute respiratory syndrome coronavirus are required for efficient assembly, trafficking, and release of virus-like particles. *J. Virol.*, **82**(22): 11318–11330.

Wagner E, Engelhardt OG, *et al.* (2000) Formation of virus-like particles from cloned cDNAs of togoto virus. *J. Gen. Virol.*, **81**: 2849–2853.