IDENTIFICATION OF ENGINEERED YEAST STRAINS FOR THE PRODUCTION OF ROTAVIRUS VP6-BASED VACCINE CANDIDATES

By

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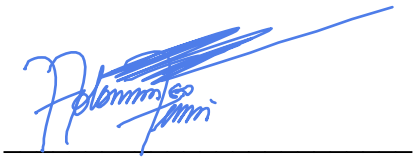
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Dedication

This PhD dissertation is fully dedicated to my family – my dearest, understanding wife and better half, my adorable daughter (MyLady) and my wonderful son (Emperor) for your care and support. I love you all. To God be the Glory!

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Sam

Conferences

2018:

i. ***Olufemi S. Folorunso,** Matshepo E. Rakaki, Jacobus Albertyn, Hester G. O'Neill: **Production of rotavirus VP6-based vaccine candidates in engineered yeast strains.** A poster presentation at the SASBMB-FASBMB, 8 – 11 July 2018 at the North-West University, Potchefstroom South Africa

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2015:

i. *MS Makatsa, **OS Folorunso**, J Albertyn, AA van Dijk, HG O'Neill: **Towards the development of non-live rotavirus vaccines: Expression of rotavirus VP6 in various yeasts.** A poster presentation at the 12th International dsRNA Virus Symposium 2015; 6-10 October 2015, Goa, India

ii. ***Olufemi S. Folorunso,** Mohau S. Makatsa, Jacobus Albertyn, Hester G. O'Neill: **Genomic integration and expression of the major virion rotavirus protein (VP6) in yeast derived from single and dual expression vectors.** A poster presentation at the Virology Africa, 2015; 30th November – 3rd December 2015 at Radisson Blu Hotel, Granger Bay, Cape Town Waterfront, South Africa

Summary

Despite two live-attenuated oral rotavirus vaccines (Rotarix®, RotaTeq®) having been used for a decade in more than 96 countries, rotavirus continues to be the major etiological agent of severe gastroenteritis and diarrhoea-related hospitalisation in infants and children less than 5 years old. Two additional live-attenuated oral vaccines (Rotavac® and RotaSIIL®) were pre-qualified for global use recently which should help expand the vaccine coverage. The highest incidence of rotavirus infection remains in low-income countries in sub-Saharan Africa and Asia. The efficacies of Rotarix® and RotaTeq® are very high in the high- to middle-income countries, however, lower efficacies have been observed in the low-income countries of sub-Saharan Africa and Asia. Notwithstanding this lower efficacy, many lives have been saved, which means a significant impact in terms of public health benefits in these two regions.

Apart from the efficacies of these vaccines, certain other shortcomings have been associated with the live-attenuated rotavirus vaccines. Intussusception, genome reassortment and viral shedding are some of the risks associated with these vaccines. Furthermore, the vaccines are quite expensive and require a cold-chain. Most of the affected, low-income countries could not implement these vaccines but for the help of GAVI (Global Alliance for Vaccines and Immunization). All these associated problems, therefore, call for the development of alternative, cost-effective and non-replicating rotavirus vaccine candidates.

Morphologically, rotavirus is an icosahedral, concentric particle with three layers of viral proteins, which encapsulate the viral genome. Capsid protein, VP6, is the most abundant and immunodominant viral protein, and together with other capsid proteins, are candidates of sub-unit, non-replicating vaccines. Rotavirus virus-like particles, either as a single- or multi-layer, have been used together with adjuvants to induce protective immune responses in animal models. Recombinantly expressed rotavirus VP6 protein has attracted attention as the safest parenterally administered vaccine candidate capable of inducing an immune response in animals. Rotavirus VP6 can induce a mucosal IgA response against rotavirus infections with heterotypic protection. Identifying a scalable expression system for production of the VP6 protein has been a major setback for the advancement of this immunoreactive protein as a vaccine candidate.

In this study, a wide-range yeast expression system was used to screen sixteen yeast strains in order to identify strain(s) capable of expressing rotavirus VP2 and VP6 encoded by three different yeast codon-optimised open reading frames (ORFs), which had been further optimised for expression by improving the Kozak consensus sequence. *Hansenula polymorpha* (UOFS_Y0915) was identified as the only yeast capable of expressing these

proteins simultaneously. Furthermore, after negative staining with uranylacetate solution, a particle of 58 nm in size was observed with transmission electron microscopy. This particle has a rough, spherical shape with a distinct single-shelled layer of rotavirus VP6 capsid enclosing the core protein, VP2.

Production of rotavirus VP6 protein in yeasts has thus far been by the intracellular expression, but to reduce the costs of downstream processing in a production facility, it would be better to produce this protein by extracellular secretion. Therefore, a recombinant wide-range expression plasmid was modified by promoter and signal sequence optimisations to enable, respectively, the expression and secretion in engineered *Kluyveromyces lactis* (UOFS_Y1167). A deep-well bioreactor screen coupled with ELISA testing evaluated VP6 expression in the growth medium by approximately one hundred *K. lactis* colonies for the first time. Therefore, as proof-of-concept, a screening protocol was developed that would allow for the identification of more yeast strains with the potential to secrete rotavirus VP6 protein. Identification of such yeasts will enable scaled-up production of this protein in bioreactors. This should, in turn, facilitate cost-effective production of rotavirus VP6 protein as a potential next generation, non-replicating rotavirus vaccine candidate.

Keywords: rotavirus, viral gastroenteritis, non-replicating vaccine candidate, heterologous gene expression in yeast, virus-like particle, secreted protein production, deep-well bioreactors

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Chapter One

1.1 Introduction

Rotavirus belongs to the family of *Reoviridae* (Flewett *et al.*, 1973; Flewett *et al.*, 1974; Yeager *et al.*, 1990). It is an icosahedral, non-enveloped, segmented dsRNA virus responsible for most of the diarrhoea-related deaths in infants and children less than 5 years (Patel *et al.*, 2013; Tate *et al.*, 2016). The genome of rotavirus is about 18 500 bp long and is made up of 11 segmented, double-stranded RNA (dsRNA) segments (Kapahnke *et al.*, 1986; Labbé *et al.*, 1994). Each of these genome segments contains one open reading frame (ORF) coding for one protein except genome segment 11, which codes for two proteins (Estes & Cohen, 1989; Prasad *et al.*, 1996). Structurally, infectious rotavirus is a triple-layered particle (TLP) with three concentric layers of structural capsid proteins enclosing the viral genome (Estes, 1996). Among the nine antigenic groups identified, rotavirus group A is the most important group responsible for most of human gastrointestinal infections caused by rotavirus. Rotavirus group A can further be classified based on genotyping of segment 9, encoding the outer glycoprotein VP7 known as the G-type, and segment 4, encoding the protease-sensitive VP4 spike protein known as the P-type (Matthijnssens *et al.*, 2008).

The efficacy and effectiveness of the live-attenuated oral rotavirus vaccines are quite remarkable against rotavirus infections (World Health Organisation, 2018b). With the introduction of the vaccines in 2006, the mortality rate due to rotavirus infections in children less than 5 years declined sequentially from 453 000 in 2008 (Tate *et al.*, 2012) to 128 500 in 2018 (Troeger *et al.*, 2018). This is possible because of the convergent efforts of the two globally licensed live-attenuated vaccines Rotarix® (GSK, Belgium) and RotaTeq® (Merck, USA) introduced in the USA by 2006 and in South Africa, by 2009. Recently, two Indian produced oral live-attenuated vaccines named Rotavac® (Bharat, India) and Rotasiil® (Serum, India) have been pre-licensed to be used globally (World Health Organisation, 2018b). Apart from these, there are regionally licensed, oral, live-attenuated vaccines such as LLR-37 (Lanzhou, China) (Li *et al.*, 2015) and Rotavin-M1® (Polyvan, Vietnam) (Le *et al.*, 2017).

Despite the outstanding reports of these vaccines from clinical trials and surveillance studies, it is important to re-emphasise that rotavirus infection is still the leading cause of death related to gastroenteritis in children less than 5 years worldwide (Tate *et al.*, 2016). Currently, 96 countries have introduced rotavirus vaccines, either sub-nationally or nationally, while 23 countries are currently planning to introduce rotavirus vaccines into their national immunization programmes. More than 71 million children/infants still lack access to rotavirus vaccines (RotaCouncil, 2018). In addition, various reports have indicated lower efficacies for

the Rotarix® and RotaTeq® vaccines in sub-Saharan Africa and parts of Asia; two regions of the world with the highest burden of rotavirus infection, accounting for more than 90% of total infections (Burnett *et al.*, 2018a; Burnett *et al.*, 2018b; Jonesteller *et al.*, 2017; Tate *et al.*, 2016). Rotavirus vaccines are expensive and therefore GAVI (Global Alliance for Vaccines and Immunizations) supported most of the low-resource countries to enable vaccine introduction, especially in Africa and Asia (Abou-Nader *et al.*, 2018). Currently, non-GAVI countries are purchasing Rotarix® at $6.20/dose and RotaTeq® at $3.62/dose as against the GAVI-qualified countries, $2.13/dose and $3.2/dose respectively (Debellut, 2018; UNICEF, 2019).

Higher rotavirus vaccine efficacies have been reported in high- and middle-income countries than in low-income countries (Msimang *et al.*, 2013; Patel *et al.*, 2011; Richardson *et al.*, 2011). Nevertheless, the impact of these vaccines, as recorded in Africa and Asia, is associated with huge reduction in the mortality rate of severe gastroenteritis and all forms of hospitalized-diarrhoea in children and infants (Mwenda *et al.*, 2010; Mwenda *et al.*, 2018). Reports from rotavirus vaccine surveillance studies attributed the lower efficacy to several factors which include the nature of the circulating rotavirus strains (Jere *et al.*, 2018), co-administration of the vaccine with oral polio vaccine (OPV) (Velasquez *et al.*, 2018), transplacentally acquired maternal rotavirus-specific antibodies by the infant (Appaiahgari *et al.*, 2014; Becker-Dreps *et al.*, 2015; Moon *et al.*, 2016), co-infections (such as norovirus and enteric bacterial infections) (Wang *et al.*, 2012), microbiota (Harris *et al.*, 2017), immune-status and general nutrition (Harris *et al.*, 2018; Harris, 2015; Mora *et al.*, 2008; Valdez *et al.*, 2014). Rotavirus infection is contagious by the faecal-oral-route only (Chandran *et al.*, 2010; Greenberg & Estes, 2009). The WHO has continued to reiterate the implementation of rotavirus vaccines as part of the comprehensive strategy to control diarrhoeal diseases, at the same time, promoting strategic prevention (including promotion of early and exclusive breastfeeding, handwashing with soap, improved water and sanitation) and treatment packages (including low-osmolarity oral rehydration salts (ORS) and zinc supplementation). The reason being that, as at the end of 2017, the global rotavirus vaccine coverage was 28% (World Health Organisation, 2018a).

All licensed rotavirus vaccines are live-attenuated vaccines capable of reassorting with circulating field strains belonging to the same group as a result of co-infection (Iturriza-Gomara & Gray, 2011; McDonald *et al.*, 2016) leading to the emergence of novel reassortant strains (Mueller & Johne, 2007; Ogden *et al.*, 2018; Payne *et al.*, 2009). Remarkably, some of these reassortants, which were formally rare (World Health Organisation, 2011), are becoming more prevalent globally due to vaccine strain selection and pressure (Dóró *et al.*, 2014). In addition,

several reports have shown the virulence of the vaccine-derived rotavirus strains (Boom *et al.*, 2012; Bowen *et al.*, 2016; Bucardo *et al.*, 2012; Donato *et al.*, 2012; Hemming & Vesikari, 2012, 2014) and this may raise a concern in the public health (Bowen *et al.*, 2016). Generally, poor seroconversion was witnessed in Africa and Asia due to transplacentally acquired IgG (Beres *et al.*, 2016; Chilengi *et al.*, 2016; Lazarus *et al.*, 2018; Moon *et al.*, 2016). Albeit at a very low rate, live-attenuated oral vaccine poses a risk of intussusception (IS) (Burnett *et al.*, 2018a; Kassim & Eslick, 2017). This risk was reported for the first prototype human-animal, reassortant, live-attenuated, oral vaccine, RotaShield® (Wyeth, USA), which was withdrawn from the market in 1999 (Murphy *et al.*, 2001). Post-marketing surveillance studies had shown an excess of 1 to 7 cases of IS at either first or second dose for the Rotarix® and RotaTeq® per 100 000 immunized children (Burnett *et al.*, 2018a; Kassim & Eslick, 2017). Similar rates have been reported for the newly licensed vaccines, Rotavac® (Bhandari *et al.*, 2014) and Rotasiil® (Kulkarni *et al.*, 2017). In all cases, the rate of intussusception is age-dependent (Buttery *et al.*, 2011; Parashar *et al.*, 2015; Simonsen *et al.*, 2005; World Health Organisation, 2013b). Several studies on RotaShield® showed that the risk of intussusception increases when the first dose was given after 3 months (Gargiullo *et al.*, 2006; Rothman *et al.*, 2006; Simonsen *et al.*, 2005) and the highest risk occurred between 4 and 9 months (Simonsen *et al.*, 2005). Because of this, the first vaccine dose of the current live-attenuated rotavirus vaccines were initially restricted to be given between 6 and 14 weeks of age when the natural incidence of intussusception is low (World Health Organization, 2007)(Parashar *et al.*, 2015). The incidence of intussusception with rotavirus vaccines needs accurate prognosis because pre-vaccination surveillance studies have shown that intussusception naturally occurs in premature, neonates, infants and children between 6 and 18 months (Bothara *et al.*, 2018; Clark *et al.*, 2019; Justice *et al.*, 2006; Tepmalai *et al.*, 2017). In the post-vaccination era, natural intussusceptions have been misconstrued for intussusception caused by the rotavirus vaccines (Clark *et al.*, 2019; Zhang *et al.*, 2019). There is no doubt that a small increase in risk of intussusception is associated with rotavirus vaccines, nevertheless, this varies geographically, is age-related, depends on the time of vaccination and dose-dependent (Bines *et al.*, 2018; Stowe *et al.*, 2016; Tate & Parashar, 2019). The total production capacity for rotavirus vaccines is, however, far below the global demand (GAVI, 2018; World Health Organisation, 2018a). Apart from these associated problems, the choice of rotavirus vaccine is constantly being restrained due to the financial status of the affected countries (majority of which are low-income countries), government policies and law-makers (World Health Organisation, 2013a).

Following the development of regional licensed vaccines (LLR-37 and Rotavin-M1®), there is an increasing awareness to produce local rotavirus vaccines matching the circulating rotavirus

strains in specific regions such as the sub-Saharan region. Apart from inactivated rotavirus particles known to be antigenic (Jiang *et al.*, 2008; Resch *et al.*, 2018), rotavirus virus-like particles (RV-VLPs) are non-replicating particles with promising immunogenic properties in animal studies (Azevedo *et al.*, 2013). VLPs are viral particles that assume the natural viral structure but lack genetic material. Hence, RV-VLPs are good alternative rotavirus vaccine candidates. Non-replicating rotavirus vaccines that are parenterally administered are in pre-clinical trials such as VP2/6/7 and VP2/4/6/7 (Burnett *et al.*, 2018a) as well as inactivated rotavirus vaccine candidates such as G1P[8] and G2P[4] (Burnett *et al.*, 2018a). Clinically, safety and immunogenicity of P2-ΔVP8-P[8] sub-unit non-replicating vaccine candidate has been carried out and the phase I and II trials of the trivalent immunocompetent formulation, P2-ΔVP8 (P[4], P[6] and P[8]), is now being prepared for (Groome *et al.*, 2017). Altogether, they have been shown with the immunogenic potential as alternative rotavirus vaccine candidates with the ability to reduce safety concerns associated with the rotavirus live-attenuated oral vaccines (Glass *et al.*, 2018).

Several of the rotavirus capsid proteins (VP2, VP4, VP6 and VP7), as well as non-structural proteins (NSP2 and NSP4), have been expressed and tested immunogenically (Glass *et al.*, 2018; Yen *et al.*, 2014). Rotavirus VP6, being immunodominant (McNeal *et al.*, 2007) and immunoreactive (Estes *et al.*, 1987), offers heterotypic immune protection against rotavirus infection (Esquivel *et al.*, 2000; Garaicoechea *et al.*, 2008; Gualtero *et al.*, 2007; Lappalainen *et al.*, 2014). Rotavirus VP6-induced, mucosal, polymeric, secretory IgA is highly efficient against rotavirus infection (Corthésy *et al.*, 2006) while the monomeric, non-neutralising IgA offers inhibition against rotavirus replication (Aiyegbo *et al.*, 2013; Feng *et al.*, 2002). However, rotavirus clearance and prevention against re-infection are centered on the ability of the VP6 protein to stimulate the $CD8^+$ T cells and B cells with mucosal homing receptors (Franco & Greenberg, 1995; Kuklin *et al.*, 2001). Adjuvant formulated rotavirus VP6 protein has been shown to induce an effective, but delayed immune response against rotavirus infection in neonatal mice (VanCott *et al.*, 2006). Generally, rotavirus VP6 protein can interact immunogenically with naïve B cells in human adults and neonatal blood via surface immunoglobulin and this improves the quality of acquired immune responses (Parez *et al.*, 2004). Rotavirus VP6 standalone (Lappalainen *et al.*, 2015) or fused with NSP4 as a bivalent vaccine (Feng *et al.*, 2017) has been observed to provide strong protection against rotavirus infection in mouse models. This, therefore, means that the rotavirus VP6 protein offers a protective immune response against heterotypic rotavirus infection by stimulating mucosal secretory IgA (Burns *et al.*, 1996). Among the parenteral administrations, intranasal administration has been observed as the most active choice of administering sub-unit rotavirus vaccine candidates (Choi *et al.*, 2002a).

Other viral proteins such as VP4 and VP7 are known to induce neutralizing IgA antibody responses (Kapikian *et al.*, 2001; Nair *et al.*, 2017). Truncated VP4 and VP8* (the cleaved product of VP4) were shown in animal studies to be highly immunogenic (Xue *et al.*, 2015). Both NSP2 and VP5 proteins have been shown to induce immune responses to rotavirus infection, although the mechanisms by which they limit viral replication are not fully understood (Kirkwood *et al.*, 2008; Nair *et al.*, 2017). IgG and/or IgA responses have been detected for VP5, VP6 and NSP2, (Nair *et al.*, 2017; Svensson *et al.*, 1987a; Svensson *et al.*, 1987b) while faecal IgA was shown to react to NSP2 and VP6 in convalescent samples (Colomina *et al.*, 1998).

Among the rotavirus VLPs, double-layered particles (DLPs) are the most attractive antigen, capable of inducing heterologous protection by stimulating the mucosal secretion of IgA in mice. This was confirmed in mice challenged with highly infectious murine rotavirus (Schwartz-Cornil *et al.*, 2002). It was proposed that the use of DLPs to prime/boost the subject prior to live-attenuated, oral vaccination will reduce the dosage regimen and boost the immune response even at first dose in a neonatal gnotobiotic pig model (Azevedo *et al.*, 2010).

Baculovirus-insect expression remains the classical model for the production of RV-VLPs (Crawford *et al.*, 1994). These insect cell producing RV-VLPs are immunogenic and capable of inducing protective immune response in animal studies, especially when used with immunostimulant in a prime/boost regimen (Azevedo *et al.*, 2010; Ciarlet *et al.*, 1998). However, this expression system is expensive and difficult to upscale. Bacterial expression systems represent a prokaryotic model of recombinant protein expression. Despite its frequent usage for recombinant expression of proteins, it cannot perform post-translational modifications (PTM) and often produces recombinant proteins as inclusion bodies. Nevertheless, this expression system has been used for the production of truncated/chimeric rotavirus capsid proteins such as ΔVP4, ΔVP8, VP7, MBP-VP6 and MBP-VP4 (MBP – maltose binding protein) and nonstructural proteins such as NSP4 (Enouf *et al.*, 2001) with promising immunogenic features (Choi *et al.*, 1999; Gil *et al.*, 2000; Li *et al.*, 2018b; Liu & Sun, 2006; Perez *et al.*, 2005; Wen *et al.*, 2012; Xue *et al.*, 2015). Transgenic plant expression systems offer a promising outcome for the rotavirus-VLPs, especially for rotavirus VP2, VP6, VP7 and VP5* proteins (Dong *et al.*, 2005; Li *et al.*, 2018a; Matsumura *et al.*, 2002; Pera *et al.*, 2015; Saldana *et al.*, 2006; Yu & Langridge, 2003), as part of edible fruits, grains, cereals or leaves but low yield and the effect of intestinal digestion may minimize the effectiveness of the antigen (Mor *et al.*, 1998; Streatfield & Howard, 2003). The biology of rotavirus has been well studied in mammalian cells such as MA104. Rotavirus capsid proteins VP6 and VP7 have been expressed in BHK-21 (Choi *et al.*, 2002b; Ito *et al.*, 1996). Although it is a versatile expression

system and capable of performing all forms of PTMs, this system is very expensive to scale up and requires technical input. More importantly is the fear of production contaminants such as porcine circovirus 1 (PCV1) DNA due to the serum used in vaccine preparation (Kuehn, 2010), as found in the current licensed oral vaccines (McClenahan *et al.*, 2011; Ranucci *et al.*, 2011).

Yeast expression is a unicellular expression system mostly used in biotechnology because yeasts are relatively simple compared to other eukaryotes and possess a highly engineerable genomic components. Various biopharmaceutical products such as recombinant vaccines, therapeutic hormones and proteins, drugs and food supplements (Walsh, 2005, 2006, 2010) have been recombinantly produced by certain species of yeasts such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Kluyveromyces lactis*, *Yarrowia lipolytica*, *Hansenula polymorpha* and *Arxula adeninivorans* (Kim *et al.*, 2015; Vieira Gomes *et al.*, 2018). With the increasing development in the recombinant vaccine production, various commercial VLPs are now being produced and licensed for use against viral infection, for example Gardasil® against human papillomavirus (McLemore, 2006), Engerix-B® (Keating & Noble, 2003), Euvax B and Recombivax used against hepatitis B virus, Ambirix and Twinrix against hepatitis A and B viruses (Kim *et al.*, 2015; Kim & Kim, 2017). Yeast requires relatively inexpensive raw materials for growth, easily scale-up for downstream processing and can be manipulated genetically for intracellular and extracellular recombinant production of proteins. Only a few reports are available for the use of a yeast expression system to produce rotavirus VLPs. The first report used *S. cerevisiae* to express the rotavirus triple-layered particles under the control of constitutive promoters in a multi-cistronic expression vector (Rodríguez-Limas *et al.*, 2011). Immunisation with the yeast extract reduced rotavirus replication and shedding in adult mice challenged with murine rotavirus strain EDIM (epizootic diarrhoea of infant mice) (Rodríguez-Limas *et al.*, 2014). In 2016, Bredell and co-workers compared the expression efficiency of rotavirus VP6 protein in *E. coli, P. pastoris* and *H. polymorpha* (Bredell *et al.*, 2016) and discovered that *H. polymorpha* outperformed *E. coli* and *P. pastoris* expression in a controlled bioreactor system for the production of intracellularly expressed rotavirus VP6 protein.

1.2 Problem identification

In addition to the lower efficacy of the rotavirus, live-attenuated, oral vaccines, especially in sub-Saharan Africa, the WHO had reported unmet global vaccine demand and low vaccine coverage at regional and international level (World Health Organisation, 2018a). In addition, rotavirus vaccines are expensive and beyond the reach of many low-income countries where the burden of rotavirus infection is very high. Currently, while the price of oral polio vaccine is less than $0.2/10-doses among the early childhood vaccines, Rotarix®, Rotavac®, RotaTeq®

and Rotasiil® are sold for $2.13/dose, $0.85/5-doses, $3.20/dose and 0.95/2-doses respectively to GAVI qualified countries. Despite these reduced prices of the rotavirus vaccines, many countries can still not afford it (Debellut, 2018; UNICEF, 2019). About 71 countries including Chad, the Democratic Republic of the Congo, Niger, Nigeria and many others with high incidence of rotavirus infection are yet to introduce rotavirus vaccines (RotaCouncil, 2018). This is due to factors such as policy making, low country income status, GAVI-eligibility, reliable cold-chain storage facilities, national conflicts and disasters (Abou-Nader *et al.*, 2018; Mwenda *et al.*, 2017; Santosham & Steele, 2017; World Health Organisation, 2013a).

Other identified problems include possible risk of genomic reassortment within the vaccine strains and between vaccine strains and the circulating field strains (Donato *et al.*, 2012; Donato *et al.*, 2014; Hemming & Vesikari, 2012, 2014; Markkula *et al.*, 2015). Reassortment is possible due to the mixed rotavirus infections that are prevalent in Africa (Mwenda *et al.*, 2010) and viral shedding by the vaccinated infants (Donato *et al.*, 2012; Ruiz-Palacios *et al.*, 2006; Vesikari *et al.*, 1986). It results in different rotavirus genotypes, which could be problematic for oral attenuated vaccines. Despite, numerous divergences in the evolution of rotavirus genotypes after the introduction of the vaccines, the efficacies of the vaccines appeared not to be affected (Bar-Zeev *et al.*, 2016; Bar-Zeev *et al.*, 2015; Velasquez *et al.,* 2014; Payne *et al.*, 2013; Steele *et al.,* 2012). However, the partial efficacy against the heterotypic strains is a concern especially for Rotarix® (Bar-Zeev *et al.*, 2016; Bar-Zeev *et al.*, 2015). A need, therefore, exists to produce an alternative, non-replicative vaccine candidate against rotavirus infection. Yeast, as a cost-effective expression system, is explored as an alternative to mammalian or insect cell expression systems.

1.3 Project rationale

With the four internationally licensed oral vaccines plus two identified regionally produced and used vaccines, the effective global vaccine coverage is 28% (World Health Organisation, 2018a). No effective treatment against rotavirus infection is available. It can be managed by oral rehydration therapy (ORT), zinc supplementation and oral ingestion of prepared immunoglobulins containing antibodies against a specific rotavirus serotype (Sarker *et al.*, 1998). Consequently, the use of rotavirus VLPs and sub-unit antigens as vaccine candidates has continued to attract much attention. Antibodies directed against VP6 neutralise rotavirus intracellularly by blocking the transcription channels of actively transcribing intracellular double-layered particles (Aiyegbo *et al.*, 2013). Rotavirus VP6 can induce humoral immunity that will enhance durable immuno-performance of B and T cells (Nair *et al.*, 2016). This

immunodominant attribute of VP6 (McNeal *et al.*, 2007) has made it an attractive target for vaccine development.

Rotavirus VP6 has been produced in insect cells (Crawford *et al.*, 1994; Heinimaki *et al.*, 2018), transgenic plants (Chung *et al.*, 2000; Feng *et al.*, 2017; Zhou *et al.*, 2010), bacteria (Lee *et al.*, 2010; Temprana *et al.*, 2018; Zhao *et al.*, 2011), yeasts (Bredell *et al.*, 2016; Rodríguez-Limas *et al.*, 2014; Rodríguez-Limas *et al.*, 2011), mammalian cells (Gonzalez & Affranchino, 1995; Nilsson *et al.*, 1998) and transgenic animals (Soler *et al.*, 2005; Soler *et al.*, 2007). With this study, yeast expression was used to produce rotavirus VLPs because it is easy to manipulate, cost-effective and requires inexpensive growth media for cultivation. To this effect, sixteen different yeast strains were engineered to express recombinant rotavirus VP2 and VP6 proteins using a previously developed, unique, wide-range yeast expression system (Albertyn *et al.*, 2011) that allows for the transformation and subsequent protein expression in any yeast. More importantly, this study also attempted to engineer the yeast strain to express and secrete rotavirus VP6 protein using a modified recombinant expression plasmid.

1.4 Preliminary data

A wide-range, yeast expression system containing the *Yarrowia lipolytica* TEF promoter, *Kluyveromyces marxianus* inulinase termination region and the hygromycin B resistance gene *(hph)* was previously developed for the transformation of any yeast and subsequent expression of recombinant proteins (Albertyn *et al.*, 2011). The expression vectors, pKM173 and pKM177, can be used for single (containing one expression gene) or dual-constructs (containing two expression genes). They possess internal transcribed spacer (ITS) regions and 18S rDNA from *K. marxianus* for homologous recombination into the susceptible transformed yeast genome. Together with the availability of the UNESCO-MIRCEN yeast culture collection, the wide-range, yeast expression system offers a unique screening tool to identify potential yeast strains capable of expressing recombinant proteins. Both resources are available at the Department of Microbial, Biochemical and Food Biotechnology, University of Free State (UFS), Bloemfontein, South Africa.

The consensus sequences encoding rotavirus VP2 and VP6 structural proteins from the South African rotavirus field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] was molecularly characterized from the stool sample of a neonate with severe gastroenteritis at the Dr. George Mukhari Hospital, Sefako Makgatho University, South Africa (Jere *et al.*, 2011). The whole genome consensus sequence of this strain was obtained using GS20/FLX technology (Potgieter *et al.*, 2009). These open reading frames (ORFs) were used to construct

recombinant, wide-range, yeast expression vectors (Makatsa, 2015) as pKM177_VP6 and pKM173_VP2/6. These VP2 and VP6 ORFs were codon-optimised for expression in *Kluyveromyces lactis* and *Arxula adeninivorans* (both purchased from GenScript®) and *Pichia pastoris/Hansenula polymorpha* (previously purchased from GeneArt® and generously provided by Prof. J. Görgens from Stellenbosch University, South Africa, under Memorandum of Transfer and Assignment, MTA).

Bacterially expressed VP2 and VP6 proteins were used as positive controls to monitor the expression of VP2 and VP6 proteins in each of the transformed yeast strains. The proteins were evaluated using western blot analysis. Despite confirmed positive genome integration, no expression was obtained in any of the selected yeast strains when transformed with pKM177 containing the VP6 ORF, optimised for expression in *K. lactis* and *A. Adeninivorans*. Expression of rotavirus VP6 was confirmed in few of the selected yeast strains when transformed with the pKM177 containing the VP6 ORF optimised for expression in *P. pastoris/H. polymorpha* (Makatsa, 2015). Rakaki in 2018 re-examined each of these recombinant expression plasmids and modified them (Rakaki, 2018). Expression of rotavirus VP6 protein was, thereafter, obtained and an oligomeric structure of this protein was evaluated (Rakaki, 2018).

In a follow-up study, a number of yeasts (*A. adeninivorans, H. polymorpha, K. lactis, P. pastoris, S. cerevisiae* and *Y. lipolytica*) tested positive for intracellular production of rotavirus VP6 encoded by the VP6 ORFs optimised for the three different yeasts (Rakaki, 2018). The presence of a second up-stream ATG was identified and removed. In addition, the Kozak sequences for improved expression were optimised. In the current study, selected yeast strains were engineered to direct the expression of rotavirus VP2 and VP6 to enable DLP production. In addition, a proof-of-concept screening protocol for the identification of secreted VP6 was developed.

1.5 Aims and objectives of the study

The aims of this work were:

i. To identify engineered yeast strains capable of intracellular co-expression of the rotavirus VP2 and VP6 proteins encoded for by the VP2 and VP6 ORFs from RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] strain
ii. To establish a screening protocol for yeasts engineered to secrete rotavirus VP6 protein

The specific objectives of this work were as follows:

i. The manipulation of the three yeast, codon-optimised expression vectors (*A. adeninivorans, K. lactis* and *P. pastoris/H. polymorpha* codon-optimised VP2 and VP6 ORFs) by removing the upstream -ATG- using site-directed mutagenesis and construction of the dual-expression plasmids for each of the yeast codon-optimised ORFs (**Chapter Three)**

ii. Screening of the UNESCO-MIRCEN yeast culture collection at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, for the recombinant, co-expression of VP2 and VP6 as coded for by the VP2 and VP6 ORFs from the dual-expression vectors. Formation of double-layered particles from the co-expressed rotavirus capsid proteins was evaluated with transmission electron microscopy (**Chapter Four)**

iii. The sequential removal of possible glycosylation motifs in VP6 optimised for expression in *P. pastoris/H. polymorpha* was carried out and followed by the intracellular expression of this mutated VP6 protein in selected yeast strains (**Chapter Five)**

iv**.** Development of a proof-of-concept protocol to assess the expression and secretion of recombinant rotavirus VP6 encoded for by the VP6 ORF optimised for *P. pastoris/H. polymorpha* in *Kluyveromyces lactis* after promoter exchange and the introduction of the signal sequence assembled with seamless cloning **(Chapter Six)**

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Chapter Two

Rotavirus: structure, infection and vaccine development

2.1 Introduction

Despite the reduction in disease burden after the introduction of rotavirus vaccines, rotavirus remains the main causative agent of severe gastroenteritis in children that are less than 5 years old (Troeger *et al.*, 2018). In 2000, an estimated 528 000 children less than 5 years old died due to rotavirus infection, whereas in 2013 this was reduced to 215 000 and by the end of 2016 an estimated 128 500 children died (Troeger *et al.*, 2018). This drastic reduction was a cooperative effort of the World Health Organisation (WHO) and other health-promoting organisations such as the Program for Appropriate Technology in Health (PATH) and Global Access to Vaccinations and Immunosations (GAVI) in reiterating the need for personal hygiene, sanitation, safe water and importantly, the introduction of rotavirus vaccines (Wang *et al.*, 2016). However, developing countries still sustain a high disease burden and 81.5 % of rotavirus deaths in 2016 occurred in sub-Saharan Africa (Troeger *et al.*, 2017; Troeger *et al.*, 2018).

Human rotaviruses were described in 1973, when viral particles were visualised by Ruth Bishop and co-workers (Bishop, 2009) in electron micrographs of thin sections of duodenal mucosa and later virus was identified in faeces by electron microscopy (Mebus *et al.*, 1976; Waters *et al.*, 2000). Flewett and co-workers suggested the name "*rotavirus*", a "wheel-like" particle, (Flewett *et al.*, 1974; Flewett & Woode, 1978) which was officially accepted by the International Committee on Taxonomy of Viruses in 1978 (Matthews, 1979). Coincidentally, in 1972 rotavirus isolated from mice was shown to be related to the virus causing diarrhoea in cattle (Rubenstein *et al.*, 1971; Woode *et al.*, 1976). A decade before the discovery of human rotaviruses, several animal viruses that caused acute gastroenteritis had been discovered (Malherbe & Harwin, 1963) with morphologies that later matched human rotavirus features (Flewett & Woode, 1978; Woode *et al.*, 1976).

2.2 Genome structure and proteins

Rotaviruses are non-enveloped, icosahedral viruses **(Figure 2.1)**. This viral particle is subdivided into an outer, middle and inner core layer. The inner core layer contains all the necessary enzymes responsible for viral mRNA synthesis (Estes & Cohen, 1989; Labbe *et al.*, 1991; Estes, 1996, 2001; Estes & Kapikian, 2007; Estes & Greenberg, 2013). It has 11-segments of double-stranded (ds)RNA and each segment codes for one functional

protein, except segment 11 which codes for two proteins (Yeager *et al.*, 1990; Estes, 2001). These proteins can either be involved in the rotavirus particle framework design (structural proteins, VP) or act as accessory proteins such as assemblage proteins and enzymes (non-structural proteins, NSP) **(Table 2.1).** The structural proteins are VP1, VP2, VP3, VP4 (VP5* + VP8*), VP6 and VP7 while non-structural proteins are NSP1, NSP2, NSP3, NSP4, NSP5/6 (Estes & Kapikian, 2007; Hu *et al.*, 2012c; Pesavento *et al.*, 2006) **(Table 2.1)**.

The rotavirus genome consists of approximately 18 550 nucleotides (Estes & Tanaka, 1989; Estes & Cohen, 1989). Segment 11 is bicistronic with 667 bp (Matsui *et al.*, 1990), the lowest number of base pairs, although the number can vary depending on the rotavirus strain. In contrast, segment 1 has the highest number of base pairs with 3302 nucleotides (Estes & Greenberg, 2013; Mattion *et al.*, 1991). Rotavirus genomes lack polyadenylation at the 3´-end of the genome segment and are rich in adenine (A) and uracil (U) (between 58% - 67%) (Estes & Greenberg, 2013; Hu *et al.*, 2012a; Imai *et al.*, 1983). The 5´-end of each of the positive sense RNA strands (coding mRNA) contain a capping sequence of $_{m}^{7}G_{ppp}GGC$ (Tao *et al.*, 2002) followed by a set of conserved sequences that form part of the 5´-non-coding sequences, an open reading frame (ORF) and conserved terminal 3´ sequences**.** Most rotavirus mRNAs end with a consensus sequence 5´-UGUGACC-3´, an important signal for gene expression and genomic replication. The extreme four-sequence 5´-GACC-3´ usually acts as translation enhancer in eukaryotic cells (Chizhikov & Patton, 2000). Furthermore, each mRNA must be signal-tagged for identification during viral packaging. The signal-like *cis*-terminal sequence (5´-UGUG-3´) is responsible for genome transcription, transportation, translation and replication.

2.3 Rotavirus structural proteins

The morphological appearance of rotavirus under the electron microscope is as a result of the structural proteins called “viral proteins”. There are six genome segments encoding six structural proteins - VP1, VP2, VP3, VP4, VP6 and VP7 (**Figure 2.1**) and their major roles/functions are discussed below:

VP1 (RNA-dependent RNA polymerase, RdRp) can synthesise dsRNA from the (+)ssRNA template (replicase activity) or synthesise mRNA from the dsRNA template (transcriptase activity) (Mansell & Patton, 1990) **(Figure 2.1)**. This means that rotavirus VP1 is a dual-function protein. The replicase function appears more complex and highly regulated than the transcriptase activity because it requires, in addition to VP1 recognising the 3´-UGUG conserved regulatory sequence, VP2, VP3 and VP6.Transcriptase activity is, however, structurally less complex because there is no need for the VP6 capsid (Lawton *et al.*, 1997a).

Table 2.1 Features of rotavirus simian agent 11 (SA11) genome segments and proteins

Genomic Segment (bp)	*Protein*	*Mw (kDa)/No of amino acids*	*Molecules/virion (% by wt/virion)*	*Post-translational modifications*	*Function*	*Reference*
1 (3 302)	VP1	124.847 (1 088 aa)	12 (2)	Myristoylation	Slightly basic inner-core protein, RNA-dependent RNA polymerase, binds ssRNA and VP3	(Lu *et al.*, 2008; Patton *et al.*, 1997; Trask *et al.*, 2012a; Valenzuela *et al.*, 1991)
2 (2 668)	VP2	102.431 (880 aa)	120 (15)	Myristoylation	Inner-core leucine zipper protein, binds RNA and VP1 for replicase activity	(Labbé *et al.*, 1994; Lawton *et al.*, 1997a, 2000; Lawton *et al.*, 1997b; Zeng *et al.*, 1994)
3 (2 591)	VP3	98.120 (835 aa)	12 (0.5)	Myristoylation	Basic inner-core protein, possesses a variable N-terminal domain, a central guanine-N7-methyltransferase with ribose-2´-O-methyltransferase domain, C-terminal guanylyltransferase with 2H-phosphodiesterase domain that cleaved 2′-5′ oligoadenylates thereby deactivating the RNase L and RNA 5´-triphosphatase domain. It can form a complex with VP1 and binds ssRNA	(Chen *et al.*, 1999; Graham *et al.*, 2003; Ogden *et al.*, 2015; Ogden *et al.*, 2014; Vasquez *et al.*, 1993)
4 (2 362)	VP4	86.782 (776 aa)	120 (1.5)	Proteolytic cleavage into VP5 (529 aa) and VP8 (247 aa)	Surface protein, hemagglutinin, P-type neutralisation antigen, protease-enhanced infectivity, virulence, homotrimer, viral attachment, neutralising antigen, putative fusion (aa 384 – 401), mature protein cleaved to VP5 (membrane-penetrating protein) and VP8 (membrane sialic acid binding)	(Denisova *et al.*, 1999; Gilbert & Greenberg, 1998; Graham *et al.*, 2003; LaMonica *et al.*, 2001; Mackow *et al.*, 1989)
5 (1 581)	NSP1	58.654 (491 aa)	-	None	Slightly basic, Zn-fingers (aa 54 – 66 and 314 – 327), interferon antagonist, binds RNA, putative viral E3 ligase activity	(Bagchi *et al.*, 2013; Bagchi *et al.*, 2010; Chung & McCrae, 2011; Liu *et al.*, 2013b; Nandi *et al.*, 2014; Nandi *et al.*, 2015)
6 (1 356)	VP6	44.816 (397 aa)	780 (51)	Myristoylation	Inner capsid protein, hydrophobic sub-group antigen, trimer, enhances transcription	(Aiyegbo *et al.*, 2014; Aiyegbo *et al.*, 2013; Clark & Desselberger, 1988; McNeal *et al.*, 2007a)
7 (1 104)	NSP3	34.600 (315 aa)	-	None	Inhibits host translation processes, binds 3´-end of viral mRNA, an acidic dimer and cellular eIF4G (eukaryotic initiation factor-4-gamma), *hsp90* (heat-shock protein), surrogate of PABP (poly(A)-binding protein)	(Chung & McCrae, 2011; Deo *et al.*, 2002; Groft & Burley, 2002; Piron *et al.*, 1998; Vende *et al.*, 2000)
8 (1 059)	NSP2	36.633 (317 aa)	-	None	NTPase (nucleoside triphosphatase), NDP (nucleoside diphosphate) kinase, RTPase (RNA triphosphatase), binds VP1 and NSP5 to form viroplasm, helix-	(Arnold *et al.*, 2012; Fabbretti *et al.*, 1999; Kattoura *et al.*, 1992; Taraporewala *et al.*, 1999; Taraporewala *et al.*, 2006; Taraporewala & Patton, 2001, 2004;

Genomic Segment (bp)	***Protein***	***Mw (kDa)/No of amino acids***	***Molecules/virion (% by wt/virion)***	***Post-translational modifications***	***Function***	***Reference***
					disruption, a basic octamer that binds RNA	Taraporewala *et al.*, 2002; Vasquez-Del Carpio *et al.*, 2006)
9 (1 062)	VP7[a] VP7[b]	37.368 (326 aa) 33.919 (297 aa)	780 (30) -	Matured cleavage of the signal peptide from the 276 amino acids, high mannose glycosylation and trimming (matured cleaved VP7 has 276 aa)	Cell attachment protein, G-type neutralisation antigen, RER (rough endoplasmic reticulum) integral membrane glycoprotein Ca^{2+}-dependent trimer with two hydrophobic NH_2-terminal regions, bicistronic gene, putative Ca2+-binding site is aa127 – 157	(Gajardo *et al.*, 1997; Graham *et al.*, 2003; Mackow *et al.*, 1988; Zambrano *et al.*, 2008)
10 (751)	NSP4	20.290 (175 aa)	-	NS29 or NS28 matured protein with the intact signal peptide is highly mannosylated	Enterotoxins secreted from infected cells, virulence, RER transmembrane glycoprotein with two hydrophobic NH_2-terminal regions, viroporin, an intracellular receptor for DLPs and morphogenesis of TLPs, interacts with viroplasm and autophagy pathway, modulates intracellular Ca^{2+} and RNA replication	(Au *et al.*, 1989; Lopez *et al.*, 2005; Meyer *et al.*, 1989; Silvestri *et al.*, 2005; Taylor *et al.*, 1996; Zambrano *et al.*, 2008)
11 (667)	NSP5	21.725 (198 aa)	-	Phosphorylation	Non-structural basic O-glycosylated phosphoprotein that binds RNA and NSP2 to form viroplasm, a protein kinase that interacts with VP2 and NSP6	(Blackhall *et al.*, 1997; Campagna *et al.*, 2007; Campagna *et al.*, 2005; Eichwald *et al.*, 2004; Fabbretti *et al.*, 1999; Vende *et al.*, 2002)
	NSP6	11.012 (92 aa)	-	Phosphorylation	A non-structural protein that interacts with RNA and NSP5 in viroplasm	(Rainsford & McCrae, 2007; Torres-Vega *et al.*, 2000; Welch *et al.*, 1989)

VP2 (core protein) is the most abundant structural protein found in the rotavirus core particles (Lazzarini *et al.*, 1981) and is the third most abundant protein in the viroplasm (Petrie *et al.*, 1982) **(Figure 2.1)**. Rotavirus VP2 can assemble itself into empty core-like particles without any other viral proteins or dsRNA (Labbé *et al.*, 1991). The two sub-units of this structural protein are VP2A, an asymmetric subunit around the icosahedral axis and VP2B, a subunit that fills the interspaces created by VP2A to form a decameric cap complex with VP1 **(Figure 2.1)**. Rotavirus VP2 is immunogenic and serum antibodies against this protein are a good indicator of previous infection (Conner *et al.*, 1988; Svensson *et al.*, 1987). In a non-specific sequence-based interaction, it binds ssRNA, dsRNA and dsDNA in preferential order (Boyle & Holmes, 1986).

VP3 (guanylyltransferase) is a minor component of the inner core of the virion known as a capping protein (Liu *et al.*, 1988) **(Figure 2.1)**. This protein catalyses the formation of the 5' cap in the post-transcriptional modification of mRNA and stabilises viral mRNA against nucleases (Cowling & Cole, 2010). Rotavirus VP3 is the the viral guanylyltransferase, which is an essential part of the transcriptase complexes (synthesis of the matured mRNA) but not the replicase complexes (synthesis of the matured dsRNA) (Wentz *et al.*, 1996). Recently, this protein has been shown to be involved in host immune evasion. VP3 exploits a 2H-phosphodiesterase domain to prevent activation of the cellular oligoadenylate synthase (OAS), which otherwise would activate the cellular RNase L pathway to degrade the viral ssRNA (Morelli *et al.*, 2015a; Morelli *et al.*, 2015b). Similarly, Ding and co-workers have reported the inhibitory effect of the rotavirus VP3 protein against the MAVS pathways, which could have induced a type III IFN induction, to initiate viral RNA degradation (Ding *et al.*, 2018).

VP1, VP2 and VP3 form a complex called the **five-fold hub** that has been postulated as a likely model for assembly of the inner core of rotavirus (McClain *et al.*, 2010). According to the model, the VP2 decamer associates with VP1 and VP3 to form the complex. The hub has three distinct functions in rotavirus assembly, replication and transcription. The first is to organise the assembly unit of the inner shell, by recruiting a copy of VP1 or the VP1-VP3 complex, probably with a segment of (+)-strand RNA. Secondly, it acts as a shaft on which the dsRNA segment, generated from the packaged (+)-strand synthesised from the *in situ* (-)-strand synthesis is coiled. Thirdly, it guides the nascent mRNA transcripts through its five-fold axis guided by the five-fold hub (Lawton *et al.*, 1997a).

The **VP4 (protease sensitive, spike-like protein)** protein is used in P-type classification and one of the virulence factors of rotavirus (Hoshino *et al.*, 2002). In the presence of intestinal trypsin, VP4 is cleaved into VP8* (28 kDa) and the VP5* (60 kDa) **(Figure 2.1)** and their roles

are to facilitate the membrane attachment and penetration, respectively (Arias *et al.*, 2002; Crawford *et al.*, 2001; Gilbert & Greenberg, 1998). The VP4 spikes, through its trimerization, interact extensively with VP6 and VP7 at type II channels (Settembre *et al.*, 2011; Trask & Dormitzer, 2006; Trask *et al.*, 2012a; Trask *et al.*, 2012b).

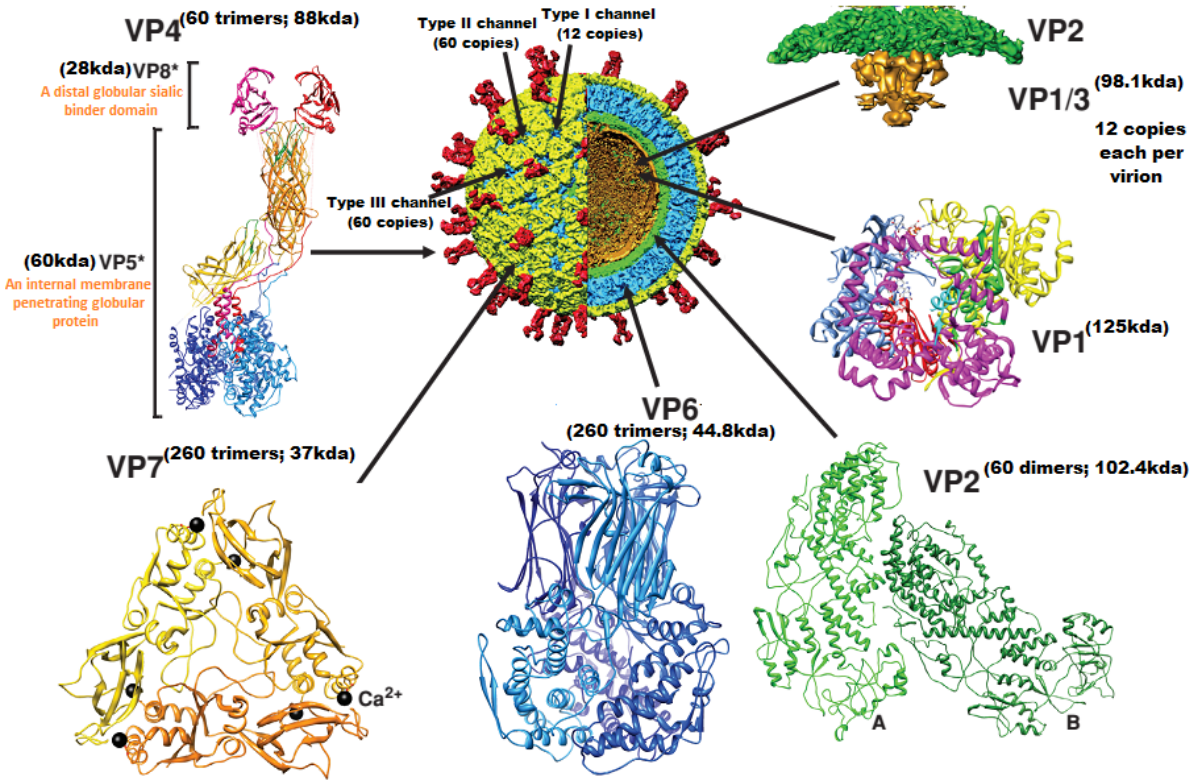


Figure 2.1: Rotavirus particle and its structural proteins. A cryo-electron microscope (cryo-EM) image of a triple-layered rotavirus particle showing the rotavirus TLP and all the associated structural proteins. VP1/3 (brown) associates with 120 dimers of VP2 (green) to form the core particle. At the inner part of the core is the multi-complex, RNA-dependent RNA polymerase, VP1 (yellow, blue, red, green and violet). The dissociated VP2 structural core protein is coated in light green (A-submit) and deep green (B-subunit), each subunit is represented by 60 dimers. VP6 (260 trimers) is found at the middle layer of the particle, coated blue. The upper part of the VP6 protein is the distal domain with an 8-stranded jelly-roll β-barrel fold that contacts VP7 and VP4, whereas the proximal domain has a cluster of 8 α-helices – a conformationally flexible loop structure, which enables it to optimally establish firm contact with the underlying VP2 subunits (McClain *et al.*, 2010). The calcium-regulated VP7 and protease-sensitive VP4 are responsible for cell attachment and membrane penetration, respectively. *Figure adapted from Estes and Greenberg, (2013)*.

VP6 (inner capsid protein) consists of 260 trimers (Gorziglia *et al.*, 1989; Sabara *et al.*, 1987), located below the VP7 trimers or on VP2 decamer (Prasad *et al.*, 1988) forming aqueous channels in the DLPs in a self-interaction T = 13 icosahedral shape (Mathieu *et al.*, 2001; Yeager *et al.*, 1990) **(Figure 2.1)**. These channels perform a specific role during rotavirus infection in the transcriptionally active rotavirus DLP (Lawton *et al.*, 1997a) **(Figure 2.1)**. The channels serve as passages for the release of the viral tanscripts during infection. VP6 forms the bulk of the capsid by weight (51% of the total capsid mass) with two domains

(the distal and the proximal domains) **(Figure 2.2)** (Baker & Prasad, 2010; Mathieu *et al.*, 2001). The interactions of VP6 with VP7 (outer) and VP2 (core) are important features of rotavirus particles (Li *et al.*, 2009; Mathieu *et al.*, 2001) because it stabilises the entire rotavirus structure and facilitates host integration, cell entry and endogenous transcription (Pesavento *et al.*, 2006) **(Figure 2.2)**. VP6 is highly antigenic and immunogenic (Heinimaki *et al.*, 2018; Lappalainen *et al.*, 2015; Malm *et al.*, 2016) compared to other structural proteins and can be used to identify rotavirus groups (Bishop, 1996). The common cross-reactive epitopes of VP6 cut across all the group A rotaviruses and is usually detected by ELISA. The VP6 protein is highly conserved with < 13% amino acid divergence between any mammalian group A rotavirus strains (Tang *et al.*, 1997). Removal of VP6 from the double-layered particles results in a loss of transcriptase activity of the particles. Addition of VP6 restores transcriptional activity (Desselberger *et al.*, 2013), however, in excess, it can inhibit transcription (Sandino *et al.*, 1986). Myristoylation observed in VP6 may be important for the formation of virus particles, their assembly and maturation (Clark & Desselberger, 1988) or for targeting double-layered particles to the ER membrane for budding (Clark & Desselberger, 1988).

VP7 (glycosylated outer coat protein) is the second most abundant rotavirus capsid protein. The activity of this protein is greatly coordinated and stabilised by Ca^{2+} (Aoki *et al.*, 2009; Chen *et al.*, 2009) **(Figure 2.1)**. However, because of its interaction with VP4, both can be dissociated from the TLP in the presence of chelating agents such as EDTA to form the DLP (Cohen *et al.*, 1979; Dormitzer *et al.*, 2000; Settembre *et al.*, 2011). Apart from its structural functions, G-type classification of rotavirus is based on the VP7 capsid protein. A 14K fragment (14 000 kDa) of VP7 has been identified as an immunodominant domain, which induces neutralising antibodies (Sabara *et al.*, 1985) and confers passive immunity in a murine model (Frenchick *et al.*, 1988). The wide variety of glycosylation sites on VP7 may affect its antigenic determinants but has little or no effect on the antibody-binding to the epitopes (Estes *et al.*, 1982).

2.4 Rotavirus non-structural proteins

There are five genome segments, four coding for one non-structural protein each (NSP1, NSP2, NSP3 and NSP4) and one (segment 11) which encodes for two nonstructural proteins (NSP5 and NSP6) (Estes & Kapikian, 2007; Estes & Greenberg, 2013; Pesavento *et al.*, 2006). Rotavirus non-structural proteins are accessory proteins expressed in the infected cell during replication and pathogenesis (Pesavento *et al.*, 2006). The interaction between the structural and non-structural rotavirus proteins is critical to viral genome replication, encapsidation and assembly (Hu *et al.*, 2012a; Hu *et al.*, 2012c; Hua *et al.*, 1993).

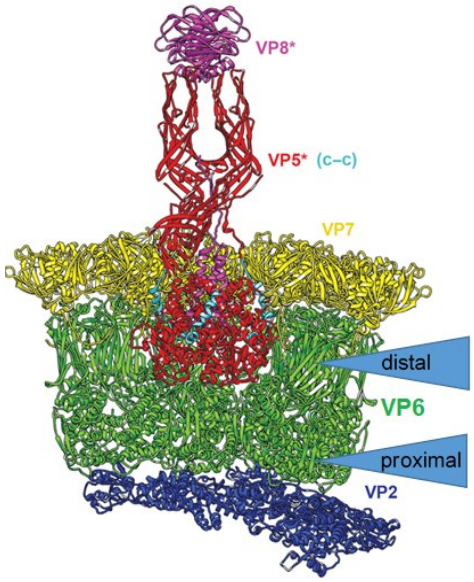


Figure 2.2: Multifunctional binding of rotavirus VP6 capsid protein with VP2 (dark blue), VP4 (VP5* + VP8*) and VP7 (yellow). The ribbon representation is showing the distal and proximal domains through which VP6 interacts with VP7 and the VP2 respectively. Each VP7 trimer forms a cover upon each trimer of the VP6 through the surface contacts in a T = 13 *l* icosahedral symmetry. Each of the VP2 decamer is overlayed with the VP6 trimer from its proximal domain. VP4 is represented in magenta as VP8* and red as coiled-coil VP5*. VP4, through the globular foot domain of coiled-coil VP5*, is anchored at the hexagonal positon in the VP6 trimer. The VP8* ensure specific sialo-receptor interaction to intiate cell attachment during infection. *Figure adapted from Settembre et al., (2011).*

Several reports have shown the antagonising roles of rotavirus nonstructural proteins such as NSP1 and NSP4. Rotavirus releases NSP1 protein to inhibit interferon production via the degradation of IRF3, IRF5, IRF7 and β-transducing repeat-containing protein (β-TrCP) (Barro & Patton, 2005; Graff *et al.*, 2009; Graff *et al.*, 2007; Graff *et al.*, 2002). Rotavirus NSP3 promotes viral mRNA translation by acting as a PABP and eIF4G binder (Chung & McCrae, 2011; Groft & Burley, 2002). The main viral enterotoxin property relies on the transmembrane, glycoprotein, NSP4 (Hu *et al.*, 2012c). The protein kinase and RNA binder, known as NSP5, can interact with NSP2 to form viroplasm together with NSP6 (Patton, 1995). Therefore, the roles of nonstructural rotavirus proteins are vital for the survival and pathogenesis of rotavirus infections.

2.5 Rotavirus morphology (Virion)

Infectious rotavirus exists as three concentric layers - **single-layered particle** (SLP)/core protein, **double-layered particle** (DLP) and **triple-layered particle** (TLP). The VP2 protein constitutes the major core protein and encloses the genomic material, capping enzyme (VP3) and the polymerase (VP1). VP6 stabilises and encloses the SLP to form the DLP **(Figure 2.3)**.

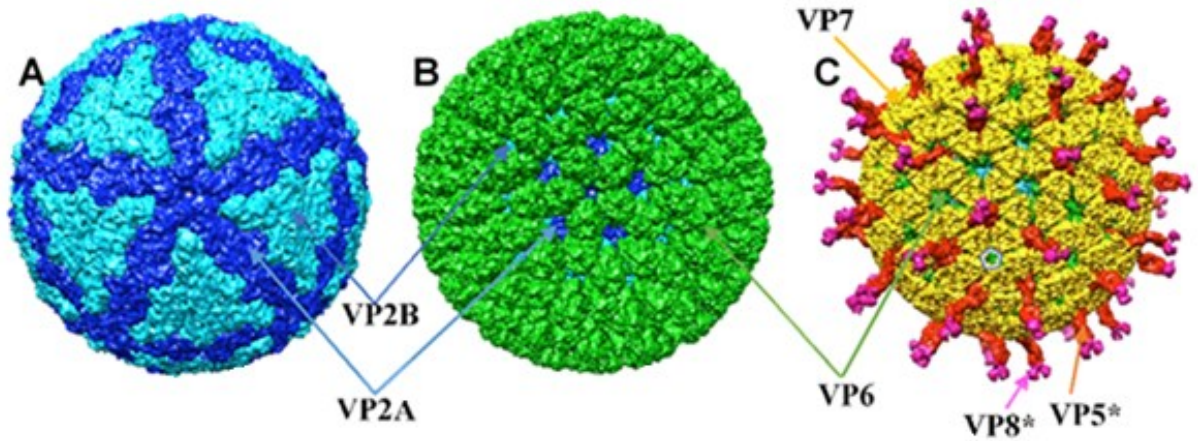


Figure 2.3: Rotavirus particles. The particles are represented as **(A)** SLP **(B)** DLP and **(C)** TLP. The VP8* and VP5* are proteolytic cleavage products of VP4 (the spike protein) (Settembre *et al.*, 2011). *Figure adapted from Harrison and Dormitzer, (2016). Figure (C) was used as a watermark on the cover page*

The icosahedral shape of the rotavirus particle is determined by the VP2 protein that forms a five-fold symmetry (Lawton *et al.*, 1997b) **(Figures 2.3)**. For each core protein, there are 260 VP6 trimers (780 monomers) enclosing the entire core protein. Out of these, 720 monomers of VP6 cover the inner layer in T (triangulation) = 13 sub-triangulated icosahedral lattices. At the icosahedral three-fold axis of the inner shell, there are three VP6 subunits of a trimer (60 monomers) with identical contacts to underlying VP2 subunits (McClain *et al.*, 2010).

There are 60 protein spikes (VP4) per triple layer capsid, which extend from the smooth surface of the outer shell (VP7) (Estes & Kapikian, 2007; Estes & Cohen, 1989; Estes & Greenberg, 2013) **(Figure 2.3)**. The integrity of the outer layer requires Ca^{2+} (Dormitzer, 2008; Dormitzer *et al.*, 2000). *In vitro* cultivation of rotaviruses requires proteolytic cleavage of the protruding outer capsid polypeptide spikes. This also facilitates the viral infectivity and replication in the cytoplasm of infected cells (Estes & Kapikian, 2007; Estes & Greenberg, 2013; Pesavento *et al.*, 2006). Addition of rotavirus VP6 to the SLP by transcapsidation can form a DLP (Kohli *et al.*, 1993b). Further transcapsidation of VP4 and VP7 to DLP, in the presence of chaotropic agents such as $CaCl_2$ can form TLPs (Chen & Ramig, 1993a; Chen & Ramig, 1993b; Trask & Dormitzer, 2006). Reversibly, a chelating agent like EDTA or EGTA (Desselberger *et al.*, 2013) can remove the Ca^{2+} and disengage VP4 and VP7 to form DLPs (Estes *et al.*, 1979) and with a sufficiently high concentration of Ca^{2+}, the VP6 layer can be removed to form the SLP core protein (Bican *et al.*, 1982) **(Figure 2.4)**.

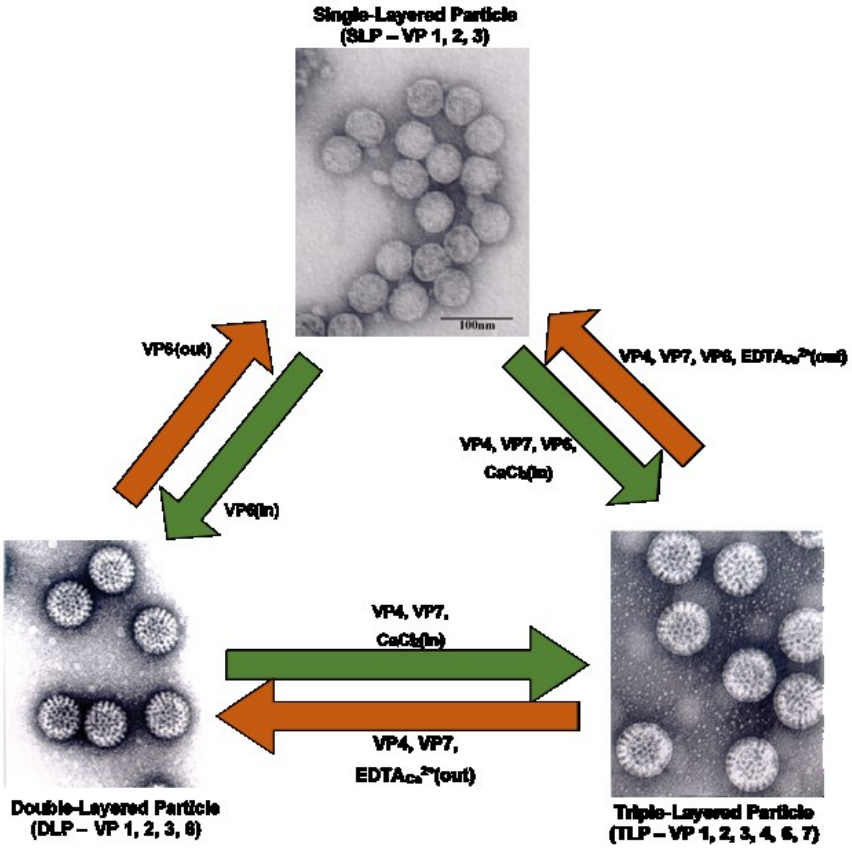


Figure 2.4: Rotavirus particle formation as viewed by transmission electron microscopy. Ionic concentration and pH determine how capsid proteins interact to form SLPs, DLPs or TLPs. VP2, VP4, VP6 and VP7 with addition of the chaotropic agent ($CaCl_2$), in the transcapsidation process leads to the formation of TLPs while a chelating agent like EDTA/EGTA generally removes the capsid layers to form SLPs (core protein). Biologically, SLPs and DLPs remain non-infectious while TLPs are infectious; SLPs and TLPs are transcriptionally inactive while DLPs are transcriptionally active (Cohen, 1977; Estes *et al.*, 1979). *Figure copied from Estes and Greenberg, (2013).*

2.5.1 Single-layered particle (SLP) formation

This is the inner layer/core protein with a T = 1 capsid (Jiménez-Zaragoza *et al.*, 2018), formed by 60 asymmetric dimers of the VP2 protein that surrounds the segmented viral genomes associated with VP1 and aggregated with VP3 at the pentameric positions (Estrozi *et al.*, 2013; Periz *et al.*, 2013). This thin SLP is an intermediate structure that is involved in the packaging and replication of the viral genome. It is the rare isolated form of rotavirus unlike the DLPs and TLPs. It is the foundational structure on which the entire virion structure is built. *In vitro* analysis showed this particle lacked polymerase activity in the absence of the viral genome (Cohen, 1977; Elias, 1977; Estes & Greenberg, 2013; Hruska *et al.*, 1978). Labbé and co-workers expressed VP2 core protein devoid of nucleic acid and heterologously interacted with VP6 to produce double-layered particles (Labbé *et al.*, 1991). Experimentally, the core-protein/open core can randomly bind dsRNA and DNA in an overlay protein blot assay (Labbé *et al.*, 1991). Full replication to produce dsRNA can be restored when heterogenous (+)ssRNA is

exogenously supplied into the open core from different rotavirus strains (Chen *et al.*, 1994; Tortorici *et al.*, 2003). The (+)ssRNA is a template for synthesising the (-)ssRNA to produce dsRNA.

2.5.2 Double-layered particle (DLP) formation

DLPs appear as rough particles when viewed using a transmission electron microscope, due to the protrusion of subunits of the trimeric inner capsid protein (Estes *et al.*, 1979). Here, VP6 stabilises the inner shell. *In vitro* analysis showed that DLPs are transcriptionally active because it contains active RNA-dependent RNA polymerase VP1, capping enzyme VP3, enclosed by the core protein VP2 followed by the core capsid VP6. Surprisingly, DLPs become infectious when transfected into susceptible cells (Bass *et al.*, 1992; Chen & Ramig, 1993b; Estes & Greenberg, 2013; Kohli *et al.*, 1993a; Lawton *et al.*, 1997b; Trask & Dormitzer, 2006). Transcapsidation of the core protein with VP6 has been shown to re-establish transcriptase activity (Kohli *et al.*, 1993a).

2.5.3 Triple-layered particle (TLP) formation

A completely infectious rotavirus particle consists of TLPs. *In vitro* analysis showed this particle to be infectious because it contains the glycoprotein VP7 and protease-sensitive VP4 responsible for host identification, attachment and penetration but transcriptionally inactive until the protective outer glycoprotein is lost during cell penetration (Cohen, 1977; Estes & Greenberg, 2013; Lawton *et al.*, 1999).

2.5.4 Rotavirus capsid channels

The icosahedral shape of rotavirus possesses a 20-sided symmetry with T (triangulation number) = 13 *l* (laevo) surface lattice for both middle (inner capsid) and outer layers while the core layer exhibits T = 1 icosahedral lattice structure (Jayaram *et al.*, 2004; Lawton *et al.*, 1997b). The channels extend through the two outer layers and link the outer surface of the capsid with the inner core protein. Classified according to their position and size, there are three distinctive conformational channels **(Figure 2.1)**. The **type I channel** (140 Å depth x ~40 Å wide) is located at the icosahedral five-fold axes (Lawton *et al.*, 1997a; Li *et al.*, 2009). These channels are passages for mRNA that first interacts with VP1 and VP3 (methyltransferase and guanylyltransferase) found in the core capsid and exit as capped transcripts (Prasad *et al.*, 1996). Each of the type I channels is surrounded by 5 type II channels. The **type II channel** (140 Å depth x 55 Å wide) is located at the quasi-six-fold axes directly adjacent to the type I channels with larger openings than type I channels. The 60 regular trimeric spikes of VP4 that protrude on the smooth outer layer of the rotavirus capsid are usually found at the edges of type II channels (peripentonal) surrounding the five-fold icosahedral axis (Yeager *et al.*, 1990).

Type II channels usually contain the globular domain of VP5* (making the length of the spike protein 20 nm) (Shaw *et al.*, 1993; Yeager *et al.*, 1994), which forms an anchor between the VP7 and VP6 capsid layers. The **type III channel** (140 Å depth x 55 Å wide) is at the six coordinated (hexavalent) positions surrounding the icosahedral three-fold axes (Pesavento *et al.*, 2006; Prasad *et al.*, 1988; Yeager *et al.*, 1990). They have a larger opening than type I channels (just like the type II channels) and is situated close to the icosahedral three-fold axes.

2.6 Rotavirus genome replication and particle assembly

Rotavirus primarily infects the mature enterocytes of the villi of the small intestine following the specific trypsin cleavage of VP4 into VP8* that interacts with sialic-gangliosides and histo-blood group antigens (HBGAs) (Delorme *et al.*, 2001; Fleming *et al.*, 2014; Haselhorst *et al.*, 2011; Haselhorst *et al.*, 2009; Rolsma *et al.*, 1998). The full replication cycle and the events leading to the formation of the viroplasm are shown in **Figure 2.5**.

It can be seen in **Figure 2.5 (steps 1 – 5)** that infectious rotavirus approaches the polarised enterocyte membrane and attaches to the cell membrane receptors with the VP4 spike protein (Crawford *et al.*, 1994). Cell entry is initiated by promoting proteolytic cleavage of VP4 into the VP8* and VP5* subunits that associate with sialo-specific membrane receptors**.** The post-attachment stage during which multistep receptor interaction occurs between the VP7 and VP5* subunit proteins with numerous integrin subunit receptors and *hsc70* proteins (Baker & Prasad, 2010) promotes cell entry. Cell entry occurs through any one of the following: Ca^{2+}-dependent cell-mediated endocytosis where VP7 dissociates due to low level of Ca^{2+} in the endosomes, round the lipid raft domain, direct invagination by pH-dependent fusion.

From **steps 6 – 10**, membrane penetration is enhanced by post-attachment conformational changes caused by the VP5* and VP7 binding, (Settembre *et al.*, 2011) and transcriptionally active DLPs are formed, which then release (+)RNAs for transcription into mRNA. First, the mRNA released can engage with cellular ribosomal complexes for translation into viral proteins. Alternatively the mRNA can be translocated to a special cytoplasmic inclusion body called the viroplasm, where replication of the viral dsRNA is orchestrated by the binding of VP1, VP3, VP2A, 2B, NSP2, NSP5 to the NSP1-mRNA-NSP3 initiation complex (Patton & Chen, 1999; Patton *et al.*, 2006; Patton *et al.*, 2004). The new (-)RNA transcripts remain attached to the (+)RNA template as dsRNA together with accessory proteins (NSP2, NSP5, NSP6 and VP1, VP2, VP3, VP6) which formed nascent DLPs inside the viroplasm and the viroplasm co-localises with lipid droplets (Borodavka *et al.*, 2018; Gridley & Patton, 2014; Lu *et al.*, 2008; McDonald & Patton, 2011; Navarro *et al.*, 2016). Further elucidation has shown that, in the viroplasm, viral (+)RNAs interact with the viral polymerase and RNA-capping

enzyme and undergo assortment. The core-shell, consisting of VP2, encloses VP1-VP3-(+)RNA complexes, that, after forming core-like structures, support dsRNA synthesis (Periz *et al.*, 2013; Prasad *et al.*, 1996). Newly formed cores interact with VP6 to form DLPs. The DLPs migrate to the endoplasmic reticulum (ER) due to the affinity of the DLP-VP6 protein for ER-transmembrane protein NSP4. The nascent DLPs bind intracellular receptor, NSP4, and bud into the ER to acquire a transient membrane/envelope. Particle maturation occurs when the outer capsid proteins (VP4 and VP7) assemble to dissociate the transient envelope. The matured particles could either leave the infected cells by Golgi-independent, non-classical vesicular transport or cell lysis.

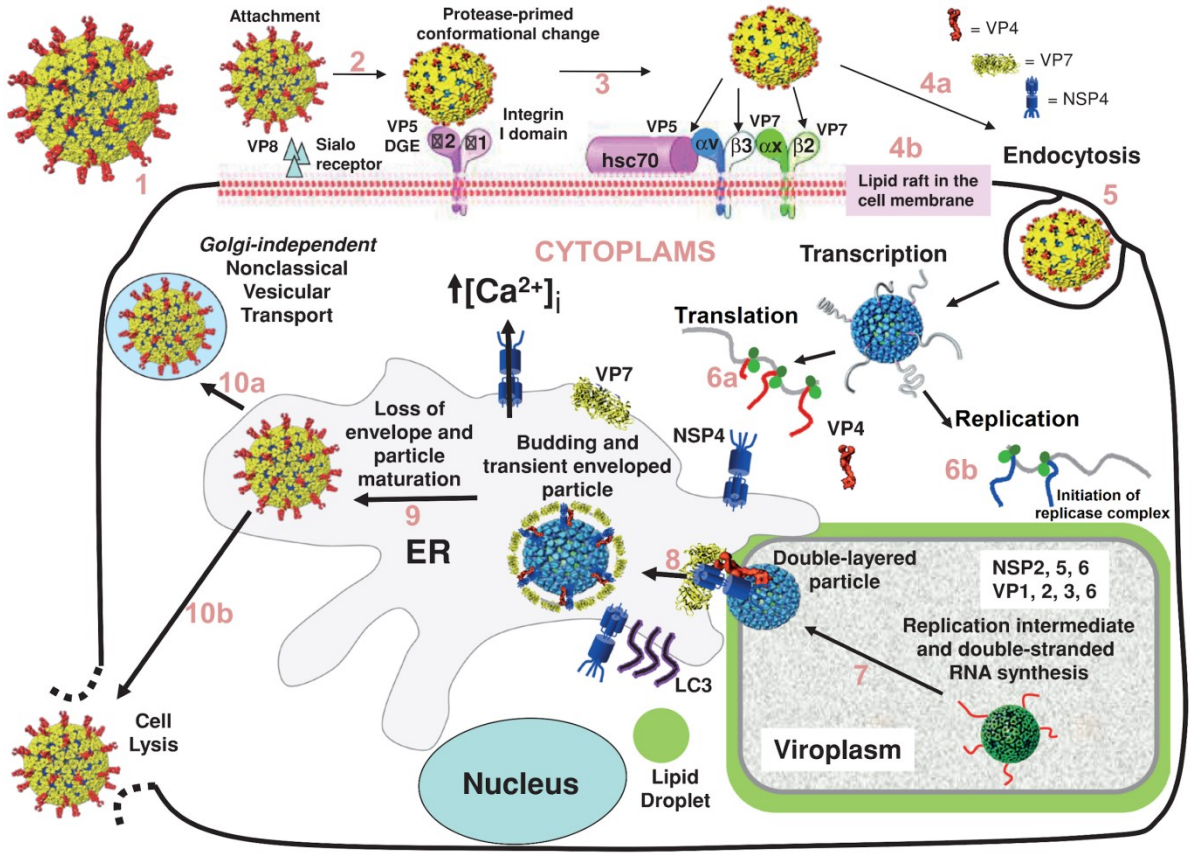


Figure 2.5: Rotavirus replication cycle. The initial stage involves proteolytic cleavage of the spike protein, VP4, into VP8* and VP5*. Together with VP7, interaction occurs with the cellular membrane receptor that leads to cellular entry by receptor-mediated endocytosis. Transcriptionally active DLPs releases (+)ssRNA for transcription and eventually translation of all accessory proteins needed for particle assembly or for the replication of dsRNA and genome packaging in the specialised cytoplasmic inclusion bodies, viroplasms. In the viroplasm, new DLPs are formed that will engage the endoplasmic reticulum membrane-bound NSP4, which will channel the VP4 and VP7 sequentially to be incorporated into nascent DLPs to form TLPs which are released by cell lysis or by nonclassical vesicular transport. *Figure adapted from Estes and Greenberg, (2013).*

2.7 Classification and molecular epidemiology of rotavirus

Taxonomically, rotaviruses are members of the family ***Reoviridae*** and the genus **Rotavirus**. It has been classified into eight different groups, rotavirus A – H (RVA to RVH) by the ICTV (International Classification and Taxonomy of Viruses). This classification is mainly based on antibody cross-reactivity with the conserved structural protein, VP6 (Matthijnssens *et al.*, 2012). Recently, novel tentative groups "I" and "J" were suggested after discoveries in Hungarian sheltered dogs (Mihalov-Kovacs *et al.*, 2015) and Schreiber's bats in Serbia (Banyai *et al.*, 2017) respectively.

Group A rotaviruses are further classified based on the nature of the glycoprotein VP7 (G-types) and the protease-sensitive VP4 (P-types) (O'Ryan, 2009; Patton, 2012). Serotyping involves the use of monoclonal antibodies against VP7 (G-serotypes) and VP4 (P-serotype) (Coulson, 1996) respectively. There is a correlation between serotype and genotype classifications based on VP7 but not for VP4. As of 29 May 2018, 36 G-types and 51 P-types have been proposed (https://rega.kuleuven.be/cev/viralmetagenomics/virus-classification).

In 2008, a whole-genome classification system was developed for RVA that assigned a specific genotype to each of the 11 genome segments (Matthijnssens *et al.*, 2008). With this approach, the genomes of individual rotavirus strains are given the complete descriptor of Gx-P[x]-Ix-Rx-Cx-Mx-Ax-Nx-Tx-Ex-Hx, where "*x*" is the number of genotypes. This descriptor corresponds to the rotavirus encoded structural protein first then to nonstructural proteins: VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3NSP4-NSP-5/6, respectively. Based on this whole genome typing, most human rotavirus A strains have either a DS-1 or Wa-like backbone. The Wa-like (G1P[8], G3P[8], G4P[8], G9P[8] and G12P[8] with non-G/P genotypes (I1-R1-C1-M1-A1-N1-T1-E1-H1) and the DS-1-like rotavirus A (G2P[4] with non-G/P (I2-R2-C2-M2-A2-N2-T2-E2-H2) are the two major groups of human rotavirus reference strains (Matthijnssens *et al.*, 2008). Most of the rotavirus gastroenteritis (RVGE) cases are caused by one of the six common genotypes of Wa-like G1P[8], G3P[8], G4P[8], G8 associated P[4]/P[6]/P[8] and G9P[8] or the DS-1-like G2P[4] strains (Banyai *et al.*, 2012; Seheri *et al.*, 2018). Recently, G12 strains in combination with P[8] or P[6], with a Wa-like backbone have also been reported (Esona *et al.*, 2010b; Miles *et al.*, 2012; Roczo-Farkas *et al.*, 2018).

2.8 Rotavirus Pathology

Rotavirus stimulates the release of serotonin/5-hydroxytryptamine (5-HT) from the human enterochromaffin (EC) cells (Hagbom *et al.*, 2011), which induces nausea and vomiting. This is suggestive of the involvement of both the enteric nervous system (ENS) and vagal afferent

stimuli of the central nervous system (CNS). Four mechanisms have been proposed in rotavirus diarrhoeal disease. These are increased secretion, decreased absorption of ions and water, altered motility and permeability of the intestinal villi (Feng *et al.*, 2008; Hennessy *et al.*, 2014; Ramani *et al.*, 2010; Weh *et al.*, 2013).

Several reports have shown that rotavirus uses the specific fragment (a114 – 135) from the NSP4 to induce diarrhoea in suckling mice (Ball *et al.*, 1996; Berkova *et al.*, 2003; Ge *et al.*, 2013; Hagbom *et al.*, 2011; Tian *et al.*, 1995). As illustrated in **Figure 2.6(A)**, the pathology of rotavirus infection involves intracellularly located NSP4 (iNSP4), which stimulates the release of $[Ca^{2+}]_i$ from the endoplasmic reticulum thereby increasing the cytoplasmic Ca^{2+} level to depolarise the cell membrane leading additional release of NSP4 and a peptide fragment (aa112 – 175) of NSP4 by a Golgi-independent pathway. In another model (middle cell) **(Figure 2.6A)**, iNSP4 disrupts tight junctions (TJ), which allows the paracellular flow of water. Another model proposes (right side cell) **(Figure 2.6A)** that eNSP4 from a previously infected cell binds to a receptor triggering a signalling cascade through PLC (phospholipase C) and inositol triphosphate (IP_3) that results in the release of Ca^{2+} and paracellular leakage with water loss (Greenberg & Estes, 2009). **Figure 2.6(B)** illustrates how rotavirus infects EC cells **(shown in red)** leading to the expression of NSP4, which increases $[Ca^{2+}]_i$ followed by the accumulation of 5-HT into secretory granules and its release. The 5-HT re-uptake transporter (SERT) regulates the 5-HT content and availability of 5-HT along the GIT and the preponderant level of 5-HT alone can cause secretory diarrhoea, which can be blocked by 5-HT3 receptor antagonists (Levine, 2012) **(shown in yellow)**. Stimulation by 5-HT from the nerves leads to the release of vasoactive intestinal peptide (VIP) from the nerve endings, which presumably increases the cAMP, water and Cl^- secretion from adjacent undifferentiated crypt cells **(shown in olive green)** (Kordasti *et al.*, 2004).

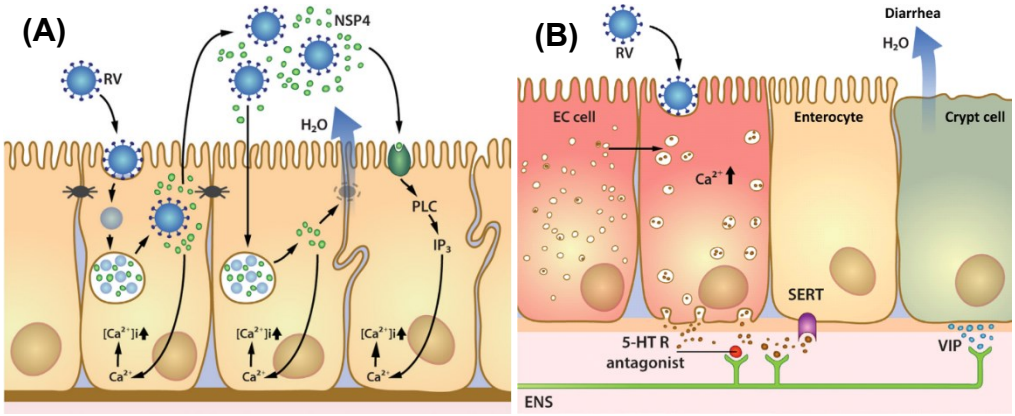


Figure 2.6: Rotavirus pathogenesis (A) The proposed mechanism by which RV-NSP4 disrupts apical tight junctions (*) in the enterocytes, induces water loss and orchestrates the influx of $[Ca^{2+}]_i$ from the ER during infection **(B)** Rotavirus infection stimulates EC cells and triggers electrolyte and water secretion from the crypt cells. *Modified figures copied from Hagbom and Svensson, (2016).*

2.9 Immune responses during rotavirus infection

Innate and adaptive immunity consist of cell-mediated (T-cell, involving lymphocytes) and humoral (B-cell, involving antibodies) immune responses to rotavirus infection. The glycoprotein VP7 and the protease-sensitive VP4 independently induce neutralising antibodies (Abs) (Estes & Kapikian, 2007). In humans, the VP4- and VP7-specific antibodies have been shown to mediate heterotypic immunity to rotavirus (Nair *et al.*, 2017).

2.9.1 Innate immune responses to rotavirus infection

Following host cell infection, most viruses trigger one or more pattern recognition receptors (PRRs) such as RIG-1, MDA-5 and TLR3, TLR9+ (Arnold *et al.*, 2013a; Arnold *et al.*, 2013b; Kawai & Akira, 2008; Kawai *et al.*, 2004; Kawai *et al.*, 2005). Viral pathogen-associated molecular patterns (PAMPs) and NF-κB recognise a virus-specific pattern like 5´-triphosphate RNA (Kato *et al.*, 2006; Malathi *et al.*, 2010; Saito *et al.*, 2008).This triggers ligand binding and subsequently, a signalling pathway is activated that drives the initiation of various antiviral responses **(Figure 2.7)**. Among the transcribed virus stress-induced genes (vSIGs) are the type I and II IFNs (Honda *et al.*, 2006), which after binding their cognate surface receptors on both the autocrine and paracrine forms, induce ligand-stimulated activation of the JAK-STAT signalling cascade (Hakim *et al.*, 2018). This cascade event along with other factors induces a signalling event, which is sent to the nucleus to initiate secondary transcription process resulting in the expression of hundreds of genes that encode antiviral proteins inhibiting multiple aspects of viral replication, assembly, encapsidation, maturation and spread (Ingle *et al.*, 2018) **(Figure 2.7)**.

Rotavirus, through the specific function of viral proteins, can inhibit innate response in several ways. For example, in addition to inhibiting the IFN III inducing pathway, rotavirus VP3 (capping enzyme) inhibits IFN-induced downstream RNAse L. This ribonuclease 4 (RNAse L) is a 2´- 5´-oligoadenylate synthetase-dependent ribonuclease, which degrades cellular RNAs. The group A rotavirus-VP3 protein, contains a C-terminal 2H-phosphodiesterase domain that can cleave 2´-5´- oligoadenylates, preventing the activation of RNAse L activity (Ogden *et al.*, 2015; Ogden *et al.*, 2014; Silverman *et al.*, 2013; Zhang *et al.*, 2013). By so doing, rotavirus genomic RNA is preserved from degradation by RNase L. Rotavirus NSP1 uses cellular E3 ligases to interfere with the NF-κB signalling pathway, suggesting that any alteration in the cullin-ring E3 ubiquitin ligase (CRLs) complexes appears to disrupt NSP1-directed β-TrCP degradation (Morelli *et al.*, 2015a; Morelli *et al.*, 2015b). In addition, as illustrated in **Figure 2.8**, viral translation of rotavirus NSP1 protein results in proteasomal degradation of the RIG-I, MAVS and IRF3 (interferon regulatory factor 3) factors as well as β-TrCP (beta-transducin repeat-containing E3 ubiquitin protein ligase). Thereafter NSP1 is itself proteasomally

degraded, the reason for this is still unknown. Furthermore, rotavirus-mediated sequestration of the NF-κB subunit p65 in viroplasms (by unknown viral factors) occurs during infection, which prevents it from co-activating the release of IFN I (Abdelhakim *et al.*, 2014; Arnold & Patton, 2011; Arnold *et al.*, 2013b; Sen *et al.*, 2009; Sen *et al.*, 2012; Sen *et al.*, 2014).

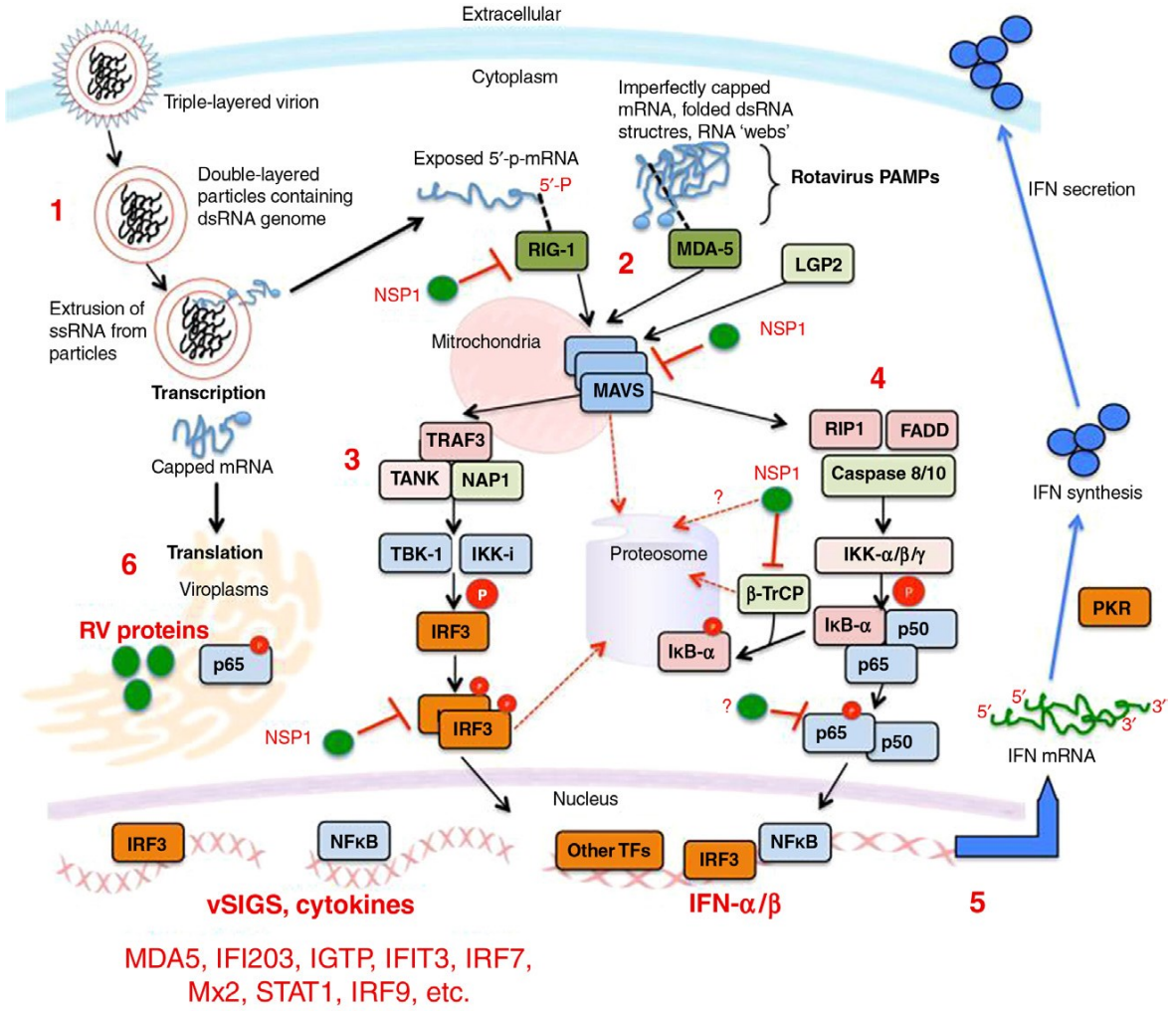


Figure 2.7: The early host interferon response to rotavirus infection. After cellular entry, which is accompanied by the removal of VP4 and VP7 to produce transcriptionally active DLPs, viral transcription (and replication) derivatives, including molecules with exposed 5´-phosphate moieties, imperfectly capped RNA, local hairpin structures and higher-order RNA oligomers are potential rotavirus PAMPs that are generated, which will activate specific signalling complexes. The complexes generated will phosphorylate and functionally activate the transcription factors (TFs) IRF3 and NF-kB. Nuclear translocation of IRF3, NF-κB and other TFs result in occupancy of promoter elements on several antiviral genes, including the type I IFNs, which are released to initiate the production of anti-rotavirus transcriptional factors. *Figure copied from Sen and Greenberg, (2016)*.

2.9.2 Acquired immunity against rotavirus infection

Rotavirus infection naturally induces acquired immune responses in B cells to produce antibodies directed against rotavirus specific proteins (Narvaez *et al.*, 2010) and in T cells to recognise T cell-specific rotavirus epitopes on the surface of infected cells. Humoral rotavirus immunity is the major immune response against rotavirus infection (Angel *et al.*, 2012;

Velazquez *et al.*, 1996), producing neutralising IgA antibodies that correlate with homotypic and heterotypic cross-protection against VP4 and VP7, especially in children after primary rotavirus infection (Johansen & Svensson, 1997). Transplacentally acquired maternal antibodies and breastfeeding have also been identified as factors for acquired immunity against rotavirus infections (Adlhoch *et al.*, 2013; Bautista-Marquez *et al.*, 2016; Friedman *et al.*, 1993; Gastanaduy *et al.*, 2016b; Goveia *et al.*, 2008; Gruber *et al.*, 2017; Rennels *et al.*, 1995; Shen *et al.*, 2018; Vesikari *et al.*, 2012). The presence of non-neutralising antibodies induced by rotavirus VP2 (Johansen *et al.*, 1994) and VP6 (Choi *et al.*, 2003; Feng *et al.*, 2002b; Nair *et al.*, 2016) as well as NSP2 and NSP4 (Ball *et al.*, 1996) have been found in the serum of convalescent patients. The implication of the non-neutralising antibodies is unknown, but numerous reports have demonstrated the protective effect of rotavirus VP6-specific IgA monoclonal antibodies against rotavirus infections (Burns *et al.*, 1996; Vega *et al.*, 2013); (Aiyegbo *et al.*, 2014; Aiyegbo *et al.*, 2013; Corthésy *et al.*, 2006; Feng *et al.*, 2002a). Specifically, rotavirus VP6-specific antibodies cross the basolateral membrane by transcytosis and are presented by a specific membrane receptor on the epithelial gut cell for protection (Schwartz-Cornil *et al.*, 2002). These antibodies can form a tight complex with the transcriptionally active DLPs in the gut (intracellular neutralization) and prevent rotavirus transcription, replication and maturation (Sapparapu *et al.*, 2014).

Cell-mediated immunity, with protection against rotavirus infection, has been observed in rotavirus-specific cell-mediated immune responses. For example, circulating B cells displaying the $\alpha_4\beta_7$ intestinal homing receptor are suggestive of localised, mucosal protection against rotavirus infection in children (Jaimes *et al.*, 2004), which is important for clearing rotavirus infection and enforce protection against reinfection in children and adults (Gonzalez *et al.*, 2003). Rotavirus-specific antibody responses usually proceed from $CD4^+$ T-cells (Franco & Greenberg, 1997). Though rotavirus prefers to replicate within the intestinal enterocytes, both intestinal and systemic primed T cells synergistically elicit immune responses (Franco *et al.*, 2006). The majority of these cells are IFN-γ producers but a few produce IFN-γ/IL (Mesa *et al.*, 2010) to offer short-term immunity due to predominant rotavirus-specific T cells.

In the intestinal homing integrin $\alpha_4\beta_7$-independent immune response, rotavirus-specific $CD8^+$ T cells offer the first line defence against primary rotavirus infection (Franco & Greenberg, 1997; Kuklin *et al.*, 2000). Unlike $CD4^+$ T cells, the $CD8^+$ cells predominantly produce IFN-γ but rarely produced IFN-γ/IL-2 (Jaimes *et al.*, 2002; Mesa *et al.*, 2010; Narvaez *et al.*, 2005; Rojas *et al.*, 2003). However, long-term protection against rotavirus infections is determined by the B cells (Franco & Greenberg, 1999), which consist of the systemic (spleen and bone marrow B cells expressing IgG and IgA), mucosal Peyer's Patches and the intestinal *lamina*

propria (LP) B cells expressing polymeric IgA (Youngman *et al.*, 2002) **(Figure 2.9)**. Intestinal localised B cells expressing homing receptors ($\alpha_4\beta_7$) mediate antiviral action that clears rotavirus infection (Williams *et al.*, 1998) in response to the action initiated by the intestinal localised effector plasma cells. Furthermore, antibody-secreting cells (ASC) in the LP are capable of mediating rotavirus expulsion (Exp) and exclusion (Exc) from the intestinal enterocytes. Exp is possible due to anti-VP6 polymeric IgA (Burns *et al.*, 1996) while Exc is due to intracellular-directed rotavirus-specific antibodies which block rotavirus transcription from the active DLPs (Aiyegbo *et al.*, 2013; Bouvet & Fischetti, 1999; Feng *et al.*, 2002a) **(Figure 2.8)**. Therefore, intestinal rotavirus IgA is the most effective mucosal antibody that is involved in protection against rotavirus infections and a good correlate of protection.

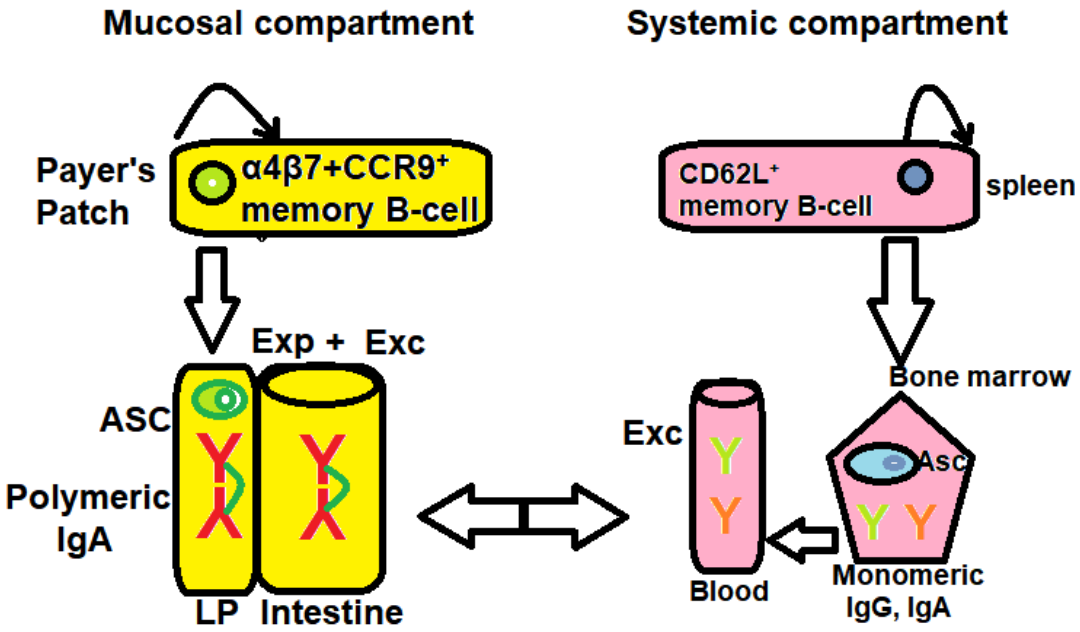


Figure 2.8: Simplified model of the two compartments stimulated by human rotavirus infection. Rotavirus antigen in Peyer's Patches generates memory B cells with homing receptors that permit these cells to circulate in the blood and return to Peyer's Patches. The antibody-secreting cells (ASC) that home to the intestinal *lamina propria* (LP) and secrete polymeric IgA that mediates viral expulsion (Exp) and exclusion (Exc) at the same time fill the luminal cavity. Systemic viral antigen stimulates memory B cells in the spleen to secrete monomeric IgA and IgG. These antibodies are the main source of serum antibodies against subsequent rotavirus infection (Bouvet & Fischetti, 1999; Grauballe *et al.*, 1981; Hjelt *et al.*, 1985; Mestecky *et al.*, 1999). *Figure adapted from Franco et al., (2006).*

2.9.3 Vitamine A deficiency (VAD) alters the immune response to human rotavirus infection or vaccines

VAD in children is a significant health concern in impoverished countries, especially those in Africa and Asia. Susceptibility to rotavirus infection increases marginally (subclinical) with VAD. Prophylactic vitamin A supplementation recommended by the WHO has reduced the incidence of diarrhoea associated morbidity and mortality in these regions of the world (Humphrey *et al.*, 1996; Imdad *et al.*, 2011). VAD increases the severity of human rotavirus-induced diarrhoea and impairs innate, mucosal and systemic adaptive immune responses to

human rotavirus infection and attenuated rotavirus vaccines (Chattha *et al.*, 2013a; Kandasamy *et al.*, 2014b).

2.10 Rotavirus vaccines and vaccination

Four live-attenuated oral rotavirus vaccines, Rotarix®, RotaTeq®, Rotavac® and RotaSIIL® are available internationally and are WHO prequalified (World Health Organisation, 2018). Historically, the first human rotavirus vaccine candidate was designed with the monovalent Nebraska Calf Diarrhoea Virus (NCDV) **BRV-RIT4237** strain G6P6[1] (Vesikari, 1983) based on the *"Jennerian concept"* (Midthun & Kapikian, 1996) **(Table 2.2)**. However, this vaccine was withdrawn due to inconsistent results in clinical trials (Bernstein, 2000; Bishop, 1988; Santosham *et al.*, 1991). A simian rotavirus vaccine called rhesus rotavirus **(RRV**-MMU**)** was developed but its protection was similar to that of BRV-RIT4237 – inconsistent and highly discrepant clinical data. Unlike BRV-RIT4237, it shared a G3 genotype with one of the dominant circulating human serotypes (Flores *et al.*, 1987). Subsequently, RRV was reassorted with genome segments encoding VP7 representing human G genotypes (G1, G2 and G4) to formthe tetravalent **RotaShield®** vaccine produced by Wyeth-Lederle Vaccines (USA) **(Table 2.2)**. RotaShield® was tested in a large, placebo-controlled trial with efficacy of more than 70% (Ward *et al.*, 1997). However, post licensing, the vaccine was withdrawn due to an increased rate of intussusception (Murphy *et al.*, 2003; Murphy *et al.*, 2001). A second bovine rotavirus vaccine candidate named **WC3** was developed initially using the G6P[5] strain. This showed heterotypic protection (Clark *et al.*, 1988), but subsequent trials provided no significant protection (Clark *et al.*, 1996). WC3 was later reassorted with the human rotavirus VP4 and VP7 encoding genome segments to produce the pentavalent **RotaTeq®** vaccine. RotaTeq® was produced by Merck and was considered safe because of its consistent protection in clinical trials (Ciarlet & Schödel, 2009; Offit & Clark, 2006; Payne *et al.*, 2015). The monovalent vaccine G1P1A[8] serotype named **Rotarix®** was also developed based on the cross-protection from multiple natural infections (Velazquez *et al.*, 1996; Vesikari *et al.*, 2009). Consequently, RotaTeq® and Rotarix® have been licensed since 2006 (Vesikari *et al.*, 2006b; Vesikari *et al.*, 2006d), followed by **Rotavac®** and **Rotasiil®** Indian made live-attenuated rotavirus oral vaccines, which were recently licensed for global used. **Rotavin-M1®** made in Vietnam and **LLR-37** from China remain licensed to be used regionally. Other oral, live-attenuated, rotavirus vaccines, which are still in phase trials are RV3-BB (prepared by Murdoch Children's Research Institute, Australia and Biofarma, Indonesia – phase II trial), **BRV-TV** (prepared by Shantha Biotech, India – Phase III trial) and **BRV-PV** (prepared by Instituto Butantan, Brazil – Phase I trial) (Burnett *et al.*, 2018) **(Table 2.2)**.

Table 2.2: Summary of live-attenuated, oral, rotavirus vaccines

Vaccine	*Strain*	*Developer/Manufacturer*	*Type of vaccine/Status*	*Remarks*
From animal rotavirus strains (First generation of rotavirus vaccines)				
RIT4237 (monovalent)	G6P6[1], BRV NCDV strain	SmithKline-RIT (Rixensart, Belgium)	Jennerian, but withdrawn	154 cell culture passages with high titre value of over 10^8 $TCID_{50}$/mL, first to be tested in human, higher efficacy in the developed countries, negative gastric effect, breast milk had no effect, suppressed OPV (Ruuska & Vesikari, 1990)
RRV-MMU (monovalent)	G3P[5] RRV strain	National Institute of Health (NIH)	Modified Jennerian -based vaccine, but withdrawn	9 and 7 passages in monkey kidney and foetal rhesus lung cells, respectively, with 10^5 PFU/dose titre level, reactogenic for fever, very virulent and later used as a backbone for RotaShield® (Flores *et al.*, 1987; Gothefors *et al.*, 1989)
WC3 (monovalent)	Wistar Calf strain G6P[7]	Institut Merieux/Wistar Institute	Jennerian, but withdrawn	20 passages with 10^7PFU/mL titre value, less efficacious compared to RRV-MMU but later used as a backbone for RotaTeq® (Bernstein *et al.*, 1990; Clark *et al.*, 1986)
LLR-37 (monovalent)	Lanzhou Lamb-derived rotavirus G10P[15]	Lanzhou Institute of Biological Products (China)	Jennerian, licensed for use in China only	Ovine attenuated vaccine, efficacious in 2 doses at 2 months and 2 years, widely available in China since 2000, replicate with faecal shedding within 15 days post-vaccination. Over 60 million doses rolled out at the end of 2014 (Fu *et al.*, 2012; Li *et al.*, 2015; Li *et al.*, 2018a)
Animal-human reassortant strains (Second generation of rotavirus vaccines) – Jennerian-based vaccines				
RotaShield® (RV4)	Tetravalent human-rhesus reassortant (G1-G4)P7[5]	Wyeth (USA)	Jennerian, but withdrawn in 1999 due to intussusception, but now in phase II trials for neonatal administration in Ghana	First reassortant prototype, stable at 25°C, formulated with G1, G2 and G4 VP7 Ags on rhesus G3P[5] backbone, reactogenic for fever, formulated with 4 x 10^4 PFU/mL with two doses and caused intussusception but tested to be safe in neonates in Ghana (Armah *et al.*, 2013), which might favour its re-introduction (Simonsen *et al.*, 2005; Vesikari *et al.*, 2006a)
RotaTeq® (RV5)	Pentavalent human-bovine reassortant G1-G4+P7[5]; G6+P1A[8]	Merck (USA)	Jennerian, licensed for global use	Formulated with 2 – 2.8 x 10^6 PFU/mL, in 3 doses with incremental immunogenicity tested in 70 000 infants during rotavirus efficacy and safety trial (REST) (Keating, 2006), overall efficacy of 95% against G1, G2, G3, G4, G9 all with P[8], exhibits heterotypic protection against G2P[4], licensed in 2006, faecal viral shedding and diarrhoea (1%) observed after vaccination (Hemming & Vesikari, 2012; Markkula *et al.*, 2015), require cold-chain transportation (2 – 8°C)
BRV-TV or BRV-PV	Bovine-human tetravalent reassortant G1-G4+P7[5]; G1-G4+G9-P7[5]	Biotecnics Instituto Butantan Shantha Biotecnics Limited Serum Institute of India (India) Wuhan (China)	Phase I trial in Brazil Phase II/III trials in India Phase III trial in China -	Started by Wyeth but discontinued at the same time RotaShield® was withdrawn, however, the prototype has been licensed by NIH to Brazil, India and China (Vesikari *et al.*, 2006c)
RotaSIIL®	Lyophilized pentavalent human-	Serum Institute of India Pvt Ltd (SIIPL)	Pre-qualified for global use	World's first cost-effective, thermostable vaccine which can be stored without refrigeration or below 25°C, bovine (UK) rotavirus NIH (USA)

Vaccine	*Strain*	*Developer/Manufacturer*	*Type of vaccine/Status*	*Remarks*
	bovine reassortant serotypes G1, G2, G3, G4 and G9			strain reassorted with human VP7 genome encoding segments (Naik *et al.*, 2017), with a lifespan of 20 months at 37°C to 7 months at 45°C
Human rotavirus strains (Second generation of rotavirus vaccines) - Modified Jennerian-based vaccines				
Rotarix® (RIX-441)	Monovalent human strain G1P1A[8]	GlaxoSmithKline (Belgium)	Licensed for global use	Most extensively used oral vaccine, strain isolated in Cincinnati, passaged 45 times in MRC-5 cells where the infectivity property was lost, 10^5 PFU/dose in the final formulation, does not cause diarrhoea but multiply effectively in children with over 60% shedding, given in 2 doses with > 90% efficacy at first dose with 60 000 infants for rotavirus efficacy and safety trial, require low level of maternal Abs, the first to be licensed in 2004 after the withdrawal of RotaShield® (Ruiz-Palacios *et al.*, 2006b; Vesikari *et al.*, 2007), exhibits heterotypic protection against G2P[4] and require cold-chain transportation (2 – 8°C)
Rotavac®	Human neonatal strain 116E G9P[11]	Bharat Biotech International Limited (India)	Licensed for global use (Tate *et al.*, 2014)	It is a naturally occurring reassortant strain G9P[11], containing one bovine rotavirus gene P[11] and ten human rotavirus genes (Bhandari *et al.*, 2014) at 10^5 FFU/dose formulation, taking in 3 single doses at 4 weeks interval starting from 6 weeks of age, vaccine licensed and introduced into the NIP (National Immunisation Program) of India by 2014 (Bhandari *et al.*, 2014) but must be stored at -20°C
Rotavin-M1®	Human strain G1P1A[8]	Polyvan (Vietnam)	Licensed to be used in Vietnam since 2012	It was derived from an attenuated G1P[8] strain (KH0118-2003) isolated from a Vietnamese infant, passaged in Vero cell at $10^{6.3}$ FFU/dose, given in 2 doses (2 month apart) or 3 doses (1 month apart), safety and immunogenicity tested in 160 infants with rotavirus-specific IgA seroconversion rate of 73% vs 58% in Rotarix® and rotavirus shedding between 44 – 48% vs 65% for Rotarix®. Over 500 000 doses already rolled out, however, it must be stored at -20°C (Le *et al.*, 2017)
RV3-BB	Human neonatal strain G3P2A[6]	Murdoch Children Research Institute, Australia and Biofarma	Phase III trial in Indonesia	RV3 is based on a neonatal G3P[6] rotavirus strain from Melbourne. A Vero cell-adapted derivative of the strain, called RV3-BB, grows to a higher titre and is more immunogenic than the original RV3 (Danchin *et al.*, 2013). Phase IIa of safety and immunogenicity trial with 30 neonatal and 27 infants with cumulative 3 doses vaccine intake showed 63% neonatal and 74% infant rotavirus-IgA seroconversion; 70% neonatal and 78% infant RV3-BB shedding rate. The vaccine is well tolerated and immunogenic at 3 doses in neonates and infants (Bines *et al.*, 2015) Recently, Phase IIb of immunogenicity and efficacy studies were done in Indonesia by employing a birth dose (<5 days of age) for neonates and 8 weeks of age for the infants in a 3-dose regime. Overall vaccine efficacy of 63% was observed for 18 months. No intussusception within the 21 days of risk after all the 3 doses but 1 intussusception episode occurred at 114th day after the 3rd dose of vaccine in the infant-schedule group (Bines *et al.*, 2018). These extremely promising efficacy results show the potential of using a neonatal rotavirus strain that was adapted to the newborn gut

2.10.1 Comparative analysis of vaccine effectiveness

The efficacy and effectiveness for Rotarix® and RotaTeq® were quite high in high-to middle-income countries ranging from 85 – 98% (Ruiz-Palacios *et al.*, 2006b; Vesikari *et al.*, 2006b; Vesikari *et al.*, 2006c; Vesikari *et al.*, 2012; Vesikari *et al.*, 1986; Vesikari *et al.*, 2015), however average performances were observed in the low-income countries in Africa and Asia with efficacies ranging from 51 – 64% (Armah *et al.*, 2016; Armah *et al.*, 2010; Breiman *et al.*, 2012; Madhi *et al.*, 2010, 2016; Madhi *et al.*, 2012; Zaman *et al.*, 2010; Zaman *et al.*, 2017).

Available reports have shown considerable data analysis in the pre-clinical trials of Rotarix® and RotaTeq®. There was a general reduction in the vaccine efficacies, especially against heterotypic infections, as observed in the low-income countries (Vesikari *et al.*, 2010; Vesikari *et al.*, 2007) **(Table 2.3)**. About 10% lower vaccine efficacies have been observed in Latin America as compared to the USA and European countries (Ruiz-Palacios *et al.*, 2006b; Vesikari *et al.*, 2009). Much-reduced efficacies were observed in Africa and Asia, especially in the second year of follow-up (Armah *et al.*, 2010; Bar-Zeev *et al.*, 2016; Madhi *et al.*, 2012; Zaman *et al.*, 2010). Though Rotavac® and RotaSIIL® were recently licensed for worldwide usage, prior pre-clinical analysis showed similar vaccine efficacy to that of Rotarix® and RotaTeq® in the low-income countries (Bhandari *et al.*, 2014; Kulkarni *et al.*, 2017) **(Table 2.3)**. There is convincing evidence that the introduction of rotavirus vaccines into more than half of Africa had greatly reduced the proportion of diarrhoea-related hospitalisations and cases of severe gastroenteritis in infants due to rotavirus infection (Mwenda *et al.*, 2018; Weldegebriel *et al.*, 2018). Vaccine effectiveness, coverage and the impact of post introduction vaccine surveillance studies were summarised in **Table 2.4**. From **Table 2.4**, despite low range vaccine coverage of 59 – 73% in the USA, more than 90% vaccine efficacy was achieved within the five years of vaccine introduction. High vaccine efficacies have also been observed in every part of Europe (Karafillakis *et al.*, 2015). Furthermore, despite high vaccine coverage in Africa, the overall vaccine efficacy against all forms of rotavirus gastroenteritis ranges from 54 – 76%. It is very important to re-emphasise that despite these average performances of these vaccines in Africa, greater proportion of the rotavirus-related gastroenteritis and hospitalisation have been averted, which is of significant perspective in public health at large. However, the need therefore, exists for alternative non-replicating sub-unit vaccines. This will be of great advantages such as complementing the existing oral vaccines in terms of cost and general production. Also, the non-replicating sub-unit vaccines will facilitate more protection without the fear of clinical risks and side effects as currently associated with the live-attenuated oral rotavirus vaccines.

Table 2.3: Comparative analysis of rotavirus vaccine efficacy in pre-clinical trials

Vaccine	*Seasonal efficacy/follow-up/doses*		*Remark*
	First	**Second**	
North America and Europe			
Rotarix® tested on 3 600 children in 5 Europian countries	96% for SRVGE 78% for any RVGE	86% for SRVGE 68% for any RVGE	Seasonal reduction observed, using Vesikari scale ≥$^{11}/_{20}$ for SRVGE 96% against G1P[8] to 86% for G2P[4] (Vesikari *et al.*, 2007)
RotaTeq® tested on almost 3 600 children	100% for SRVGE 72% for any RVGE	94% for SRVGE 58% for any RVGE	With REST (rotavirus efficacy and safety trial) (Vesikari *et al.*, 2006d) and Clark scale $^{16}/_{24}$ for SRVGE. The first year followed-up VE was 95%. For up to 3 years follow-up in Finland, 95 – 96% against G1, G3, G4, G9 with P[8] including G2P[4], which was 83% were observed (Vesikari *et al.*, 2009; Vesikari *et al.*, 2010)
Latin America			
Rotarix® trial of 20 000	85% for RVI but 86% when co-administered with OPV		With $^{11}/_{20}$ Vesikari scale, lower VE against G2P[4] (Ruiz-Palacios *et al.*, 2006b)
RotaTeq® with less data for pivotal trial	90% for RVGE		Like the USA and European data but wider confidence interval. Generally, the VE of currently licensed RVV in Latin America is about 10% lower compared to Europe and affluent Asia (Hongkong, Singapore and Taiwan) (Vesikari *et al.*, 2009)
Africa			
Rotarix®	61.2%-point estimate between 2 and 3 doses and overall efficacy of 76.9% in South Africa and 49.4% efficacy in Malawi at first-year follow-up (Madhi *et al.*, 2010). Though, higher efficacy was reported in South Africa more episodes of severe rotavirus gastroenteritis was prevented in Malawi than in South Africa (Madhi *et al.*, 2010)		No difference in VE against G1 and non-G1 (G2, G8, G12) (Madhi *et al.*, 2012)
RotaTeq®	83%-point estimate in Kenya, 65% in Ghana but no efficacy observed in Mali at first-year follow-up	Reduced efficacy at the end of the second year follow-up (Armah *et al.*, 2010)	At Vesikari scale ≥$^{11}/_{20}$, although the VE was generally low in the second-year follow-up but effective in reducing RVGE cases in these regions
RotaSIIL®	66.7% for SRVGE and 78.8% VSRVGE in Niger		Reasonably efficacious against morbidity and mortality from RVI, BRV-PV offers a low cost-effective and heat-stable oral vaccine but large-scale surveillance will be needed to establish its safety (Isanaka *et al.*, 2017)
Southeast Asia			
RotaTeq®	73% for SRVGE in Vietnam and 45% in Bangladesh		Combined efficacy in Asia was 61% (Zaman *et al.*, 2010)
India			
Rotavac®	53.6% for SRVGE in 3 single doses based on ≥ $^{11}/_{20}$ Vesikari scale and 56.4% in the first year (Bhandari *et al.*, 2014)		It may be difficult to compare this licensed vaccine since 116E has not been tested outside India and no other vaccine has been tested for efficacy in India save RotaSIIL®
RotaSIIL®	39.5% for SRVGE at 2 years follow-up per protocol (PP); 38.8% for intent to treat (ITT) and 54.7% against VSRVGE (PP)		BRV-PV well tolerated and safe vaccine in Indian infants (Kulkarni *et al.*, 2017) but the efficacy is very low especially against SRVGE

VSRVGE = very severe rotavirus gastroenteritis; **SRVGE** = severe rotavirus gastroenteritis; **RVGE** = rotavirus gastroenteritis; **VE** = vaccine efficacy; **RVV** = rotavirus vaccines; **RVI** = rotavirus infection; **BRV-PV** = bovine rotavirus pentavalent vaccine

Table 2.4: General impact of oral live-attenuated vaccines, Rotarix® and RotaTeq®

Country	*Surveillance Year(s)*	*Coverage*	*Impact (reduction of all forms of RVGE)*
USA	2010 – 15	59 – 73%	> 90% VE against RVGE (Payne *et al.*, 2013)
Austria	2008 – 11	72 – 74%	95 – 96% VE for the full course of a vaccine against RVGE hospitalisations (Paulke-Korinek *et al.*, 2013)
Belgium	2007 – 09	85 – 90%	91% for at least 1 dose, overall of 90% against RVGE, 91% against SRVGE and 66%against mild-moderate rotavirus (Braeckman *et al.*, 2012)
Finland	2010 – 12	95 – 97%	92% VE overall against RVGE hospitalisations (Vesikari *et al.*, 2013)
Germany	2008 – 10	11 – 58%	Coverage was 58% moderate to 22% low in the Eastern Federal States and 11 % very low for the Western Federal States, all with 80 % overall VE (Adlhoch *et al.*, 2013; Dudareva-Vizule *et al.*, 2012)
Moldova	2012	35 – 55%	79 % against rotavirus hospitalisations and 84% against moderate-to-severe RVGE (Gheorghita *et al.*, 2016)
Armenia	2012	94%	79 % against more severe rotavirus disease across all age groups up to 2 years (Abou-Nader *et al.*, 2018; Immunisation Financing, 2017; Sahakyan *et al.*, 2016)
Mexico	2007-08	>70%	42% reduction in mortality due to RVGE (Richardson *et al.*, 2011)
Nicaragua	2006 - 07	61 – 82%	45% overall VE, which declined with age/genotype; 50% against SRVGE (Becker-Dreps *et al.*, 2011; Patel *et al.*, 2009)
Bolivia	2008	90%	59% overall VE (Pringle *et al.*, 2016; Smith *et al.*, 2011)
Rwanda	2012-13	98 – 99%	75% VE with 61 & 70% reduced-hospitalisation due to RVI (Ngabo *et al.*, 2016)
South Africa	2010-12	66.5 – 100.6%	60-64% reduction in RVI, with 76.9% VE (Groome *et al.*, 2014b)
Mozambique	2016-17	76 – 89%	Rotavirus prevalence before vaccine introduction was 40.2% in 2014 and 38.3% in 2015, but reduced to 12.2% and 13.5% in 2016 and 2017 respectively after vaccine introduction (de Deus *et al.*, 2018; de Deus *et al.*, 2017)
Malawi	2013 – 14	26 – 92%	58.3% VE (Bar-Zeev *et al.*, 2016; Bar-Zeev *et al.*, 2015; Madhi *et al.*, 2016)
Botswana	2012	70%	54% overall VE against rotavirus diarrhoea requiring hospitalisation (Gastanaduy *et al.*, 2016b)
Ghana	2012 – 08	90 – 94%	65% VE full course against SRVGE (Pecenka *et al.*, 2018)
Bangladesh	2017-18	94-97%	48% VE full course against SRVGE (Pecenka *et al.*, 2018)

VE = vaccine efficacy; **SRVGE** = severe rotavirus gastroenteritis; **RVGE** = rotavirus gastroenteritis

Rotavirus vaccines also offer translational protection as high as 26% in herd immunity for non-vaccinated children have been reported (Pietsch & Liebert, 2019). This is a highly profitable effect of wider vaccine coverage. According to the WHO, 96 countries worldwide have introduced rotavirus vaccines by the end of 2018 and 46 were supported by GAVI. Africa has continued to be the leading continent in the implementation of rotavirus vaccines with 35 out of 47 countries in sub-Saharan Africa introducing rotavirus vaccines into their national immunisation programmes (Tate *et al.*, 2016).

2.10.2 Factors affecting rotavirus vaccine efficacy

Poor rotavirus vaccine absorption in the gut has been predicted as a major underlying factor affecting vaccine efficacy, particularly in Africa and southeast Asia (Angel *et al.*, 2012; Clarke & Desselberger, 2015; Franco *et al.*, 2006; Kirkpatrick *et al.*, 2015; Lopman *et al.*, 2012). **Breastfeeding** has been predicted as a factor against the efficacy of rotavirus vaccines but with inconclusive evidence because some reports indicated the interaction of the rotavirus vaccines with non-antibody milk components, whereas in a controlled study, there was no significant difference regarding the role of breastfeeding before/after/abstinence during

vaccination (Goveia *et al.*, 2008; Groome *et al.*, 2014a; Gruber *et al.*, 2017; Moon *et al.*, 2013; Rongsen-Chandola *et al.*, 2014; Vesikari *et al.*, 2012). Rotavirus-specific IgG obtained **transplacentally** has been shown to reduce seroconversion after rotavirus vaccination (Appaiahgari *et al.*, 2014; Becker-Dreps *et al.*, 2015; Chen *et al.*, 2017; Moon *et al.*, 2016; Rongsen-Chandola *et al.*, 2014).

The quality of **microbiota/probiotic** diversities in children ranging from high- to middle- to low-income countries have been comprehensively compared (Dethlefsen *et al.*, 2007; Lin *et al.*, 2013) in terms of how it affects the immune system against gut infections (Chinen & Rudensky, 2012; Gallo & Hooper, 2012; Kamada *et al.*, 2013a; Kamada *et al.*, 2013b; Praharaj *et al.*, 2015). This has been considered critical to immunity against rotavirus infection based on animal studies (Gallo *et al.*, 2016; Harris *et al.*, 2018; Harris, 2015; Harris *et al.*, 2017; Kandasamy *et al.*, 2014a; Twitchell *et al.*, 2016; Vlasova *et al.*, 2013). The presence of gut commensals such as *Lactobacillus rhamnosus GG*, *L acidophilus*, *L reuteri*, *Bifidobacterium lactis Bb12* decreases the clinical symptoms of RV disease (Chattha *et al.*, 2013b; Liu *et al.*, 2013a; Vlasova *et al.*, 2013; Zhang *et al.*, 2008). A significant correlation between the composition of the infant gut microbiome and response to rotavirus vaccination has been observed in Ghana (Harris *et al.*, 2017).

General **malnutrition** such as lack of vitamins A and D and poor serum zinc levels is common in sub-Saharan Africa and Asia. Poor nutritional status contributes to the lower efficacy of rotavirus vaccines in these regions (Gastanaduy *et al.*, 2016a; Gruber *et al.*, 2017; Hoest *et al.*, 2014) because of the associated innate and adaptive dysfunctions (Bosomprah *et al.*, 2016; Bucak *et al.*, 2016; Colgate *et al.*, 2016; Prendergast, 2015; Surman *et al.*, 2016; Zhao *et al.*, 2014; Zitt *et al.*, 2012).

Studies have shown the negative impacts of **co-infections** at the time of rotavirus vaccination. Such infections include enterovirus infection (Taniuchi *et al.*, 2016) and bacterial enteric pathogens (Bhavnani *et al.*, 2012; Hung *et al.*, 2009; Vasco *et al.*, 2014). Co-infections with rotavirus generally aggravate the clinical symptoms and prolong hospitalisation (Lee *et al.*, 2012; Valentini *et al.*, 2013). In another scenario, the **juvenile immune system** (innate and acquired) particularly associated with infants (Simon *et al.*, 2015) plays a role in rotavirus vaccine efficacies. As controversial as it seems, a report has shown a fundamental reduction in the efficacy of the vaccines against rotavirus infection in the low-income countries due to the immature immune system of the infants (Lopman *et al.*, 2012). However, infants' immune systems are generally immature and suffer rotavirus infection, but compounding factors such as poor nutrition, co-infections and low-quality microbiota, as frequently found in Africa and

Asia, may aggravate the infection. These poor intestinal conditions can sometimes lead to **Environmental enteropathy (EE)**, characterised by several gastrointestinal-dysfunctions such as intestinal inflammation, reduced intestinal absorption and gut barrier dysfunction. These conditions are more common in African children (Campbell *et al.*, 2003a; Campbell *et al.*, 2003b) and it has been shown to be associated with a poor response to rotavirus vaccines (Becker-Dreps *et al.*, 2017; Naylor *et al.*, 2015).

Variation in the developmental and **genetic distribution of HBGAs** (histo-blood group antigens) causes susceptibility variation to rotavirus infection either by vaccination or natural infection (Kazi *et al.*, 2017; Ramani *et al.*, 2016). Available evidence showed strain selection in terms of the preferred membrane receptors with either sialic acid or HBGAs for cellular attachment and penetration (Hu *et al.*, 2012b; Imbert-Marcille *et al.*, 2014; Ramani *et al.*, 2013; Ramani *et al.*, 2016; Venkataram Prasad *et al.*, 2014). Evidence from Nordgren and co-workers in 2014 showed that P[8] rotavirus strains infect only Lewis and secretor positive children while P[6] predominantly infect Lewis-negative children and to some extent Lewis-positive children irrespective of the secretor status. This is the reason there was a resistance against G1P[8] because there are a high percentage of Lewis-negative children in Africa. This also explains why there is a prevalence of P[6] rotavirus strain in Africa (Nordgren *et al.*, 2014). Nevertheless, more insights are needed to unravel vaccine efficacies and antigenic receptor distribution in individuals. Lastly, **co-administration** with oral polio vaccine (OPV) has been shown to suppress rotavirus vaccine efficacy as indicated by the low level of serum RV IgA but not vice versa (Giammanco *et al.*, 1988; Patel *et al.*, 2012).

2.10.3 Problems associated with live-attenuated rotavirus vaccines

Intussusception (IS) is a rare clinical symptom of speculative etiology in infants manifested as intestinal convolution against the free flow in the gut caused by the invagination along the intestinal tract (Loukas *et al.*, 2009; Mannai *et al.*, 2017; Slam & Teitelbaum, 2007; Ueki *et al.*, 2004). This clinical condition is rather very common among the neonates and the preterms (Loukas *et al.*, 2009) and naturally occurs at a rate of 1 – 4 in 2000 children between 6 and 18 months of age (Justice *et al.*, 2006; Tepmalai *et al.*, 2017). Sadly, this clinical condition has been shown to be related with the rotavirus vaccination (Tate & Parashar, 2019). Though, while clinical trials re-assured the efficacies and safety of the rotavirus vaccines (Ruiz-Palacios *et al.*, 2006a; Vesikari *et al.*, 2006d), the small increased risk of intussusception associated with the rotavirus vaccines cannot be rule out in some settings (Tate & Parashar, 2019). Clark and co-workers reported the case-fatality ratios (CFRs) in Africa as 1 death in every 10 hospital admissions due to intussusception caused by rotavirus vaccine and the rest of the world, which is fewer than 1 death in every 100–2000 hospital admissions (Clark *et al.*, 2019).

RotaShield® was withdrawn from the market because of the high rate of IS (1:10 000), which occurred 3 – 7 days after administration of the first dose of vaccine (Murphy *et al.*, 2001; Simonsen *et al.*, 2005). All the current live-attenuated vaccines have been reported to cause IS with Rotarix® and RotaTeq® reportedly having an occurrence of upwards of 1 to 7 cases per 100 000 immunizations (Yen *et al.*, 2016). Porcine circovirus (PCV) has not been reported to infect humans, but has been found in the vaccine formulation of the two globally licensed rotavirus vaccines (McClenahan *et al.*, 2011; Victoria *et al.*, 2010). Whether this may cause a significant effect is not known for now, but there are PCV genomic material transferred each time an infant is vaccinated. The cumulative effect of this may induce an unusual immune response. Despite this possible effect, the recommendation to use these vaccines remains (Ranucci *et al.*, 2011) and with changes in the manufacturing process, PCV-free vaccine should be made available (Ranucci *et al.*, 2011).

Live-attenuated rotavirus vaccines reassort to virulence strains (Hanley, 2011) in two ways. Firstly, with the circulating field strain and secondly, within attenuated rotavirus components of a polyvalent vaccine such as RotaTeq®. In either of the cases, a new strain emerges with partial or complete genomic constellation, which can be more virulent. A few instances have been reported post-vaccination, for example, the discovery of vaccine-derived G1P[8] from RotaTeq® (Boom *et al.*, 2012; Donato *et al.*, 2012; Hemming & Vesikari, 2014; Payne *et al.*, 2010) and Rotarix® (Boom *et al.*, 2012; Rose *et al.*, 2013) and heterotypic G2P[4] from RotaTeq® (Donato *et al.*, 2014). This poses a serious threat against vaccine efficacy (Dóró *et al.*, 2014). This is, in fact, one of the main risks associated with the use of replicating live-attenuated vaccines and underlines the need for alternative, non-replicating vaccine candidates.

The majority of live-attenuated vaccines are cold-chain storage vaccines (Bhandari *et al.*, 2014; Cortese & Parashar, 2009; Le *et al.*, 2017; Naik *et al.*, 2017) **(Table 2.2)**. This involves various logistical issues such as storage, transportation and distribution, which may be difficult to implement in low-income countries (Levin *et al.*, 2007; Matthias *et al.*, 2007). Inadequate storage and transportation facilities for these cold-chain vaccines could lead to massive wastage of the vaccine products (Naik *et al.*, 2017; Pecenka *et al.*, 2018).

2.10.4 Impact of rotavirus vaccines on the genotype distribution pattern

The massive rollout of rotavirus vaccines in recent years, especially Rotarix® and RotaTeq®, has resulted in strain selection due to vaccine-induced selective pressure (Roczo-Farkas *et al.*, 2018). In Australia for example, before vaccine introduction G1 was the most prevalent genotype followed by G2, G4, G3 and G9 (Roczo-Farkas *et al.*, 2018). After vaccine

introduction G1, G2, G3, G4, G9 and G12 were reported (Roczo-Farkas *et al.*, 2018). The emergence of G12P[8], equine-like G3P[8] and the increased frequencies of G8 and G10 represented the diverse population of genotypes circulating following vaccination in Australia (Roczo-Farkas *et al.*, 2018). The emergence of G12P[8] and equine-like G3P[8] were identified in regions with more than 90% coverage for the RotaTeq® vaccine (Arana *et al.*, 2016; Cowley *et al.*, 2016; Delogu *et al.*, 2015; Dóró *et al.*, 2016; Luchs & Timenetsky Mdo, 2016; Markkula *et al.*, 2017; Markkula *et al.*, 2015; Neves *et al.*, 2016; Ogden *et al.*, 2018; Wylie *et al.*, 2014). Unfortunately, lower vaccine efficacies have been reported against these emergent strains (Cowley *et al.*, 2016; Roczo-Farkas *et al.*, 2018). The emergence of G2P[4] strains has been recorded by regions with the increasing use of Rotarix® (Luchs & Timenetsky Mdo, 2016; Roczo-Farkas *et al.*, 2018) and being a heterotypic, emerging human strain, consistent low vaccine efficacies have been recorded against G2P[4] (Clarke & Desselberger, 2015; Pitzer *et al.*, 2015).

Generally, the pre-vaccination genotype strains in Africa were G1P[8], G2P[4], G2P[6], G9[8], G12P[8], G8P[4], G1P[6], G3P[6] with minor atypical strains but the following genotypes have been observed after the introduction of the vaccine: G1P[8] being the most frequent, followed by G2P[4], G8P[4], G12P[8], G2P[6], G4P[8], G3P[6] and G9P[8] coupled with a few low frequency, mixed serotypes (Seheri *et al.*, 2018; Simwaka *et al.*, 2018) and atypical genotypes such as G1P[4], G2P[8], G9P[4] and G9P[12]. This report was from fifteen countries across Eastern and Southern Africa (Seheri *et al.*, 2018; Simwaka *et al.*, 2018).

2.10.5 Non-replicating rotavirus vaccine candidates

Non-replicating rotavirus vaccine candidates are seen as next generation vaccines, capable of eliciting both humoral and innate immune responses. Despite the efficacies of oral, live-attenuated rotavirus vaccines against all forms of rotavirus gastroenteritis and hospitalisation, the vaccines are quite expensive and associated with risks such as intussusception, viral shedding (which is contagious) and reassortments. The route of administration of these vaccines also may result in mild to severe diarrhoea in the infants. In light of these, however, the use of non-replicating rotavirus vaccine candidates can offer advantages in terms of production, co-administration, immune response and route of administration. There are three different non-replicating rotavirus vaccine candidates namely, inactivated rotavirus particles, rotavirus VLPs and sub-unit proteins.

A thermally or chemically **inactivated rotavirus vaccine** (IRV) has several advantages over live-attenuated oral vaccines in terms of the cost, route of administration and the associated risks. This vaccine candidate has shown high efficacy in animal studies, lack of IS, ease of

administration and co-administration with other vaccines such as oral polio vaccine (OPV) or inactivated polio vaccine (IPV) and cost-effective production (Jiang *et al.*, 2008a; Jiang *et al.*, 2013; Jiang *et al.*, 2008b; Resch *et al.*, 2018; Wang *et al.*, 2010; Yen *et al.*, 2016). An inactivated rotavirus vaccine, for example CDC-9, a heat-inactivated G1P[8] with segment 3 derived from G2P[4] (Esona *et al.*, 2010a), has attracted much attention because of its high immunogenic properties, with or without adjuvants, in animal models (Resch *et al.*, 2018; Wang *et al.*, 2010; Wang *et al.*, 2019; Yuan *et al.*, 2001; Yuan & Saif, 2002; Zhang *et al.*, 2014). Other IRVs reportedin literature are iEDIM (McNeal *et al.*, 1998), iSA11 (Jiang *et al.*, 1999), iRRV (Johansen *et al.*, 2003), iYK-1 (Jiang *et al.*, 2008b), iRIT 4237 (Zissis *et al.*, 1983), iWa (Yuan *et al.*, 1998).

Generally, virus-like particles (VLPs) are antigenic capsid layers (single- or multi-layer) of viral proteins with natural resemblance of the virion but without nucleic acids and, therefore, cannot replicate in the susceptible hosts. **Rotavirus virus-like particles** (RV-VLPs) can contain the core capsid, VP2 (SLP), the double-layered particles (VP2/6) (Redmond *et al.*, 1993) or triple-layered particles (VP2/6/7 or VP2/4/6/7) (Crawford *et al.*, 1994). Various combinations of these VLPs have been prepared with or without adjuvants and immunogenicity was tested in different animal models (Blazevic *et al.*, 2011; Blazevic *et al.*, 2016; Ciarlet *et al.*, 1998; Conner *et al.*, 1996; Istrate *et al.*, 2008; Marashi *et al.*, 2014; Yuan *et al.*, 2000). Many of these reports provided evidence that VLPs are a potential alternative vaccine candidate against rotavirus infection (Azevedo *et al.*, 2010) because of their well-defined structures (Choi *et al.*, 1999; Choi *et al.*, 2002a; Istrate *et al.*, 2008; Jere *et al.*, 2014; Matsumura *et al.*, 2002; O'Brien *et al.*, 2000; Yu & Langridge, 2003), parenteral and mucosal route of administration (Ciarlet *et al.*, 1998; Istrate *et al.*, 2008; Lappalainen *et al.*, 2015), which allows it to by-pass gastrointestinal and proteolytic effects and efficient uptake by the dendritic cells for the activation of innate and adaptive immune responses (Bertolotti-Ciarlet *et al.*, 2003; Sharma *et al.*, 2012). Rotavirus VLPs are generally expressed in baculovirus insect cell expression systems (Azevedo *et al.*, 2013; Bertolotti-Ciarlet *et al.*, 2003). Other, less widely used, expression systems such as transgenic plants (Pera *et al.*, 2015; Yang *et al.*, 2011), yeasts (Rodríguez-Limas *et al.*, 2011), mammalian cells (Choi *et al.*, 2002b), and transgenic animals (Soler *et al.*, 2005; Soler *et al.*, 2007) have also been used to produce RV-VLPs.

Various structural and non-structural rotavirus proteins have been recombinantly produced as **sub-unit vaccine candidates**. The tubular structures of VP6 capsid have been confirmed to be highly immunogenic (Fingas *et al.*, 2018; Lappalainen *et al.*, 2015; Lappalainen *et al.*, 2013; Vega *et al.*, 2013) and to reduce viral shedding by 70% with a single dose in mice models (Pastor *et al.*, 2014). Rotavirus VP6 can induce non-neutralising antibodies that can block

rotavirus replication (Aiyegbo *et al.*, 2013; Lappalainen *et al.*, 2014; Trask *et al.*, 2012a). High levels of serum IgA antibodies against rotavirus VP6 protein seems to explain the rotavirus vaccine-induced protection (Cheuvart *et al.*, 2014; Patel *et al.*, 2013). Immunocompetent preparations of rotavirus VP6 has displayed remarkable induction of protective immune responses against rotavirus infection and gastroenteritis. These preparations include VP6 with other VLP (like Norovirus VLP) (Blazevic *et al.*, 2011), rotavirus NSP4 (Afchangi *et al.*, 2018), VP2 (Lopez-Guerrero *et al.*, 2018), VP6 protein as an adjuvant (Blazevic *et al.*, 2016; Malm *et al.*, 2016), VP6 protein formulated with adjuvants (Choi *et al.*, 2005; McNeal *et al.*, 2007b; Smiley *et al.*, 2007) and VP6 as nanoparticles (Li *et al.*, 2019; Rota *et al.*, 2018; Ward & McNeal, 2010).

VP4 and VP7 capsids are type-specific antigens inducing protective, neutralising antibodies against natural rotavirus infections (Desselberger & Huppertz, 2011). Oral immunization with a rotavirus VP7-cholera toxin B subunit (CTB) fusion protein expressed in transgenic *Arabidopsis thaliana* induced antigen-specific IgA and IgG and passive protection in mice (Li *et al.*, 2018b). It has been shown that the immunogenicity of rotavirus VP4 is higher than that of VP5* and VP8* (Dunn *et al.*, 1995; Li *et al.*, 2018c). The production of a P2-VP8* protein candidate of human rotavirus genotype P[8], P[4] or P[6] expressed in *E. coli* (Wen *et al.*, 2012; Wen *et al.*, 2015) is a remarkable event in the use of prokaryotes for vaccine prodcution. The sub-unit is a truncated ΔVP8* containing only the core region, aa64 – 223, from the Wa strain (G1P[8]) of human rotavirus fused with a universal tetanus toxoid $CD4^+$ T cell epitope P2 and expressed in *Escherichia coli* (Wen *et al.*, 2014). This sub-unit induced neutralising immune responses and with the P2 epitope, exhibited a strong Th cell function (Kirkwood *et al.*, 2017; Wen *et al.*, 2014; Wen *et al.*, 2015). With the success of the first trial of the monovalent vaccine candidate (P2-VP8*-P[8]) (Fix *et al.*, 2015), a phase II trial of a trivalent P2-VP8*-(P[4], P[6] and P[8]) subunits will be investigated at three sites in South Africa (Groome *et al.*, 2017). Rotavirus NSP4 enterotoxin protein induced a more heterotypic antibody response during natural rotavirus infection in children (Ray *et al.*, 2003). As an adjuvant to DLPs and VP6, NSP4 increases the immunogenicity of these vaccine candidates when administrated in mice (Afchangi *et al.*, 2018; Feng *et al.*, 2017; Kavanagh *et al.*, 2010).

Rotavirus **DNA vaccines** provide an adequate immune response against rotavirus infection and this has been confirmed in rotavirus encoding genes for VP2, VP4, VP6 and VP7 (Chen *et al.*, 1997; Chen *et al.*, 1998; Choi *et al.*, 1997; Herrmann *et al.*, 1996a; Herrmann *et al.*, 1996b; Herrmann *et al.*, 1999; Yang *et al.*, 2001). Intramuscular co-administration of VP2 and VP6 encoding genes induced rotavirus-specific serum antibodies but not intestinal rotavirus-specific IgA (Lopez-Guerrero *et al.*, 2018). A rotavirus VP2 DNA vaccine given intranasally to

mice showed increased cytokine levels (IFN-γ and IL-4) with the production of anti-VP2 IgG (Pourasgari *et al.*, 2008). Protective immunity was induced in a murine model by oral immunisation with a VP6 DNA vaccine encapsulated in poly(lactide-coglycolide) (PLG) microparticles (Chen *et al.*, 1998). As attractive as DNA vaccination is, there has not been any trials in humans against rotavirus infections due to potential problems, which may occur in humans. These include insertional mutagenesis, chromosomal instability and activation/inactivation of tumour suppressor genes. Furthermore, the development of rare autoimmune diseases and more commonly autoantibodies against the exogenous DNA and its adjuvants may also occur (Kutzler & Weiner, 2008). Because the initial propagation of the plasmid takes place in bacteria with antibiotic resistance, the possibility exists to transfer such gene(s) together with the recombinant DNA into the host (Kutzler & Weiner, 2008).

2.11 Heterologous gene expression systems

The quest for non-replicating rotavirus vaccine candidates is a task that requires expression systems. Heterologous expression system offers a platform by which exogenous DNA encoding protein is propagated and expressed in a living cell to produce proteins/antigens. Some of these heterologous expression systems include bacterial cells (prokaryotes) others eukaryotes, which include insect cells, transgenic plants, yeasts, mammalian cells and transgenic animals.

Each expression system has its own unique strengths and weaknesses when it comes to characteristics such as production cost, expression rate and effective post-translational modification **(Table 2.5)**. Generally, VLPs have been expressed and purified from a number of prokaryotic and eukaryotic systems. The approximate percentage of the total VLP expression done in each system are: bacterial - 28%, yeast - 20%, plant - 9%, insect - 28% and mammalian - 15% (Zeltins, 2013). Bacterial cells are the cheapest and fastest method for recombinant protein expression, but they do not glycosylate proteins and often fold mammalian proteins incorrectly **(Table 2.5)**. Due to the poor solubility and weak immunogenic properties of recombinant antigens from bacteria, many expressed proteins are truncated or trimmed to minimize this effect (Liu & Sun, 2006). Many recombinant rotavirus subunit vaccine candidates such as VP7 (Perez *et al.*, 2005), VP6 (Bredell *et al.*, 2016), truncated VP4* (Li *et al.*, 2018c), VP8* (Marelli *et al.*, 2011; Xue *et al.*, 2015) and NSP4 (Enouf *et al.*, 2001) have been produced in bacteria such as *E. coli, Bacillus subtilis* and *Lactococcus lactis*.

Expression of rotavirus VLPs as foreign antigens in plants is an effective way of producing inexpensive and scalable subunit vaccines delivered as edible fruit (Haq *et al.*, 1995; Mor *et al.*, 1998) **(Table 2.5)**. So far, all forms of rotavirus capsid proteins have been successfully

expressed in transgenic plants with promising immunogenic properties (Matsumura *et al.*, 2002; O'Brien *et al.*, 2000; Pera *et al.*, 2015; Saldana *et al.*, 2006; Yang *et al.*, 2011). However, such expression has been characterised by poor yield, perishable plant product formulations and policies against plant products (Streatfield, 2005; Streatfield & Howard, 2003).

Using an engineered baculovirus *Autographa californica* nuclear polyhedrosis virus in *Spodoptera frugiperda* (Sf9 or Sf21) cells or *Trichoplusia ni* (High-Five®) has been the gold-standard for expressing RV-VLPs and subunit proteins with high immunogenic properties (Azevedo *et al.*, 2013; Bertolotti-Ciarlet *et al.*, 2003; Ciarlet *et al.*, 1998; Crawford *et al.*, 1994; Estes *et al.*, 1987; Labbé *et al.*, 1991; O'Neal *et al.*, 1997; Parez *et al.*, 2006). However, as versatile as this expression system is, the insect cell baculovirus expression vector system (IC-BVEVS) is expensive to scale-up production and co-production of enveloped baculovirus particles with the recombinant proteins (Buonaguro *et al.*, 2006; Palomares & Ramirez, 2009), have been identified as disadvantages of this expression system **(Table 2.5)**.

Mammalian cell expression systems are the most advanced system for protein expression suitable for VLP production. It can perform appropriate mammalian-type post-translational modifications with high integrity of recombinant protein assembly (Earnest-Silveira *et al.*, 2016a; Earnest-Silveira *et al.*, 2016b; Kumar *et al.*, 2016). Choi and co-workers used a Semliki forest virus (SFV)-based expression system to express rotavirus recombinant bovine-VP6 and human-VP7 in BHK-21 cells (Choi *et al.*, 2002b). A recombinant vaccinia virus system has been used to express DLPs in monkey kidney CV-1 cells (Gonzalez & Affranchino, 1995). There was a successful co-expression of NSP4 and VP7 using a bicistronic adenovirus vector in MA104 cells, which induced both humoral and cell-mediated immune responses efficiently and provides potential efficacy for protection against rotavirus disease (Xie *et al.*, 2015). Despite this high profile of mammalian cells, this expression system is highly technical, time-consuming and expensive. In the same trend, transgenic animals are now being used to express and secrete rotavirus VLPs with high immunogenic properties. Most of the transgenic recombinant expression and secretion occur in the milk of transgenic animals like mice and rabbits (Li *et al.*, 2019; Soler *et al.*, 2005; Soler *et al.*, 2007).

Yeasts such as *Saccharomyces cerevisiae, Pichia pastoris, Kluyveromyces lactis, Hansenula polymorpha, Yarrowia lipolytica* and *Arxula adeninivorans* have been used in the recombinant production of proteins of biological value. Several recombinantly produced VLPs from yeasts have been licensed for commercial use as vaccines against viral infection. For example, Merck and Co produces Engerix-B® (an intramuscular injectable recombinant vaccine against hepatitis B virus), Hepavax-Gene® (an intramuscular injectable recombinant vaccine against

hepatitis B virus), Recombivax HB® (an intramuscular injectable recombinant vaccine against all sub-types of hepatitis B virus) and Gardasil® (an intramuscular injectable recombinant vaccine against human papillomavirus (HPV) in girls and women aged 9 – 45 years against cervical/vaginal/anal cancers or genital warts (Masavuli *et al.*, 2017). Yeast expression system offers hope for the production of VLPs, especially on a large-scale. For example, different strains of foot-and-mouth disease virus (FMDV) VP1, VP2, VP3 and VP4 capsid proteins have been produced in *P. pastoris* while the infectious bursal disease virus (IBDV) VP2 capsid protein was produced in *K. lactis,* as reviewed in (Rajasekaran *et al.*, 2018). These recombinant VLPs have been used in the management of these diseases in cattle and chickens respectively (Rajasekaran *et al.*, 2018). In addition, Rodríguez-Limas and co-workers first reported the use of *S. cerevisiae* to produce immunogenic rotavirus DLPs and TLPs with multicistronic expression vectors (Rodríguez-Limas *et al.*, 2014; Rodríguez-Limas *et al.*, 2011) and in 2016, Bredell and co-workers successfully express rotavirus VP6 in *P. pastoris* and *H. polymorpha* (Bredell *et al.*, 2016).

Remarkable success has been observed in all the expression systems listed in **Table 2.5**. However, certain shortcomings have been identified in each of the expression systems. These include inefficient post-translational modifications with bacterial expression systems, plant expression is limited with poor yield and unconventional post-translational modifications of

Table 2.5: Features of different expression systems

Expression system	*Advantage*	*Disadvantage*
Bacteria	It requires inexpensive growth medium, simple expression system and genome content (chromosomal DNA and plasmids), fast doubling time, for example *E. coli* has 20 min, (Sezonov *et al.*, 2007), *Lactobacillus acidophilus* has 66 – 87min and *Streptococcus lactis* in milk has 26 min (Todar, 2018), high-level of expression, genetic stability, simple process to scale-up but secretes into the periplasm (Liew *et al.*, 2010; Marcekova *et al.*, 2009; Yin *et al.*, 2010)	Lack mammalian-like of (PTMs) post-translational modifications (Roldao *et al.*, 2010) hence refolding may be required (Rosano & Ceccarelli, 2014), antigen expressed elicits poor/weak immunogenicity, presence of host cell-derived endotoxin contamination (Schädlich *et al.*, 2009), incapable of extracellular secretion (Edman *et al.*, 1981), expression mostly as inclusion bodies and difficult to purify (Michel & Tiollais, 2010)
Yeast	Less expensive compared to insect expression, high-density bioreactor cultivation, moderately rapid expression (about 90 min) depending on the promoter and the nature of the yeast, support most protein folding and PTM such as N- and O-glycosylation; phosphorylation; acetylation; acylation, myristylation but cannot carboxylate. It can be engineered for both intracellular and extracellular protein secretion with easy scale-up (Kim *et al.*, 2015; Kim & Kim, 2017; Kim *et al.*, 2004; Lin-Cereghino *et al.*, 2013; Park *et al.*, 1997; Song *et al.*, 2007)	High mannose modification (Conde *et al.*, 2004; Harrison & Jarvis, 2006; Tang *et al.*, 2016; Wildt & Gerngross, 2005), sometimes misfolding of secretory proteins, leading to proteasomal degradation of the engineered antigens (Gasser *et al.*, 2008; Liu *et al.*, 2012)

Insect	Support most protein folding and higher eukaryotic-type post-translational modifications of the expressed protein (can naturally N-and O-glycosylate; phosphorylate; acetylate; acylate, myristylate, high efficiency for non-enveloped and enveloped multilayered VLPs, secretes into the growth medium, free of mammalian pathogens (van Oers, 2011; Yamaji, 2014).	High cost, hence difficult to scale-up (Chen, 2008; Chen, 2011; Chen & Lai, 2013; Lai & Chen, 2012), incomplete modification of recombinant proteins especially sialylytion but can carboxylate and fucosylate proteins, low-level expression, contamination of the product with enveloped baculovirus particles which can induce an immune response and time-consuming expression system (18 – 24 hours) (Virag *et al.*, 2009)
Mammalian	Perform appropriate complex mammalian-type PTMs (all forms of PTMs), perform authentic assembly and folding of recombinant proteins (Walsh, 2006), high efficiency for non-enveloped and enveloped multilayered VLPs (Zhu, 2012), secretes into the medium (Chen & Lai, 2013)	High cost hence difficult to scale-up (Kato *et al.*, 2012), lengthy expression time (over 24 hours), low yield, vulnerable to infection with mammalian pathogens/adventitious pathogens (Zhu, 2012)
Plant	Greater delay in expression as compared to insect cells, highly scalable but more expensive, free of mammalian/adventitious pathogens (Chen, 2011; Faye & Gomord, 2010), support most protein folding and eukaryotic-type PTMs (almost all PTMs) (Chen & Lai, 2013; Giorgi *et al.*, 2010) and possibility of oral therapy by feeding with edible plants (Santi *et al.*, 2006)	Low yield, technical and regulatory issues, low VLP expression (Saldana *et al.*, 2006), plant-specific glycosylation of glycoproteins (Gomord *et al.*, 2010) and inefficient multilayered VLP assembly (Pera *et al.*, 2015; Yang *et al.*, 2011).

the recombinant proteins, recombinant animal and plant products are continually hampered by regulations and policies. Insect cell expression on the other hand, is an excellent expression system for rotavirus VLPs but it is expensive and not easily scaled up to increase production. Despite the widely reported production of rotavirus VLPs in insect-based expression systems, data on any clinical trial is yet to be published.

Identifying a yeast expression system as an alternative system has attracted attention. Yeast expression is widely used in the biotechnology industries for large scale production of biopharmaceutical products such as peptide hormones, enzymes, vaccines and food aditives. Bredell and co-workers reported that yeast outperformed bacteria in a bioreactor process to produce rotavirus VP6 (Bredell *et al.*, 2016). Also, yeast requires inexpensive growth media, be it natural or synthetically formulated (Kottmeier *et al.*, 2010).

In this study, a wide-range yeast expression system previously developed in the Department of Microbial, Biochemical and Food Biotechnology at the University of the Free State (UFS), Bloemfontein, South Africa, was used. This expression plasmid can accommodate either single or double heterologous protein expression in a variety of yeast strains (Albertyn *et al.*, 2011). This expression system was used in this study to construct expression vectors containing the VP2 and VP6 ORFs (open reading frames), codon-optimised for three different yeast strains. The VP2 and VP6 ORFs were from the South African G9P[6] rotavirus field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] (Jere *et al.*, 2011; Potgieter *et al.*, 2009). The wide-range yeast expression system was used in combination with the UNESCO-MIRCEN Yeast Culture Collection at UFS, which consists of more than 3000 yeast strains, to screen yeast strains capable of producing DLPs and secreting VP6.

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Chapter Three

Construction of dual-expression vectors for co-expression of rotavirus VP2 and VP6 proteins in various yeasts

3.1 Introduction

Yeast cells contain various subcellular organelles with unique microenvironments, each providing favourable conditions for different metabolic pathways. Historically, *Saccharomyces cerevisiae* was the dominant yeast for heterologous protein production (Nielsen, 2013). However, other yeasts such as *Arxula adeninivorans, Hansenula polymorpha, Kluyveromyces lactis, Pichia pastoris* and *Yarrowia lipolytica*, otherwise referred to as Crabtree-negative yeasts, have emerged as competitive hosts for heterologous protein production (Löbs *et al.*, 2017; Porro *et al.*, 2005). These yeasts favour respiration over fermentation thus they possess phenotypes that are particularly useful for recombinant production (Löbs *et al.*, 2017).

The use of yeast to produce virus-like particles (VLPs) has attracted much attention recently. *Saccharomyces cerevisiae, Pichia pastoris* and *Hansenula polymorpha* have been used to produce single-layered VLPs of different viruses derived from single, nucleocapsid proteins (Noad & Roy, 2003; Stockmann *et al.*, 2009). Single-layered VLPs can be produced by secretion from yeasts, especially methylotrophic yeasts, however, production of multi-layered VLPs is usually intracellular as reviewed in Kim and Kim, (2017). Most of these studies produced VLPs in *S. cerevisiae,* followed by methylotrophic yeasts such as *P. pastoris* and *H. polymorpha*. Of interest was the immunogenic, multi-layered, rotavirus VLPs produced intracellularly by co-expressing rotavirus VP2, VP6 and VP7 capsid proteins in *S. cerevisiae* (Rodríguez-Limas *et al.*, 2011). There is no doubt that *S. cerevisiae* remains the prototype yeast to produce many types of VLPs. However, expression in *S. cerevisiae* is characterised by hyperglycosylation (Conde *et al.*, 2004), in particular, hypermannosylation (Kniskern *et al.*, 1994). This is undesirable for vaccine production since hyperglycosylation could result in reduced immunogenicity. Therefore, alternative yeast expression hosts are needed.

Previously, single- and dual-expression vectors were constructed using wide-range yeast expression vectors and rotavirus VP2 and VP6 open reading frames (ORFs) (Makatsa, 2015). The two ORFs were obtained from the South African rotavirus field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6]. These VP2 and VP6 encoding sequences were obtained in 1999 (Potgieter *et al.*, 2009) and originated from the stool sample of a neonate suffering from a gastrointestinal infection (Jere *et al.*, 2011; Potgieter *et al.*, 2009). The GR10924 strain is a local South African strain which has a DS-1-like or genogroup 2 backbone. Rotarix®, which is based on Wa (genogroup 1) backbone, is the choice of rotavirus vaccine that is used

extensively in South Africa, therefore, deriving sub-unit non-replicating vaccine from an alternative genogroup could compliment the current rotavirus vaccine.

The VP2 and VP6 ORFs were optimised for expression in three different yeast strains: *Arxula adeninivorans* (AO)*, Kluyveromyces lactis* (KO) and *Pichia pastoris/Hansenula polymorpha* (PO). The VP6 ORF was cloned into pKM177 as a single-expression vector and the VP2 and VP6 ORFs were cloned into pKM173 as a dual-expression vector (Makatsa, 2015).

Expression of the VP6 protein optimised for *P. pastoris/H. polymorpha* was confirmed. There was, however, no expression of the other two strain-optimised VP6 ORFs. In addition, no expression of VP2 was observed for any of the three optimised dual-expression vectors transformed in various yeast strains (Makatsa, 2015). Inspection of the single-expression vector for the VP6 ORF revealed an additional ATG upstream of the ORFs for both *A. adeninivorans* and *K. lactis* codon-optimised ORFs, hence there was no expression of VP6. Expression was only obtained for the *P. pastoris/H. polymorpha* VP6 codon-optimised ORF because the additional -ATG- observed was in-frame with the VP6-encoding ORF. It was, therefore, speculated that the expression, which was obtained for this recombinant plasmid may have additional amino acid residues at the N-terminal of the expressed protein. These additional amino acid residues could, in turn, affect the protein folding and ultimately, formation of the oligomeric structure. The additional -ATG- codons were subsequently removed. To improve expression, an optimised Kozak motif was introduced. These two corrections drastically improved the expression of the VP6 proteins from all three optimised ORFs when transformed in various yeast strains (Rakaki, 2018). In this chapter, similar manipulations of both the expression vectors containing the different codon optimised VP2 and VP6 ORFs, were performed to enable co-expression of VP2 and VP6 and the subsequent production of DLPs.

3.2 Materials and Methods

3.2.1 Molecular reagents, chemicals, restriction enzymes and molecular enzymes

The following enzymes and molecular reagents were purchased from Thermo Fisher Scientific (USA): Phusion® High-Fidelity DNA polymerase, restriction endonucleases (unless otherwise stated), T4 ligase, polynucleotide 5'-hydroxyl-kinase (PNK), 1 kb DNA ladder, DNA Gel Loading Dye (6X), 10 mM ATP, BigDye® Terminator v3.1 Cycle Sequencing Kit. Kapa Biosystems (South Africa) supplied 10 mM dNTP mix. Merck (Germany) supplied: bacterial agar, peptone, NaCl (sodium chloride), EDTA (ethylenediaminetetraacetate), isopropanol, Tris base (Tris(hydroxymethyl)aminomethane), glacial acetic acid, absolute ethanol, yeast extract,

manganese II chloride, potassium acetate, calcium chloride, glycerol, hygromycin B. SeaKem® LE Agarose was purchased from Lonza (Switzerland). Macherey-Nagel (Germany) was the manufacturer of NucleoSpin® Gel and PCR clean-up kits and NucleoSpin® plasmid extraction kits. The Shrimp alkaline phosphatase (*rSAP)* enzyme was manufactured by New England BioLabs (USA). Calbiochem® (USA) supplied: kanamycin sulphate and ampicillin while Sigma-Aldrich (USA) supplied $RbCl_2$ and MOPS (3-(N-morpholino)propanesulfonic acid. All primers used in this study, unless otherwise stated, was synthesised by Integrated DNA Technologies, IDT (USA).

3.2.2 Plasmids

The plasmids pKM173 (7548bp) and pKM177 (7566bp) were obtained from Prof J Albertyn, Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein (Albertyn *et al.*, 2011) **(Figure 3.1)**. All the plasmids used in this work are listed in **Table 3.1**. The VP2 and VP6 ORFs of RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] cloned in pUC57 and optimised for expression in *Kluyveromyces lactis* and *Arxula adeninivorans* were purchased from GenScript™ (USA). The VP2 and VP6 ORFs of RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] optimised for expression in *Pichia pastoris/Hansenula polymorpha* (GeneArt™, Germany) was a generous gift from Prof. J. Görgens, Stellenbosch University, South Africa and were obtained under a material transfer agreement (MTA). The *in-silico* maps for all the plasmids are presented in **Appendix A**. The -ATG- to be deleted is indicated with an arrow at the 3´-end of the promoter sequence before the *Xhol*-site **(Figure 3.1)**.

3.2.3 Bacterial strains and cultivation

Escherichia coli Top 10 cells, with genotype (F- *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15Δ *lac*X74 *rec*A1 *ara*D139Δ(*araleu*)7697 *gal*U *gal*K *rps*L(StrR) *end*A1 *nup*G) previously transformed with recombinant plasmids (pKM173, pKM177, pKM173/AOVP2, pKM173/KOVP2, pKM173/POVP2, pKM177/AOVP6, pKM177/KOVP6, pKM177/POVP6, pUC57/AOVP2 and pUC57/KOVP6) were revived and plasmids extracted **(Section 3.2.4.2)**. NEB® Stable Competent *E. coli* (High Efficiency) cells were purchased from New England BioLabs (USA) with the genotype [F' *proA+B+ lacI*^{q} Δ*(lacZ)M15 zzf::Tn10* (Tet^R) Δ*(ara-leu) 7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14- Φ80dlacZΔM15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1* Δ*(mrr-hsdRMS-mcrBC)*] and used for the propagation of the unmethylated, ligated plasmids. Cells were cultured in Luria-Bertani (LB) media (1 L LB broth: 10 g tryptone, 5 g yeast extract, 10 g NaCl or 1 L LB agar: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar). Selective LB medium contained 100 µg/mL kanamycin or 100 µg/mL ampicillin depending on which resistance gene was present **(Table 3.1)**.

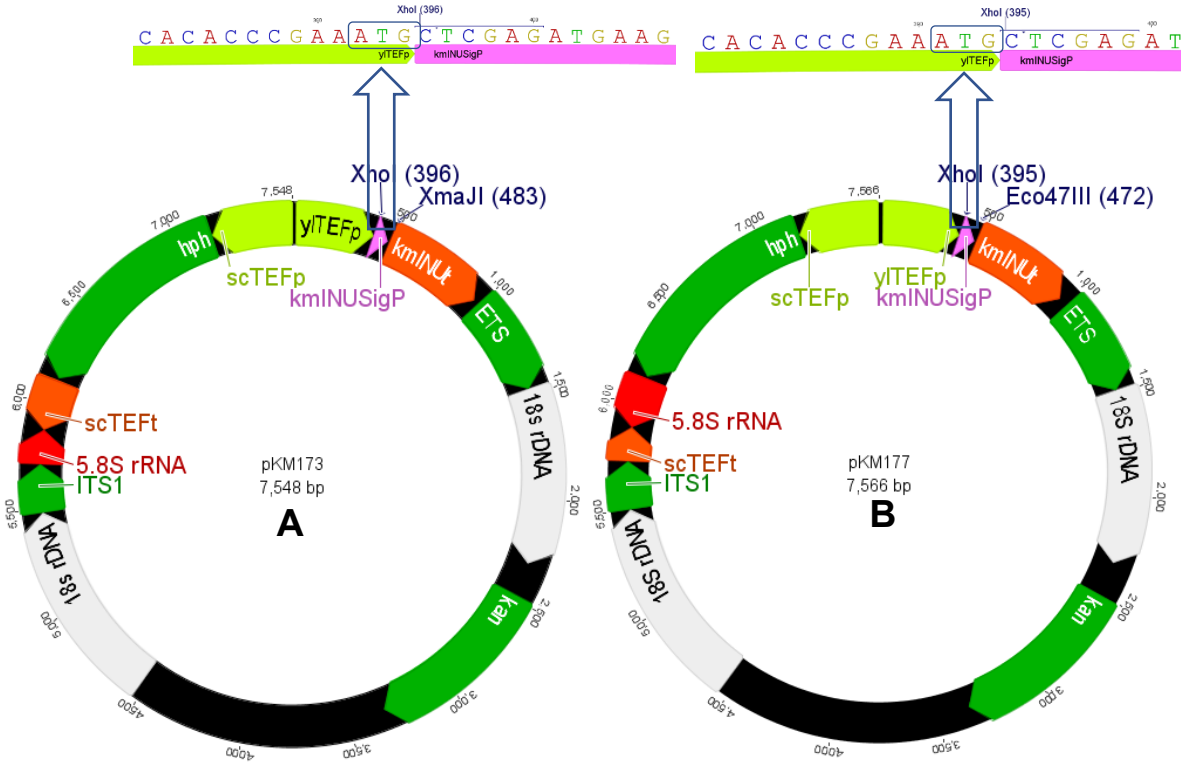


Figure 3.1: The expression vectors used in this study: (A) pKM173 and (B) pKM177. The ATG indicated by an arrow above each plasmid is the target -ATG- at the cloning site that was suspected to have affected the previous expression (Makatsa, 2015). Important features of the wide-range, yeast expression vectors include the *XhoI-XmaJI* and the *XhoI-Eco47III* cloning sites in pKM173 and pKM177 respectively, *Kluyveromyces marxianus* signal sequence encoding the signal peptide, yITEFp – *Yarrowia lipolytica* transcriptional elongation factor EA-α1 promoter, kmINUt - *Kluyveromyces marxianus* inulase terminator region, genes for the selective markers, which are Kan^R (kanamycin for bacterial selection) and hygromycin B (*hph* – hygromycin-B-phosphotransferase gene under the *Saccharomyces cerevisiae* TEF promoter and terminator control regions for yeast selection), 18S rDNA (ribosomal DNA) ITS (inter-spacer sequence) for homologous integration into the yeast chromosomal DNA.

Table 3.1: Details of the plasmids used in this study

Plasmid name	***Size (bp)***	***Antibiotic resistance***	***Application in the study***	***Reference***
pKM173	7 548	*Kan^R* and *hph*	Wide-range yeast expression vector; removal of additional -ATG- upstream of ORF; used for dual-expression of VP2 and VP6	Albertyn *et al.*, (2011)
pKM177	7 655	*Kan^R* and *hph*	Wide-range yeast expression vector; removal of additional -ATG- upstream of ORF; used for expression of VP6	Albertyn *et al.*, (2011)
pKM173/AOVP2	10 108	*Kan^R* and *hph*	pKM173 containing the VP2 ORF optimised for expression in *A. adeninivorans*, removal of additional -ATG- upstream of ORF	Makatsa, (2015)

Plasmid name	***Size (bp)***	***Antibiotic resistance***	***Application in the study***	***Reference***
pKM177/AOVP6	8 692	*Kan*^{R} and *hph*	pKM177 containing the VP6 ORF optimised for expression in *A. adeninivorans*, removal of additional -ATG- upstream of ORF	Makatsa, (2015)
pKM177delATG/AOVP6	8 689	*Kan*^{R} and *hph*	pKM177 containing the VP6 ORF optimised for expression in *A. adeninivorans*, additional -ATG- upstream of ORF previously removed; used for the construction of a dual expression vector	Rakaki, (2018)
pKM173/KOVP2	10 108	*Kan*^{R} and *hph*	pKM173 containing the VP2 ORF optimised for expression in *K. lactis;* removal of additional -ATG- upstream of ORF	Makatsa, (2015)
pKM177/KOVP6	8 692	*Kan*^{R} and *hph*	pKM177 containing the VP6 ORF optimised for expression in *K. lactis;* removal of additional -ATG- upstream of ORF	Makatsa, (2015)
pKM177delATG/KOVP6	8 689	*Kan*^{R} and *hph*	pKM177 containing the VP6 ORF optimised for expression in *K. lactis*, additional -ATG- upstream of ORF previously removed; used for the construction of a dual expression vector	Rakaki, (2018)
pKM173/POVP2	10 108	*Kan*^{R} and *hph*	pKM173 containing the VP2 ORF optimised for expression in *P. pastoris/H. polymorpha;* removal of additional -ATG- upstream of ORF	Makatsa, (2015)
pKM177/POVP6	8 692	*Kan*^{R} and *hph*	pKM177 containing the VP6 ORF optimised for expression in *P. pastoris/H. polymorpha;* removal of additional -ATG- upstream of ORF	Makatsa, (2015)
pUC57/AOVP2	5 363	*Amp*^{R}	Shuttle vector containing the VP2 ORF optimised for expression in *A. adeninivorans*; used in construction of pKM173delATG	GenScript™
pUC19	2 686	*Amp*^{R}	Used to determine bacterial transformation efficiency of bacteria	Thermo Fisher Scientific™

3.2.4. Recombinant DNA techniques

3.2.4.1 Bacterial competent cells and transformation

NEB® Stable Competent *E. coli* (High Efficiency) cells were used to prepare chemically competent cells using the rubidium chloride ($RbCl_2$) method first described by Hanahan, (1983). The competency of the bacterial cells was determined with the pUC19 plasmid and found to be 1.2 x 10^6 CFU/µg DNA. The prepared bacterial competent cells (200 µL) were transformed with the recombinant DNA (Hanahan, 1983). The transformed cells were plated on kanamycin- or ampicillin-containing LB plates and incubated overnight at 37˚C.

3.2.4.2 Bacterial plasmid extraction

Plasmids were extracted from the previously transformed and cultured recombinant bacteria **(Section 3.2.3)**. A 5 mL preinoculum was prepared for each of the recombinant bacteria and incubated overnight in a shaker at 37˚C. The plasmids were extracted and purified using a NucleoSpin® Plasmid Kit and the high-copy plasmid DNA extraction protocol of the manufacturer was followed. The plasmids were quantified with a NanoDrop 1 000 UV-Vis spectrophotometer (Thermo Fisher Scientific).

3.2.4.3 Mini plasmid preparations

A rapid mini-preparation of bacterial plasmid DNA was carried out for the screening of recombinant bacterial colonies (Holmes & Quigley, 1981). The plasmid DNA was dried and resuspended in a 20 µL solution of 0.1x TE buffer (1x TE: 1 M Tris-base and 0.5 M EDTA, pH 8.0) and RNase (0.4 mg/mL).

3.2.4.4 Restriction enzyme digestions

Restriction enzyme digestions of the extracted plasmids (either by miniprep or NucleoSpin® kit) were performed according to the manufacturer's protocols specific for each restriction enzyme using 1x enzyme buffer. A web-based double digest calculator from Thermo Fisher Scientific (http://www.thermoscientificbio.com/webtools/doubledigest/) was used to determine the optimal buffer for double digestions.

3.2.4.5 Agarose gel electrophoresis

Samples were prepared by mixing 5x sample volume with 1x sample buffer volume prior to loading onto the gel. Electrophoresis was carried out at 120 V for 35 – 40 minutes using a 1 % w/v agarose gel in TAE buffer (40 mM Tris buffer pH 8.3, 2 mM EDTA and 20 mM glacial acetic acid) plus 5 mg/mL EtBr to give a total concentration of 0.5 µg/mL of EtBr. Gels were visualised with the ChemiDoc™ MP Imaging System (Bio-Rad, USA). The 1 kb GeneRuler was included as a DNA size standard.

3.2.4.6 Agarose gel purification of DNA fragments

Separated DNA fragments **(section 3.2.4.5)** were viewed under blue light (Dark Reader, Clare Chemical Research), excised and purified with the NucleoSpin® Gel and PCR Clean-up kit according to the manufacturer's protocol. A minor modification was applied in that the final elution step was performed with deionised water instead of the NE buffer provided in the kit.

3.2.4.7 Sanger sequencing

Sanger sequencing was carried out using the BigDye™ Terminator Cycle Sequencing Kit v3.1, according to the manufacturer's instructions. Primers used are listed in **TABLE A1 in APPENDIX A**. The samples were analysed on a 313*xl* Genetic Analyzer (Applied Biosystems™). The nucleotide sequences obtained were processed, trimmed and compared using Geneious 6.1.8 (Biomatters).

3.2.5 Site-directed mutagenesis

3.2.5.1 Deletion of additional upstream -ATG-

A list of the primers used for deletion of the -ATG- are provided in **TABLE A1 in APPENDIX A**. The expression plasmids were amplified with Phusion® High-Fidelity DNA polymerase. The PCR reaction mixture contained Phusion HF buffer, 200 µM dNTP mix, 0.5 µM each of the forward and reverse primer, 10 ng of the purified plasmid, 1.0 U Phusion® HiFi DNA Polymerase and nuclease-free water to a final volume of 50 µL. The reaction was subjected to 30 amplification cycles following Phusion® High-Fidelity DNA polymerase instructional manual with a G-Storm GS04822 Thermal Cycler (Gene Technologies Ltd). PCR amplification was verified with agarose gel electrophoresis **(Section 3.2.4.5)**. Overnight *DpnI* digestion of the PCR product was subsequently carried out to ensure that the template DNA was completely degraded in order to prevent interference with the subsequent bacterial transformation. The *DpnI* digest was done in a final volume of 50 µL following the manufacturer's protocol. To facilitate ligation, the digested PCR amplicon was gel purified **(Section 3.2.4.6)** and subsequently phosphorylated and ligated in a combined reaction using T4 ligase buffer, 1 mM ATP, 7.5 Weiss U/µL T4 ligase and 15 U/µL PNK. The reaction mixture was incubated for 3 hours at room temperature. NEB® Stable Competent *E. coli* cells were transformed with the ligation mixture **(Section 3.2.4.1)**. Deletion of the ATG was verified using Sanger sequencing **(Section 3.2.4.7)**.

3.2.5.2 Modification of Kozak motif

To enable optimal expression, the Kozak motif for two plasmids, pKM173delATG/POVP2 and pKM177delATG/POVP6 were modified. Two directly overlapping primers **(TABLE A1 in APPENDIX A)** were designed for each plasmid that exchanged -ACC- for -GAG- preceding

the -ATG- at the 5' end of the VP2 or VP6 ORF. PCR amplification of the entire plasmid was done with KAPA® High-Fidelity DNA polymerase (Kapa Biosystems) according to the manufacturer's instructions using a G-Storm GS04822 Thermal Cycler (Gene Technologies Ltd). PCR amplification was verified with agarose gel electrophoresis **(Section 3.2.4.5)**, followed by overnight digestion with *DpnI,* DNA purification, phosphorylation and ligation as described in **Section 3.2.5.1**. The ligation mixture was transformed **(Section 3.2.4.1)** and mutagenesis confirmed using Sanger sequencing **(Section 3.2.4.7)**.

3.2.6 Molecular cloning

3.2.6.1 Construction of the single expression vector, pKM173delATG/AOVP2

Plasmid, pUC57/AOVP2 **(Table 3.1**, GenScript™**)**, was digested with *SalI* and *XmaJI* to release the AOVP2 ORF. At the same time, pKM173delATG **(Section 3.2.5.1)** was digested with *XhoI* and *XmaJI* to linearise the plasmid while the kmINUSigP gene was removed. The linearised vector (-pKM173delATG-) was ligated with the insert in a 1:3 molar ratio (vector:insert). To this mixture, T4 ligase buffer and 7.5 Weiss U/µL T4 ligase were added after which it was incubated at 4˚C for 48 hours. The ligated, recombinant plasmid DNA was transformed **(Section 3.2.4.1)**, minipreps were performed **(Section 3.2.4.3)** and screened with restriction enzyme digestion **(Section 3.2.4.4)**. The minipreps were followed by plasmid extraction **(Section 3.2.4.2)** and confirmation of successful cloning by Sanger sequencing **(Section 3.2.4.7)**.

3.2.6.2 Construction of the dual expression plasmids containing VP2 and VP6 ORFs

To construct the dual expression vectors containing the respective ORFs for expression in *Kluyveromyces lactis, Arxula adeninivorans* and *Pichia pastoris/Hansenula polymorpha*, each of the single-expression vectors containing VP2 (pKM173delATG/VP2) and VP6 ORF (pKM177delATG/VP6) was digested with *I-SceI* enzyme. Therefore, pKM173delATG/VP2 was linearised and for pKM177delATG/VP6 the VP6 ORF-containing expression cassette (flanked by the promoter and terminator regions) was removed **(Figure 3.2**). The vector was dephosphorylated with shrimp alkaline phosphatase (*rSAP)* enzyme and ligated with the VP6 ORF-containing expression cassette at a molar ratio of 3:1 (insert to vector). The ligated, recombinant plasmid was transformed **(Section 3.2.4.1)**, minipreps were performed **(Section 3.2.4.3)** and screened with restriction enzyme digestion **(Section 3.2.4.4)**, followed by plasmid extraction **(Section 3.2.4.2)** and confirmation of successful cloning by Sanger sequencing **(Section 3.2.4.7)**.

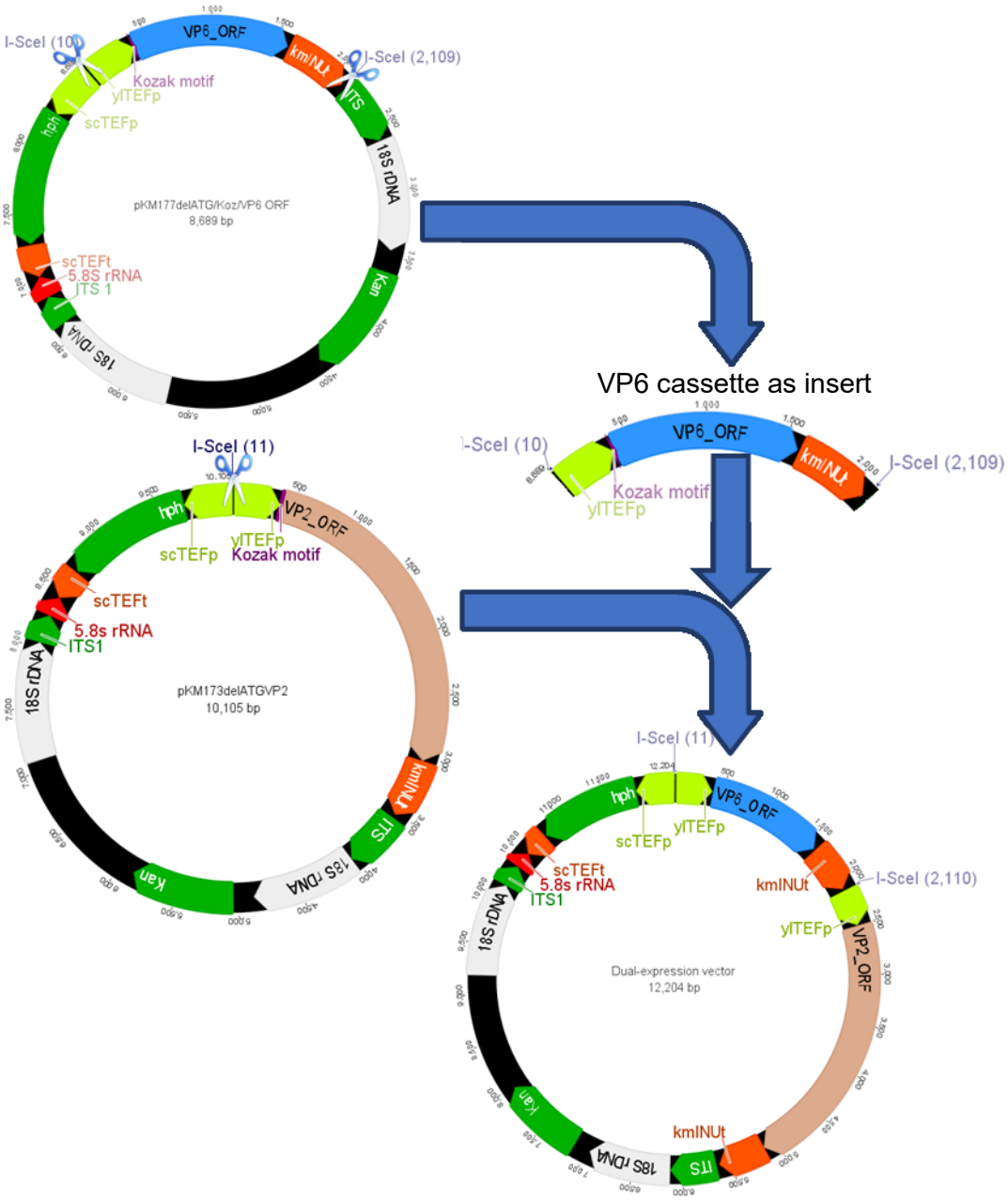


Figure 3.2: Schematic representation of the cloning strategy for the construction of the dual-expression plasmids.

3.3 Results

ATG/AUG is a universal start/initiation codon for translation. In eukaryotes, translation usually starts at the first AUG in the mRNA. The expression vectors used in this study were observed to contain an additional ATG upstream of the inserted ORFs. The additional, upstream -ATG- as shown in **Figure 3.3A,** was likely recognised first resulting in the detection of rotavirus VP6 expression in yeasts transformed with pKM177/POVP6, although the protein most likely contained three additional amino acids at the N-terminal end. However, in the case of

pKM177/AOVP6 and pKM177/KOVP6 the -ATG- was out-of-frame with the VP6 ORF **(Figure 3.3B)**, and resulted in non-detection of VP6 (Makatsa, 2015).

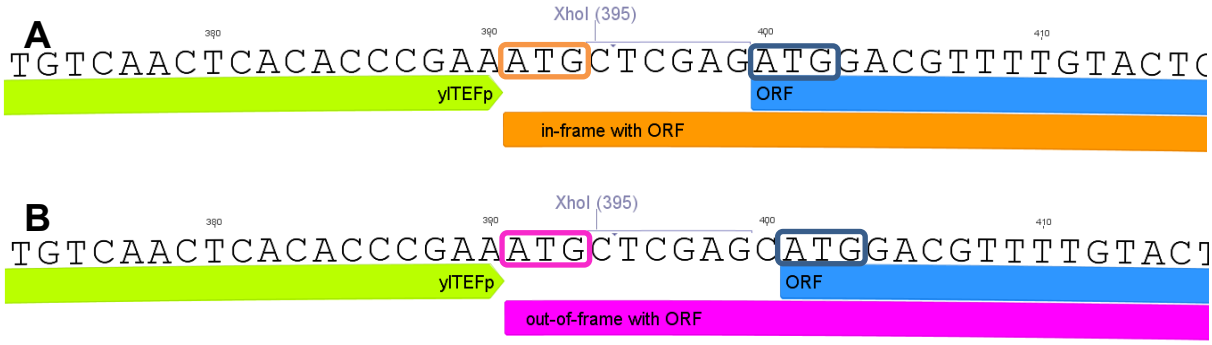


Figure 3.3: Schematic representation of the additional -ATG- upstream of the cloned ORF (*blue*), in-frame with cloned ORF - *orange* (A) and out-of-frame with cloned ORF - *pink* (B).

3.3.1 Modification of single-expression plasmids containing optimised VP2 and VP6 encoding ORFs

To ensure expression of VP2 and VP6 by the dual expression plasmids, the additional -ATG- had to be removed. Therefore, the -ATG- sequence was deleted from pKM173/KOVP2, pKM173/POVP2 and pKM177/POVP6 to form pKM173delATG/KOVP2, pKM173delATG/POVP2 and pKM177delATG/POVP6, respectively. In addition to these constructs, pKM173delATG/AOVP2 was generated by sub-cloning the AOVP2 ORF from pUC57/AOVP2 **(Table 3.1)** into pKM173delATG **(Section 3.3.1.2)**.

3.3.1.1 Deletion of additional ATG from pKM173/KOVP2, pKM173/POVP2 and pKM177/POVP6

The alignment of the primers designed to remove the -ATG- from the **pKM173/POVP2** is shown in **Figure 3.4**. PCR amplification **(Section 3.2.5.1)** using the aforementioned primers produced the expected amplicon size of 10.1 kb, which was digested with *DpnI* **(Section 3.2.5.1)**, purified **(Section 3.2.4.2)**, ligated **(Section 3.2.5.1)** and transformed into bacteria **(Section 3.2.4.1)**.

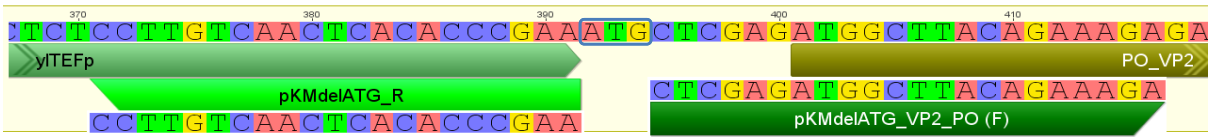


Figure 3.4: The primer pair flanking the -ATG- targeted for removal in the pKM173/POVP2 (10 108 bp). PCR amplification using the primers resulted in the exclusion of the -ATG-.

Figure 3.5 shows the agarose gel electrophoretic analysis of the restriction digests of nineteen plasmids prepared from the transformed bacteria. The digestion of the pKM173delATG/POVP2 with *SphI* was expected to produce two fragments of 7 021 bp and 3

091 bp. Thirteen clones were identified as possible positive clones, see **Figure 3.5** because the observed fragments corresponded to the expected fragment sizes. Sanger sequencing results of one of the representative clones identified with restriction enzyme digestion are shown in **Figure 3.6**. The sequencing confirmed the removal of the additional ATG. Two out of 13 sequenced clones had the desired deletion of -ATG- to create pKM173delATG/POVP2. Similarly, the additional, upstream ATG was removed in the expression plasmids, **pKM173/KOVP2 and pKM177/POVP6.** Results are presented in **APPENDIX A3**.

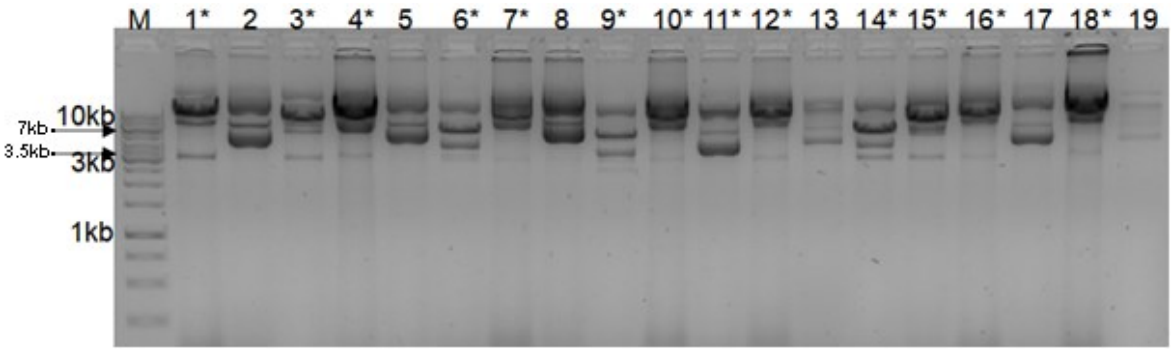


Figure 3.5: Agarose gel analysis of the *SphI* restriction enzyme digests of mini-prep extractions for pKM173delATG/POVP2 (10.1kb). Expected band sizes were 7 021 bp and 3 091 bp. The clones labelled with an asterisk were sequenced to verify the deleted -ATG-. "M" is the GeneRuler™ 1 kb DNA Ladder.

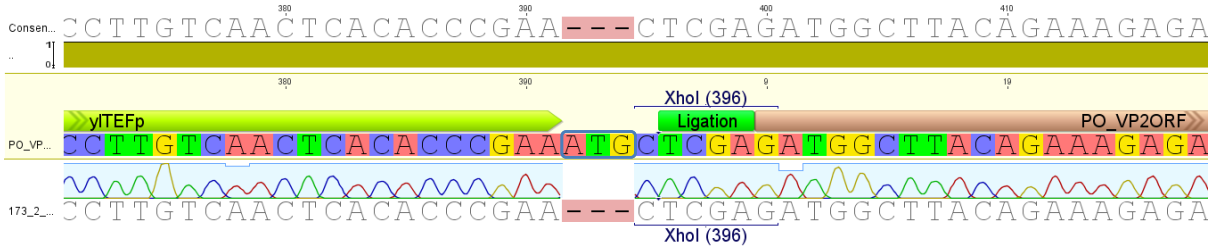


Figure 3.6: Alignment of the *in-silico* sequence of **pKM173/POVP2** (middle) and Sanger sequencing result (bottom) indicating the removed -ATG- as shown in the consensus (top). The *XhoI* recognition site at the ligation site between the POVP2ORF and the 3´-end of the promoter (yITEFp) are also shown.

3.3.1.2 Construction of pKM173delATG/AOVP2

Since the removal of the additional -ATG- from pKM173/AOVP2 proved problematic, it was decided to generate pKM173delATG/AOVP2 by sub-cloning into pKM173delATG. The wide-range expression vector, pKM173, was therefore mutated.

The targeted -ATG- was removed using PCR amplification **(Section 3.2.5.1).** The primer binding sites, as well as the -ATG- being excluded are shown in **Figure 3.7**. The PCR product was digested overnight with *DpnI,* purified, ligated **(Section 3.2.5.1)** and transformed into the bacteria **(Section 3.2.4.1)**.

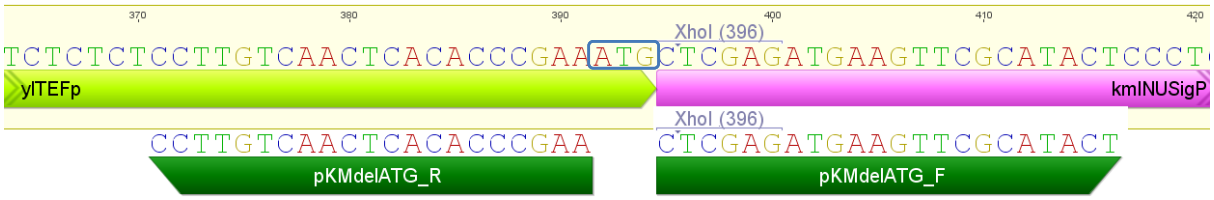


Figure 3.7: The primer pair flanking the -ATG- to be removed at the cloning site of pKM173. The primers were designed in such a way that the -ATG- targeted for removal was excluded during PCR amplification. The restriction enzyme recognition sites were not affected by the site-directed mutagenesis.

Nineteen plasmids isolated via mini-prep from the bacterial transformation were screened with *NdeI* **(**New England BioLabs, USA) digestion. The screening was considered positive if two fragments with expected band sizes of 5 215 bp and 2 337 bp were obtained (**Figure 3.8)**. As can be seen in **Figure 3.8**, 12 clones corresponded to the expected sizes and these were selected for Sanger sequencing. The results of the Sanger sequencing to verify the -ATG- deletion is shown in **Figure 3.9**. Three out of 12 sequenced clones had the desired removal of -ATG- to create pKM173delATG.

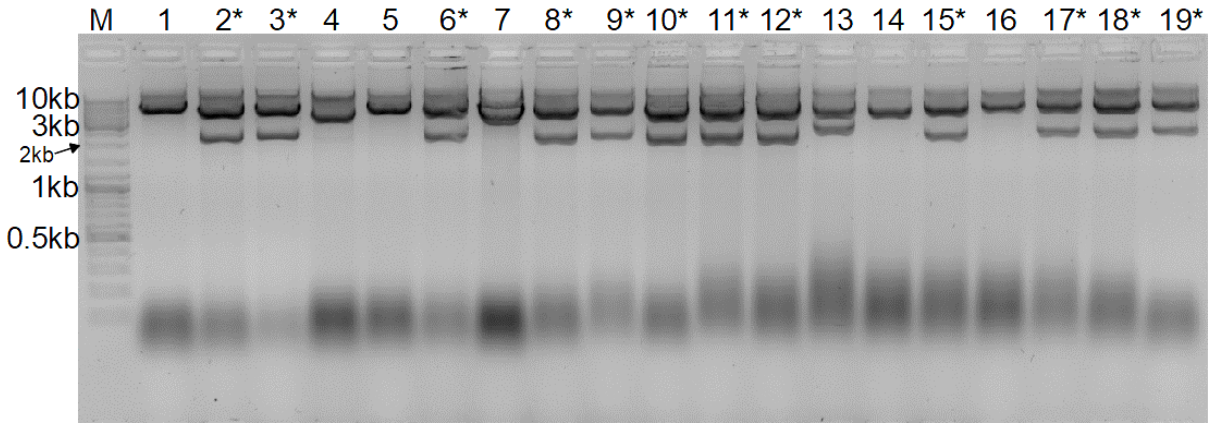


Figure 3.8: Agarose gel analysis of the *NdeI* restriction enzyme digests of mini-prep extractions for the pKM173 plasmid. Expected band sizes were 5 215 bp and 2 337 bp. The 12 clones labelled with an asterisk were suspected positive clones and were subjected to sequencing to verify deletion of the targeted -ATG-. "M" is the GeneRuler™ 1 kb DNA Ladder.

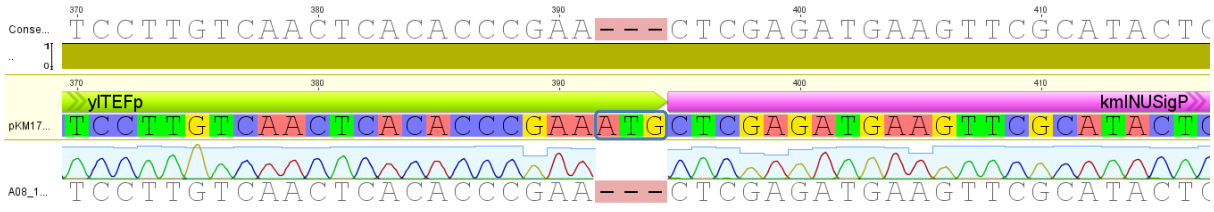


Figure 3.9: Alignment of the pKM173 *in-silico* sequence (middle) with the Sanger sequencing result (bottom). The Sanger sequence showed that the -ATG- was deleted from the expression plasmid as confirmed by the consensus sequence **(top)**.

During the construction of pKM173delATG/AOVP2, *XhoI* and *SalI* were used to generate compatible overhangs at the 5' end for the vector and insert, respectively. This approach was used since the insert contained two internal *XhoI* recognition sites. To screen for the successful insertion of the AOVP2 ORF, the construct, pKM173delATG/AOVP2 was digested with *XhoI* (**Figure 3.10A).** Since the one *XhoI* recognition site was lost during cloning, screening was based on the two internal *XhoI* recognition sites with expected fragment sizes of 8 726 bp and 1 390 bp. These expected fragments were observed, suggesting successful cloning. As a secondary confirmation, the construct was also digested with *XhoI* and *XmaJI*, **Figure 3.10B.** *XmaJI* was the second cloning enzyme and this restriction site remained intact. The expected fragment sizes of 8 046 bp, 1 390 bp and 684 bp were observed. Successful insertion was confirmed with Sanger sequencing (**Figure 3.11)**.

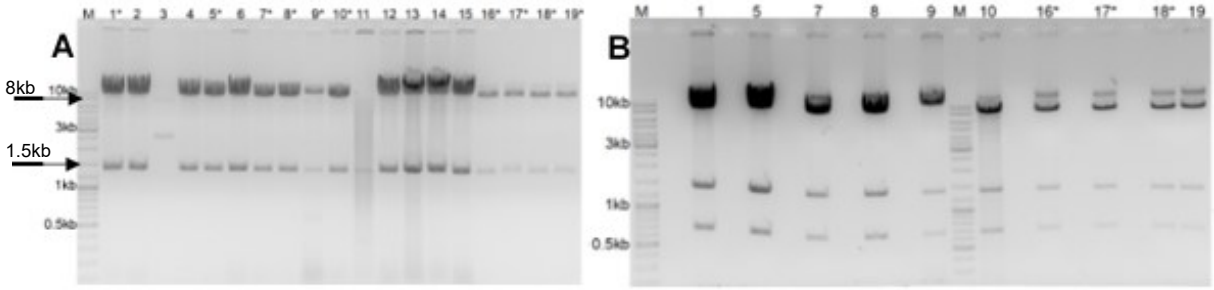


Figure 3.10: Agarose gel analysis following ligation and transformation with pKM173delATG/AOVP2. The mini-prep plasmids were digested with *XhoI* with expected fragment sizes of 8 726 bp and1 390 bp **(A).** Suspected positive clones (asterisks) in **(A)** were further digested with *XhoI* and *XmaJI* restriction enzymes with expected fragments of 8 046 bp, 1 390 bp and 684 bp **(B)**. "M" is the GeneRuler™ 1 kb DNA Ladder.

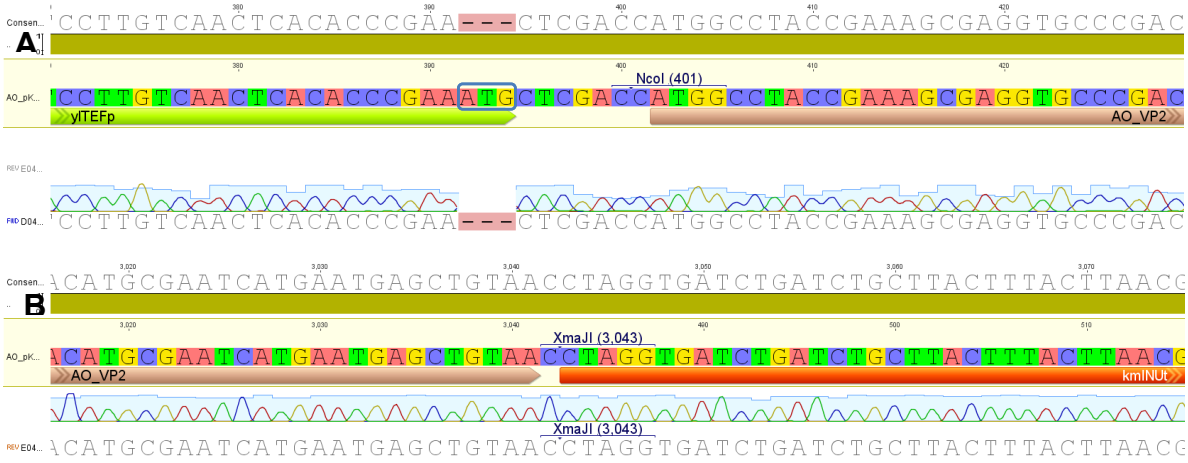


Figure 3.11: Alignment of the pKM173/AOVP2 *in-silico* sequence with the Sanger sequencing results at the 5´-end (A) and at the 3´-end (B) of the pKM173delATG/AOVP2. The deleted -ATG- is shown in the contig at the 5´-end **(A)**.

3.3.2 Optimisation of Kozak consensus sequence for the expression plasmids

To enhance the efficiency of recombinant expression of the optimised VP2 and VP6 ORFs in the selected yeast strains, each of the preceding sequences in the ORFs was compared to the preferred consensus Kozak sequence **(Figure 3.12)**. The Kozak consensus sequences for the VP2- and the VP6-containing ORFs optimised for *A. adeninivorans* and *K. lactis* corresponded with the preferred consensus sequence, except at the -2 position. A deviation from the consensus sequence was, however, observed for the pKM173delATG/POVP2 and pKM177delATG/POVP6 constructs, specifically the presence of the G at position -1 and most importantly another G at position -3. Strong bias for A at position -3 is usually preferred. It was therefore decided to change the Kozak consensus sequence for the *P. pastoris/H. polymorpha* codon-optimised ORFs from -GAG- to -ACC-.

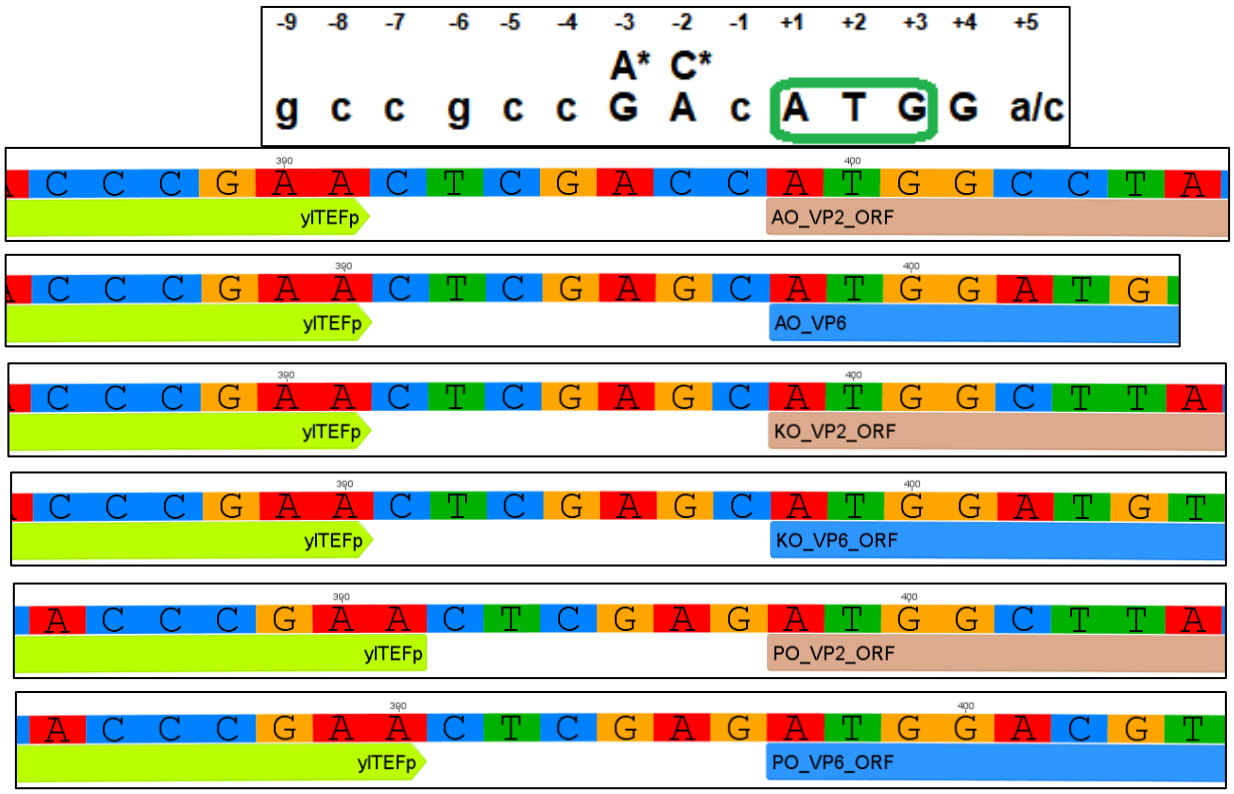


Figure 3.12: Comparison of the Kozak consensus sequence (Nakagawa *et al.*, 2007) preceding the codon-optimised rotavirus VP2 and VP6 ORFs.

To obtain the desired changes, overlapping primers were designed to replace –GAG- with -ACC- (**Figure 3.13)**. pKM173delATG/Koz/POVP2 and pKM177delATG/Koz/POVP6 (results presented in **APPENDIX A4)** were engineered. Confirmation of pKM173delATG/Koz/POVP2 was obtained with *NcoI* and *XmaJI* digestions with five fragment sizes of 4 077 bp, 2 646 bp, 1 332 bp, 1 144 bp and 925 bp expected. Further confirmation was carried out by digesting the plasmid with *XhoI-XmaJI* enzymes. This was crucial digestion that was expected to

produce only one fragment of 10 108 bp. The introduction of -ACC- to replace -GAG- removed the *XhoI* recognition site at the 5´-end but *NcoI* recognition site was introduced. Agarose gel electrophoresis was used to assess the successful introduction of the altered Kozak motif, the results are shown in **Figure 3.14**. The sequencing results also confirmed the removal of the *XhoI* recognition site and the introduction of the *NcoI* site **(Figure 3.15)**.

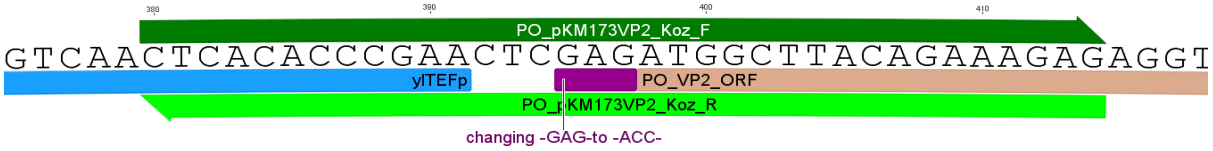


Figure 3.13: Overlapping primers (dark green – forward primer; light green – reverse primer) on the pKM173delATG/POVP2ORF to introduce Kozak motif by changing the -GAG- to -ACC-. Successful mutagenesis should replace the *XhoI* recognition site (-CTCGAG-) with the *NcoI* recognition site (-CCATGG-).

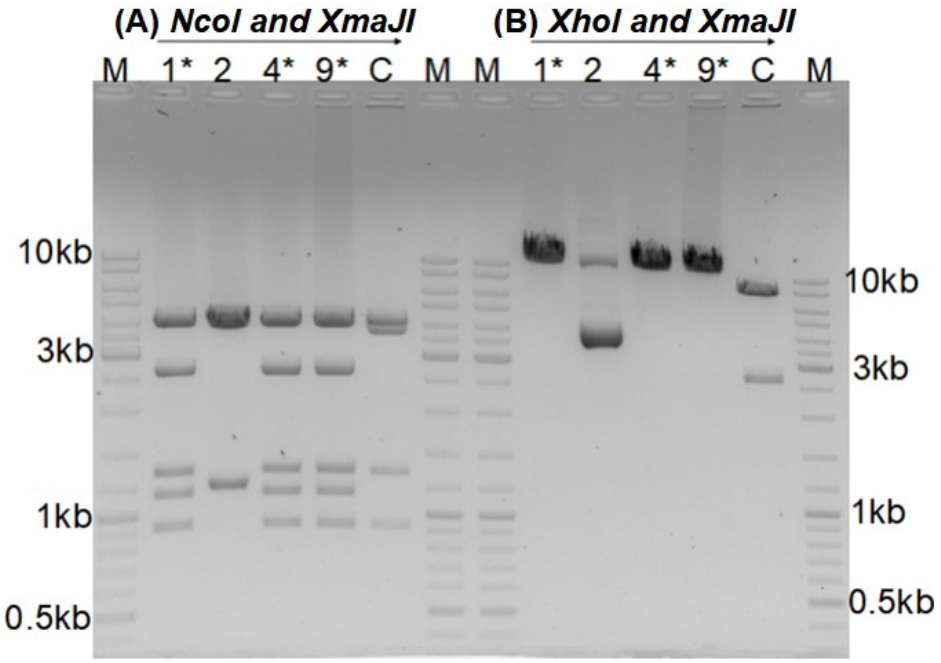


Figure 3.14: Agarose gel analysis with the fragments obtained from the restriction enzyme screening of pKM173delATG/Koz/POVP2. (A) Digestion with *NcoI* and *XmaJI* with five expected fragments (4 077 bp, 2 646 bp, 1 332 bp, 1 144 bp and 925 bp) but only 4 fragments were expected for the control plasmid "C" (pKM173delATG/POVP2) - 4 077 bp, 3 786 bp, 1 332 bp and 925 bp. **(B)** *XhoI-XmaJI* digestion produced only one band for the pKM173delATG/Koz/POVP2 (10 108 bp) because the *XhoI* recognition site has been removed with the introduction of the optimised Kozak sequence while the control plasmid "C" produces two fragments (7 462 bp and 2 650 bp) because the *XhoI* recognition site was still retained upstream of the ORF. "M" is the GeneRuler™ 1 kb DNA Ladder.

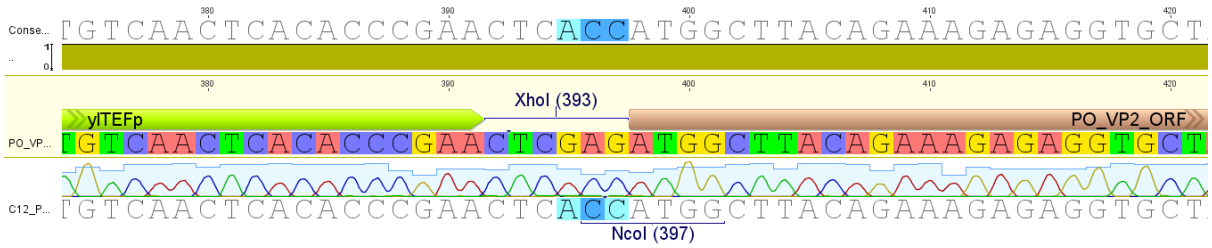


Figure 3.15: Sanger sequencing showing the replacement of -GAG- with -ACC- to introduce an optimised Kozak motif. The -GAG- shown in the *in-silico*, unmutated sequence **(middle)** was replaced with -ACC- shown in the Sanger sequencing result **(bottom)** and confirmed in the consensus sequence **(top)**.

3.3.3 Construction of the dual expression plasmids containing optimised VP2 and VP6 encoding ORFs

The strategy for the construction of the dual expression vectors is shown in **Figure 3.2**. pKM173delATG/Koz/AOVP2-6 was constructed by digesting pKM177delATG/Koz/AOVP6 and pKM173delATG/Koz/AOVP2 with *I-SceI* **(Sections 3.2.4.4 – 3.2.4.7)**. **Figure 3.16** shows the agarose gel analysis of the PCR linearised pKM173delATG/Koz/AOVP2 **(Figure 3.16, A-1)**, with an expected fragment size of 10.1 kb after *I-SceI* digestion. Also shown is pKM177delATG/Koz/AOVP6 digested to release the AOVP6 ORF-containing expression cassette (including the ylTEFp and the kmINUt) with a fragment size of 2 106 bp **(Figure 3.16, A-2)**. The linearised pKM173delATG/Koz/AOVP2 **(Figure 3.16, B-3)**, as well as the AOVP6 expression cassette **(Figure 3.16, B-4),** was purified. To prevent self-ligation, the former was dephosphorylated prior to purification.

Figure 3.16C shows pKM173delATG/Koz/AOVP2-6 plasmids digested with *NcoI* and *XmaJI* which should produce 7 fragments with expected sizes of 4 077 bp, 2 546 bp, 2 353 bp, 1 332 bp, 925 bp, 498 bp and 401 bp. Insertion of the VP6 ORF-containing expression cassette was confirmed with Sanger sequencing at the 5´-end **(Figure 3.17A)** and the 3´-end **(Figure 3.17B)**. Results for the construction of pKM173delATG/Koz/KOVP2-6 and pKM177delATG/Koz/POVP2-6 are presented in **APPENDIX A5**. Plasmid maps for the three dual expression plasmids, each containing one of the three optimised ORFs (*A. adeninivorans*, *K. lactis* and *P. pastoris/H. polymorpha*) are shown in **Figure 3.18**.

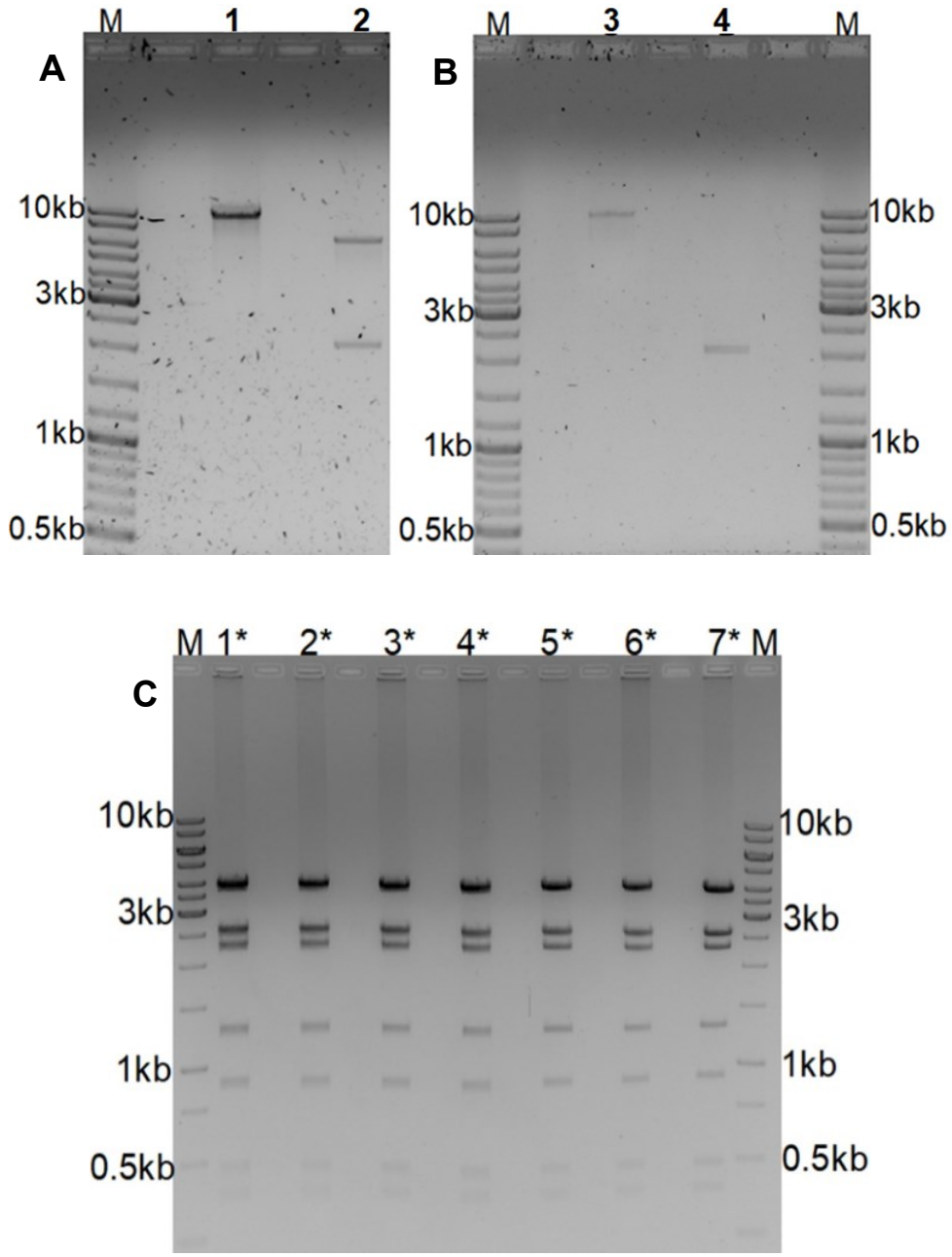


Figure 3.16: Agarose gel analysis of the fragment generated during the construction of the dual expression vector, pKM173delATG/Koz/AOVP2-6. (A) *I-SceI* digestion of the vector to linearise pKM173delATG/Koz/AOVP2 (10. 1 kb) (lane 1) and digestion of pKM177delATG/Koz/AOVP6 to release the AOVP6 ORF-containing cassette (2.1 kb) as insert, which include its promoter (ylTEFp) and terminator (kmINUt) (lane 2). **(B)** dephosphorylated and purified pKM173delATG/Koz/AOVP2 (10.1 kb) **(Vector)** (lane 3) and gel purified AOVP6 ORF-containing expression cassette (2.1 kb) as **Insert** (lane 4) followed by ligation and transformation. **(C)** Mini-prep of plasmids digested with *NcoI* and *XmaJI*, as indicated by the asterisks, all plasmids analysed returned positive results. "M" is the GeneRuler™ 1 kb DNA Ladder.

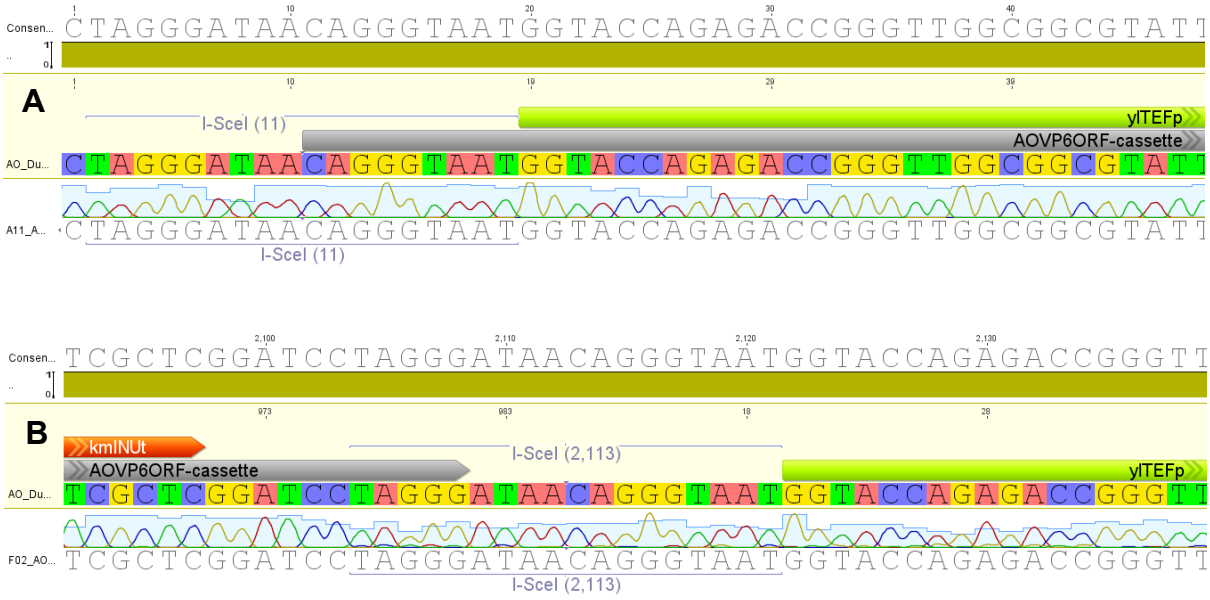


Figure 3.17: Sanger sequencing for the pKM173delATG/Koz/AOVP2-6 confirming the 5´-end ligation (A) and the 3´-end ligation (B) of the inserted expression cassette. All the sequenced clones matched the *in-silico* sequence (middle) and were thus confirmed to have the correct insertion of the expression cassette.

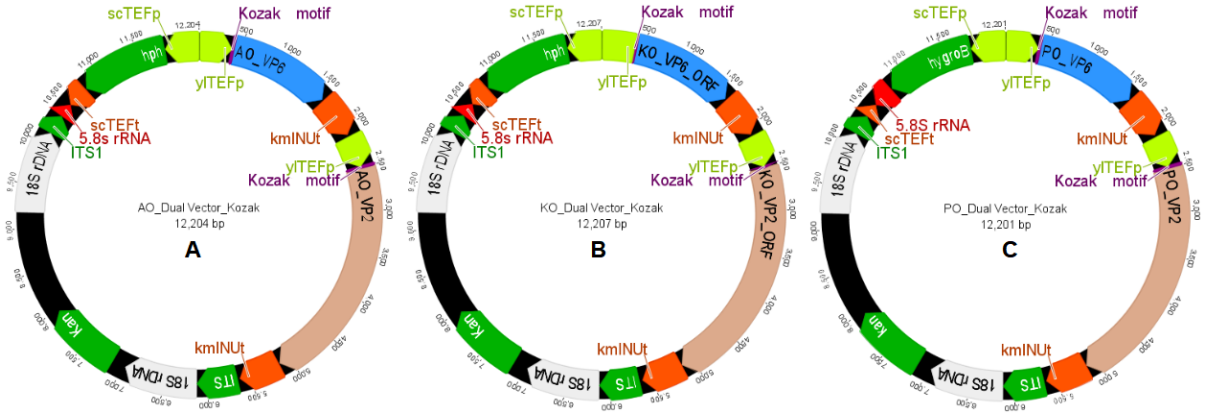


Figure 3.18: Dual expression vector containing rotavirus VP2 and VP6 ORFs optimised for *A. adeninivorans* (A), *K. lactis* (B) and *P. pastoris/H. polymorpha* (C) constructed for expression in different yeast strains.

3.4 Discussion

When genes from one organism are expressed in other host cells, there is often a problem with codon usage bias (Chu *et al.*, 2019), which can usually be overcome through nucleotide optimisation. For example, UCU is the most frequently used codon for serine in *S. cerevisiae* and *Homo sapiens*. However, when comparing the frequency of usage, 76.7% in the *S. cerevisiae* against 23.3% in *Homo sapiens* (Volkova *et al.*, 2006). Therefore, optimisation of coding sequences is needed if a human protein is to be expressed in yeast. Codons are read by transfer RNA and when a codon is seldom used, lower levels of the specific tRNA will be present. Consequently, to optimise protein production, codon usage must be considered either

by changing redundant codons or exogenously supplying the rare tRNA. This technique has been used in expressing codon-optimised, truncated/full-length rotavirus VP6 protein in *E. coli* (Choi *et al.*, 2004), insect cells (Jere *et al.*, 2014) and yeast (Bredell *et al.*, 2016). In this study, the VP2 and the VP6 ORFs of a South African rotavirus field strain, RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6], was codon-optimised for three different yeast species: *A. adeninivorans, K. lactis and P. pastoris/H. polymorpha.* In addition, the additional, upstream -ATG- was deleted from pKM173/KOVP2, pKM173/POVP2, pKM177/POVP6 and pKM173/AOVP2. Previously, the additional, upstream -ATG- was removed from pKM177/AOVP6 and pKM177/KOVP6 (Rakaki, 2018).

Understanding the regulatory mechanisms of translation in expression hosts are essential for constructing recombinant expression plasmids. It was proposed that when the AUG codon is in the context of **GCC GCC (A/G)CC AUG G** (AUG represents the translation initiation codon), which is called the *'Kozak consensus sequence'*, the efficiency of translation initiation is enhanced (Kozak, 1978, 2002) **(Figure 3.12)**. However, an extensive nucleotide comparison upstream and downstream of the initiation codon for different eukaryotes including animals, fungi, plants and protists showed nucleotide diversities among these species, which are evolutionarily related (Nakagawa *et al.*, 2007). There was a strong, commonly observed bias for A/G at position -3, C at position -1 and A/C at position +5 in all species examined (Nakagawa *et al.*, 2007; Volkova *et al.*, 2006). Several reports have shown that -3(A) plays the most crucial role in enhancing translation initiation in eukaryotes (Kochetov, 2004; Kozak, 1986; Lukaszewicz *et al.*, 2000; Pesole *et al.*, 2000) by interacting with eIF2α (Pisarev *et al.*, 2006). Apart from determining the second amino acid, +5(A/C) has been shown to enhance the efficiency of translation (Grünert & Jackson, 1994; Kozak, 1997; Niimura *et al.*, 2003; Sawant *et al.*, 2001) along with biased +4(G) (Kozak, 1997; Lukaszewicz *et al.*, 2000; Pisarev *et al.*, 2006). The diversity of preferred nucleotide sequences around the initiation codon for eukaryotes might be explained by differences in relative contributions from two distinct patterns, (strong) **GCC A/GCC AUG** and (weak) **AAA AAA AUG**, which implies the presence of multiple molecular mechanisms for controlling translation initiation (Nakagawa *et al.*, 2007).

For yeast, *S. cerevisiae* prefers **AAA AAA AAA AUG TC** (Cavener & Ray, 1991; Cigan & Donahue, 1987; Yun *et al.*, 1996), which is generally regarded as the yeast Kozak sequence (Romanos *et al.*, 1992). Interestingly, for *S. cerevisiae* nucleotide substitutions at position -3 did not substantially affect the rate of translation initiation (Cigan & Donahue, 1987; Yun *et al.*, 1996). In **Table 3.2,** the Kozak consensus sequence of five yeasts is presented and these are, with a few exceptions, very similar to that of *S. cerevisiae*. For example, *P. pastoris* and *Y.*

lipolytica would prefer to use -ACC- instead of -ACA- or -AAA- at position -3, -2, -1 respectively (Nakagawa *et al.*, 2007).

Table 3.2: Commonly used nucleotides flanking the translation initiation codon -ATG- in yeast genomes (Nakagawa *et al.*, 2007)

Yeast	-5	-4	-3	-2	-1	-ATG-	+3	+4	+5
S. pombe	**T/A**	**C**	**A**	**A**	**A**		**G**	**C**	**T**
S. cerevisiae	**C**	**A**	**A**	**A/C**	**A**		**T**	**C**	**T**
K. lactis	**C**	**A**	**A**	**C**	**A**		**T**	**C**	**T**
D. hansenii	**C**	**A/C**	**A**	**A**	**A**		**T**	**C**	**T**
Y. lipolytica	**C**	**C**	**A**	**C**	**C**		**T**	**C**	**A**

The nucleotide sequence flanking the initiation codon -ATG- for each of the single-expression vectors was compared with the strong Kozak sequence **(Figure 3.12)**. Detailed analysis showed that pKM173delATG/Koz/AOVP2 contained G, A, C, C at -4, -3, -2, -1 comparing to the Kozak motif C, **A**/G, **C**/A, C at position -4, -3, -2, -1 with bias for the bolded nucleotides. This means the construct resembled the Kozak consensus sequence. Similarly, pKM177delATG/Koz/AOVP6 showed the same trend except for G at position -2. This deviation was insignificant as the function of the nucleotide at position -2 is not known irrespective of the nucleotide therein (Nakagawa *et al.*, 2007). Similar sequences were observed for the pKM173delATG/Koz/KOVP2 and pKM177delATG/Koz/KOVP6. Both pKM173delATG/POVP2 and pKM177delATG/POVP6 deviated, however, at -1 and -3 positions **(Figure 3.12)**. Although the deviation at -1 is inconsequential, the change at -3 is significant since bias towards nucleotide “A” has been observed for various yeast species (Nakagawa *et al.*, 2007).

To conclude, the dual expression vectors were constructed from the individual single expression vectors. Each of the dual expression vectors contained both the VP2 and the VP6 ORFs optimised for expression in the various yeasts. The ORFs were also preceded by an optimised Kozak motif. These dual expression vectors will allow for simultaneous expression of both VP2 and VP6 capsid proteins to enable DLP formation.

3.5 Reference

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Chapter Four

Co-expression of rotavirus VP2 and VP6 in various yeasts

4.1 Yeast expression system

Yeast expression is a well-established platform for recombinant protein production. Historically, *Saccharomyces cerevisiae* was the dominant yeast host for heterologous production of therapeutic proteins such as insulin and glucagon (Nielsen, 2013). As mentioned earlier, a number of internationally licensed vaccines are produced using yeast-based expression systems, which includes Engerix-B®, Hepavax-Gene®, Recombivax HB® and Gardasil® (Merck and Co) (Masavuli *et al.*, 2017). Yeasts such as *Kluyveromyces lactis* (Donnini *et al.*, 2004; Van Ooyen *et al.*, 2006), *Yarrowia lipolytica* (Madzak *et al.*, 2004), *Hansenula polymorpha* (Kulkarni *et al.*, 2006), *Pichia pastoris* (Cregg *et al.*, 2000; Zhou *et al.*, 2006) and *Arxula adeninivorans* (Terentiev *et al.*, 2004) among others have, however, emerged as competitive non-conventional yeasts (Gellissen *et al.*, 2005). Most of these yeasts have been used to express human insulin, human serum albumin, hepatitis A, B and C vaccine candidates, trypsin, collagen and many others (Gasmi *et al.*, 2011; Ling *et al.*, 2012; Lixin *et al.*, 2019; Salunkhe *et al.*, 2010; Vassileva *et al.*, 2001b; Wang *et al.*, 2001; Yamaguchi *et al.*, 1998; Zhu *et al.*, 2012; Zhu *et al.*, 2009). These yeasts are Crabtree-negative that under respiratory conditions do not lose carbon by producing ethanol, which results in higher biomass formation and consequently more recombinant protein production (Löbs *et al.*, 2017; Nielsen, 2013; Porro *et al.*, 2005). The availability of genetic tools plays a prominent role when choosing a host for heterologous production. Most synthetic biology tools for yeast have been developed in *S. cerevisiae* due to its well-annotated genome, the ability to genetically manipulate the genome and overall ease of use (Wagner & Alper, 2016).

Several virus-like particles (VLP), as vaccine candidates, have been produced either as single-layered or multi-layered VLPs. An example is hepatitis B (enveloped virus) surface antigen (HBsAg) produced as intracellular single-layered VLP in *S. cerevisiae* (Yamaguchi et al., 1998), but as intracellular (Cregg *et al.*, 1987) and secretory (Vassileva *et al.*, 2001a; Vassileva *et al.*, 2001b) single-layered protein in *P. pastoris*. Xu and co-workers have produced PreS2-S antigen intracellularly as a single-layered protein in *H. polymorpha* (Xu *et al.*, 2014). L1 antigen from human papillomavirus (a nonenveloped virus) has been intracellularly produced in *S. cerevisiae* (Hofmann *et al.*, 1996), *P. pastoris* (Bazan *et al.*, 2009) and *H. polymorpha* (Liu *et al.*, 2015) and L1, L2 multilayered produced intracellularly in *S. cerevisiae* (Hofmann *et al.*, 1995). Norovirus (nonenveloped virus) VP1 was produced intracellularly (Xia *et al.*, 2007)

and extracellularly (Tomé-Amat *et al.*, 2014) in *P. pastoris* as a single-layered antigen. Production of the E1 antigen of dengue virus (enveloped) was achieved in *P. pastoris* as a single-layered antigen (Mani *et al.*, 2013). The premembrane protein and E1 as multi-layered VLPs was produced intracellularly (Sugrue *et al.*, 1997) and in a secreted form (Liu *et al.*, 2010) in *P. pastoris*. Furthermore, the VP1, VP2, VP3 and VP4 proteins of enterovirus 71 (nonenveloped virus) were produced intracellularly in *S. cerevisiae* (Wang *et al.*, 2016), while VP0, VP1 and VP3 was produced intracellularly in *S. cerevisiae* (Li *et al.*, 2013) and *P. pastoris* (Zhang *et al.*, 2015).

The first report of a yeast-based expression system for rotavirus virus-like particles (VLPs) was in 2011 when *S. cerevisiae* was used to express the multilayer recombinant rotavirus triple-layered particle (TLP) containing VP2, VP6 and VP7 with a multicistronic expression vector (Rodríguez-Limas *et al.*, 2011) **(Figure 4.1)**.

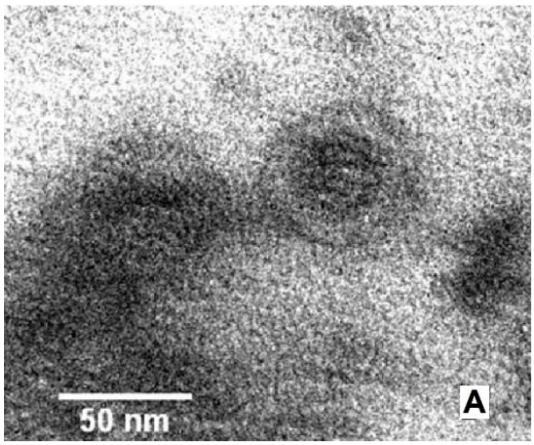


Figure 4.1: Ultrastructural analysis of the rotavirus triple-layered particles recovered by caesium chloride ultracentrifugation gradients. Samples were stained with 3% uranyl acetate and viewed under a transmission electron microscope. Magnification of 250 000x. *Figure adapted from Rodríguez-Limas et al., (2011).*

Although an approximate 70 nm particle size was obtained for the TLP, the image did not show the ultrastructural of the particles as often observed for particles produced by insect cells (Crawford *et al.*, 1994). Despite this milestone achievement, there are various problems associated with recombinant protein production in *S. cerevisiae*. First, this yeast has been known for low yields during recombinant proteins production (Cregg *et al.*, 1993; Faber *et al.*, 1995) due to product feedback inhibition resulting in cellular stress (Mattanovich *et al.*, 2004). Secondly, *S. cerevisiae* accumulates ethanol during cultivation (Mattanovich *et al.*, 2012) and lastly, this yeast is known for hypermannosylation (Bretthauer & Castellino, 1999; Löbs *et al.*, 2017; Miura *et al.*, 2004). These problems necessitate the use of other Crabtree-negative non-conventional yeasts as an alternative expression host to *S. cerevisiae* since most of these yeasts are genetically alike to *S. cerevisiae*. In addition, these yeasts have a capacity for

higher production in terms of biomass and recombinant protein expression (Cregg *et al.*, 1993; Faber *et al.*, 1995). The biological properties of *S. cerevisiae* and a few non-conventional yeasts are described in **Table 4.1**. This table details the properties of each yeast as expression host in terms of their safety, promoter usage, cultivation, post-translational modifications, product accumulation and production.

Table 4.1: General characteristics and genomic information of various yeasts developed as hosts for recombinant protein production (Kim *et al.*, 2015)

Yeast species	*General features*
S. cerevisiae	GRAS (generally regarded as safe) status, adjustable to both classical genetics and modern recombinant DNA techniques, versatile vector systems (episomal, integrative and copy-number regulated) with a wide range of mutant strains, well-established fermentation and downstream processes, hypermannosylation with immunogenic terminal α-1,3-linked mannose residues (Camirand *et al.*, 1991)
P. pastoris (Komagataella phaffii)	Methylotrophic yeast, tightly regulated, methanol-inducible alcohol oxidase (AOX) promoters, a Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes, grow rapidly on inexpensive media at high cell densities, integrative vectors developed for genetic stability and application in continuous and large-scale fermentation processes, well-established commercial vector systems and host strains, hypermannosylation occurs at a lesser extent than *S. cerevisiae* with no terminal α-1,3-linked mannose residues (Bretthauer, 2003; Bretthauer & Castellino, 1999)
H. polymorpha (P. angusta)	GRAS status, methylotrophic, stringently regulated strong promoters such as methanol oxidase (MOX) and formate dehydrogenase (FMDH), a Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes, stable and multi-copy integration of foreign DNA into chromosomal locations, thermo-tolerant (growth up to 45^{o}C), resistant to heavy metals and oxidative stress, can assimilate nitrates, hypermannosylation to a lesser extent compared to *S. cerevisiae* with no terminal α-1,3-linked mannose residues (Kang *et al.*, 1998; Kim *et al.*, 2004)
Y. lipolytica	GRAS status, an oleaginous dimorphic yeast due to its ability to accumulate large amounts of lipids (Madzak *et al.*, 2004), can grow in hydrophobic environments, can metabolize triglycerides, fatty acids, n-alkanes and n-paraffins as carbon sources for the bioremediation of environments contaminated with oil spills (Bankar *et al.*, 2009; Coelho *et al.*, 2010; Madzak *et al.*, 2004), can secrete a variety of proteins via co-translational translocation and efficient secretion signal recognition like higher eukaryotes, salt tolerance, hypermannosylation to lesser extent than *S. cerevisiae,* lack immunogenic terminal α-1,3-mannose linkages (Song *et al.*, 2007), pre- and probiotics, recombinant enzymes replacement therapies (ERTs), high level of secretion of native and heterologous proteins (Beckerich *et al.*, 1998)
K. lactis	GRAS status, a Crabtree-negative yeast allowing for high dilution rates and high biomass yields in fermentation processes (Van Ooyen *et al.*, 2006), lactose-fermenter with LAC4-inducible promoter, very high cell density, able to use both integrative and episomal expression vectors, terminal N-acetylglucosamine and no mannose phosphate (Lopez-Avalos *et al.*, 2001), used industrially to secrete β-galactosidase (for lactose-free products) and more than 20 recombinant proteins for foods and pharmaceuticals, metabolize hexoses via glycolysis and pentose phosphate pathway (Spohner *et al.*, 2016).
A. adeninivorans	GRAS status, dimorphic (dual-growth: mycelial/hyphae formation at temperature > 42^{o}C and budding at temperature < 42^{o}C) (Wartmann *et al.*, 2002), thermal- and salt-resistant yeast (Wartmann *et al.*, 2002; Wartmann *et al.*, 1995; Yang *et al.*, 2000), haploid, non-pathogenic, ascomycetous, anamorphic, arthroconidia yeast with some unusual biochemical characteristics, higher secretion of heterologous protein in mycelial (Rösel & Kunze, 1995; Stoltenburg *et al.*, 1999).
D. hansenii	Extremophilic oleaginous non-pathogenic probiotic yeast (Adler *et al.*, 1985; Fleet & Balia, 2006; Fleet, 2007), possess similar genetic system than *P. pastoris* (Mattanovich *et al.*, 2009) but with high genomic redundancy like *S. cerevisiae*, which have undergone genome duplications followed by partial genome losses during evolution (Dujon *et al.*, 2004). It has been used in the expression of *Aequoria victoria*-derived *GFP* (Steinborn *et al.*, 2006) using the wide-range vector CoMeD^{TM}. Its various biotechnological potential and utility in a diversity of areas have been reported by (Breuer & Harms, 2006).

In this chapter, various yeast strains were selected for recombinant expression of VP2 and VP6 rotavirus capsid proteins. Competent yeast cells were prepared, transformed and screened for recombinant expression of rotavirus VP2 and VP6. Proteins were detected with the western blot and the DLP particle formation was evaluated with a transmission electron microscope (TEM).

4.2 Materials and Methods:

4.2.1 Molecular reagents, chemicals, restriction enzymes and molecular enzymes

All the primers used were purchased from Integrated DNA Technologies. Restriction endonucleases enzymes were from Thermo Scientific (unless otherwise stated) as well as the DreamTaq DNA polymerase, DreamTaq Green PCR Master Mix, DNA Gel Loading Dye (6X), Spectra™ Multicolor Broad Range Protein Ladder, PageRuler™ Prestained Protein Ladder and Slide-A-Lyzer™ G2 Dialysis Cassettes with 20K MWCO. Chemical reagents for SDS-PAGE, yeast transformation and western blot analysis (except the organic solvent and antibodies), bicine, D-(+)-glucose monohydrate, ammonium persulfate (APS), sorbitol, sucrose, caesium chloride were obtained from Sigma-Aldrich, USA. Lonza (USA): SeaKem® LE Agarose. Abcam (UK): primary Goat polyclonal antibody raised against the Nebraska calf diarrhoea virus (NCDV) and the secondary Donkey polyclonal anti-Goat IgG antibody. Calbiochem® (USA): kanamycin sulphate, sodium dodecyl sulphate (SDS). Merck (USA): hygromycin B, Tris(hydroxymethyl)aminomethane, yeast extract, peptone, methanol, NaCl.

4.2.2 Recombinant bacterial strains

Recombinant *E. coli* BL21 (DE3) cells [F–, ompT, hsdSB (rB–, mB–), dcm, gal, λ(DE3); Stratagene] containing a recombinant plasmid, pColdTFVP2/TF, expressing rotavirus VP2 fused to the molecular chaperone called triggered-factor (total molecular weight of fused protein = 150.4 kDa) was used as a positive control for VP2 expression. In addition, BL21 cells harbouring the pCold II vector containing rotavirus VP6 ORF fused to the translational enhancing element (TEE) and His-Tag (total molecular weight of tagged VP6 = 47.5 kDa) was used as positive control for expression of VP6 (pColdII/VP6). These two recombinant bacterial cultures were generously provided by Prof A. van Dijk, North-West University, Potchefstroom, South Africa.

4.2.3 Yeast strains

The following yeast strains were obtained from the UNESCO-MIRCEN Yeast Culture Collection at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State: *Arxula adeninivorans* (UOFS_Y1219 and UOFS_Y1220), *Debaryomyces*

hansenii (UOFS_Y0610), *Kluyveromyces lactis* (UOFS_Y1167, UOFS_Y0841), *Hansenula polymorpha* (UOFS_Y0915 and UOFS_Y1507), *Pichia pastoris* (UOFS_Y1552T), *Yarrowia lipolytica* (UOFS_Y0097, UOFS_Y2221, UOFS_2415 and UOFS_Y2355). The following yeast strains were provided by Prof. J. Albertyn from the Pathogenic Yeast Laboratory, Department of Microbial, Biochemical and Food Biotechnology, UFS: *A. adeninivorans* (LS3) and *S. cerevisiae* (CENPK). *Pichia pastoris* (GS115) is a commercial strain (Invitrogen, USA) and was jointly provided by Prof. M. Smit and Prof. D. Opperman from the Biocatalysis Laboratory, Department of Microbial, Biochemical and Food Biotechnology, UFS. In addition, Prof. Smit also provided *Y. lipolytica* (Po1f). All yeast strains were cultured in either yeast extract-peptone-dextrose (YPD) agar plates or broth and incubated at 30˚C (except *H. polymorpha* at 37˚C). A litre of YPD agar contained 20 g peptone, 20 g glucose, 10 g yeast extract and 15 g agar. Agar was excluded for the YPD broth. Selective YPD media contained hygromycin B at either 600 µg/mL or 800 µg/mL.

4.2.4 Yeast competent cells and transformation

All yeast competent cells were prepared according to the bicine-NaOH protocol described by Klebe and co-workers (Klebe *et al.*, 1983). All the competent yeast cells were kept at -80ºC until use. Each of the dual-expression vectors, pKM173delATG/Koz/AOVP2-6, pKM173delATG/Koz/KOVP2-6 and pKM173delATG/Koz/POVP2-6 **(from Chapter Three)** were digested (1.0 µg) with *NotI* to linearise the plasmid. The linearised plasmid was mixed with pre-heated sheared Salmon sperm DNA (10 mg/ml) in a ratio of 3:1 by volume respectively and transformed in each of the selected yeast strains following the bicine-NaOH protocol (Chen *et al.*, 1997) with some modifications. All the yeasts were heat-shocked at 37˚C for 10 minutes except *H. polymorpha* which was heat-shocked at 42ºC for 10 minutes. Transformed yeast cells were plated on 600 µg/mL hygromycin B YPD plates and incubated at 30˚C (except *H. polymorpha* incubated at 37˚C) for 3 – 5 days for colony formation. Colonies were transferred to 800 µg/mL hygromycin B YPD plate for more stringent selection.

4.2.5 Colony PCR

Colony PCR was performed to confirm successful integration of the expression cassettes containing coding sequences for VP2 and VP6 using the protocol described by Ward, (1992). DreamTaq DNA polymerase in 50 µL reaction mixture was used for the partial amplification of AO_VP2/6_ORFs (AO-dual-expression cassette) and DreamTaq Green PCR Master Mix (2x) in 10 µL reaction mixture was used for KO_VP2/6_ORFs (KO-dual-expression cassette) and PO_VP2/6_ORFs (PO-dual-expression cassette). All the primers used, expected amplicon size and their annealing temperature are shown in **Table 4.2**. The PCR reactions were performed according to the manufacturer's instructions. Amplicons were verified on 1 % w/v

agarose gel electrophoresis using x1 TAE buffer with ethidium bromide at 50 µg/mL and viewed under the ChemiDoc™ MP Imaging System (Bio-Rad, USA).

Table 4.2: Primers used for partial amplification targeting each of the ORFs from all the dual-expression vectors transformed in the selected yeast

Primer name	*Sequence (5´ to 3´)*	*Tm (ºC)*	*Purpose*
ArxVP2F3	CCTTGACTACCGAGAAGCTC	57.4	Screening of yeast strains transformed with pKM173delATG/Koz/AOVP2-6; Expected amplicon size 536 bp
ArxVP2R3	GTAATCTGGGCGTAATCTCC	55.4	
pKM_Seq_F1	GTCAAGACTGTCAAGGAGGG	57.5	Screening of yeast strains transformed with pKM173delATG/Koz/AOVP2-6; Expected amplicon size 534 bp
ArxVP6Seq AR1	CTTATCTCGGGCATCCTTCAG	58.0	
KluyVP2_F2	CTGCATCAATTTGTGATGCTAT	55.5	Screening of yeast strains transformed with pKM173delATG/Koz/KOVP2-6; Expected amplicon size 891 bp
VP2_Int_R2	GTCCGTTTCTGATGTTATCG	54.3	
KOpKM173dC_FOR	ATGGATGTATTGTATTCATTGAGTAAAAC	57.2	Screening of yeast strains transformed with pKM173delATG/Koz/KOVP2-6; Expected amplicon size 493 bp
VP6Seq KR2	GTGAAAGATGCAGAGTAAGGGAAG	59.4	
PO_pKM173VP2_Koz_F	CTCACCATGGCTTACAGAAAGAGAG	54.4	Screening of yeast strains transformed with pKM173delATG/Koz/POVP2-6; Expected amplicon size 723 bp
VP2_Int_R1	AGTTAACAACGTTTCTGTCAG	61.1	
PO_VP6Seq_PF1	GGACGAAATGGTTAGAGAATCTC	61.1	Screening of yeast strains transformed with pKM173delATG/Koz/POVP2-6; Expected amplicon size 213 bp
PO_VP6Seq_PR2	CTGTTCAAGGTGAAAGAAGC	54.4	

pKM_Seq_F1 was obtained from Inqaba Biotec, South Africa

4.2.6 Evaluation of expression of rotavirus VP2 and VP6 in selected yeast strains

Selected yeast colonies that tested positive by colony PCR for integration of the respective expression cassettes containing rotavirus VP2 and VP6 ORFs were selected and evaluated for the expression of rotavirus VP2 and V6 proteins.

4.2.6.1 Bacterial expression of rotavirus VP2 and VP6 as positive controls

The recombinant *E. coli* BL21 previously transformed with pColdTF/VP2 and pColdI/VP6 **(Chapter Three, Table 3.1)** were revived on 100 µg/mL ampicillin LB plates and incubated overnight at 37ºC. A colony from each plate was transferred to 5 mL LB broth containing 100 µg/ml ampicillin and incubated overnight in a 37ºC shaker as pre-inoculum. The recombinant

protein was expressed according to the pCold™ Cold Shock Expression System protocol (TaKaRa, Cold Shock Expression System pCold™ DNA). Bacterial cells were transformed and incubated for 24 hours at 15°C. The cells were harvested at 10 000 x g for 10 minutes at 4°C. The cell pellet was resuspended in lysis buffer containing 1 % w/v SDS and using 1x TE buffer, pH 8.0 at 3 mL/g wet pellet. The lysis buffer contained the Roche cOmplete™ ULTRA Tablets Protease Inhibitor Cocktail (Roche Diagnostic GmbH) and incubated for 30 minutes at room temperature. The insoluble cell debris was separated from the soluble fraction by centrifugation at 16 000 *xg* for 20 minutes at 4˚C. The supernatant which contained the soluble fraction of the recombinant proteins was transferred to a clean 1.5 mL microfuge tube and was analysed by western blot. The total protein analysed with SDS-PAGE ranged from 7 – 10 µg per well.

4.2.6.2 Yeast expression of rotavirus VP2 and VP6

A pre-inoculum was prepared for each of the yeast colonies in 5 mL YPD media, incubated at 30˚C in a shaker for 24 hours except for *H. polymorpha,* which was incubated at 37˚C. A volume of 5 µL of the pre-inoculum was transferred to 50 mL YPD media in a 500 mL Erlenmeyer flask. The culture was incubated at 30˚C (37˚C for *H. polymorpha*) in a shaker for 18 - 24 hours. The cells were harvested at 10 000 *xg* for 10 minutes at 4˚C and resuspended in 50 mM Tris-HCl buffer pH 7.4 at 3 mL buffer per wet gram cell pellet. The buffer contained Pierce Protease Inhibitor Tablets (Thermo Scientific™). The cells were lysed with the Constant Cell Disruption System (Constant Systems, UK) at 35 *kpsi*. The lysate was centrifuged at 20 000 *xg* for 1 hour at 4˚C. The supernatant which contained the soluble fraction was transferred to a clean sterile 1.5 mL microfuge tube and the recombinant proteins were analysed by western blot. The total protein analysed with SDS-PAGE ranged from 7 – 10 µg per well. Bacterial-expressed total protein lysates were included as controls for VP2 at 0.017 µg and VP6 at 0.0042 µg.

4.2.6.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Total protein concentration was determined with the Pierce™ BCA protein assay kit (Thermo Scientific™ Pierce™) according to the manufacturer's instructions. The protein sample was mixed with Laemmli buffer in a 1:1 ratio. Samples were loaded along with the 5 µL of the PageRuler™ Prestained Protein Ladder (Thermo Scientific). Proteins were separated on a 10% SDS-PAGE gel according to Green and Sambrook, (2012).

4.2.6.4 Western blot analysis

Following SDS-PAGE, the gel was transferred to a nitrocellulose membrane (BioTrace™ NT Nitrocellulose Transfer Membrane, Pall Corporation) for 1 hour at 100 V in transfer buffer containing 0.025 M Tris, 0.2 M glycine and 20% methanol (pH 8.4). The efficiency of blotting was confirmed with Ponceaus S (0.1 %w/v in 1 % glacier acetic acid) staining for 10 minutes and rinsed with distilled water. The membrane was blocked with blocking buffer (5 %w/v skimmed milk (Nestle) prepared with 0.2 %v/v Tween 20™ in x1 Tris-HCl buffer saline solution, pH 7.5 (50 mM Tris-Cl, pH 7.5. 150 mM NaCl) and incubated overnight at 4˚C. The membrane was subsequently washed three times with 0.2 % TBS-T for 10 minutes each time and incubated for 90 minutes at room temperature with primary polyclonal Goat antibody raised against the Nebraska Calf Diarrhoea Virus (NCDV) in blocking buffer at a dilution of 1:1 000. The primary antibody was removed and the membrane washed three times for 10 minutes each to remove unbound primary antibodies. The membrane was subsequently incubated for 60 minutes at room temperature with the secondary polyclonal Donkey anti-Goat IgG antibody HRP in blocking buffer at a 1:10 000 dilutions. The membrane was again washed to remove the unbound antibodies. Recombinant protein expression was detected with the ECL substrate according to the manufacturer's protocol and the membrane was viewed under the ChemiDoc™ MP Imaging System (Bio-Rad, USA).

4.2.7 Evaluation of DLP formation using the transmission electron microscope

4.2.7.1 Optimisation of growth conditions to produce DLPs in yeast

The recombinant yeast, *H. polymorpha,* containing the AO-dual-expression cassette was used for preliminary optimisation of expression, first using shake-flask cultivation and, subsequently, in the Sixfors bioreactor (Infors, Switzerland). Eight long arm Erlenmeyer flasks containing 50 mL Syn-6-MES mineral media were inoculated with 5 mL of recombinant yeast, *H. polymorpha* pre-inoculum. The flasks were incubated at 37˚C in a shaker at 200 rpm. The recombinant yeast culture was harvested (10 mL per harvest) at 24 and 48 hours. The sample was centrifuged and the pellet processed with the Constant Cell Disruption System (Constant Systems, UK) at 35 *kpsi*. Recombinant VP6 production was estimated using the Premier Rotaclone® EIA kit (Meridian Bioscience, USA). This is an enzyme immunoassay commercially developed to detect VP6 antigen in the susceptible biological sample.

Syn-6-MES mineral media, used for shake-flask experimentation and bioreactor cultivation, were prepared according to the protocol described in Kottmeier *et al.*, (2010) with some modification. All the chemical reagents were purchased from Merck (USA) unless otherwise stated **(Table 4.3)**. Detailed information regarding the chemical names and Catalogue Number

(Cat #) of all the chemical components of Syn-6-MES media are shown in **SUPPLEMENTARY A1**. The primary carbon sources and the secondary substrates formulation are described in **Table 4.4**. Apart from the two carbon sources used (glucose and glycerol), secondary substrates/metabolites, phosphate, potassium and magnesium, were varied for optimised conditions to produce rotavirus VP2 and VP6 proteins.

The two best conditions identified with the shake-flask experiment were used in the Sixfors bioreactor with standard YPD medium as a control. The Sixfors bioreactor was maintained at 37 °C incubation temperature, 300 rpm stirring to enhance aeration, pH 6.4, 100 % pO_2 and 5 mL pre-inoculum in 240 mL medium in each of the bioreactors. A 10 mL volume of each of the yeast cultures was harvested at 8, 12, 18, 24 and 30 hours, centrifuged at 10 000 *xg* at 4 °C for 10 minutes and the wet weight was measured (Radwag®) as biomass per time. Each sample was processed following **Section 4.2.6.2**, SDS-PAGE was analysed following **Section 4.2.6.3** and the western blot analysis was carried out following **Section 4.2.6.4** to detect the expressed proteins. The best conditions for the production of rotavirus VP2 and VP6 proteins were used to set up a Biostat B bioreactor (Sartorius Stedim Biotech, Germany) with 1 L working volume, 37°C, pH 6.4, pO% controlled above 30 % with increasing agitation, airflow 1 slpm. The yeast culture was harvested after 24 hours and processed for analysis using transmission electron microscopy (TEM).

4.2.7.2 Sucrose-density gradient and ultracentrifugation

Sucrose gradients (10 – 60 % w/v) were prepared in Tris-HCl-NaCl-$CaCl_2$ (TNC) buffer containing 10 mM Tris (pH 7.4), 140 mM NaCl, 10 mM $CaCl_2$ and the clarified homogenate, fractionated, analysed with SDS-PAGE. Purification was done according to the procedures described by Lappalainen and co-workers (Blazevic *et al.*, 2016b; Lappalainen *et al.*, 2013) with some modifications. The fractionated sample was extensively dialysed with PBS at 4 °C with Slide-A-Lyzer™ G2 Dialysis Cassettes with 20K MWCO (molecular weight cut-off). The protein sample was concentrated and subjected to CsCl isopycnic ultracentrifugation.

4.2.7.3 Caesium chloride (CsCl) isopycnic separation and ultracentrifugation

Isopycnic CsCl separation of the crude rotavirus particle was carried out on two gradient layers of CsCl at two different mass concentrations - 55 and 40 % w/v of CsCl in TNC buffer. This was prepared to purify the sucrose-density DLP-containing sample **(Section 4.2.7.2)** using a procedure described by Nakagomi, (1998). The gradient was centrifuged with an Optima™ L-100 XP Ultracentrifuge (Beckman Coulter) using the SW 32Ti rotor at 120 000 *xg* for 30 hours at 4°C. The gradient was fractionated and each the fractions evaluated with SDS-PAGE.

Table 4.3: Composition of Syn-6-MES mineral media (Kottmeier *et al.*, 2010)

Stock Solution	***Components***	***Final g L^{-1}***	***Preparation***	***Volume, (mL)***	***mL/flask***
Basal Salts (pH 6.4)	$C_6H_5O_7Na_3$	1.00	All the chemicals were dissolved one by one in the order presented in acidified distilled water	1000	30
	$(NH_4)_2SO_4$	3.00			
	NaCl	0.30			
	MES hydrate *(Sigma)*	27.30			
Vitamins (100x)	D(+) Biotin *(Sigma)*	0.0004	Both vitamins were dissolved in distilled water, filter sterilized and kept at 4°C	100	0.5
	Thiamine HCl *(Sigma)*	0.1334			
Microelements (100x)	EDTA	0.0667	All the components were dissolved in the order presented in distilled water, filter sterilized and kept at 4°C	100	0.5
	$(NH_4)_2FeSO_4.6H_2O$	0.0667			
	$MnSO_4.H_2O$	0.0267			
	$ZnSO_4.7H_2O$	0.0200			
	$CuSO_4.5H_2O$	0.00534			
Trace elements (100 x)	$NiSO_4.6H_2O$	0.0267	All the components were dissolved in the order presented in distilled water, filter sterilized and kept at 4°C	100	0.5
	$CoCl.6H_2O$	0.000333			
	$Na_2MoO_4.2H_2O$	0.000333			
	KI	0.000333			
	H_3BO_3	0.000333			
Calcium (10x)	$CaCl_2.2H_2O$	1.00	Dissolved in distilled water and autoclaved	100	5
Carbon	Glucose *(Sigma)*	10.00	Each of the carbon sourses was dissolved in distilled water and autoclaved	100	5
	Glycerol	10.22		100	
Mg (20x)	$MgSO_4.7H_2O$	0.50	Dissolved separately in distilled water and autoclaved	100	0.025
K (20x)	KCl	0.50		100	0.025
P (20x)	$(NH_4)_2HPO_4$	0.85		100	0.25
Water added			Autoclaved		8.2

Table 4.4: Formulation of primary and secondary substrates/metabolites (Kottmeier *et al.*, 2010)

Flask	*Carbon Source*	*P*	*K*	*Mg*	*Basal salt (mL)*	*$CaCl_2.2H_2O$ (mL)*	*H_2O (mL)*	*Carbon*		*Phosphate*	*Potassium*	*Magnesium*	*Vitamin (mL)*	*Micro-element (mL)*	*Trace-element (mL)*
								Glucose (mL)	*Glycerol (mL)*	*$(NH_4)_2HPO_4$ (mL)*	*KCl (mL)*	*$MgSO_4$ (mL)*			
1	**Glucose**	**Limited**	**Limited**	**Limited**	**30**	**5**	8.2	**5**	**0**	**0.25**	**0.025**	**0.025**	**0.5**	**0.5**	**0.5**
2	**Glycerol**	**Limited**	**Limited**	**Unlimited**	**30**	**5**	5.7	**0**	**5**	**0.25**	**0.025**	**2.5**	**0.5**	**0.5**	**0.5**
3	**Glucose**	**Unlimited**	**Limited**	**Unlimited**	**30**	**5**	3.5	**5**	**0**	**2.5**	**0.025**	**2.5**	**0.5**	**0.5**	**0.5**
4	**Glycerol**	**Unlimited**	**Limited**	**Limited**	**30**	**5**	6.0	**0**	**5**	**2.5**	**0.025**	**0.025**	**0.5**	**0.5**	**0.5**
5	**Glucose**	**Limited**	**Unlimited**	**Unlimited**	**30**	**5**	3.3	**5**	**0**	**0.25**	**2.5**	**2.5**	**0.5**	**0.5**	**0.5**
6	**Glycerol**	**Limited**	**Unlimited**	**Limited**	**30**	**5**	5.7	**0**	**5**	**0.25**	**2.5**	**0.025**	**0.5**	**0.5**	**0.5**
7	**Glucose**	**Unlimited**	**Unlimited**	**Limited**	**30**	**5**	3.5	**5**	**0**	**2.5**	**2.5**	**0.025**	**0.5**	**0.5**	**0.5**
8	**Glycerol**	**Unlimited**	**Unlimited**	**Unlimited**	**30**	**5**	1.0	**0**	**5**	**2.5**	**2.5**	**2.5**	**0.5**	**0.5**	**0.5**

In this table, a limited supply of secondary nutrients represents a concentration of 0.25 mg/L for each of magnesium (Mg) and potassium (K) and 4.25 mg/L for phosphate (P). The unlimited supply was 100x the limited concentrations for Mg and K and 10x for P. Detail of the different media compositions are shown in **Table 4.3** and all comprised of 50 mL.

Fractions for which VP2 and VP6 could be detected were pooled and concentrated at room temperature. The final concentrated samples were submitted for TEM analysis at the Centre for Microscopy Unit, The Aaron Klug Centre for Imaging and Analysis, University of Cape Town, South Africa, using the protocol described in Lappalainen and co-workers (Lappalainen ***et al.***, 2013).

4.2.8 Statistical analysis

The specific data obtained in this study were analysed with GraphPad Prism v. 7 and the result was presented as average mean±SEM (standard error mean) and the average mean was considered statistically different at $p<0.05$ using Tukey's multiple comparison test for one- and two-ways ANOVA analysis.

4.3 Results

4.3.1 Integration of the VP2 and VP6 ORFs containing expression cassette in the genome of various yeast strains

The wide-range yeast expression plasmid used in this study allowed integration by homologous recombination into the yeast genomes which was confirmed with colony PCR (Ward, 1992). The amplicons (fragments) for pKM173delATG/Koz/AOVP2-6, pKM173delATG/Koz/KOVP2-6 and pKM173delATG/Koz/POVP2-6 are shown in **Figures 4.2, 4.3** and **4.4,** respectively. Only representative gels are shown for the sixteen yeast strains screened, following transformation with each of the dual expression vectors.

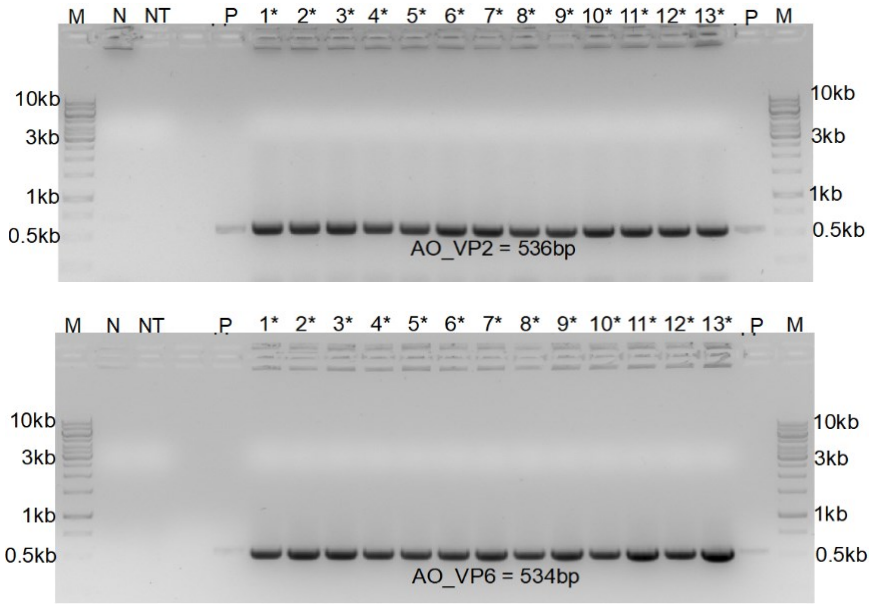


Figure 4.2: Agarose gel analysis of fragments obtained from the partial amplification of the AO/VP2 and AO/VP6 expression cassettes. "M" – GeneRuler™ 1 kb DNA Ladder, "N" – untransformed yeast colony as negative control, "NT" – no template control, "P – positive control: pKM173delATG/Koz/AOVP2-6, * – positive colonies with confirmed integration of the AO-dual-expression cassette (pKM173delATG/Koz/AOVP2-6) among the screened colonies.

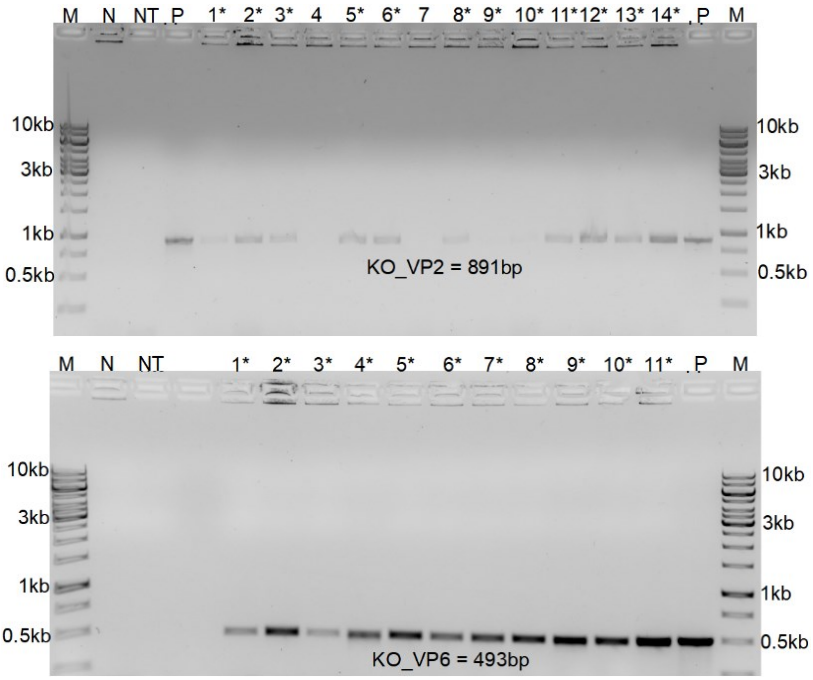


Figure 4.3: Agarose gel analysis of fragments obtained from the partial amplification of the KO/VP2 and KO/VP6 expression cassette. "M" – GeneRuler™ 1 kb DNA Ladder, "N" – untransformed yeast colony as negative control, "NT" – no templated control, "P" – positive control: pKM173delATG/Koz/KOVP2-6, * – positive colonies with confirmed integration of the KO-dual-expression cassette (pKM173delATG/Koz/KOVP2-6) among the screened colonies.

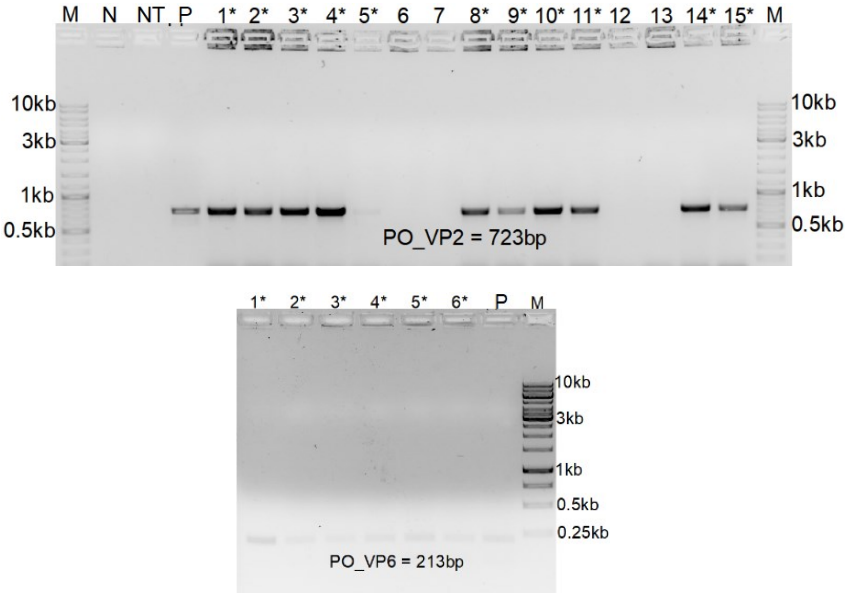


Figure 4.4: Agarose gel analysis of fragments obtained from the partial amplification of the PO/VP2 and PO/VP6 expression cassettes. "M" – GeneRuler™ 1 kb DNA Ladder, "N" – untransformed yeast colony as negative control, "NT" – no templated control, "P" – positive control, pKM173delATG/Koz/POVP2-6, * – positive colonies with confirmed integration of the PO-dual-expression cassette (pKM173delATG/Koz/POVP2-6) among the screened colonies.

From the colony PCR **(Section 4.2.5),** the expected fragment sizes were as follows: AO/VP2 and AO/VP6, 536 bp and 534 bp; KO/VP2 and KO/VP6, 891 bp and 493 bp and PO/VP2 and PO/VP6, 723 bp and 213 bp respectively. These fragments confirmed genomic integration of the expression cassette. The expression cassette consists of the ORF with its promoter and the terminator sequences. The number of positive colonies showing the integration of the recombinant dual-expression cassette containing the two ORFs is shown in **Table 4.5.**

Generally, there was an average efficiency observed for the chromosomal integration of all three dual-expression cassettes in the selected yeasts as confirmed by colony PCR. The integration in *P. pastoris* (GS_115 and UOFS_Y1552T) and *Y. lipolytica* (Po_1f, UOFS_Y0097, UOFS_Y2221, UOFS_2355 and UOFS_Y2415) was poor for all three dual-expression cassettes **(Table 4.5)**. It was discovered that the KO-dual-expression cassette integrated poorly in all the yeasts except in *A. adeninivorans, D. hansenii* and *K. lactis* (UOFS_Y1167). There was no successful transformation of KO-dual-expression cassette in any of the *P. pastoris* strains. Generally poor integration of the single-expression vector pKM177delATG/Koz/KOVP6 was also observed in a parallel study with a similar set of yeast strains (Rakaki, 2018). In *Y. lipolytica,* many of the strains selected in this study, showed a high number of colony formation after transformation but very poor integration of the recombinant dual-expression cassette **(Table 4.5)**. Correspondingly, the majority of the yeasts that showed integration failed to express these recombinant proteins; however, the opposite was the case for *H. polymorpha.* The integration of all the recombinant dual-expression cassettes in the *H. polymorpha* (UOFS_Y0915 and Y1507) was high as compared to the rest of the yeasts. In total, the highest percentage efficiency of over 97% was observed for the two strains of *H. polymorpha*. Relatively high integration was also observed in *A. adeninivorans* (LS3 and UOFS_Y1219), *D. hansenii, K. lactis* and *S. cerevisiae.*

4.3.2 Expression of rotavirus VP2 and VP6 ORFs in various yeast strains

Western blot analysis was performed to test the expression of the VP2 and VP6 proteins. Bacterial expressed rotavirus VP2_TF and VP6_EEF_HisTag proteins **(Figure 4.5)** were used as positive controls. Results from the western blot analysis **(Section 4.2.6.4)** confirmed the expression and detection of rotavirus capsid proteins VP2 and VP6 proteins as shown in **Figures 4.6 – 4.10**.

Variation in the expression among the yeast strains was observed. Unlike the bacterial expressed VP2 and VP6, which were fused with other proteins, yeast expressed VP2 and VP6 proteins were not fused to any other proteins, hence the molecular sizes of the expected proteins were 102.4 kDa for VP2 and 44.9 kDa for VP6 proteins (**Figures 4.6 – 4.10)**.

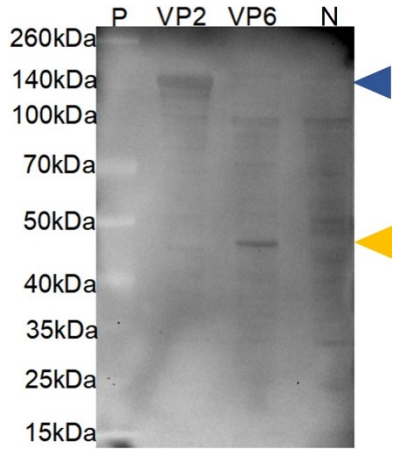


Figure 4.5: Western blot analysis to detect the recombinant bacterial-expressed rotavirus VP2_TF (150.4kDa; blue arrow) and VP6_EEF_His-Tag (47.5kDa; yellow arrow). "P" – Spectra™ Multicolor Broad Range Protein Ladder, "N" – untransformed BL21 bacteria as a negative control.

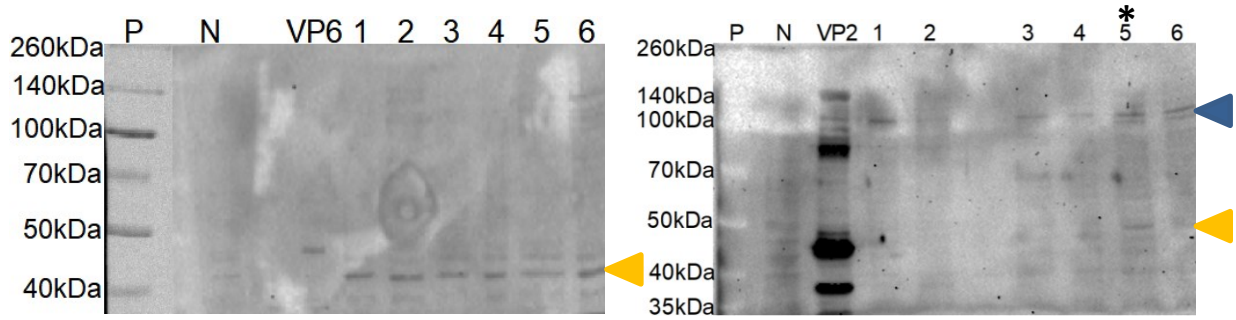


Figure 4.6: Western blot analysis to detect rotavirus VP2 and VP6 proteins expressed in the *A. adeninivorans* (LS3) transformed with pKM173delATG/Koz/AOVP2-6. Only colony 5 was observed with co-detection of the two proteins. Each lane is a colony screened. "P" – Spectra™ Multicolor Broad Range Protein Ladder, "N" – untransformed yeast colony as a negative control, "VP2 and VP6" – bacterial-expressed VP2 and VP6 proteins as a positive control.

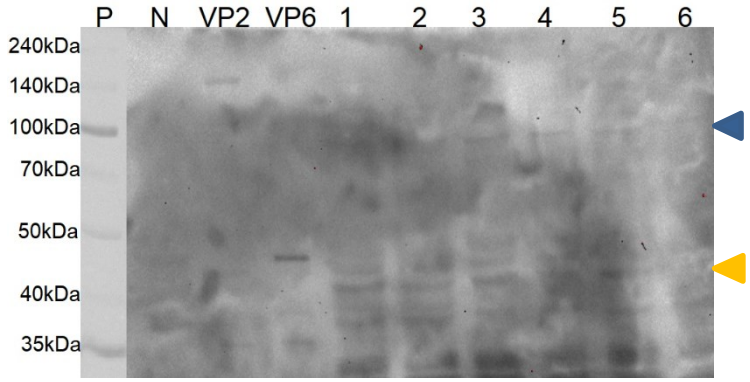


Figure 4.7: Western blot analysis to detect rotavirus VP2 and VP6 proteins expressed in the *A. adeninivorans* (UOFS_Y1220) transformed with pKM173delATG/Koz/AOVP2-6. Each lane is a colony screened. "P" – Spectra™ Multicolor Broad Range Protein Ladder, "N" – untransformed yeast colony as a negative control, "VP2 and VP6" – bacterial-expressed VP2 and VP6 proteins as a positive control.

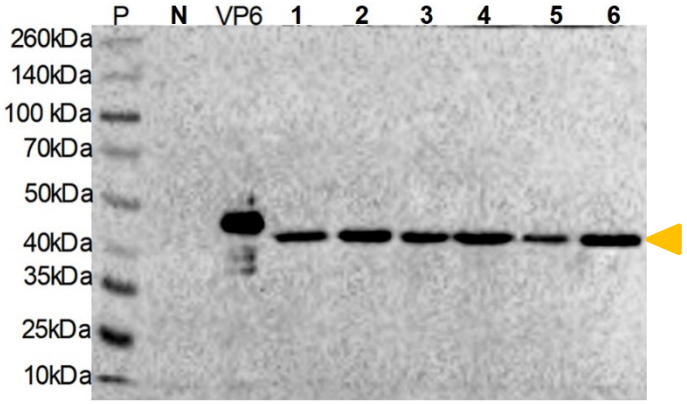


Figure 4.8: Western blot analysis to detect rotavirus VP2 and VP6 proteins expressed in the *A. adeninivorans* (UOFS_Y1219) transformed with pKM173delATG/Koz/AOVP2-6, but only VP6 protein was detected. Each lane is a colony screened. "P" – Spectra™ Multicolor Broad Range Protein Ladder, "N" – untransformed yeast colony as a negative control, "VP6" – bacterial-expressed VP6 protein as a positive control.

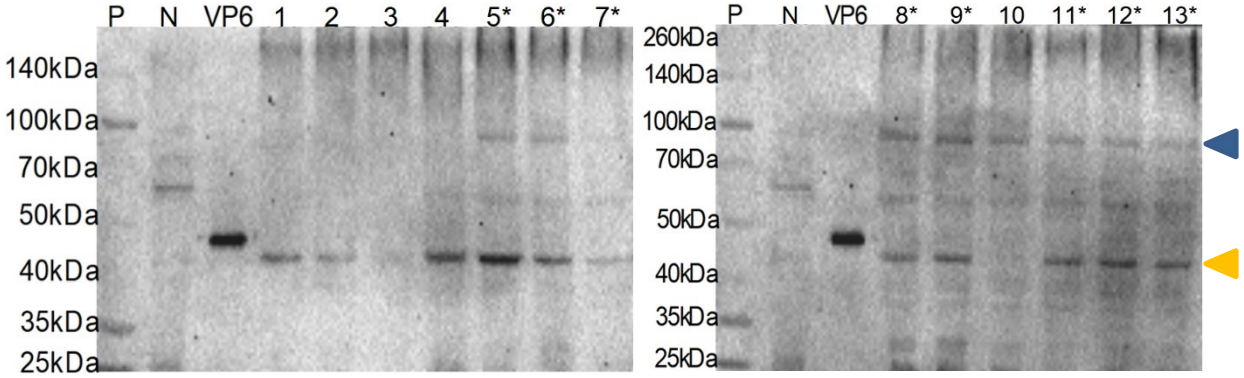


Figure 4.9: Western blot analysis to detect rotavirus VP2 and VP6 proteins expressed in *H. polymorpha* (UOFS_Y0915) transformed with pKM173delATG/Koz/AOVP2-6. Each lane is a colony screened. "P" – Spectra™ Multicolor Broad Range Protein Ladder, "N" – untransformed yeast colony as a negative control, "VP6" – bacterial-expressed VP6 protein as a positive control.

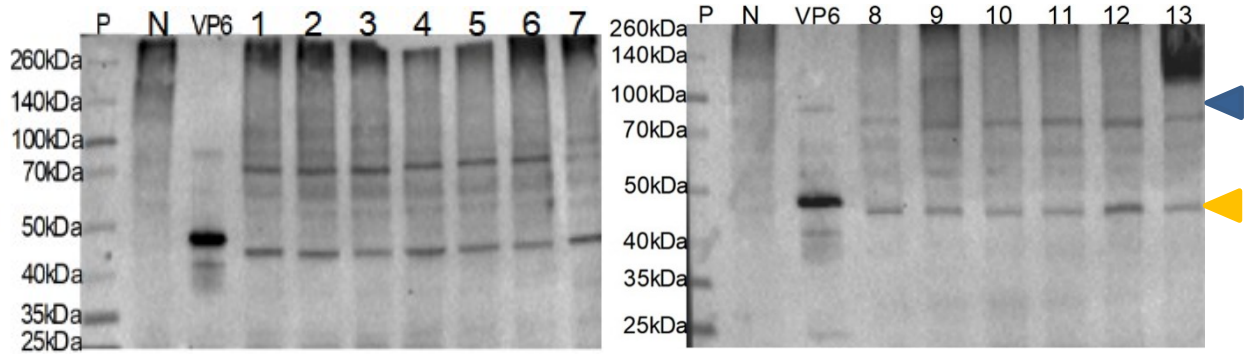


Figure 4.10: Western blot analysis to detect rotavirus VP2 and VP6 proteins expressed in the *H. polymorpha* (UOFS_Y1507) transformed with pKM173delATG/Koz/AOVP2-6. Each lane is a colony screened. "P" – Spectra™ Multicolor Broad Range Protein Ladder, "N" – untransformed yeast colony as a negative control, "VP6" – bacterial-expressed VP6 protein as a positive control.

In this study, bacterial-expressed rotavirus VP2 and VP6 proteins were used as positive controls having a molecular size 150.4 kDa and 47.5 kDa because of the fused triggered-factor and TEE-His-Tag respectively **(Figure 4.5)**. The initial western blot analysis of *A. adeninivorans* (LS3) and (UOFS_Y1220) transformed with AO-dual-expression cassette showed expression for both VP2 (102.4 kDa) and VP6 (45 kDa) **(Figure 4.6** and **4.7)**. The expression level was very low in these selected yeast strains **(Figure 4.6** and **4.7)** and this made co-detection of the two proteins difficult especially in the LS3, except for the clone 5 **(Figure 4.6)**. This may probably be the reason for losing the expression in the two yeast strains (*A. adeninivorans* LS3 and UOFS_Y1220) after three months of storage at – 80 ºC. Recombinant plasmid ejection was confirmed when no amplicons, i.e. the 536 bp fragment of AO/VP2 ORF, were obtained following colony PCR **(Section 4.2.5) (Figure 4.11)**.

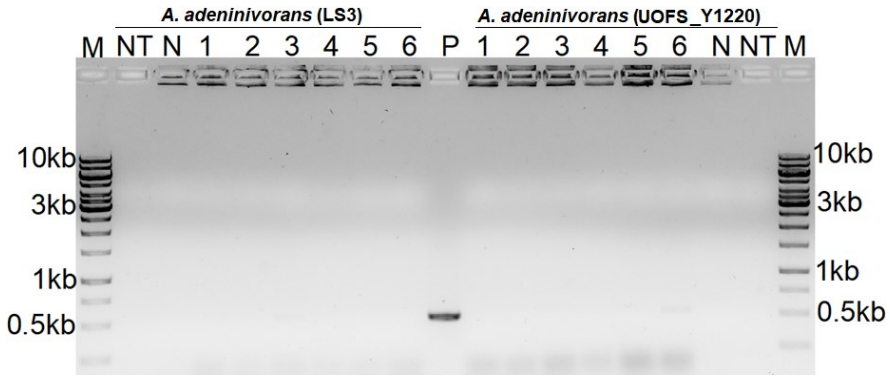


Figure 4.11: Agarose gel analysis of fragments obtained from the partial amplification of the AO/VP2 from the AO/dual-expression cassette previously transformed in *A. adeninivorans* (LS3 and **UOFS_Y1220). "**M" – GeneRuler™ 1 kb DNA Ladder, "N" – untransformed yeast colony as a negative control, "NT" – no templated control, "P" – positive control, pKM173delATG/Koz/AOVP2-6.

The third strain of *A. adeninivorans* (UOFS_Y1219), expressed only VP6 **(Figure 4.8)** for all the tested colonies when transformed with the AO-dual-expression cassette. Similarly, all colonies tested for *D. hansenii* (UOFS_Y0610) and *P. pastoris* (GS_115) transformed with AO-dual-expression cassettes expressed only the VP6 protein. Also, *D. hansenii* (UOFS_Y0610) and *K. lactis* (UOFS_Y1167) exclusively expressed rotavirus VP6 when transformed with the PO-dual-expression cassettes. Strains like *P. pastoris* (UOFS_Y1552T), *S. cerevisiae* (CENPK) and all the *Y. lipolytica* showed no expression for any of the proteins **(Table 4.5)**. However, there was an expression of the rotavirus VP2 and VP6 proteins by the two strains, UOFS_Y0915 and

UOFS_Y1507, of *H. polymorpha,* evaluated in this study. Strain UOFS_Y0915 expressed both proteins from each of the optimised ORFs. Of the transformed colonies tested, 2.6% showed expression for VP2 protein, 76% for VP6 protein, while 21% of the colonies showed expression for the VP2 and VP6 proteins when transformed with the AO-dual-expression cassette **(Table 4.5)**. When transformed with the KO-dual-expression cassette, 65% of the colonies showed expression for the VP2 and VP6 proteins, while 33% showed expression for the VP2 protein. All the colonies tested showed expression for VP2 and VP6 when transformed with the PO-dual-expression cassette **(Table 4.5)**. Similarly, for UOFS_Y1552T, all the tested colonies showed expression for the VP2 and VP6 proteins when transformed with each of the yeast codon-optimised ORFs (AO, KO and PO-dual-expression cassettes) **(Table 4.5)**. However, only the VP6 protein had the correct size of about 45 kDa while the VP2 had a smaller protein size as compared to the expected size of 102.4 kDa **(Figure 4.10)**. This difference in size of the VP2 protein was observed for all the UOFS_Y1552T colonies tested for all the three expression cassettes. In contrast, strain UOFS_Y0915 expressed the correct sizes of 102.4 kDa and 45 kDa for VP2 and VP6, respectively **(Figures 4.9)** and was identified as the best producing strain for the two proteins.

P. pastoris, strains GS_115 and UOFS_Y1552T, performed poorly in terms of expression, although integration levels were on par with that obtained with *A. adeninivorans, K. lactis* and *D. hansenii* when transformed with the AO and PO-dual-expression cassettes. Transformation with KO-dual-expression cassette in *P. pastoris* strains GS_115 and UOFS_Y1552T yielded no colonies. Only strain GS_115 was able to express the VP6 protein when transformed with the AO-dual-expression cassette.

Poor integration was observed with the *S. cerevisiae* (CENPK) and the strains of *Y. lipolytica* selected in this study. Less than 50% of the colonies tested showed integration for *S. cerevisiae,* and no expression was observed for any of these colonies. Similarly, none of the limited number of positive colonies obtained for integration in *Y. lipolytica,* strains Po_1f and UOFS_Y0097, was able to express any of the two proteins. The integration performances of *Y. lipolytica* strains UOFS_Y2221, Y2355 and Y2415 was also generally poor as they showed no colonies or colonies without integrations **(Table 4.5)**. *H. polymorpha,* strain UOFS_Y0915 show the highest degree of genomic integration and the expression of the rotavirus VP2 and VP6 proteins was confirmed with correct protein size. This strain was therefore selected for the evaluation of the DLP.

Table 4.5: Screening of transformed yeast strains for genomic integration of the dual-expression cassettes and expression of rotavirus VP2 and VP6 proteins

Yeast	Strain	Dual-expression vector (pKM173delATG/Koz/VP2-6)	Genomic integration	Expression of V2 and VP6 in selected colonies
A. adeninivorans	LS3	*A. adeninivorans optimised*	8/15	6/6 (expressed VP2/6 before but lost later)
		K. lactis optimised	10/11	0/7 (no expression)
		P. pastoris/H. polymorpha optimised	9/10	0/3 (no expression)
A. adeninivorans	UOFS_Y1219	*A. adeninivorans optimised*	6/21	6/6 (expressed VP6 only)
		K. lactis optimised	9/14	0/2 (no expression)
		P. pastoris/H. polymorpha optimised	6/10	0/3 (no expression)
A. adeninivorans	UOFS_Y1220	*A. adeninivorans optimised*	8/52	6/6 (expressed VP2/6 before but lost later)
		K. lactis optimised	14/39	0/2 (no expression)
		P. pastoris/H. polymorpha optimised	4/10	0/4 (no expression)
D. hansenii	UOFS_Y0610	*A. adeninivorans optimised*	6/36	6/6 (expressed VP6 only)
		K. lactis optimised	11/14	0/2 (no expression)
		P. pastoris/H. polymorpha optimised	6/10	6/6 (expressed VP6 only)
K. lactis	UOFS_Y1167	*A. adeninivorans optimised*	2/3	0/2 (no expression)
		K. lactis optimised	11/13	0/2 (no expression)
		P. pastoris/H. polymorpha optimised	10/10	10/10 (expressed VP6 only)
H. polymorpha	UOFS_Y0915	*A. adeninivorans optimised*	38/39	38/38 (1 for VP2; 29 for VP6; 8 for VP2/6)
		K. lactis optimised	3/3	3/3 (1 expressed VP2; 2 expressed VP2/6)
		P. pastoris/H. polymorpha optimised	11/15	3/3 (all expressed VP6, weak detection for VP2)
H. Polymorpha	UOFS_Y1507	*A. adeninivorans optimised*	38/39	38/38 expressed VP2/6 but VP2 suspected to be truncated
		K. lactis optimised	5/5	2/2 expressed VP2/6 but VP2 suspected to be truncated
		P. pastoris/H. polymorpha optimised	14/14	3/3 (expressed VP2/6 but VP2 suspected to be truncated
P. pastoris	GS_115	*A. adeninivorans optimised*	3/8	3/3 (expressed VP6 only)

Yeast	Strain	Dual-expression vector (pKM173delATG/Koz/VP2-6)	Genomic integration	Expression of V2 and VP6 in selected colonies
		K. lactis optimised	No colonies	-
		P. pastoris/H. polymorpha optimised	5/10	0/3 (no expression)
P. pastoris	UOFS_Y1552T	*A. adeninivorans optimised*	3/7	0/3 (no expression)
		K. lactis optimised	No colonies	-
		P. pastoris/H. polymorpha optimised	4/4	0/4 (no expression)
S. cerevisiae	CENPK	*A. adeninivorans optimised*	23/66	0/23 (no expression)
		K. lactis optimised	5/17	0/2 (no expression)
		P. pastoris/H. polymorpha optimised	8/10	0/3 (no expression)
Y. lipolytica	Po_1f	*A. adeninivorans optimised*	0/73	-
		K. lactis optimised	4/13	0/2 (no expression)
		P. pastoris/H. polymorpha optimised	1/10	0/1 (no expression)
Y. lipolytica	UOFS_Y0097	*A. adeninivorans optimised*	10/39	0/10 (no expression)
		K. lactis optimised	10/13	0/3 (no expression)
		P. pastoris/H. polymorpha optimised	0/10	-
Y. lipolytica	UOFS_Y2221	*A. adeninivorans optimised*	0/39	-
		K. lactis optimised	0/12	-
		P. pastoris/H. polymorpha optimised	No colonies	-
Y. lipolytica	UOFS_Y2355	*A. adeninivorans optimised*	No colonies	-
		K. lactis optimised	2/14	0/2 (no expression)
		P. pastoris/H. polymorpha optimised	ND	-
Y. lipolytica	UOFS_Y2415	*A. adeninivorans optimised*	No colonies	-
		K. lactis optimised	0/13	-
		P. pastoris/H. polymorpha optimised	0/10	-

ND = not determined; UOFS = the University of the Free State as part of the yeast nomenclature from the UNESCO-MIRCEN Yeast (Y) Culture Collection; LS3, GS_115 and Po_1f are commercial strains

4.3.3 Evaluation of DLP formation in *H. polymorpha* (UOFS_Y0915)

To evaluate double-layered (DLP) formation, growth conditions were optimised for *H. polymorpha* (UOFS_Y0915) to increase recombinant protein production by growing the yeast in bioreactors. A representative clone of *H. polymorpha* transformed with pKM173delATG/Koz/AOVP2-6 was selected. Initially, shake-flask experimentation was carried out in YPD media and TEM analysis showed only a few small sphere-like particles representing rotavirus VP6 **(SUPPLEMENTARY A2)**. These sphere-like particles obtained were in the range of 25 – 40nm. Due to pleomorphic nature of the rotavirus VP6 capsid protein, different particulate sizes ranging from 20 – 60nm of this protein have been reported based on the ionic strength, buffer and pH (Blazevic *et al.*, 2016a; Bugli *et al.*, 2014; Lepault *et al.*, 2001; Malm *et al.*, 2017).

Subsequently, the shake-flask experiment was repeated, this time using a factorial designed experiment to improve the co-expression of the VP2 and VP6 proteins and *H. polymorpha* specific medium, Syn-6-MES. Each of the flasks contained Syn-6-MES media formulated with either glucose or glycerol as a carbon source and varying concentrations of nutrients P (phosphorus), K (potassium) and Mg (magnesium), either as limited or unlimited **(Table 4.4)**.

The Premier Rotaclone® EIA kit, used to quantify VP6 protein in each of the shake-flasks, utilises monoclonal antibodies in a solid phase sandwich-type enzyme immunoassay (EIA) to capture the VP6 protein, which is the group specific antigen for all known human rotaviruses. The kit was designed in such a way that any sample having a value higher or equal to the positive control is considered positive but lower than the positive is considered negative to VP6 protein. In this study, VP6 protein encapsulating the VP2 core protein was used as an index for quantifying the DLPs produced in each flask at 24- and 48-hours **(Figure 4.12)**.

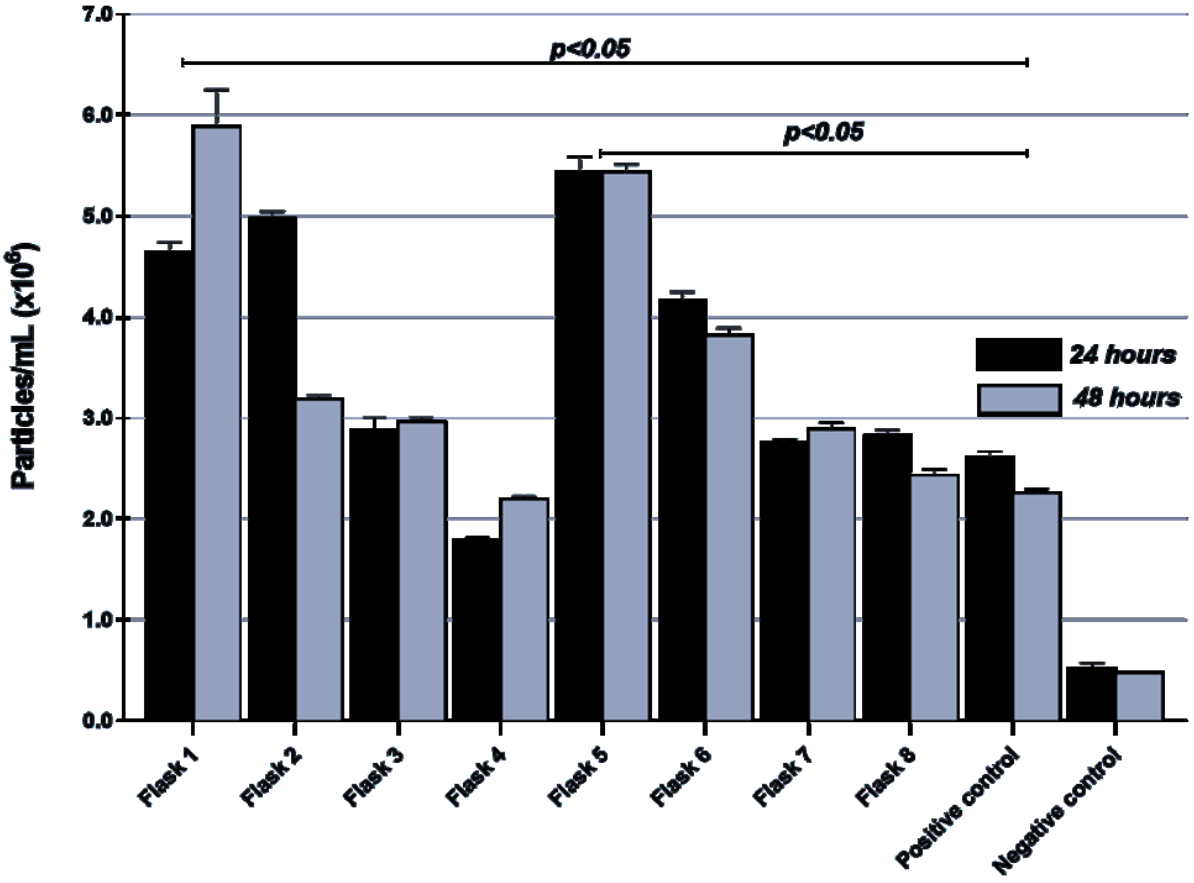


Figure 4.12: Detection of recombinant proteins produced in shake-flask cultures of *H. polymorpha* (UOFS_Y0915) transformed with pKM173delATG/Koz/AOVP2-6 and harvested at 24 and 48 hours. The quantification was carried out with the Premier Rotaclone® EIA kit. The statistical difference was observed at *p<0.05* by comparing to the positive control in terms of the total average production of VP6 protein.

The highest yield of recombinant VP6 protein was observed in flasks 1, 2, 5 and 6 as compared to the positive control. Flasks 1 and 5 contained glucose as carbon source while Flasks 2 and 6 contained glycerol as a carbon source. The highest yields were observed in Flask 1 at 48 hours (5.9 ± 0.150 x 10^6 particles/mL) and Flask 5 at 24 hours (5.4 ± 0.071 x 10^6 particles/mL). There was an increase in VP6 yield from 24 to 48 hours in Flask 1, however, the yield remained the same at 24 and 48 hours in Flask 5. Apart from both Flasks utilising glucose as a source of carbon, Flask 1 contained a limited supply of all the nutrients (P, K and Mg), but Flask 5 only has a limited supply of "P" while the "K" and "Mg" were continuously supplied unlimitedly.

Interestingly, in Flask 2, there was a significant decrease (1.7-fold) in the VP6 produced from 24 hours (5.0 ± 0.029 x 10^6 particles/mL) to 48 hours (3.2 ± 0.012 x 10^6 particles/mL) *(p<0.05).* There was no statistical difference in the level of the recombinant VP6 protein detected in the Flasks 3, 4, 7 and 8 as compared to the positive control *(p>0.05).* Therefore, the formulated Syn-6-MES mineral media in the Flasks 1 and 5 were selected as growth media in Sixfors

bioreactors. Each of these cultures was compared with YPD as a positive control growth medium to cultivate recombinant *H. polymorpha* (UOFS_Y0915).

Figure 4.13 shows the biomass production of the 10 mL harvest at 8, 12, 18, 24 and 30 hours during Sixfors bioreactor. Biomass production increased steadily in the YPD growth medium as compared to Flasks 1 and 5. There was no observed difference in the biomass production for the two flasks up to the 18 hours of cultivation. After 18 hours, there was an increase in the biomass production in Flask 1 compared to Flask 5 and this remained steady up to 30 hours of cultivation. The biomass produced in Flask 1 was still less compared to that produced in the YPD growth medium **(Figure 4.13)**.

At 8, 12, 18, 24 and 30 hours of cultivation, the VP6 protein produced was quantified with the Premier Rotaclone® EIA kit **(Figure 4.14)**. By comparing the VP6 protein produced during cultivation in YPD medium, Flask 1 and Flask 5, a significant amount of recombinant VP6 protein was detected in YDP (flask 1) medium compared to the Syn-6-MES mineral media *($p<0.05$),* flasks 3 and 5*.* Generally, there was an increase in VP6 production in YPD from 8 to 24 hours with some reduction in the VP6 yield at 30 hours. However, this reduction was not statistically different from the VP6 protein produced at 18 and 24 hours *($p<0.05$).* A lag in VP6 production was observed during cultivation in the growth conditions of Flask 1 and Flask 5, compared to VP6 production in YPD (Flask 1). For the latter, an approximately 1.5-fold increase in VP6 production (compared to positive control) was observed at 8 hours **(Figure 4.14)**. The VP6 yield in Flasks 1 and 5 increased from 8 to 30 hours, although a lower amount of VP6 was detected in the Flask 5 compared to Flask 1 between 8 and 18 hours. Comparative production of VP6 was thereafter observed, in Flask 1 (YPD) and Flask 5 growth media at 24 and 30 hours (no statistical differences in VP6 yield *($p<0.05$)*). An increase in VP6 production was observed for Flask 1 at 18 hours *($p<0.005$)* as compared to the positive control **(Figure 4.14)**. The yield was as high as an expression at 8 and 12 hours in YPD medium. A lower yield was, however, detected at 24 hours but increased again at 30 hours. Thus, no significant difference *($p<0.05$)* in VP6 yield was detected at 30 hours in all the experimental flasks, suggesting that harvesting between 24 – 30 hours might be the optimal time of harvest **(Figure 4.14)**.

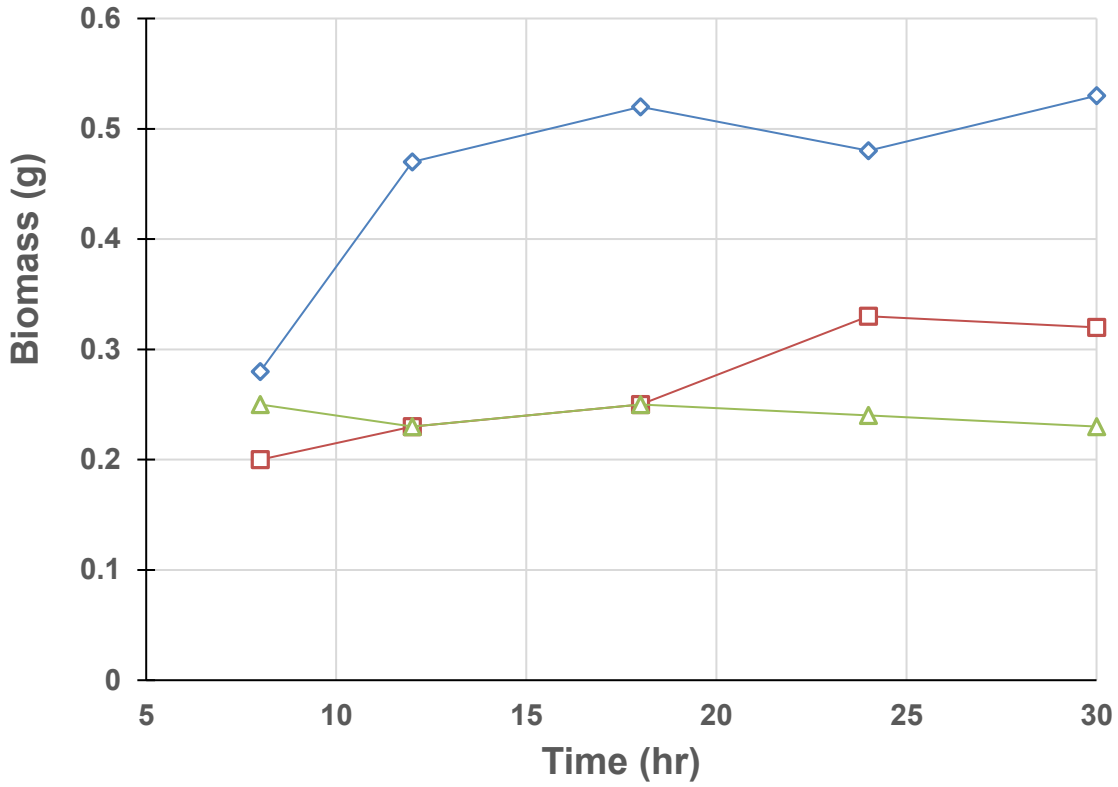


Figure 4.13: Biomass production per time during the cultivation of *H. polymorpha* (UOFS_Y0915) transformed with pKM173delATG/Koz/AOVP2-6 dual-expression cassette containing the two ORFs. Biomass production in YPD is indicated with blue diamonds, orange squares represent biomass production in Flask 1-growth conditions and grey triangles indicate biomass production in Flask 5-growth conditions.

Premier Rotaclone® EIA kit could only detect rotavirus VP6 protein. Western blot analysis **(Section 4.2.6.4)** was carried out at 8, 12, 18, 24 and 30 hours to evaluate the production of VP2 and VP6 proteins in each of the samples **(Figure 4.15)**. The VP6 expression was detected as early as 8 hours in YPD growth medium and Flask 1 growth conditions, whereas the first detection of VP6 for Flask 5 growth conditions was observed at 12 hours. VP2 detection was observed at 18 hours for yeast cultivation in YPD medium, but no VP2 expression could be observed in either of the Syn-6-MES culture conditions in Flask 1 or Flask 5. YPD media was therefore used to cultivate the recombinant yeast, *H. polymorpha* (UOFS_Y0915) transformed with the AO-dual-expression cassette, in a 1L bioreactor to evaluate DLP production.

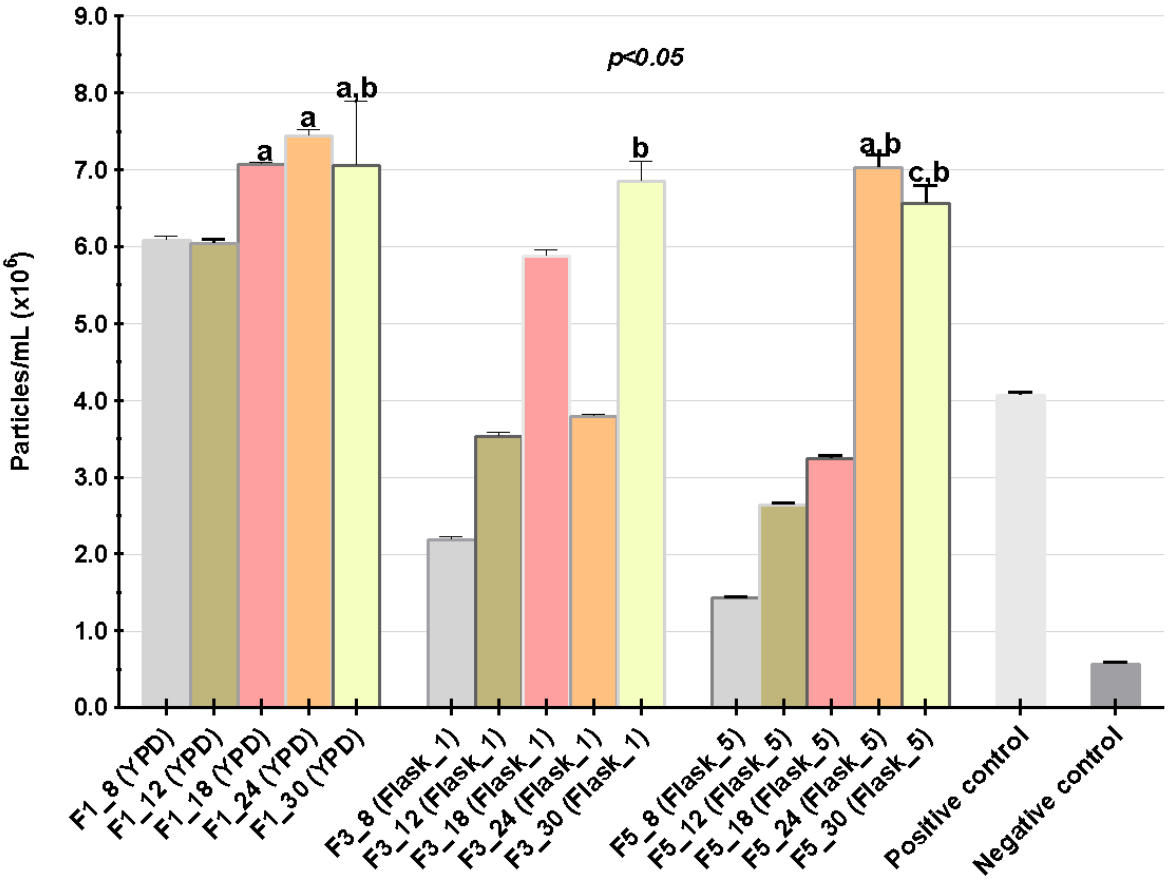


Figure 4.14: Graph showing production of rotavirus VP6 by *H. polymorpha* (UOFS_Y0915) transformed with pKM173delATG/Koz/AOVP2-6 at different time points and cultivated in the Sixfors bioreactor with 3 different culture conditions: **Fermenter 1 (F1),** YPD culture with 10 mL harvested at 8, 12, 18, 24 and 30 hours. **Fermenter 3 (F3),** Flask 1 culture conditions **(Table 4.4)**, glucose as the carbon source with a limited supply of phosphate, potassium and magnesium. **Fermenter 5 (F5),** Flask 5 culture conditions **(Table 4.4)** glucose as the carbon source with a limited supply of phosphate but an unlimited supply of potassium and magnesium. According to the manufacturer, the **positive control** is an inactivated Simian rotavirus SA-11 in saline buffer with 0.02% thimerosal as a preservative while the sample diluent (saline buffer) preserved with 0.02% thimerosal served as the **negative control**.

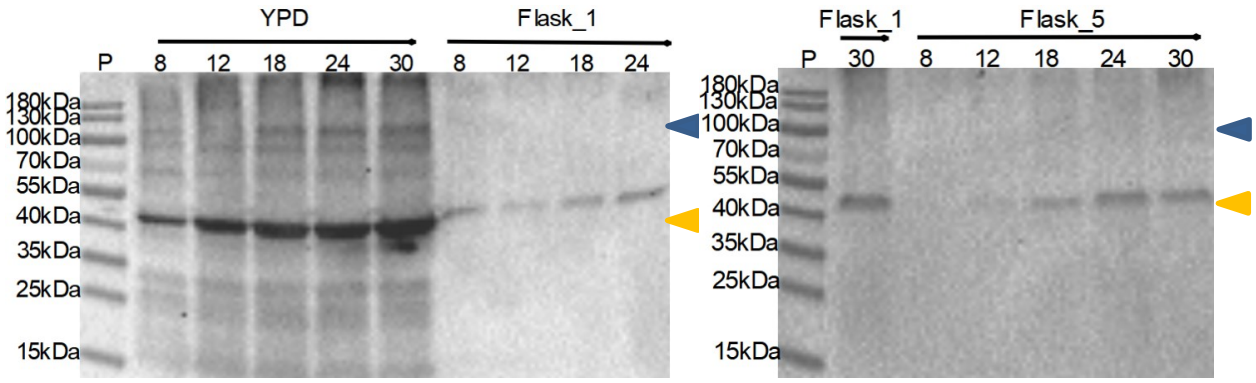


Figure 4.15: Western blot analysis showing the detection of rotavirus VP2 and VP6 proteins at 8-, 12-, 18-, 24- and 30-hours following bioreactor cultivation of the recombinant yeast *H. polymorpha* (UOFS_Y0915) transformed with pKM173delATG/Koz/AOVP2-6. "P" is the PageRuler™ Prestained Protein Ladder. The yellow arrow indicates the VP6 protein while the blue arrow indicates the VP2 protein.

H. polymorpha (UOFS_Y0915) expressing AO-dual-expression cassette was cultivated for 24 hours in a 1 L bioreactor in YPD medium to evaluate DLP formation using TEM. SDS-PAGE analysis suggested the presence of VP2 and VP6 following sucrose gradient ultracentrifugation **(Figure 4.16)** in fractions 13 – 18. These fractions were pooled, dialysed **(Section 4.2.7.2)** and western blot analysis **(Section 4.2.6.4)** confirmed the expression of VP2 and VP6 **(Figure 4.17)**.

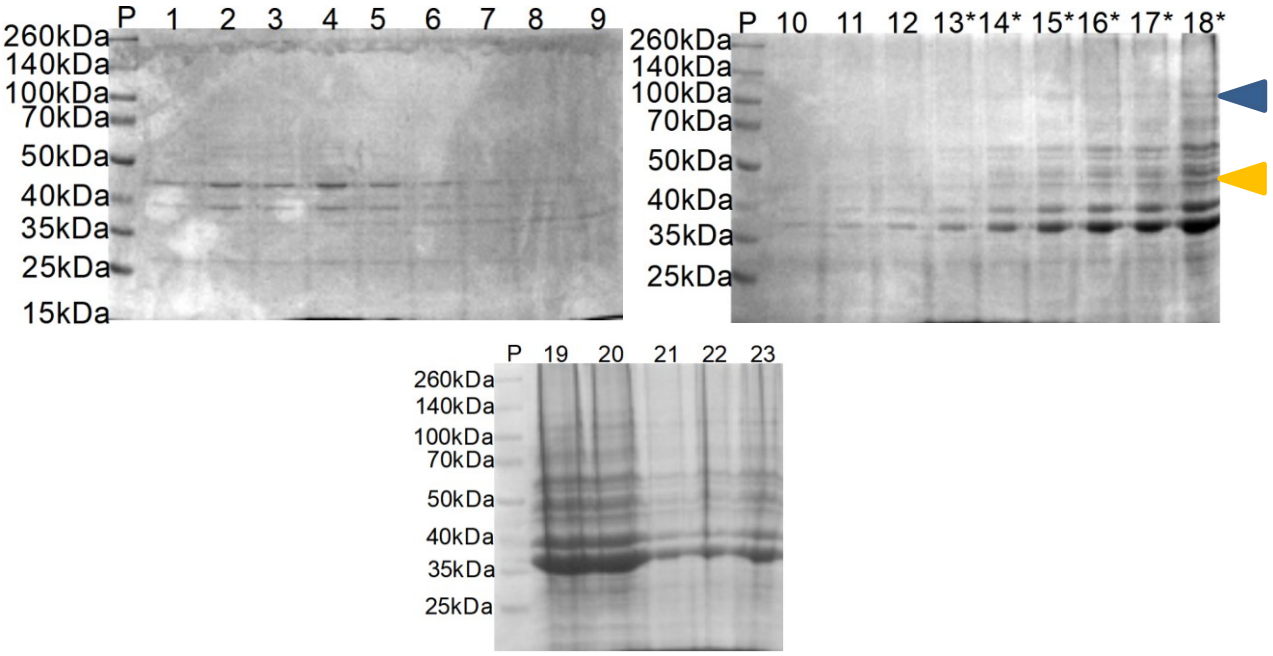


Figure 4.16: SDS-PAGE analysis of the 23 fractions obtained following the sucrose gradient ultracentrifugation and fractionation. Asterisk lanes are the suspected fractions with the recombinant VP2 and VP6 proteins. "P" is the PageRuler™ Prestained Protein Ladder.

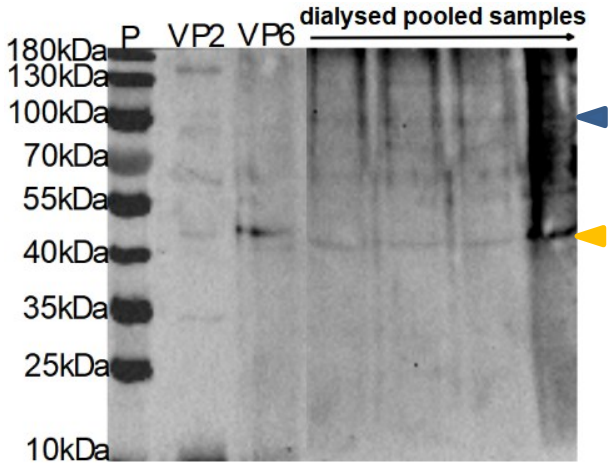


Figure 4.17: Western blot analysis showing the detection of VP2 and VP6 in the pooled and dialysed fractions (10 – 18). "P" is the PageRuler™ Prestained Protein Ladder; VP2 and VP6 proteins are the bacterial-expressed positive control.

After isopycnic CsCl gradient purification, a translucent layer of rotavirus particles was observed **(Figure 4.18)**, which was carefully fractionated and evaluated for DLP formation using TEM (**Figure 4.19)**. The size of the particle observed was about 58 nm, which was within the size range reported for rotavirus DLPs (Estes & Kapikian, 2007; Labbé *et al.*, 1991; Soler *et al.*, 2005). From this TEM image, few properties of rotavirus DLPs were observed such as the rough edges of the particle due to the VP6 trimeric subunit.



Figure 4.18: Translucent layers of rotavirus particles as observed after 30 hours of CsCl isopycnic gradient ultracentrifugation at 32 000 rpm and 4 ºC.

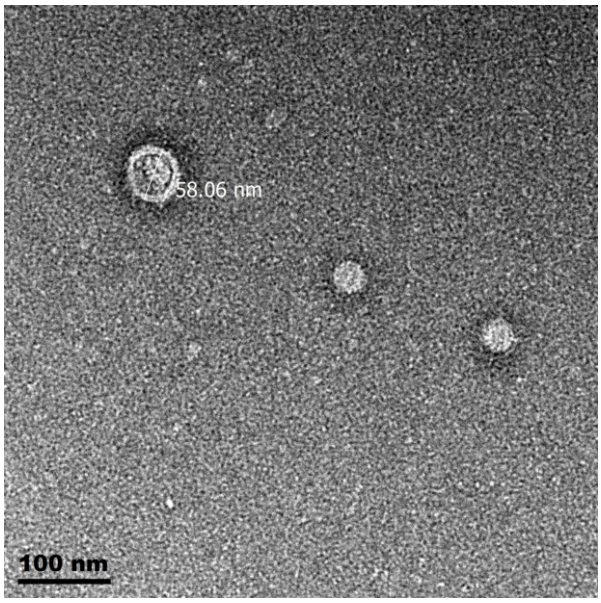


Figure 4.19: TEM image obtained from separation by isopycnic CsCl ultracentrifugation followed by negative staining with 3 % uranyl acetate solution. The particle was measured at 58 nm in size.

4.4 Discussion

In this study, 15 different yeast strains were screened for the heterologous expression of rotavirus VP2 and VP6 proteins. These yeasts were obtained from the UNESCO-MIRCEN yeast culture collections at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein. This resource offers more than 3 000 yeast strains and in combination with the wide-range yeast expression system (Albertyn *et al.*, 2011),

offered an extensive screening approach. The criteria for selection of yeast strains used in this study were based on their frequent use in heterologous expression of proteins, fast growth rate and, most importantly, none of the strains used was pathogenic. In addition, the exploration of non-*S. cerevisiae* yeasts in the recombinant production of heterologous protein is continually increasing (Kottmeier *et al.*, 2010; van der Heide *et al.*, 2003) especially the methylotrophic Crabtree-negative yeasts, which prefer respiration over fermentation and fail to accumulate ethanol, which could reduce the efficiency of recombinant expression (Vieira Gomes *et al.*, 2018). Theron and co-workers previously reported a similar screening approach using these yeasts and a wide-range expression vector for heterologous expression of recombinant enzymes (Theron *et al.*, 2018; Theron *et al.*, 2014). Similar to their results, different expression levels among the yeasts were observed in this study and this variance may be attributed to the nature of the wide-range yeast expression cassette constructed and the efficiency of chromosomal integration. In general, the level of integration by the three dual-expression cassettes (AO, KO and PO-dual-expression cassettes) differed in different yeast strains as confirmed with the colony PCR **(Table 4.5)**. However, confirmation of integration of the cassette did not correlate with the expression and detection of the VP2 and VP6 proteins.

Percentage integration and positive expression analysed indicated that *A. adeninivorans* (UOFS_Y1220) (transformed with AO-dual-expression cassette), *Y. lipolytica* strain Po_1f (transformed with PO-dual-expression cassette) and strain UOFS_2355 (transformed with KO-dual-expression cassette) showed the lowest percentage integration – 15, 10 and 14% respectively **(Table 4.5)**. Although initial results indicated that *A. adeninivorans* strain UOFS_Y1220 expressed both proteins, no expression was observed in these clones following storage. *S. cerevisiae* and *P. pastoris* performed poorly in terms of expression while *Y. lipolytica* performed poorly in terms of integration (except for the KO-dual-expression cassette) and expression. PO-dual-expression cassettes transformed in *K. lactis* (UOFS_Y1167) and *H. polymorpha* (UOFS_Y1507) showed 100% integration. However, while *K. lactis* (UOFS_Y1167) expressed only the VP6 protein, *H. polymorpha* (UOFS_Y1507) expressed both the VP2 and VP6 proteins. Generally, integration and expression of all the three expression cassettes were better in the *H. polymorpha* in comparison to any of the other yeast species. Overall there was no correlation between chromosomal integration of any of the three dual-expression cassettes (as confirmed by the colony PCR) and the level of gene expression except for the *H. polymorpha* strains where a positive correlation was observed between the integration and recombinant expression of the two proteins.

Chromosomal integration and expression of the rotavirus VP2 and VP6 proteins with correct protein sizes were only observed in *H. polymorpha* (UOFS_Y0915), though occasional

inconsistent protein bands were observed in the western blot due to multiple usage of the primary polyclonal Goat antibody. The effect of strain preference was observed as the *H. polymorpha* strain UOFS_Y0915 was able to express the two proteins correctly based on their molecular size. Although the expected size for VP6 was detected for strain UOFS_Y1507 in the western blot, the protein size observed for VP2 was smaller than expected. This smaller size might be attributed to proteolytic cleavage of the VP2 protein or processivity errors that can lead to the lower molecular weight of the recombinant protein (Belcourt & Farabaugh, 1990; Dong & Kurland, 1995). Improper protein folding, excessive protein production or toxic protein usually induces cellular events that target such protein for proteolytic cleavage either in an ubiquitin- or non-ubiquitin-dependent pathways (Berner *et al.*, 2018; Kimata *et al.*, 2018). These mechanisms are found in the yeasts analysed and might be responsible for the suspected lower molecular weight observed for the rotavirus VP2 protein expressed in the *H. polymorpha* (UOFS_Y1507). Surprisingly, *H. polymorpha* strain CBS_4732 was previously shown to be a poor host for heterologous co-expression of benzoate-para-hydroxylase CYP53B1 with different cytochrome P_{450} reductases (Theron *et al.*, 2018). Poor integration and expression were observed for *Y. lipolytica* and *S. cerevisiae,* but these were the best candidates identified during the screening study by Theron and co-workers for benzoate-para-hydroxylase with cytochrome reductase (Theron *et al.*, 2018; Theron *et al.*, 2014). Therefore, differential expression can be observed for the same host using the same expression plasmid containing different insertions during recombinant protein production. In another scenario, variable activities could be observed when different host strains are used for different heterologously expressed proteins. There are reports indicating diverse results for recombinant protein expression in *H. polymorpha* (Böer *et al.*, 2007). Recombinant expression of IL-6 in *H. polymorpha* and *S. cerevisiae* had been reported to be N-terminally truncated compared to the full-length polypeptide expressed in *A. adeninivorans* (Böer *et al.*, 2007). However, *H. polymorpha* was a better producer of alcohol dehydrogenase than *A. adeninivorans*.

Selective integration was observed with the KO-dual-expression cassette. This expression cassette integrated quite well in the *A. adeninivorans, K. lactis, H. polymorpha* and *Y. lipolytica* (Po_1f), however, its non-integration in *P. pastoris* was very surprising. Poor integration of KO/VP6 ORF (pKM177delATG/Koz/KOVP6 ORFs) into the *P. pastoris* (GS_115) had been reported in a parallel study involving the screening of yeast strains for the production of rotavirus VP6 protein (Rakaki, 2018). Similarly, genetic instability was suspected in *A. adeninivorans* (LS3 and UOFS_Y1220) as it lost its expression after 3 months of storage at –80 °C. This might mean that the *Arxula* strains could not sustain long-term mitotic stability of the expression cassette. So far there is no evidence of mitotic instability in this yeast strain

especially for expression vectors with endogenous promoters like the *Arxula adeninivorans* TEF promoter (Steinborn *et al.*, 2006; Steinborn *et al.*, 2007; Wartmann *et al.*, 2002; Wartmann *et al.*, 1995; Yang *et al.*, 2000).

Of the fifteen yeasts screened, only *H. polymorpha* (UOFS_Y0915) could be identified to stably express both VP2 and VP6. A number of reasons can be proposed for the poor expression observed in the majority of the yeasts tested. Firstly, the size of the recombinant expression cassette (about 10 kb) may be too large for the yeast to maintain. Secondly, the use of a strong constitutive promoter like yITEFp used in this dual-expression cassette may have imposed cellular pressure as it promotes a high rate of transcription of heterologous genes (Partow *et al.*, 2010). Cellular pressure can lead to protein products that can activate the unfolded protein response (UPR) due to cellular stress (Cavallius *et al.*, 1993). It has been revealed that random chromosomal integration by homologous recombination may lead to unnecessary metabolic stress and terminal truncation (Aw & Polizzi, 2013; Böer *et al.*, 2007; Löbs *et al.*, 2017) – a case suspected in *H. polymorpha* strain UOFS_Y1507. Lastly, inefficient homologous chromosomal integration can occur which can lead to the gene of interest being lost while the marker gene will be integrated for cellular survival (Kawai *et al.*, 2010; Makatsa, 2015; Vieira Gomes *et al.*, 2018).

Previously, the rotavirus VLP production in *S. cerevisiae* had been reported (Rodríguez-Limas *et al.*, 2011) but the transmission electron microscope (TEM) image was too indistinctive and unclear to see the monomeric units of the particles compared to the insect cell expression (Crawford *et al.*, 1994). In addition, there was a low yield of the rotavirus VP7, which could have compromised the inclusive structural formation of the rotavirus particles (Rodríguez-Limas *et al.*, 2011). Being a glycoprotein, its formation is greatly influenced by cellular conditions related to glycosylation motifs, vesicular transportation and sorting (Rodríguez-Limas *et al.*, 2011) **(Figure 4.1)**. The expression of rotavirus VP2 and VP6 capsid proteins in this study enabled the formation of an oligomeric structure that resembles rotavirus DLP. In this study, an attempt was made to optimise the growth condition of the recombinant yeast for DLP production by comparing the formulated Syn-6-MES mineral media (Kottmeier *et al.*, 2010) with the YPD but the YPD was found relatively better in terms of the biomass and protein production. With bioreactor cultivation, rotavirus DLP was obtained from *H. polymorpha* (UOFS_Y0915), which measured 58 nm and correlated with reported DLP sizes. The size of rotavirus DLPs varies and is highly heterogeneous ranging between 50 – 70 nm (Boudreaux *et al.*, 2015; Long & McDonald, 2017; Soler *et al.*, 2005). Estes and Kapikian, while working on SA11 strain, reported 55 nm for DLPs (Estes & Kapikian, 2007) and Labbe and co-workers

reported 60 nm for DLPs (Labbé *et al.*, 1991). Morphologically, the TEM image, as observed in this study, has a roughly spherical shape, which is similar to those reported in the literature.

In this study, the use of a methylotrophic yeast was reported for the first time to express rotavirus VP2 and VP6 proteins using a wide-range yeast expression vector. The same yeast had been used before to express rotavirus VP6 (Bredell *et al.*, 2016) in bioreactor cultivation. From the results obtained in this study, it is recommended that a comprehensive evaluation of the DLPs produced by *H. polymorpha* be made. The recombinant yeast transformed with AO-dual-expression cassette should be analysed alongside the same yeast transformed with an expression plasmid containing no insert to confirm that the formation of the observed oligomeric structure is indeed derived from the AO-dual-expression cassette. In addition, particle formation should also be evaluated in parallel to *H. polymorpha* containing only the AO/VP2ORF (pKM173delATG/Koz/AOVP2). It is important to establish that the *H. polymorpha*-produced rotavirus VP2 protein can form a single-layered particle (SLP) of 45 nm size (Labbé *et al.*, 1991). The formation of the SLP is critical for the formation of DLP since underproduction of one of the proteins would compromise particle formation.

In conclusion, one of the objectives of this study was to produce DLPs in yeast. A yeast strain, *H. polymorpha* (UOFS_Y0915) transformed with the pKM173delATG/Koz/AOVP2-6 (AO-dual-expression cassette), was identified in this study as the only yeast strain capable of expressing rotavirus VP2 and VP6 proteins to enable the formation of DLPs. From the TEM analysis, the particle structure of 58 nm size was observed, which morphologically resembles rotavirus DLPs as reported in the literature.

4.5 Reference

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Chapter Five

Removal of glycosylation motifs for secretion of rotavirus VP6

5.1 Introduction

Several reports have shown the immunogenic and antigenic properties of rotavirus VP6 capsid protein (Colomina *et al.*, 1998; Ishida *et al.*, 1996; Svensson *et al.*, 1987a; Svensson *et al.*, 1987b; Tang *et al.*, 1997a; Tang *et al.*, 1997b). More importantly, rotavirus VP6 protein can retain its native conformation (trimers) and conserve the immunoreactive determinant when expressed recombinantly (Desselberger *et al.*, 2013; Estes *et al.*, 1987; Kohli *et al.*, 1993a; Kohli *et al.*, 1992; Kohli *et al.*, 1993b; McNeal *et al.*, 2007a). It is, therefore, considered a potential rotavirus vaccine candidate and several reports have shown its immunogenic properties in animal models (Burns *et al.*, 1996; Choi *et al.*, 2004; Choi *et al.*, 2002; Corthésy *et al.*, 2006; Esquivel *et al.*, 2000; Lappalainen *et al.*, 2014; Malm *et al.*, 2016; McNeal *et al.*, 2007b; Pastor *et al.*, 2014; Temprana *et al.*, 2018). By targeting the mucosal and systemic immune responses (Feng *et al.*, 2017), rotavirus VP6 protein was shown to induce heterotypic protection against rotavirus infection by stimulating B and T lymphocytes (Choi *et al.*, 1999; McNeal *et al.*, 2002; Parez *et al.*, 2004; Pastor *et al.*, 2014) and at the same time induces secretory monomeric (Feng *et al.*, 2002) and polymeric mucosal IgA (Corthésy *et al.*, 2006) that confer protection and reduce faecal viral shedding.

Previously, six yeast strains have been engineered with the wide-range yeast expression system to produce rotavirus VP6 proteins intracellularly (Rakaki, 2018). These yeasts were *Arxula adeninivorans, Hansenula polymorpha, Kluyveromyces lactis, Pichia pastoris, Saccharomyces cerevisiae* and *Yarrowia lipolytica.* Since the overall objective of the greater project is to produce a cost-effective rotavirus VP6-based vaccine, extracellular production of VP6 would offer more advantages for downstream processing than intracellularly produced protein. Processing of intracellular, yeast-expressed rotavirus VP6 protein entails cell lysis and purification of the protein from the cell lysate that increases production costs. Therefore, engineering the yeast to enable secretion of VP6 will reduce downstream processing steps and ultimately, production costs.

Extracellular production directs the protein through the secretory pathway, which includes the glycosylation pathway in the endoplasmic reticulum and Golgi apparatus **(Figure 5.1)**. The secretory signal and glycosylation (N- and O-linked) direct the vesicular transport and protein trafficking to the plasma membrane through the secretory pathway (Ahmad *et al.*, 2014; Kim *et al.*, 2015; Kim & Kim, 2017; Lin-Cereghino *et al.*, 2013; Park *et al.*, 1997). Accurate processing of the N-terminal secretory signal from the protein of interest is an integral part of

efficient secretion. This cleavage is usually performed by proteolytic enzymes called dipeptidase, *Kex2p* and *Ste13p,* which are found in the Golgi apparatus (Brake *et al.*, 1984; Van Ooyen *et al.*, 2006; Vitale & Denecke, 1999) **(Figure 5.1)**.

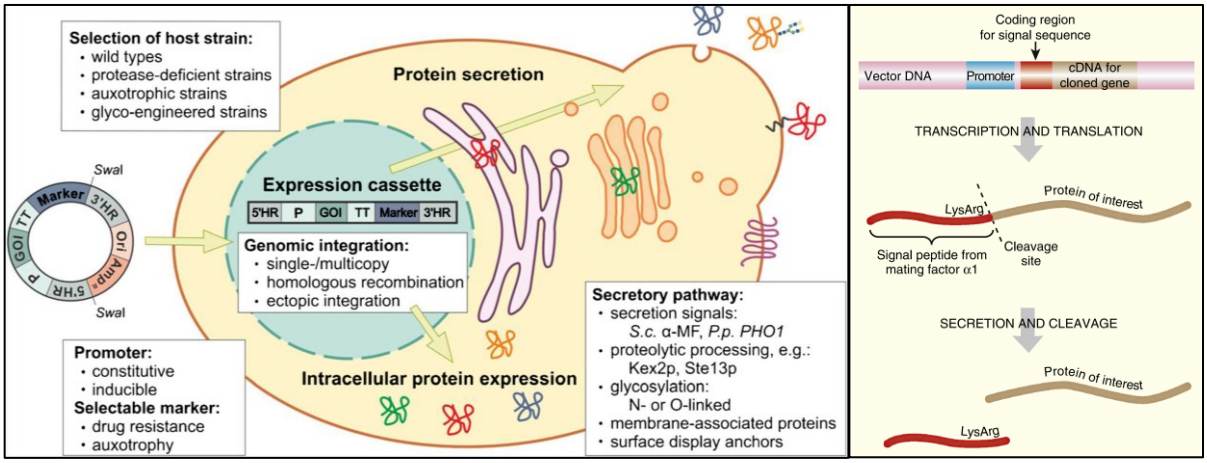


Figure 5.1: Intracellular and extracellular secretory pathways needed for the recombinant expression of heterologous genes in yeast. Expression plasmids containing gene(s) of interest (*GOI*) are linearized prior to transformation. The expression level of the protein of interest depends on the number of chromosomal integration and type and strength of the promoters. Protein secretion is under the influence of the secretory signal, which direct vesicular transport and trafficking of the protein towards the extracellular environment. *Figure adapted from (Ahmad et al., 2014).*

Secretion of rotavirus VP6 protein in the milk of transgenic rabbits has been described by Soler *et al.*, (2005). In this study, the glycosylation motifs, N-X-T/S/C (N – asparagine, X – any amino acid except proline, T – threonine, S – serine and C – cysteine), in the VP6 protein were identified and altered to prevent glycosylation during secretion. This mutated rotavirus VP6 protein was co-expressed with the VP2 protein and an immunogenic DLP was secreted into mammary milk produced by the transgenic rabbit (Soler *et al.*, 2005). Furthermore, intrarectal administration of the partially purified VP2 and non-glycosylated VP6 from the transgenic rabbit milk fused with the *Escherichia coli*-derived toxin LT(R192G) (mLT) conferred almost a complete protection against rotavirus faecal shedding in the mouse model when challenged with the murine EDIM rotavirus strain (Soler *et al.*, 2007). Eventually, this vaccine formulation induced rotavirus-specific faecal secretory IgA, systemic IgG and IgA and a rotavirus-specific Th1 response. Thus, removing any possible glycosylation motifs in the rotavirus VP6 protein does not have any significant impact on the biological functions of the protein. Therefore, the objective of this study was to identify the putative glycosylation sites in the rotavirus VP6 codon-optimized ORF, modify the identified motifs to prevent glycosylation and evaluate the intracellular production of VP6 in the yeasts. With this, the impact of the consecutive mutagenesis in the rotavirus VP6 was evaluated through the expression and antibody detection.

Based on the previous work that directed the secretion of non-glycosylated rotavirus VP6 protein in the milk of a transgenic animal (Soler *et al.*, 2005; Soler *et al.*, 2007), the VP6 encoding ORF optimised for expression in *P. pastoris/H. polymorpha* (pKM177delATG/Koz/POV6) was, therefore, used to identify potential glycosylation sites. The identified motifs were subsequently modified by replacing the potentially glycosylated asparagine (N) with glutamine (Q). This modified expression cassette was expressed intracellularly in two selected yeast strains and expression confirmed with the polyclonal antibody raised against the Nebraska Calf Diarrhoea Virus (NCDV).

5.2 Materials and Methods

5.2.1 Molecular reagents, chemicals, restriction enzymes, molecular enzymes

The following enzymes and molecular reagents were purchased from Thermo Fisher Scientific (USA): DreamTaq® Green MasterMix DNA polymerase, all restriction endonucleases used unless otherwise stated, T4 DNA ligase, polynucleotide 5'-hydroxyl-kinase (PNK), 1kb DNA ladder, DNA Gel Loading Dye (6X), 10mM ATP, Spectra™ Multicolor Broad Range Protein Ladder, PageRuler™ Prestained Protein Ladder and BigDye® Terminator v3.1 Cycle Sequencing Kit. Bio-Rad: 10x TGS buffer. KAPA BIOSYSTEMS (South Africa): 10mM dNTPs mix. Merck (Germany): bacterial agar, peptone, NaCl, ethylenediaminetetraacetate (EDTA), isopropanol, Tris(hydroxymethyl)aminomethane, glacial acetic acid, absolute ethanol, yeast extract, $MnCl_2$, CH_3COOK, $CaCl_2$, glycerol, hygromycin B. Lonza (Switzerland): SeaKem® LE Agarose. Macherey-Nagel (Germany): NucleoSpin® Gel and PCR clean-up kits and NucleoSpin® plasmid extraction kits. Calbiochem® (USA): Kanamycin sulphate and Ampicillin. Sigma-Aldrich (USA): Ponceaus S, Tween 20™, glucose, $RbCl_2$, MOPS (3-(N-morpholino)propanesulfonic acid. Integrated DNA Technology, IDT (USA): all primers used in this study.

5.2.2 Plasmids and yeast selection

The construction of pKM177delATG/Koz/POV6 has been described in **Chapter Three (Section 3.2.4, sub-sections 3.2.4.1 – 3.2.4.7)**. *The VP6 ORFs of* RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] *optimised for expression in* P*ichia pastoris/Hansenula polymorpha* (*GeneArt™, Germany)* was a generous gift from *Prof. J. Görgens, Stellenbosch University, South Africa, under a material transfer agreement (MTA).* P*ichia pastoris* (UOFS_Y1552T) and *Hansenula polymorpha* (UOFS_Y0915) were obtained from the UNESCO-MIRCEN Yeast Culture Collection Centre at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State.

5.2.3 Recombinant DNA techniques

All the procedures used in this section have been discussed in **Chapter Three, Section 3.2.4.1,** which described the preparation of bacterial competent cells and transformation procedures; **Section 3.2.4.2,** which described bacterial plasmid extraction; **Section 3.2.4.3,** which described mini-prep plasmid preparations; **Section 3.2.4.4,** which described restriction digestion of the plasmids; **Section 3.2.4.5,** which described agarose gel electrophoresis; **Section 3.2.4.6,** which described agarose gel purification of DNA fragments; **Section 3.2.4.7,** which described Sanger sequencing and **Section 3.2.5.2,** which described the *DpnI* digestion, fragment phosphorylation and ligation.

5.2.4 Site-directed mutagenesis (SDM)

N-linked glycosylation motifs in the rotavirus VP6 ORF were identified. Using a split-PCR reaction, these motifs were altered to remove the N-linked glycosylation motifs and at the same time introduce a silent mutation that allowed for screening of the mutated DNA (**Figure 5.2**).

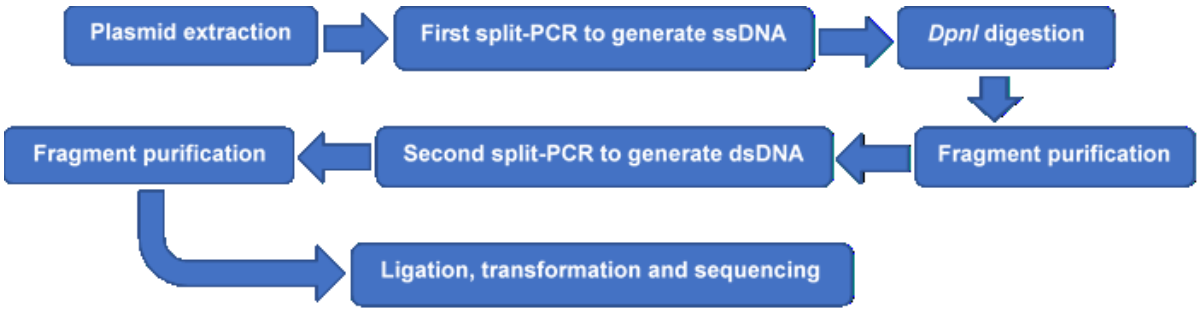


Figure 5.2: Overview of the SDM used in this study

5.2.4.1 Identification of potential motifs for post-translational modifications in VP6

The VP6 of the South Africa field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] (GenBank number FJ183358.1) was used to predict potential post-translational modifications. The VP6 protein sequence was submitted to a web tool at the Technical University of Denmark *(http://www.cbs.dtu.dk/)* for analysis of possible post-translational modifications. Possible N-terminal acetylation of methionine (Met) was investigated by making use of the NetAcet 1.0 Prediction Server webtool (Kiemer *et al.*, 2004), the presence of possible O-linked glycosylation motifs was investigated by making use of NetOGlyc 4.0 Prediction Server webtool (Steentoft *et al.*, 2013) and lastly, with NetNGlyc 1.0 Prediction Server (Gupta *et al.*, 2004), potential N-glycosylation site was predicted.

5.2.4.2 Primer design

Overlapping pair of primers were designed to introduce glutamine (Q) in place of asparagine (N). These primers were submitted to an online web tool, Watcut *(http://watcut.uwaterloo.ca/)*, which offers an online tool for restriction analysis, silent mutation scanning and SNP-RFLP analysis. In addition, silent mutations were introduced into these primer sets to create a new restriction endonuclease site at the point of mutation, which will allow for screening. The resulting primers and the new restriction sites are presented in **Table 5.1.** The approach was that the codon in "red" represents the glutamine (Q) residue. This will replace the -AAC- or -AAT- codon, which code for asparagine (N) in the rotavirus VP6 ORF with either -CAG- or -CAA-. Furthermore, the nucleotides in bold represent the silent point mutations introduced to generate new restriction enzyme sites (underlined).

Table 5.1: Primer sets used in the site-directed mutagenesis and silent mutations

Primer	*Sequence (5´ to 3´)*	*Restriction enzyme*	*Purpose*
PO_N-26_F:	GCACCTTGTACTC**G**CA**G**GT**G**TCTGACTTGATCC	*AarI*	To introduce glutamine (Q) in place of asparagine (N) at residue 26 at 51 °C annealing temperature
PO_N-26_R:	GGATCAAGTCAGA**C**AC**C**TG**C**GAGTACAAGGTGC		
PO_N-131_F:	GAGAATCAACTTCGACCAATC**G**TCTGAATACATCG	*Eam1105I*	To introduce glutamine (Q) in place of asparagine (N) at residue 131 at 60 °C annealing temperature
PO_N-131_R:	CGATGTATTCAGA**C**GATTGGTCGAAGTTGATTCTC		
PO_N-167_F:	CTTCTTTCACCTTGCAG**C**G**C**TCCCAACCAGCTCAC	*Eco47III*	To introduce glutamine (Q) in place of asparagine (N) at residue 167 at 62 °C annealing temperature
PO_N-167_R:	GTGAGCTGGTTGGGA**G**C**G**CTGCAAGGTGAAAGAAG		
PO_N-345_F:	GAAACCATGTTGGCTCAGGTTACCTCTGTTAGAC	*BPu10I*	To introduce glutamine (Q) in place of asparagine (N) at residue 345 at 63 °C annealing temperature
PO_N-345_R:	GTCTAACAGAGGTAACCTGAGCCAACATGGTTTC		
PO_N-366_F:	GTTTTCCCACCAGG**A**ATGCAATGGACCGACTTGATC	*BsmI*	To introduce glutamine (Q) in place of asparagine (N) at residue 366 at 64 °C annealing temperature
PO_N-366_R:	GATCAAGTCGGTCCATTGCAT**T**CCTGGTGGGAAAAC		

5.2.4.3 Split-PCR, *DpnI* digestion, phosphorylation, ligation and transformation

Split-PCR was used to amplify the recombinant plasmid following the overview shown in **Figure 5.1**. The PCR was carried out with KAPA® HiFi DNA polymerase, as stated in **Chapter Three, Section 3.2.5, sub-section 3.2.5.1**. The purified fragment from this PCR was used as

a template for the second round of PCR to generate a complete dsDNA. This product was phosphorylated, ligated, transformed into competent bacterial cells, screened and sequenced following the procedures highlighted in **Section 5.2.3. Table 5.2** shows the list of primers used for Sanger sequencing. Site-directed mutagenesis was performed sequentially, i.e. after confirmation of the successful mutagenesis using Sanger sequencing for N-26-Q, a positive clone for N-26-Q was used to construct N-131-Q. A positive clone for N-26-Q and N-131-Q was subsequently selected for N-167-Q. A clone positive for the prior three mutations was selected to prepare N-345-Q, and finally, a positive clone to N-345-Q that also contained the previous four mutations was used for N-366-Q mutagenesis.

Table 5.2: Primer sets used for Sanger sequencing at each mutagenic site

Primers	Sequence (5´→3´	Tm (°C)	Purpose
PO_pKM177VP6_KozF	CTCACACCCGAACTCACCATGGACGTTTTGTACTCTTTG	68.3	Used to sequence the mutagenic sites and the entire VP6 ORF
PO_VP6Seq PF1	GGACGAAATGGTTAGAGAATCTC	56.8	
PO_VP6Seq PR2	CTGTTCAAGGTGAAAGAAGC	54.8	
PO_VP6Seq PF2	CAACACCTACCAAGCTAGA	53.8	

5.2.4.4 Yeast expression

Procedures for competent yeast cell preparation, yeast transformation and colony PCR have been described in **Chapter Four, Sections 4.2.4** and **4.2.5.** The primer set used for colony PCR is shown in **Table 5.3**. Bacterial expression of the rotavirus VP6 as a positive control has been discussed in **Chapter Four, Section 4.2.6, sub-section 4.2.6.1.** Yeast expression, preparation of samples for SDS-PAGE and western blot analysis were provided in **Chapter Four, Section 4.2.6, sub-sections 4.2.6.2 – 4.2.6.4.** This was so because the objective of this chapter was to evaluate the intracellular expression of the rotavirus VP6 protein whose glycosylation sites have been altered.

Table 5.3: Primer set used for colony PCR targeting 1 052 bp fragment

Primer	*Sequence (5´ to 3´)*	*Fragment*	*Purpose*
PO_N-26_F:	GCACCTTGTACTC**G**CA**G**GT**G**TCTGACTTGATCC	1 052 bp	Colony PCR to confirm integration of the expression cassette
PO_N-366_R:	GATCAAGTCGGTCCATTGCAT**T**CCTGGTGGGAAAAC		

5.3 Results

5.3.1 Identification of possible post-translation motifs in RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] VP6

Rotavirus VP6 is a multifunctional core capsid protein interacting with VP2, VP4 and VP7 to produce rotavirus triple-layered particle (Settembre *et al.*, 2011). This protein can self-polymerise to form the oligomeric structure, which is highly immunogenic (Lappalainen *et al.*,

2016; Pastor *et al.*, 2014). With the aid of web tools, possible post-translation modifications in the VP6 protein of the South Africa field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] was evaluated. No acetylation of the N-terminal methionine was predicted **(APPENDIX B – B1)**. Furthermore, no O-linked glycosylation of rotavirus VP6 protein was predicted because none of the predicted sites had a threshold probability higher than or equal to 0.5 **(APPENDIX B – B2)**. However, seven potential N-linked glycosylation sites were identified for this protein **(Table 5.4)**. Three of these had a high threshold probability above or equal to **0.5** and were identified as 1+ or 3+ **(Table 5.4** and **Figure 5.3)**. Additionally, two other sites with threshold proximity close **0.5** were regarded as additional sites **(Figure 5.3A)**. In total, five potential N-linked glycosylation sites were therefore identified for this VP6 protein: N-26, N-131, N-167, N-345 and N-366 **(Figure 5.3B)**.

5.3.2 Mutagenesis of the glycosylation sites on the VP6 encoding ORF

The five potential glycosylation sites on the VP6 encoding ORF was sequentially subjected to site-directed mutagenesis converting asparagine coding sequences to glutamine coding sequences and introducing new restriction site for screening **(Table 5.1; APPENDIX B – B3)**.

Figure 5.4A shows the restriction digestion to confirm the introduction of the first mutation, N-26-Q, where -AAC- (asparagine, N) was changed to -CAG- (glutamine, Q) at residue 26 in the encoding optimised VP6 ORF. At the same time, an *AarI* restriction enzyme site was introduced. Digestion with *AarI* and *Eco47III* restriction enzymes resulted in 7 561 bp and 1 131 bp as expected indicating the successful introduction of the silent mutations. This result also suggested the successful change of –AAC- to –CAG- (N to Q) which was confirmed with Sanger sequencing **(Figure 5.4B)**. For each of the subsequently predicted motifs, restriction enzyme digests confirmed the introduction of silent mutations **(APPENDIX B – B3)**. Subsequently, Sanger sequencing confirmed the successful exchange of the codons resulting in the mutation of N-131-Q, N-167-Q, N-345-Q and N-366-Q (**Figure 5.5)**.

Table 5.4: NetNGlyc 1.0 prediction of N-linked glycosylation motifs in the VP6 protein of the rotavirus field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6]

SeqName	Position	Potential (Threshold = 0.5)	Jury	N-Glycosylation
Sequence	26 NVSD	0.7840	(9/9)	+++
Sequence	131 NSSE	0.4848	(4/9)	-
Sequence	167 NRSQ	0.6386	(8/9)	+
Sequence	299 NMTP	0.1510	(9/9)	- - -
Sequence	345 NVTS	0.7540	(9/9)	+++
Sequence	366 NWTD	0.4704	(6/9)	-
Sequence	373 NYSP	0.1328	(9/9)	- - -

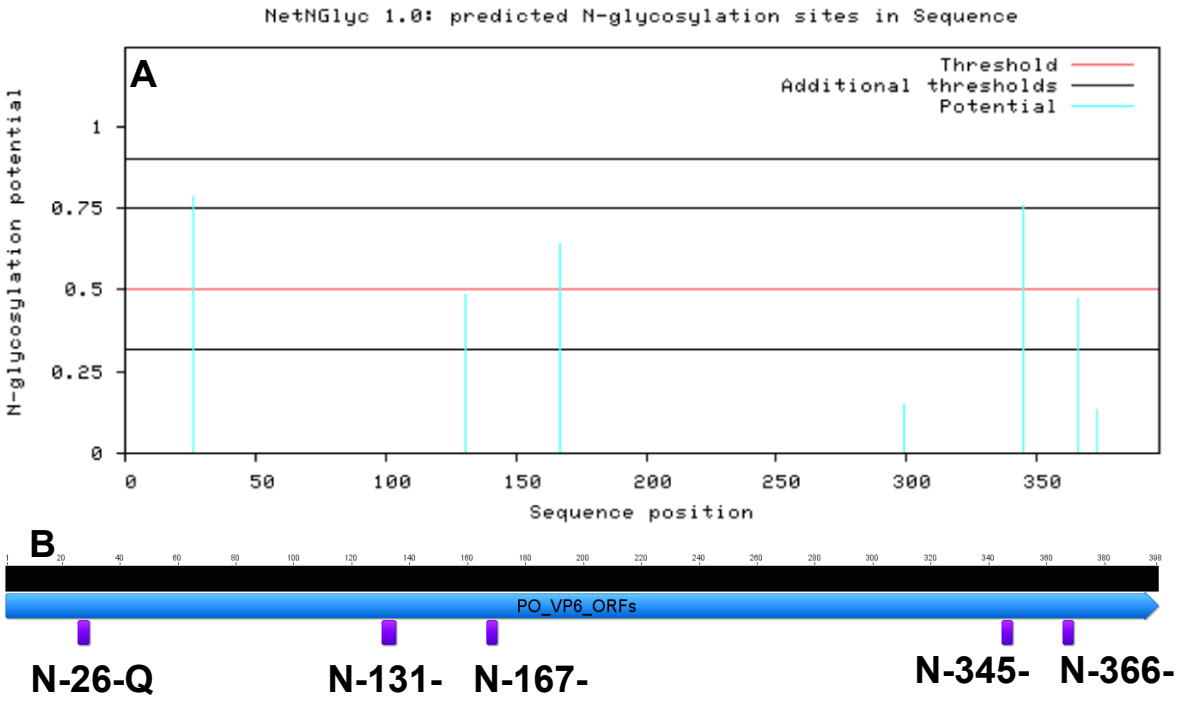


Figure 5.3: NetNGlyc prediction result for the N-glycosylation of asparagine in the VP6 protein of the South Africa field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6]. (A) Each of the vertical light blue lines represents the potential glycosylation site, with the threshold ≥ **0.5**. **(B)** Based on the threshold value, asparagine (N) at position 26, 131, 167, 345 and 366 will likely be glycosylated.

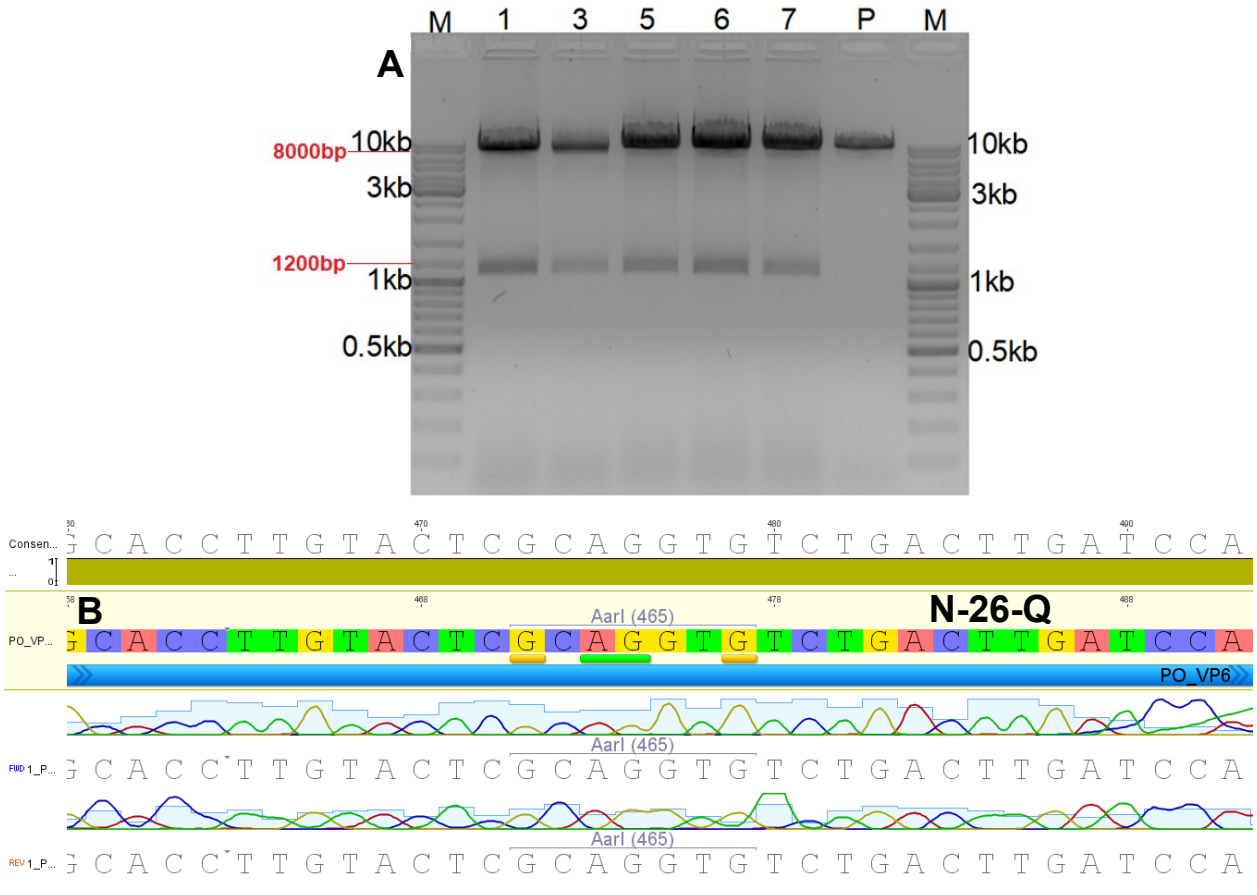


Figure 5.4: Site-directed mutagenesis of -AAC- to -CAG- (N-26-Q) in pKM177delATG/POVP6 (A) Five clones from the miniprep plasmid were screened with *AarI* and *Eco47III* restriction enzymes. Expected fragments of 7 561 bp and 1 131 bp were observed for all the clones (1, 3, 5, 6 and 7). The specific use of *AarI* restriction confirmed that the N-26-Q mutagenesis was successful. **(B)** Confirmation of AAC to CAG change was performed with Sanger sequencing. "M" is the GeneRuler™ 1 kb DNA Ladder and "P" is the pKM177delATG/Koz/POVP6 plasmid.

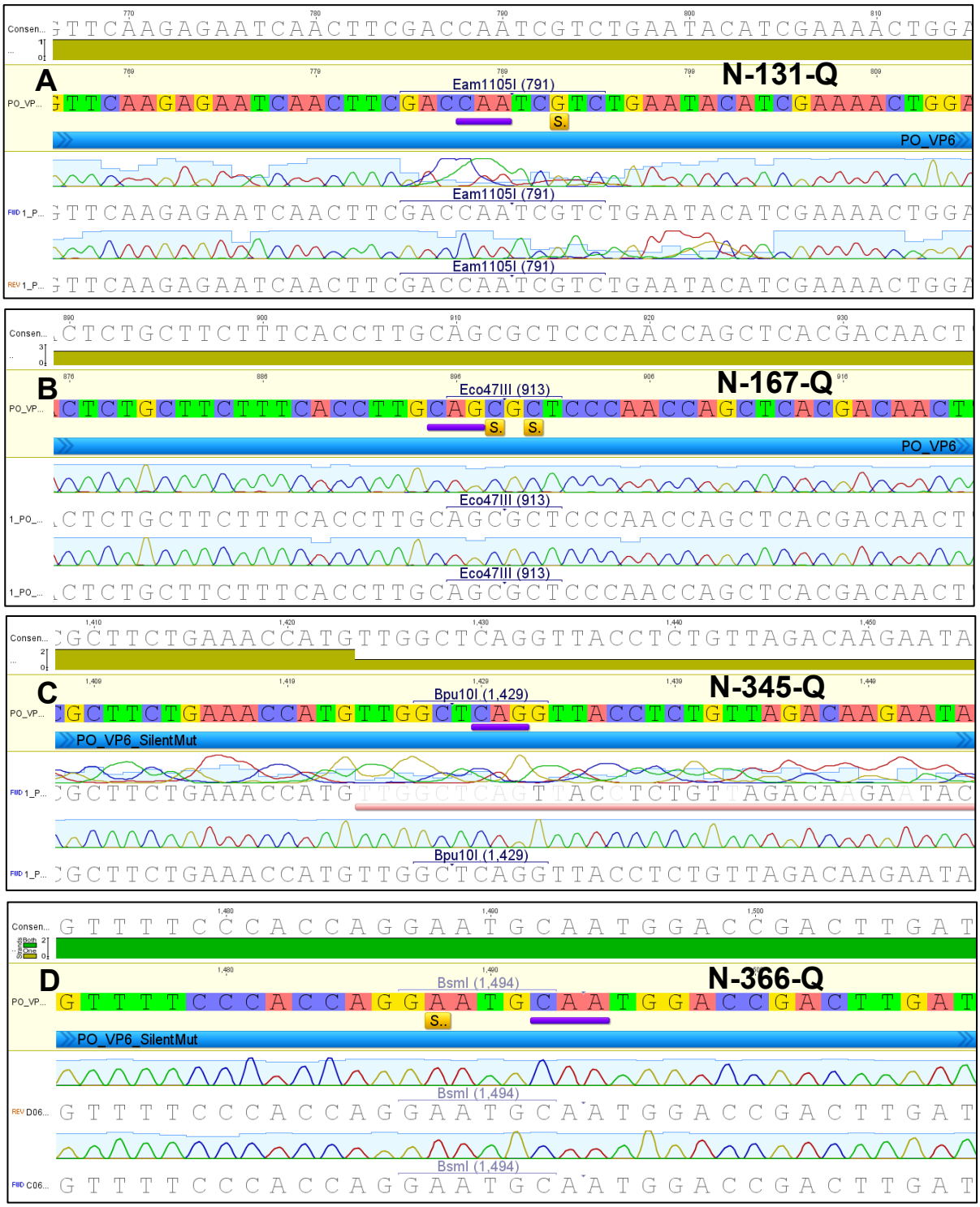


Figure 5.5: Sanger sequencing confirming successful site-directed mutagenesis at each of the glycosylation sites in the rotavirus VP6 ORF. (A) -AAC- changed to -CAA- as underlined in purple for N-131-Q mutagenesis **(B)** -AAC- changed to -CAG- as underlined in purple for N-167-Q

mutagenesis **(C)** -AAC- changed to -CAG- as underlined in purple for N-345-Q mutagenesis **(D)** -AAC- changed to -CAA- as underlined in purple for N-366-Q mutagenesis

5.3.3 Expression of the mutagenic recombinant plasmid in yeast

Following the successful removal of the N-linked glycosylation sites in the rotavirus VP6 protein by mutating the encoding nucleotides such that asparagine was changed for glutamine, the mutated plasmid (pKM177delATG/Koz/POVP6_mut) was transformed into *P. pastoris* (UOFS_Y1552T) and *H. polymorpha* (UOFS_Y0915). **Figure 5.6** shows the primer binding site for colony PCR targeting 1 052 bp and the resolved gel for each of the yeast strains, confirming genomic integration of the expression cassette. Low transformation efficiency was observed for the selected two yeasts. In fact, only one colony was formed for *H. polymorpha* (UOFS_Y0915) and fewer than 10 colonies were obtained for *P. pastoris* (UOFS_Y1552T).

With western blot analysis, the mutated VP6 protein was detected in *H. polymorpha* (UOFS_Y0915) only. There was no detection in all the *P. pastoris* (UOFS_Y1552T) colonies tested **(Figure 5.7)**. Furthermore, the expression of the mutated rotavirus VP6 protein from this *H. polymorpha* (UOFS_Y0915) was also compared with the expression of the unmutated protein from the same yeast strain and a highly comparable expression and detection were observed **(Figure 5.8)**.

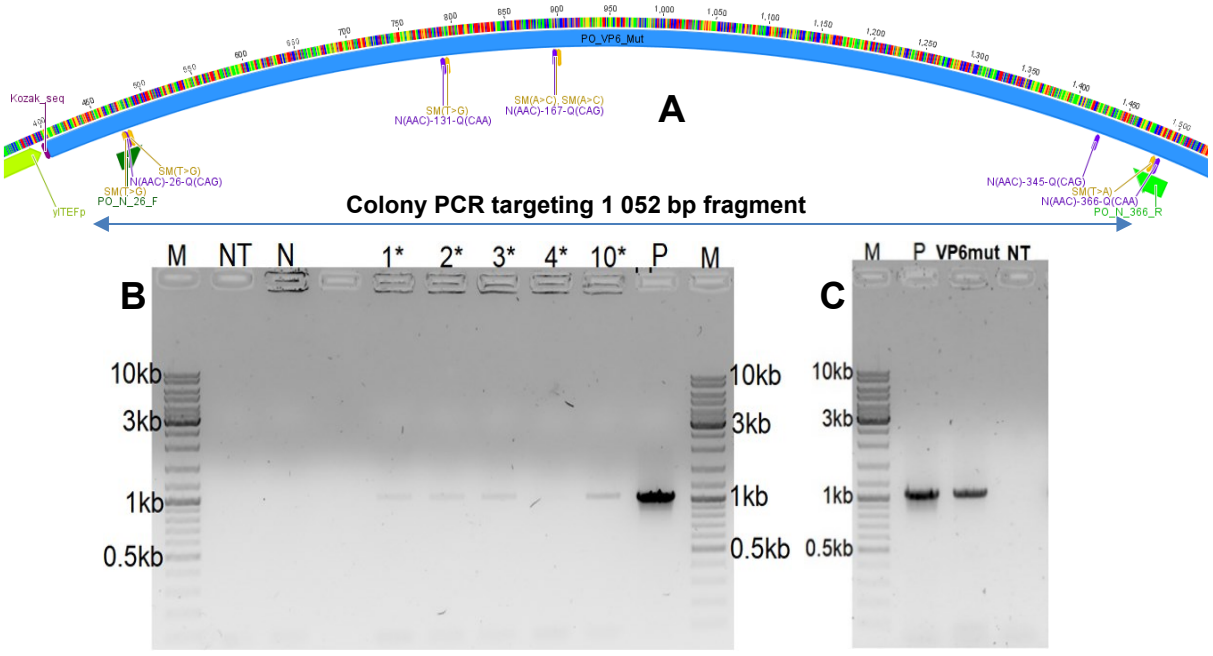


Figure 5.6: Overview of colony PCR screen to identify genomic integration. (A) the primer binding site within the mutated VP6 ORF targeting a 1 052 bp fragment to confirm yeast genomic integration of the expression cassette containing the mutated rotavirus VP6 ORF. **(B)** colony PCR amplified fragments from *P. pastoris* (UOFS_Y1552T) and **(C)** from *H. polymorpha* (UOFS_Y0915). "M" is the GeneRuler™ 1 kb DNA Ladder, "NT" is the negative control (no template control), "N" is the untransformed yeast as negative and "P" is the mutated recombinant plasmid as a positive control.

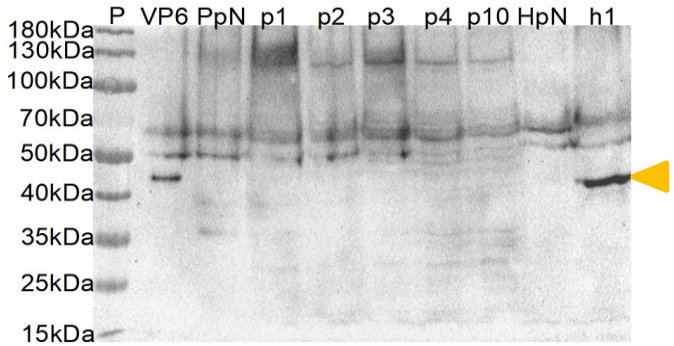


Figure 5.7: Western blot analysis showing the detection of the recombinant mutated rotavirus VP6 protein. Expression in *P. pastoris* (UOFS_Y1552T) is represented as samples – p1, p2, p3, p4 and p10 with PpN as a negative, untransformed yeast and *H. polymorpha* (UOFS_Y0915) has one sample – h1 and HpN as the negative, untransformed yeast. "P" is the PageRuler™ Prestained Protein Ladder. "VP6" is the bacterial-expressed VP6 protein as a positive control.

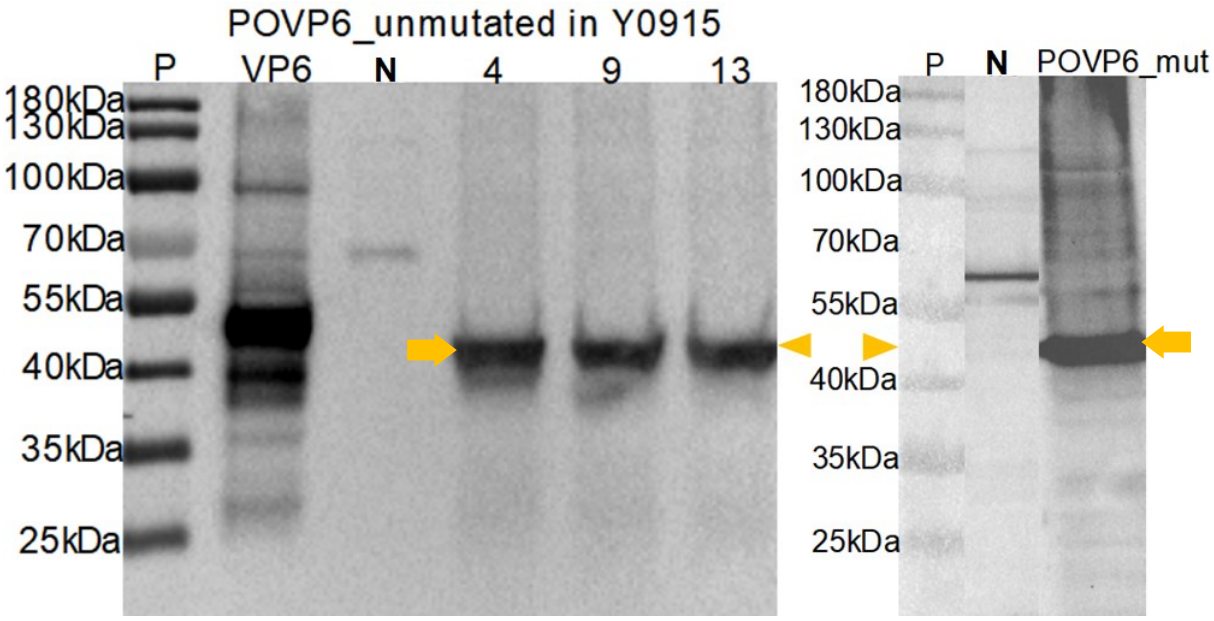


Figure 5.8: Western blot analysis comparing the expression of the unmutated rotavirus VP6 protein with mutated VP6 protein from *H. polymorpha* (UOFS_Y0915). "P" is the PageRuler™ Prestained Protein Ladder, "VP6" is the bacterial-expressed VP6 protein as a positive control, "N" is the untransformed yeast strain as a negative control, unmutated colonies 4, 9, 13 compared with the only one mutated colony in the western blot.

5.4 Discussion

Previously, rotavirus SA11 VP6 protein has been analysed and characterised at the N- and C-terminal for the most probable PTMs (Emslie *et al.*, 2000). With **ExPASy FindMod tool**, PTM of the rotavirus SA11 VP6 showed N-terminal acetylation of methionine which was also confirmed with post source decay and electrospray ionisation mass spectrometry (Emslie *et al.*, 2000). Further confirmation was the blockage of N-terminal methionine analysis to Edman sequencing due to acetylation (Emslie *et al.*, 2000). In contrast, based on the predictive web tools used in this study, it was predicted that the N-terminal methionine of RVA/HUMAN-

wt/ZAF/GR10924/1999/G9P[6] VP6 protein (GenBank number FJ183358.1) was likely not to be acetylated **(ADDENDUM - A)**. It was further revealed that acetylation of the N-terminal methionine usually prefers short R-group amino acids residues within the next three downstream of the N-terminal methionine. From this evaluation, it was clearly observed that the next four amino acid residues downstream of the N-terminal methionine were aspartate, valine, leucine and tyrosine. These are amino acid residues with relatively large R-groups that are acidic, neutral and basic in nature.

In this study, it was observed that rotavirus field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] VP6 protein lacked motifs for O-glycosylation and as such, no potential O-glycosylation was predicted for the rotavirus VP6 capsid protein used in this study. However, there were 7 potential N-glycosylation sites predicted for this protein. The N-glycosylation motif is N-X-T/S/C, where **N** is asparagine, **X** is any amino acid except proline, T is threonine (the most common amino acid in this motif), **S** is serine and **C** is cysteine. Three potential sites (26-NVSD, 167-NRSQ and 345-NVTS) out of these seven showed a high predictive threshold > 0.5 for glycosylation if the protein is to be secreted. There were 2 (131-NSSE and 366-NWTD) additional glycosylation sites with competitive threshold values close to **0.5** and these were added as potential N-glycosylation sites. **(Table 5.4** and **Figure 5.3)**. With Sanger sequencing, removal of the potential N-glycosylation sites on the rotavirus VP6 protein was confirmed **(Figure 5.5)**.

In-depth knowledge into the role of glycosylation of rotavirus proteins has shown that glycosylation plays important roles during rotavirus pathogenesis, capsid assembly and antibody recognition (Cornaglia *et al.*, 1991; Estes & Greenberg, 2013; Graham *et al.*, 1984; Tian *et al.*, 1995; Venkataram-Prasad *et al.*, 2014). By consensus, both O- and N-glycosylation share similar glycosylation motifs (Wilson *et al.*, 1991). Different strains of rotavirus show different patterns of glycosylation and that VP6 epitopes may be carbohydrate-dependent especially in animal rotavirus strains (Cornaglia *et al.*, 1991). From this study, the VP6 capsid of the human rotavirus A G9P[6] field strain was predicted with the web tool to lack O-glycosylation but possessed N-glycosylation motifs. However, monoclonal and polyclonal antibody characterisations of animal rotavirus VP6 capsid protein from bovine (NCDV, Quebec Q17 and 678) and porcine (OSU) have been used to predict the presence of immunogenic O-glycosylation epitopes but no N-glycosylation (Cornaglia *et al.*, 1991). N-glycosylation occurs co-translationally in the rough endoplasmic reticulum under the influence of the signal peptide (Kornfeld & Kornfeld, 1985) while O-glycosylation occurs post-translationally in the Golgi apparatus (Roth, 1987). This may probably explain the conservative nature of the rotavirus VP6 epitopes, the proximity of the DLPs with endoplasmic reticulum to acquire glycosylated VP7 during capsid assembly and antigenic variations among the rotavirus strains.

Rotavirus VP6 capsid is highly conserved and immunogenic and capable of eliciting protection against rotavirus shedding and infection (Choi *et al.*, 2004; Choi *et al.*, 2000). The protective efficacy of rotavirus VP6 capsid derived from *in vivo* delivery gene against rotavirus challenge in animal studies has also been proven in DNA-vaccine technology with or without priming/boosting (García-Díaz *et al.*, 2004; Yang *et al.*, 2001; Yuan *et al.*, 2005). In this study, intracellular expression and detection of the mutated rotavirus RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] VP6 protein were achieved in *H. polymorpha* (UOFS_Y0915) after removal of five possible glycosylation sites by changing the asparagine (N) to glutamine (Q) for each of the motifs. The detection was achieved with polyclonal antibody raised against Nebraska Calf Diarrhoea Virus (NCDV) particles. In this study, there was no expression of the mutated recombinant rotavirus VP6 protein from *P. pastoris* (UOFS_Y1552T). This result seemed to confirm the inability of this strain of *P. pastoris* to express the "wild-type" intracellularly in **Chapter Four (Table 4.5)**. Furthermore, inability of this yeast to express recombinant gene may sometimes be as a result of premature termination of the transcript, which may occur in *P. pastoris* if the recombinant DNA contains "A+T rich regions" (Henikoff & Cohen, 1984; Irniger *et al.*, 1991; Scorer *et al.*, 1993; Zaret & Sherman, 1984). As reported in Estes and Cohen, (1989), all the rotavirus encoding gene sequences are A+T rich (ranging 58 to 67%) and the codon usage is biased against CGN and NCC codons (N represents any nucleotide), as in many eukaryotic and other viral genes (Porter *et al.*, 1979). Though the VP6 encoding ORF optimised for *P. pastoris/H. polymorpha,* as used in this study contained about 56% A+T, it may therefore be suspected that either the expression level was lower and beyond the antibody detection due to low integrated copy number of the recombinant plasmid into the yeast genome **(Figure 5.6 (B) vs (C))** due to inefficient transformation or random termination of the transcripts, which can prevent the expression of a complete detectetable rotavirus VP6 protein from the *P. pastoris,* has occurred.

In a similar fashion, Soler and co-workers performed five consecutive mutations to prevent glycosylation during secretion of the rotavirus bovine RF strain VP6 protein co-expressed with the VP2 encoding ORFs. With all the mutations carried out on the VP6 ORF, immunogenic DLPs were obtained by secretion in the milk of transgenic rabbits (Soler *et al.*, 2005). These DLPs induced 100% seroconversion and subsequent reduction in viral antigen shedding in mice gavaged with this transgenic milk (Soler *et al.*, 2005). This means mutation of the VP6 ORF to prevent glycosylation may not hinder the biological functions of rotavirus VP6 from being a non-replicating alternative vaccine candidate against rotavirus infection and at the same time enabling extracellular secretion for large-scale production of VP6.

Multiple alignments of VP6 proteins from different strains have indicated residues 32 – 64, 155 – 173, 208 – 274, 295 – 311 and 380 – 397 as regions containing group specific antigenic

sites (Buragohain *et al.*, 2008; Kohli *et al.*, 1993a; Kohli *et al.*, 1992). Comparing with the glycosylation motifs of rotavirus VP6 protein used in this study, four out of the five mutagenic sites did not occur within these antigenic domains. The exception was N-167, which was found within the 155 – 173 antigenic region. Because this mutation had already been tested in the VP6 protein that successfully formed DLPs with VP2 capsid and induced immune response in mice, this one-point mutation, therefore, may not have an effect on the antigenicity of the rotavirus VP6 protein (Soler *et al.*, 2005), however, the ultrastructure of this protein can only be predicted with the use of transmission electron microscope.

P. pastoris and *H. polymorpha* yeasts selected for intracellular expression of the mutated rotavirus VP6 protein should be able to express this recombinant mutated protein because the encoding ORF was specifically optimised for these yeasts. However, poor transformation efficiency was observed for the two yeasts. While *H. polymorpha* (UOFS_Y0915) produced only one colony, fewer than ten colonies were obtained for *P. pastoris* (UOFS_Y1552T). Surprisingly, the single colony of *H. polymorpha* (UOFS_Y0915) was able to express the mutated rotavirus VP6 protein **(Figures 5.7** and **5.8)**. The reason for this outcome was not clear but it is possible that the bicine-NaOH method was insufficient to propagate the transformation in these yeasts. Transformation by electroporation is better and may be recommended for future work (Becker & Guarente, 1991; De Backer *et al.*, 1999; Suga & Hatakeyama, 2003; Thompson *et al.*, 1998; Wu & Letchworth, 2004). There was already an improved optimisation for the transformation of yeast based on the use of lithium chloride and polyethylene glycol as reviewed by Kawai *et al.*, (2010) instead of bicine-NaOH and polyethylene glycol used in this study. Furthermore, the work of Sagt *et al.*, (2000), suggested that targeting the *his4 locus* in *P. pastoris* for chromosomal integration improves stable genome integration.

In conclusion, five potential glycosylation sites on the VP6 encoding ORF was sequentially removed to engineer a mutated protein without any glycosylation motifs. This is the first report describing the engineering of a recombinant, rotavirus VP6 protein that was mutated to prevent glycosylation during secretion by yeast. Further research is recommended to verify the ultrastructure of this mutated rotavirus VP6 protein in the form of tubule formation and *in vivo* immunological studies to confirm the immunogenicity of the protein.

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Chapter Six

Secretion of rotavirus VP6 by *Kluyveromyces lactis*

6.1 Introduction

Constitutive or inducible promoters are capable of driving expression to not only achieve moderate expression levels but also an overproduction of recombinant proteins expression (Partow *et al.*, 2010). Constitutive promoters offer relatively constant levels of expression, though generally low quantities are obtained. Inducible promoters are commonly used when separation of growth and production is required to direct high-level expression of heterologous genes (Partow *et al.*, 2010). Promoter exchange allows for optimisation of heterologous protein production in yeast. Weak promoters have a low transcriptional yield while strong promoters can lead to excessive transcription of a gene causing cellular stress and death due to the unfolded protein response (UPR) (Partow *et al.*, 2010).

Recombinant protein production using the wide-range expression vector used in this study is driven by the ylTEFp (transcriptional elongation factor EF-1α) from *Yarrowia lipolytica* (Albertyn *et al.*, 2011) **(Figure 6.1)**. The TEF promoter is a strong promoter, but may not be recognised equally across different yeast species. Therefore, promoter usage was optimised in this chapter by exchanging it for a homologous promoter that matches the specific yeast strain. The objective of this is to enhance recombinant gene expression.

Targeting a recombinant protein to the secretory pathway for secretion is mainly determined by the signal sequence attached to the N-terminal of the polypeptide. This signal peptide guides the recombinant protein to the endoplasmic reticulum for posttranslational modification including glycosylation, removal of the pre- and pro-regions of the signal peptide and protein folding and then into the Golgi apparatus where the signal peptide is finally processed before vesicular trafficking of the recombinant protein to the extracellular milieu (Lin-Cereghino *et al.*, 2013). The most widely used heterologous signal peptide is the α-mating factor (α-MF) secretion signal from *S. cerevisiae*. This signal peptide is first processed in the endoplasmic reticulum with an *endopeptidase* and then in the Golgi apparatus with two consecutive enzymatic reactions involving *Kex2 endopeptidase* and *Ste13* (Brake *et al.*, 1984; Kurjan & Herskowitz, 1982).

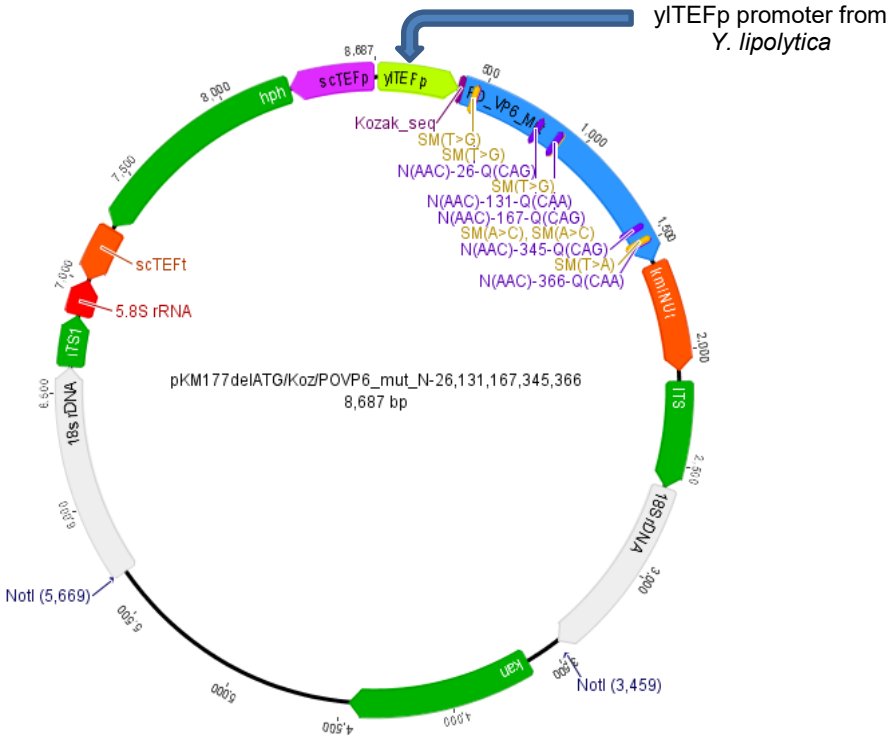


Figure 6.1: Features of the pKM177delATG/Koz/POVP6_mut expression plasmid. This is the expression vector containing the mutated ORF for rotavirus VP6 optimised for *P. pastoris/H. polymorpha* expression. The 5 consecutive mutations removed the potential glycosylation sites from the ORF (Chapter 5). *NotI* cleavage site allows for expression plasmid linearisation prior to transformation.

This α-MF secretion signal from *S. cerevisiae* (also known as yeast mating pheromone α-factor peptide) has been used across many different yeast species such as *P. pastoris, H. polymorpha, Y. lipolytica* and *K. lactis* (Brake *et al.*, 1984; Hsieh & Da Silva, 1998; Lin-Cereghino *et al.*, 2013) with different levels of secretion for recombinant proteins obtained (Vieira Gomes *et al.*, 2018). Factors such as the nature of the recombinant protein, the pro-peptide sequence, trafficking proteins and proteolytic cleavage greatly determine the secretion level of recombinant proteins in yeasts (Hou *et al.*, 2013; Hou *et al.*, 2012a; Hou *et al.*, 2012b; Tomimoto *et al.*, 2013). The use of native signal peptides is more efficient and productive (Rocha *et al.*, 1996; Vadhana *et al.*, 2013) than the synthetic or heterologous signal peptides. Some examples of signal peptides are SUC2 (invertase from *S. cerevisiae*), PHO1 (acid phosphatase from *S. cerevisiae* or *P. pastoris)* and PHO5 (acid phosphatase from *K. lactis*).

In **Chapter Five,** the expression vector, pKM177delATG/Koz/POVP6, containing the VP6 codon-optimised ORF for *P. pastoris/H. polymorpha* was mutated to prevent glycosylation of the rotavirus VP6 protein. This protein was expressed intracellularly in the yeast and expression of VP6 was detected. In this chapter, promoter optimisation was carried out by exchanging the heterologous ylTEFp from *Y. lipolytica* **(Figure 6.1)** with a homologous klLAC4p promoter from *K. lactis* to enhance recombinant expression of the mutated rotavirus

VP6 protein. *K. lactis* α-mating factor (α-MF_KL) was also introduced upstream of the mutated VP6 ORF. The newly constructed expression plasmid was transformed into *K. lactis* (UOFS_Y1167). Secreted non-glycosylated rotavirus VP6 protein was detected with ROTADIAL™ ELISA Kit (INTA - Argentina). Part of the work presented in this chapter was conducted at the Instituto Nacional de Tecnologia Industrial (INTI), Bueno Aires, Argentina during a scientific visit from 5 November to 1 December 2018.

6.2 Materials and Methods

6.2.1 Molecular reagents, chemicals, restriction enzymes and molecular enzymes

The following enzymes and molecular reagents were purchased from Thermo Fisher Scientific (USA): all restriction endonucleases unless otherwise stated, DH5α competent *E. coli,* GeneRuler™ 1 kb DNA ladder, DNA Gel Loading Dye (6x) and Phusion® High-Fidelity DNA polymerase. Applied Biosystems (USA): BigDye® Terminator v3.1 Cycle Sequencing Kit. Merck (Germany): bacterial agar, peptone, NaCl, ethylenediaminetetraacetate (EDTA), isopropanol, Tris(hydroxymethyl)aminomethane, glacial acetic acid, absolute ethanol, yeast extract, glycerol. Lonza (Switzerland): SeaKem® LE Agarose. Macherey-Nagel (Germany): NucleoSpin® Gel and PCR clean-up kits and NucleoSpin® plasmid extraction kits. Calbiochem® (USA): Kanamycin sulphate. Sigma-Aldrich (USA): glucose. Integrated DNA Technology, IDT (USA): all primers used in this study unless otherwise stated. Instituto Nacional de Tecnología Agropecuaria (INTA, Argentina): ROTADIAL™ ELISA Kit. New England BioLabs Inc: NEB® 10-beta Competent *E. coli* (High Efficiency), NEBuilder® HiFi DNA Assembly Cloning Kit. Invitrogen: hygromycin B (100 mg/mL). PB-L Productos Bio-Logicos®: Ladder 1 kpb *plus* (ready-to-use).

6.2.2 Plasmids

Previously, **pKM177delATG/Koz/POV6** (8 689 bp) was constructed in **Chapter Three** and its VP6 encoding ORF mutated in **Chapter Five** to prevent possible glycosylation during secretion. These two plasmids together with pKLAC2 were used in this study **(Table 6.1).**

Table 6.1: List of plasmids used in this study

Name and size	Information	Usage
pKLAC2 (9 107 bp)	New England BioLabs® (USA)	The *K. lactis* promoter, klLAC4p, that drives expression of the *LAC4* gene that encodes β-galactosidase in the yeast, and klα-MF secretion signal sequence were amplified from this plasmid
pKM177delATG/Koz/POVP6	Constructed in Chapter Three	The ylTEFp promoter in this plasmid was exchanged with the klLAC4p promoter and the klα-MF was introduced as the signal sequence.
pKM177delATG/Koz/POVP6mut	Constructed in Chapter Five	The ylTEFp promoter in this plasmid was exchanged with the klLAC4p promoter and the klα-MF was introduced as the signal sequence

Prof. J. Albertyn (Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein Campus) provided pKLAC2, a commercial plasmid from New England BioLabs (USA).

6.2.3 Primers

Primers were specifically designed using the NEBuilder webtool. All the primers used are listed in **Tables 6.2, 6.3 and 6.4**.

Table 6.2: Specific use of the primers for fragments generation required for NEBuilder assembly

Primer name	Purpose	Fragment
pKM177/POVP6_F pKM177/POVP6_R	Primer pair used to amplify pKM177delATG/Koz/POVP6 and pKM177delATG/Koz/POVP6mut, excluding the ylTEFp and Kozak sequence	8 309 bp
klLAC4p_F klLAC4p_R	Primer pair used to amplify klLAC4 promoter from pLAC2 plasmid	1 529 bp
klα-MF_F klα-MF_R	Primer pair used to amplify klα-MF signal sequence, including Kozak sequence (-GCCACC-), from pLAC2 plasmid	273 bp

Table 6.3: NEBuilder primers and the overlapping genes

Primer name	Overlaps	Sequence (5´-end to 3´-end)	Anneals
pKM177/POVP6_R	klpLAC4p	ATTACCCTGTTATCCCTAGATCTGTTTAGCTTG	pKM177/POVP6 at 60.9 ºC
klLAC4p_F	pKM177/POVP6	tctagggataacagggtaatGGGGATCGACTCATAAAATAG	klLAC4p at 60.3 ºC
klLAC4p_R	klα-MF	agaatttcatggtggcGCTTTTGTCTCTTCACCTTTTC	klLAC4p at 60.3 ºC
klα-MF_F	klLAC4p	agacaaaagcgccaccATGAAATTCTCTACTATATTAGCCG	klα-MF at 59.9 ºC
klα-MF_R	pKM177/POVP6	aaagagtacaaaacgtccatAGCTTCAGCCTCTCTTTTC	klα-MF at 59.9 ºC
pKM177/POVP6_F	klα-MF	ATGGACGTTTTGTACTCTTTG	pKM177/POVP6 at 60.9 ºC

Nucleotides in "uppercase" are gene-specific primer sequences while "lowercase" nucleotides represent overlapping regions.

Table 6.4: Primers used for Sanger sequencing

Primer name	Sequence	Tm (ºC)	Use
pKM_Seq_F1	5´-GTCAAGACTGTCAAGGAGGG-3´	57.5	Primers used to confirm the introduction of the new promoter and signal sequence
VP6_Seq_PR1	5´-GACAAAGAGTACAAAACGTCC-3´	55.1	

pKM_Seq_F1 primer was obtained from Inqaba Biotec™, South Africa

6.2.4 Recombinant DNA techniques

NEB® 10-beta Competent *E. coli* (High Efficiency) that accompanied the NEBuilder® HiFi DNA Assembly Master Mix/NEBuilder HiFi DNA Assembly Cloning Kit, with genotype Δ*(ara-leu) 7697 araD139 fhuA* Δ*lacX74 galK16 galE15 e14-* ϕ*80*d*lacZ*Δ*M15 recA1 relA1 endA1 nupG rpsL* (Str^R) *rph spoT1* Δ*(mrr-hsdRMS-mcrBC),* was used for the propagation of NEBuilder recombinant expression plasmids. Also, DH5α competent *E. coli* was used to propagate the recombinant plasmid.

All molecular procedures used have been described in **Chapter Three, Section 3.2.3,** which includes the preparation of bacterial growth culture and selective media; **Section 3.2.4.1,** the preparation of the bacterial competent cells and transformation procedures; **Section 3.2.4.2,** bacterial plasmid extraction; **Section 3.2.4.3,** mini-prep plasmid preparation by boiling; **Section 3.2.4.4,** restriction digestion of plasmids; **Section 3.2.4.5,** agarose gel electrophoresis; **Section 3.2.4.6,** agarose gel purification of DNA fragments and **Section 3.2.4.7,** Sanger sequencing, with the primers used listed in **Table 6.4**.

6.2.4.1 Seamless cloning

Fragments were generated from different plasmid sources and were ligated in a seamless cloning approach using the NEBuilder® HiFi DNA Assembly Cloning Kit. The overall strategy for generating promoter sequences and signal peptide encoding sequences is shown in **Figure 6.2.** pKM177delATG/ylTEFp/Koz/POV6, constructed in **Chapter Three,** and pKM177delATG/ylTEFp/Koz/POV6mut constructed in **Chapter Five** were amplified using PCR, excluding the ylTEFp region as well as the Kozak sequence. The promoter region and the signal sequence were introduced to construct either the "wild-type" pKM177delATG/klLAC4/Koz/klα-MF/POVP6 and "mutated" pKM177delATG/klLAC4/Koz/klα-MF/POVP6mut recombinant plasmids following the procedure displayed in **Figure 6.2**. Dr Danilo Legisa (Instituto Nacional de Tecnologia Industrial (INTI), Bueno Aires, Argentina) prepared the "wild-type" construct during a month-long visit to UFS in 2018.

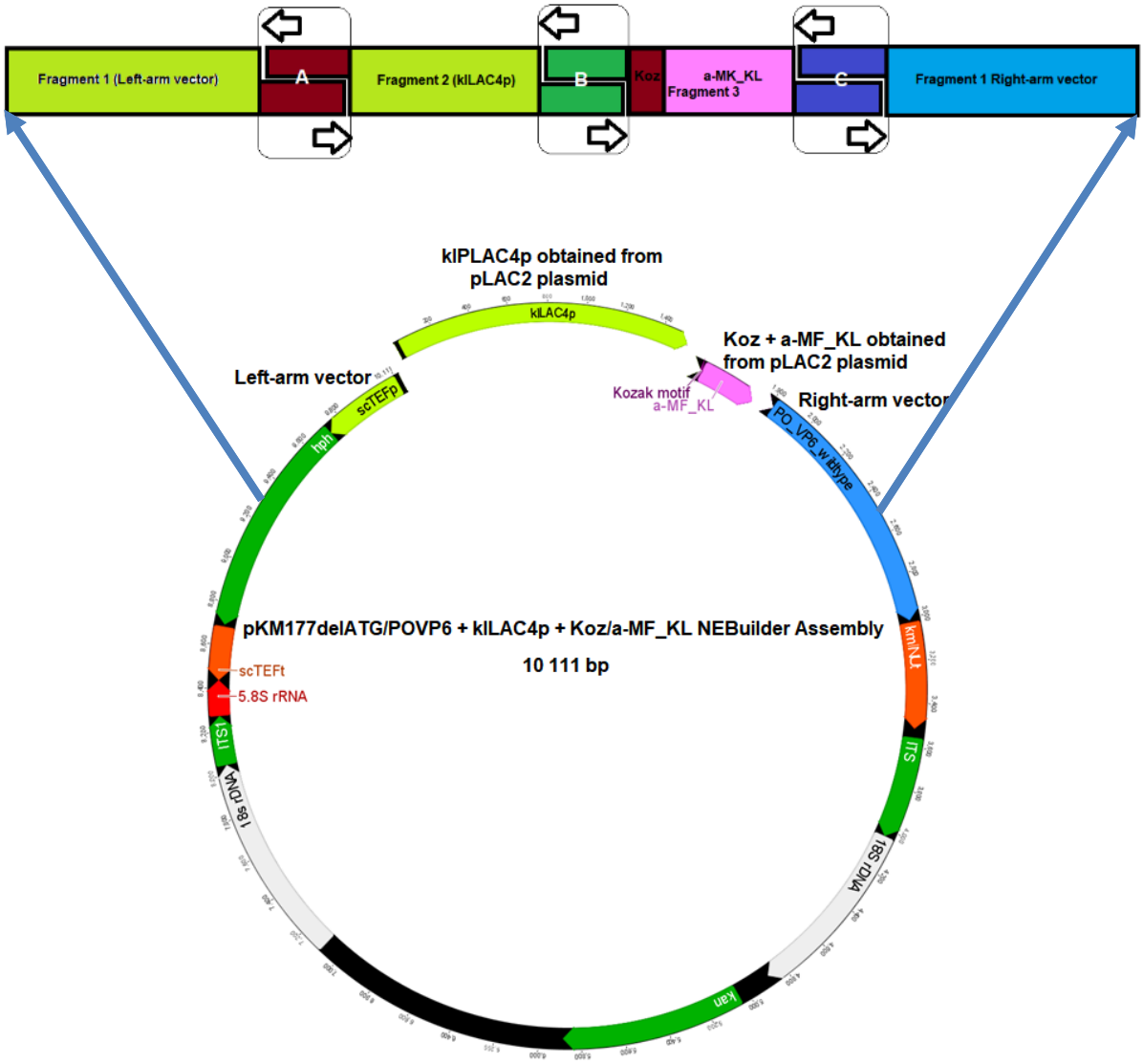


Figure 6.2: Overview of the NEBuilder assembly for the "wild-type" pKM177delATG/klLAC4/Koz/klα-MF/POVP6 and "mutated" pKM177delATG/klLAC4/Koz/klα-MF/POVP6mut plasmids**.** The NEBuilder assembly in this study consists of three different DNA fragments. The vector with its two open arms, one to the LEFT (green) and the other to the RIGHT (blue) making the fragment 1. Fragment 2 is the klLAC4p (green) obtained from the pLAC2 plasmids and fragment 3 is the kla-MF obtained from the pLAC2 plasmid as well. The overlapping arrangement is that fragment 1 at its LEFT arm overlaps with the fragment 2 (region in dark red - **A**), then fragment 2 overlaps with fragment 3 (a region in green - **B**) and the fragment 3 overlapping with the fragment 1 at its RIGHT arm (a region in blue - **C**). The 5´-exonuclease and polymerase proceed in either of the direction of the arrow. Nicks created are sealed by the ligase.

The promoter and the signal sequence regions in the plasmids, as well as the plasmid to obtain a vector without the ylTEFp, were amplified with Phusion® High-Fidelity DNA polymerase. The PCR reaction mixture contained 1x Phusion HiFi buffer, 200 µM dNTPs mix, 0.5 µM each of the forward and reverse primer, 10 ng of the purified plasmid, 0.5 µL of Phusion® HiFi DNA Polymerase at 0.02 U/µL and nuclease-free water to a final volume of 50 µL. The reaction was subjected to 30 cycles using the G-Storm GS04822 Thermal Cycler

(Gene Technologies Ltd), according to the manufacturer's instructions. The PCR amplification was verified with agarose gel electrophoresis **(Section 6.2.4.6)**. Overnight *DpnI* digestion of the PCR product was subsequently carried out and purified **(Section 6.2.4.7)** following the manufacturer's instruction to ensure that the template DNA was completely degraded to prevent interfering with the subsequent bacterial transformation. The NEBuilder® HiFi DNA Assembly Master Mix/NEBuilder HiFi DNA Assembly Cloning Kit (New England BioLabs) was used to assemble the DNA fragments according to the manufacturer's instructions. The kit is based on the Gibson Assembly **(Figure 6.2)**.

6.2.5 Yeast expression and deep-well screening – INTI, Argentina

6.2.5.1 Yeast selection

K. lactis (UOFS_Y1167) received from the UNESCO-MIRCEN Yeast Culture Collection Centre at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State (UFS), was selected for the expression. Previously, *K. lactis* (UOFS_Y1167) was among the six yeasts engineered to produce rotavirus VP6 intracellularly (Rakaki, 2018). The yeast was shipped to INTI, Argentina under a Materials Transferred Agreement (MTA) from UFS to INTI.

6.2.5.2 *NotI* digestion and phenol-chloroform purification of the fragments

The plasmids, pKM177/klLAC4p/Koz/klα-MF/POVP6 (wild-type) and pKM177/klLAC4p/Koz/klα-MF/POVP6mut (mutated) constructed in this study were transported to INTI, Argentina, under MTA using a shipment procedure described by (Rosman & Miller, 1990). The plasmids were recovered (Rosman & Miller, 1990), transformed into DH5α competent *E. coli* cells **(Section 6.2.4.2)**, extracted **(Section 6.2.4.3)** and digested with *NotI.* The *NotI* digested plasmids were purified prior to yeast transformation using the phenol-chloroform method, first described by Neumann and co-workers (Neumann *et al.*, 1992) and revised in (Sambrook & Russell, 2006).

6.2.5.3 Electrocompetent yeast cells and transformation

Electrocompetent cells of *K. lactis* (UOFS_Y1167) was prepared and transformed according to the Transformation Protocol (Protocol No. 4308 915.545–03/2004, Multiporator/Eppendorf Eporator® – Eppendorf, Germany). YPD (yeast extract 10 g/L, peptone 20 g/L, dextrose 20 g/L and agar 15 g/L) selective plates used for yeast transformation were prepared with hygromycin B with 600 µg/mL and 800 µg/mL.

6.2.5.4 Deep-well screening for yeast expression

Deep-well screening in 96-well plates was carried out while visiting the Instituto Nacional de Tecnología Industrial (INTI). After yeast transformation by electroporation (**Section 6.2.5.3)**, all the colonies from the 600 µg/mL hygromycin B selective YPD plates were transferred to 800 µg/mL hygromycin B selective YPD plates. The plates were incubated at 30˚C for 24 hours and each colony from each plate was inoculated in deep-well plates containing 500 µL YPD broth per well. The deep-well plate was incubated at 30˚C in a shaker (Certomat® BS-1) at 350 rpm for 24 hours. A volume of 100 µL culture was transferred from each of the deep-wells into a microplate well and the cell turbidity was measured at OD_{600nm}. The remaining culture was centrifuged at 6 000 x g (IEC/Centra® GP8) for 10 minutes at room temperature. A volume of 100 µL of supernatant from each well was pooled and the average secretion of rotavirus VP6 was assessed with the ROTADIAL™ ELISA Kit (INTA, Argentina) following the manufacturer's instructions. The values were relatively quantified with the negative control from the ELISA kit, which measured ≤ 0.130 at OD_{450nm}. Any sample having a value less than or equal to the negative value was considered negative.

6.2.6 Statistical analysis

Data obtained in ELISA quantification was analysed with GraphPad Prism v.7. The data were presented as mean ± SEM and the mean values were statistically different at $p<0.05$ using Tukey's multiple comparison test for one-way ANOVA analysis. Each sample data was read in duplicate.

6.3 Results

Previously, it was predicted that the rotavirus strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] VP6 protein contain five potential N-glycosylation sites. These sites were mutated in **Chapter Five** to prevent glycosylation during secretion by the engineered yeast strain. Furthermore, the intracellular expression of this mutated VP6 was confirmed in *H. polymorpha* (UOFS_Y0915). Secretion of rotavirus VP6 by yeast has not been reported before, though intracellular production of VP6 and multi-layered VLPs have been reported (Bredell *et al.*, 2016; Rodríguez-Limas *et al.*, 2014; Rodríguez-Limas *et al.*, 2011). It was, therefore, proposed that the use of a suitable promoter to enhance the recombinant expression together with a leader peptide, which will guide the recombinant protein through the secretory pathway, will improve rotavirus VP6 protein production through secretion by the yeast.

6.3.1 Promoter exchange and signal peptide introduction

In order to optimise the expression of the rotavirus VP6 protein in the selected yeast strain, *K. lactis* (UOFS_Y1167), the promoters of expression vectors - pKM177delATG/ylTEFp/Koz/VP6 and pKM177delATG/ylTEFp/Koz/VP6mut containing ylTEFp (**Figure 6.2)** was exchanged with klLAC4p and at the same time introduced klα-MF to generate pKM177/klLAC4p/Koz/klα-MF/POVP6 **(wild-type)** and pKM177/klLAC4p/Koz/klα-MF/POVP6mut **(mutated)**, respectively. This NEBuilder assembly system also offered the opportunity to introduce the Kozak sequence -GCCACC- along with the secretory signal sequence at the 5´-end prior to assembly. **Figure 6.3** shows the digestion of the mutated plasmid obtained after mini-prep preparation **(Section 6.2.4.4)**. This mutated plasmid, digested with *BstBI,* was expected to yield five fragments 5 178 bp, 2 335 bp, 1 533 bp, 755 bp and 320 bp. Two suspected clones were confirmed with another digest using *BsmI* as shown in **Figure 6.4***.* This digestion was important because *BsmI* was one of the enzymes used previously to screen the site-directed mutagenesis performed at N-345-Q **(Chapter Five)**. As expected, digestion with *BsmI* yielded four fragments 4 800 bp, 2 368 bp, 2 159 bp and 792 bp.

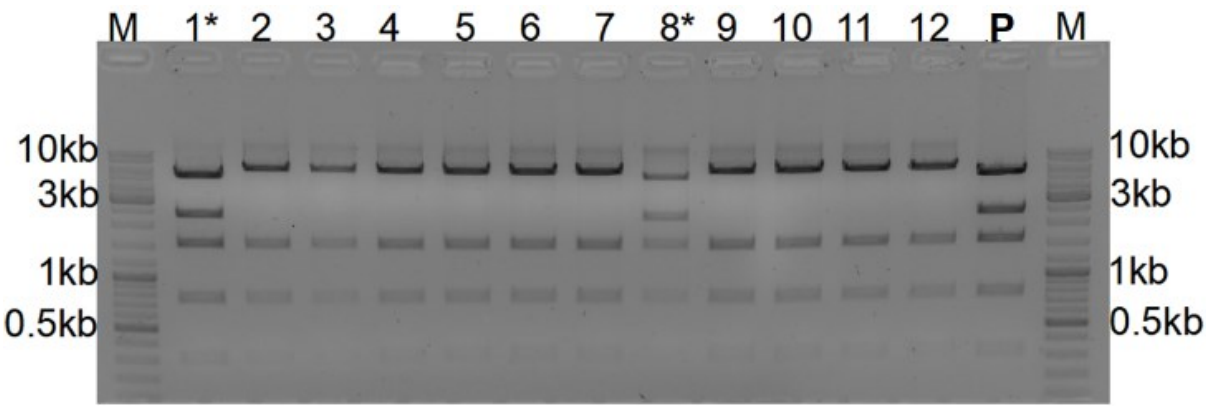


Figure 6.3: Agarose gel analysis of *BstBI* digest from the pKM177/klLAC4p/Koz/klα-MF/POVP6mut mini-prep plasmids. Five fragments are expected 5 178 bp, 2 335 bp, 1 533 bp, 755 bp and 320 bp. Clones 1 and 8 are suspected as possible positive clones due to the detection of 5 fragments in each of their digestion. "P" is the "wild-type" plasmid (pKM177/klLAC4p/Koz/klα-MF/POVP6 ORF) as a positive control. "M" is the GeneRuler™ 1 kb DNA Ladder.

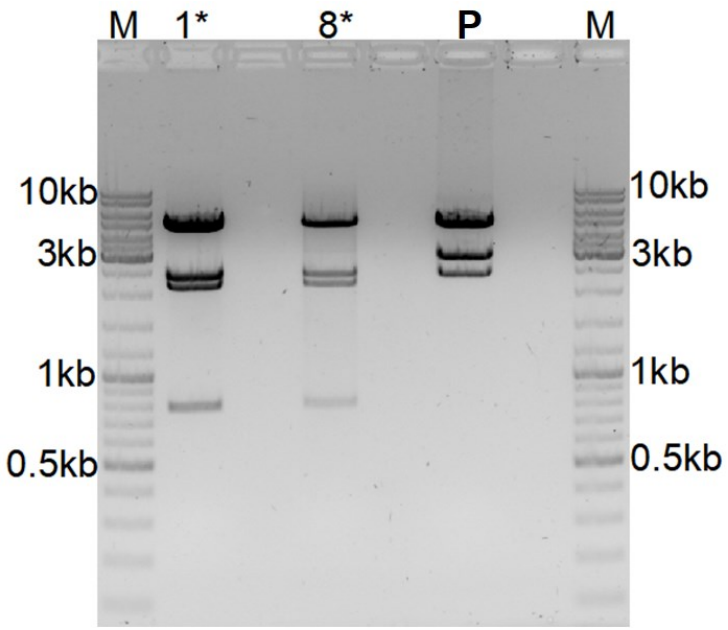


Figure 6.4: Agarose gel analysis of pKM177/klLAC4p/Koz/α-MF_KL/POVP6mut, clones 1 and 8, further digested with *BsmI.* The four expected fragments of digestion are 4 800 bp, 2 368 bp, 2 159 bp and 792 bp. "P" is the wild-type plasmid that will generate only three fragments from *BsmI* digestion – 4 800 bp, 2 949 bp and 2 368 bp as expected. "M" is the GeneRuler™ 1 kb DNA Ladder.

Clones 1 and 8 were sequenced and aligned with the *in-silico* **pKM177/klLAC4p/Koz/α-MF_KL/POVP6mut** construct **(Figure 6.5)**. From **Figure 6.5,** the Sanger sequencing confirmed the correct insertion and accurate assembly of the promoter and the signal sequence. As stated earlier, the "wild-type" expression plasmid (pKM177/klLAC4p/Koz/klα-MF/POVP6) was constructed by Dr Danilo Legisa. Sanger sequencing was carried out to confirm promoter exchange and insertion of the signal sequence as shown in **Figure 6.6,** similar to **Figure 6.5**. The plasmid map for both the wild-type and the mutated constructs are shown in **Figure 6.7**. The preliminary result of the electrocompetent transformation of *K. lactis* showed a well over 100 colonies in the selective plate for wild-type and the mutated expression cassettes. The average turbidity of this yeast at OD_{600nm} was 0.76 for wild-type and 1.45 for mutated and 96 colonies from each of the expressions (wild-type and mutated) were screened in a deep-well bioreactor.



Figure 6.5: Alignment of the *in-silico* sequence of the pKM177/kILAC4p/Koz/klα-MF/POVP6mut (mutated) – middle and the Sanger sequencing results (bottom) indicating the correct insertion and accurate assembly of the promoter and the signal sequence as shown in the consensus sequence (top), with the 5´-end of the kILAC4p (A) and the ligation junction between the kILAC4p – Koz – klα-MF (B).

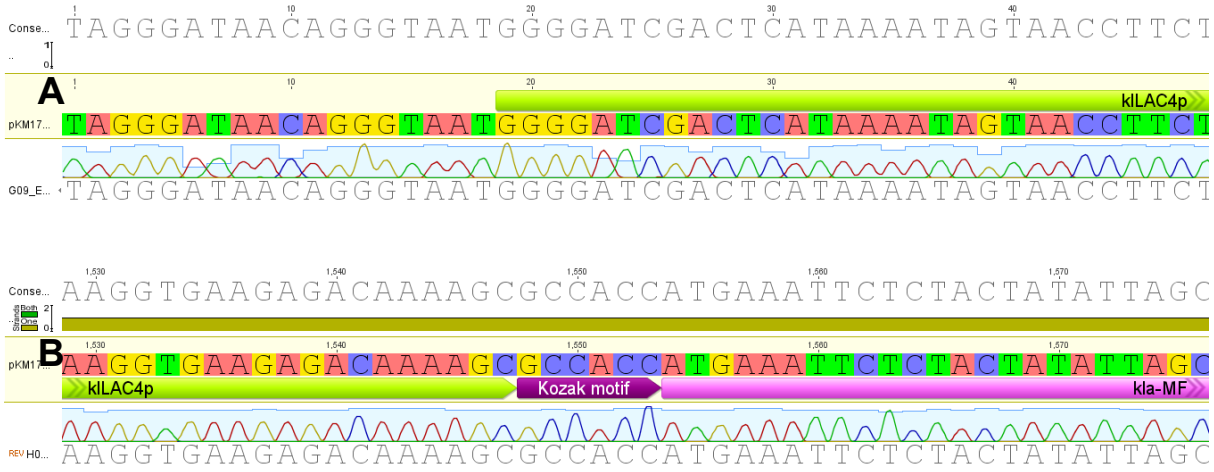


Figure 6.6: Alignment of the *in-silico* sequence of the pKM177/kILAC4p/Koz/klα-MF/POVP6 (wild-type) – middle and the Sanger sequencing results (bottom) indicating the correct insertion and accurate assembly of the promoter and the signal sequence as shown in the consensus sequence (top), with the 5´-end of the kILAC4p (A) and the ligation junction between the kILAC4p – Koz – klα-MF (B).

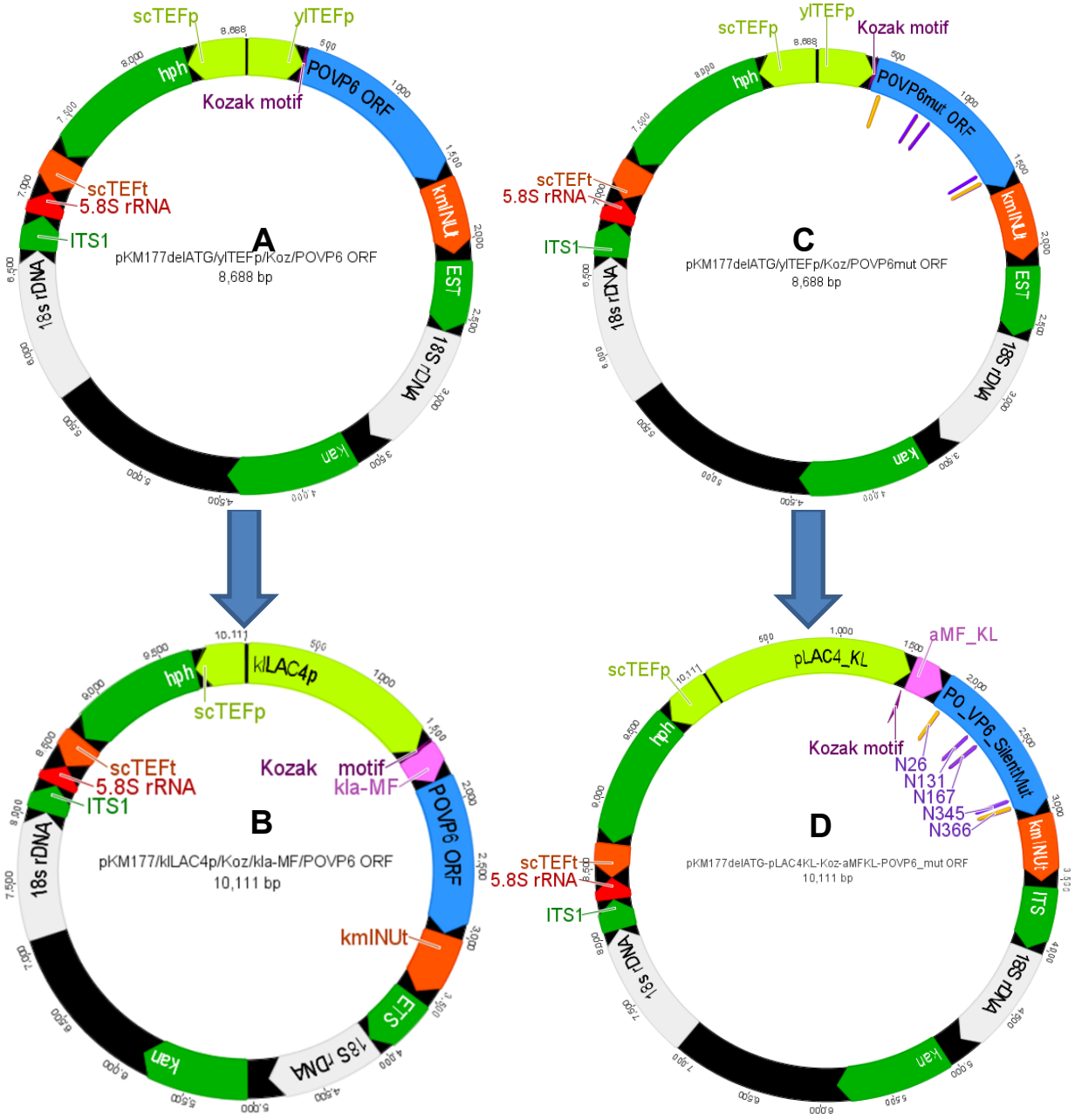


Figure 6.7: Promoter exchange and signal sequence introduced using the NEBuilder® HiFi DNA Assembly Cloning Kit. The ylTEFp promoter in the recombinant plasmid, pKM177delATG/ylTEFp/Koz/VP6 **(A)** was exchanged with klLAC4p and the klα-MF signal sequence was introduced, together with the Kozak sequence, to produce pKM177/klLAC4p/Koz/klα-MF/POVP6 - wild-type **(B)**. Similarly, the ylTEFp in the pKM177delATG/ylTEFp/Koz/VP6mut **(C)** was replaced with klLAC4p and klα-MF signal sequence, together with the Kozak sequence, to produce pKM177/klLAC4p/Koz/klα-MF/POVP6mut - mutated **(D)**. In each case, every other accessory gene on the modified expression plasmid remained.

6.3.2 Deep-well bioreactor screening

The deep-well bioreactor process in biotechnology allows for massive parallel screening using different culture conditions. The volume range for the deep-well bioreactor screen used in this study was between 100 – 500 µL per well. The 96-deep-well plate was used to analyse and relatively quantify the secreted rotavirus VP6 protein (wild-type and mutated) from *K. lactis*

(UOFS_Y1167) and the result for the ELISA assay is shown in **Figure 6.8**. The assay showed the average secretion of VP6 by *K. lactis* transformed with pKM177/klLAC4p/Koz/klα-MF/POVP6 (118 colonies) and pKM177/klLAC4p/Koz/klα-MF/POVP6mut (110 colonies). During the analysis, the relative value obtained for the negative control was less than 10% of the positive control **(Figure 6.8)**. By implication, this means the limiting detection capacity of the ELISA. Any sample, even if it contains rotavirus VP6 antigen, may not likely to be detected except the amount of the antigen exceeds or equals to the one-tenth of the positive control.
By comparing the average secretion level of rotavirus VP6 protein by *K. lactis* (UOFS_Y1167) transformed with plasmids containing either the wild-type or the mutated ORFs with the negative control, a significant difference was observed. Secreted VP6 protein quantified in the wild-type and the mutated expression was significantly higher than the negative control *(****p<0.00001* and ****p<0.0001* respectively*).* A two-fold increase in secreted rotavirus VP6 protein was observed in the wild-type as compared to the mutated *(***p<0.0001)* **(Figure 6.8)**. An extremely low value obtained in the YPD broth as an internal control showed that the ELISA was very sensitive to rotavirus VP6 protein. This, therefore, means that the engineered *K. lactis* (UOFS_Y1167) was able to secrete rotavirus VP6 derived from either the wild-type ORF as well as the mutated ORF.

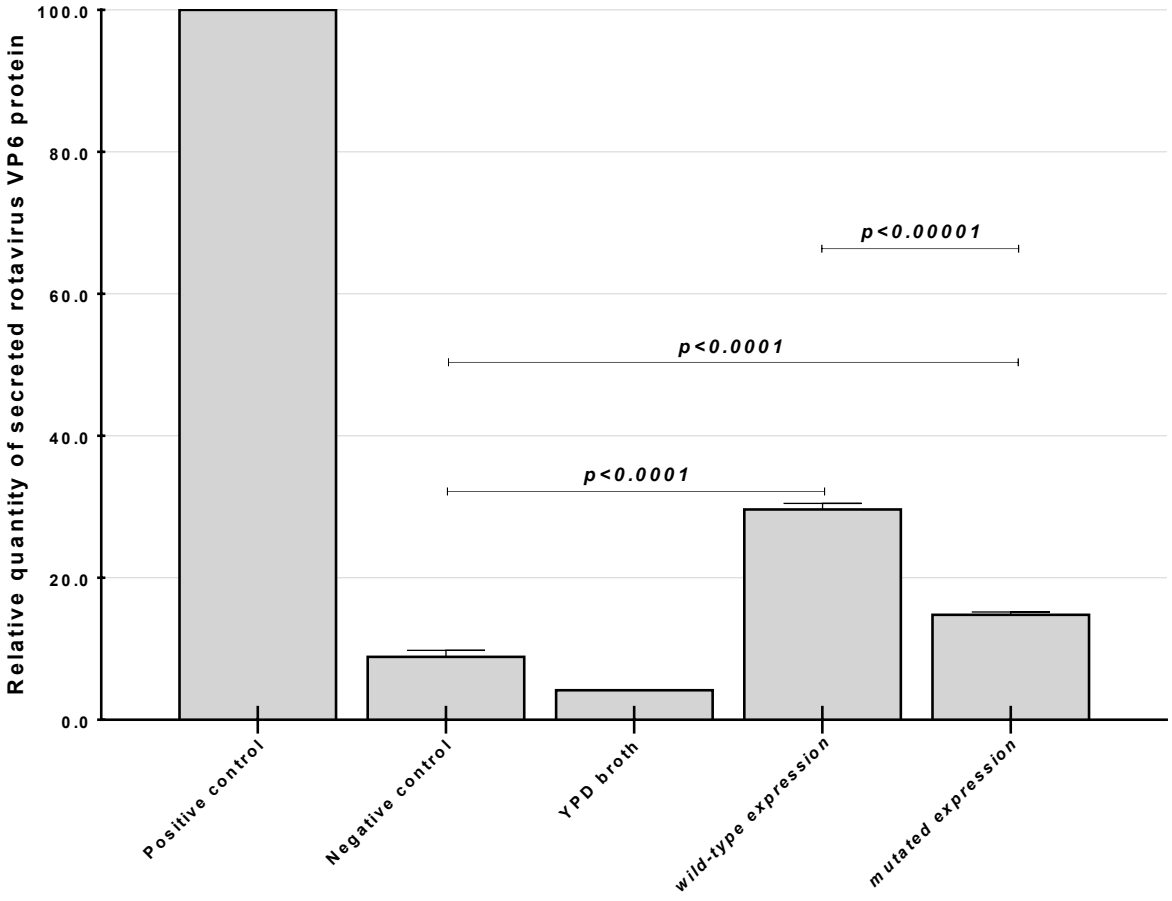


Figure 6.8: Graph showing the relative quantification of the average secretion of rotavirus VP6 protein from *K. lactis* (UOFS_Y1167) transformed with pKM177/klLAC4p/Koz/klα-MF/POVP6 (wild-type secretion) or pKM177delATG/ylTEFp/Koz/VP6mut (mutated expression) using the ROTADIAL™ ELISA Kit. The negative control from the ELISA is the benchmark to determine whether a sample is positive or negative as measured at OD_{450nm}.

6.4 Discussion

Rotavirus VP6 capsid is a single immunodominant antigen (McNeal *et al.*, 2007) and has been shown in animal models to be immunogenic and induce protective immune responses (Choi *et al.*, 2004; Choi *et al.*, 2002a; Choi *et al.*, 2002b; Lappalainen *et al.*, 2015; Lappalainen *et al.*, 2014). It has therefore received much attention as potential sub-unit vaccine candidate or as an adjuvant in a chimeric immunocompetent vaccine preparation (Afchangi *et al.*, 2018; Afchangi *et al.*, 2019; Lappalainen *et al.*, 2015; Lappalainen *et al.*, 2014; Lappalainen *et al.*, 2013). However, finding a cost-effective suitable expression system for a large-scale production has been a major setback.

Rotavirus VP6 protein has been expressed and produced in insect cells (Heinimaki *et al.*, 2018), bacterial cells (Bredell *et al.*, 2016; Choi *et al.*, 2004; Temprana *et al.*, 2018), plant cells (Feng *et al.*, 2017), mammalian cells (Nilsson *et al.*, 1998) and even transgenic animal (Soler *et al.*, 2005; Soler *et al.*, 2007). Only a few reports are available for the expression of rotavirus VP6 in yeast (Bredell *et al.*, 2016; Rodríguez-Limas *et al.*, 2011) despite the potential of yeast for large production of recombinant proteins. Previously, six yeast strains have been engineered to express and produce rotavirus VP6 protein intracellularly. These yeasts include *A. adeninivorans, H. polymorpha, K. lactis, P. pastoris, S. cerevisiae* and *Y. lipolytica* (Rakaki, 2018). For large-scale production and cost-effective downstream processing, extracellular secretion is preferred.

In this study, *K. lactis* (UOFS_Y1167) was explored to secrete rotavirus VP6 protein because it has been identified as one of the recombinant yeasts capable of expressing rotavirus VP6 protein (Rakaki, 2018). The choice of this yeast is also based on the fact that it is a non-methylotrophic yeast that can be fermented with a high cell density without losing its productivity (Swinkels *et al.*, 1993). This yeast can stably maintain exogenous recombinant DNA (Müller *et al.*, 1998) and capable of secreting high molecular weight heterologous recombinant proteins (Wésolowski-Louvel *et al.*, 1996). The recombinant expression vector was engineered prior to transformation. To improve the recombinant expression of the rotavirus VP6 protein, the strong heterologous ylTEFp promoter obtained from *Y. lipolytica* (Albertyn *et al.*, 2011) in the expression cassette was exchanged for the strong homologous klLAC4p promoter obtained from *K. lactis*. Furthermore, the native signal sequence was incorporated into the expression vector to enhance the secretory production of the recombinant VP6 protein (Van Ooyen *et al.*, 2006).

With the two sets of expression cassettes – the "wild-type" rotavirus VP6 ORF and the "mutated" ORF-containing cassettes, *K. lactis* (UOFS_Y1167) was successfully engineered to express and secrete rotavirus VP6 protein **(Figures 6.7 and 6.8)**. Higher secretion of the VP6 protein was obtained in the wild-type as compared to the mutated VP6 despite the two-fold increase in the turbidity of the recombinant *K. lactis* transformed with the mutated expression plasmid as compared to the *K. lactis* transformed with the wild-type expression plasmid. This suggests that active secretion of the recombinant protein does not necessarily depend on the accumulated biomass of the yeast.

During this study, these two expressed proteins were not characterised in terms of glycan content. However, the proteins were analysed with the ELISA assay, which was specifically designed to capture rotavirus VP6 antigens (Garaicoechea *et al.*, 2008). The ELISA antibody can only recognise the native conformational epitope of rotavirus VP6 antigen (Garaicoechea *et al.*, 2008), which in this case, was exhibited by the two expressed proteins. It means, then, that either the proteins were not glycosylated (mutated expression) or the wild-type expressed protein was minimally glycosylated, which did not affect its conformational structure for the ELISA detection.

Furthermore, human rotavirus VP6 protein is generally produced as a non-glycosylated protein during rotavirus replication unlike animal rotavirus VP6 (Choi *et al.*, 2002c). In this study, potential N-glycosylation sites were identified in the VP6 protein. Naturally, the lack of a signal peptide prevents it from being co-translationally glycosylated in the endoplasmic reticulum of the infected host cell. Therefore, introduction of the signal peptide may expose it to glycosylation during extracellular secretion in the yeast. However, the mutagenesis performed in the encoding ORF could have prevented glycosylation during secretion. Careful comparison of all the mutagenic sites with the conserved antigenic site of the rotavirus VP6 showed that glycosylation during secretion may not affect ELISA detection because all the potential glycosylation sites did not occur within the conserved epitope regions, except N-167 **(Chapter Five)**. It is possible that the wild-type VP6 protein may have been minimally glycosylated during secretion. Detection of the wild-type VP6 during secretion suggests that glycosylation may have little or no effect on the overall conformation of the protein since it was successfully detected with ELISA or that minimal (if at all it occurs) glycosylation occurred (Cornaglia *et al.*, 1991).

In conclusion, with the use of deep-well bioreactors in combination of the ROTADIAL^{TM} ELISA Kit, which is based on the *in vitro* neutralising activity of the Llama-derived single-chain antibody fragment that offered protection when directed against rotavirus VP6 antigen in mice

(Garaicoechea *et al.*, 2008), a screening tool to confirm the secretion of rotavirus VP6 protein was established. With *K. lactis* (UOFS_Y1167) used in this study, secretion of rotavirus VP6 protein was confirmed. The ability of *K. lactis* (UOFS_Y1167) to be engineered for secretion of rotavirus VP6 protein is a proof-of-concept that could be applied to other yeasts to test their ability to secrete this protein. Nevertheless, further investigation of the secreted proteins is needed. This includes determining if the wild-type secreted protein is glycosylated or not; if the N-terminal signal peptide is correctly processed and most importantly if these proteins will induce a protective immune response in an animal model.

6.5 References

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Chapter Seven

Conclusion and future recommendations

7.1 Conclusion

The continual recommendations by the WHO to incorporate rotavirus vaccines into the National Expanded Programme on Immunisation for every country point to the fact that rotavirus infection still leads in causing severe gastroenteritis in infants and children worldwide (World Health Organisation, 2013, 2018a, 2018b). Despite this, evidence has shown a drastic reduction in mortality and diarrhoea-hospitalisation cases in many countries that implemented rotavirus vaccines (Arnold, 2018; Steele *et al.*, 2016; Troeger *et al.*, 2017; Troeger *et al.*, 2018).

The four live-attenuated oral rotavirus vaccines licensed and recommended to be used are effective against rotavirus infection (Bhandari *et al.*, 2014; Desselberger, 2017; Jonesteller *et al.*, 2017), however, few issues surrounding the use of live-attenuated oral vaccines against rotavirus infections seem insurmountable. Many of the low-income countries with the highest burden of this infection could not procure these vaccines because of their high cost (Bhandari *et al.*, 2014). Safety is another concern with these vaccines. Genome reassortments leading to more virulent strains (Arana *et al.*, 2016; Bowen *et al.*, 2016; Jere *et al.*, 2018; Lartey *et al.*, 2018; Ogden *et al.*, 2018; Ouermi *et al.*, 2017; Seheri *et al.*, 2018; Simwaka *et al.*, 2018) and the risk of intussusception (Bowen *et al.*, 2016; Buttery *et al.*, 2011; Tate & Parashar, 2019; Tate *et al.*, 2008; Yen *et al.*, 2016) are negative aspects associated with the use of live-attenuated oral vaccines against rotavirus infection. Vaccination with the live-attenuated oral vaccines results in mild to acute diarrhoea which can lead to faecal viral shedding. The efficacy of these vaccines is lower in low-income countries in Africa and Asia and is constantly being affected by co-administration with OPV, maternally transferred rotavirus-specific antibodies, nutritional status, gut probiotics of the infants and disruption in the cold-chain. GAVI (Global Vaccine Alliance) has continued to help many of these low-income countries to implement vaccination but, the question remains, what happens after the end of the tenure agreement with GAVI as countries are then required to carry the cost of vaccination. Collectively, this underlines the need for an alternative and cost-effective rotavirus vaccine.

The use of non-replicating rotavirus virus-like particles (RV-VLPs) as next-generation vaccine candidates against rotavirus infection has attracted much attention in recent times (Engeroff & Bachmann, 2019). Among these next-generation vaccine candidates are the inactivated virion, RV- VLPs and sub-unit vaccine candidates. In this study, the focus was on rotavirus VP6-based vaccine candidates due to their ability to induce protective immune responses in

various animal models (Lappalainen *et al.*, 2015; Lappalainen *et al.*, 2014; Pastor *et al.*, 2014; Vega *et al.*, 2013). Rotavirus-directed VP6-specific antibodies can mediate intracellular neutralization by binding to a quaternary structure in the transcriptional pore on the DLPs thereby blocking the RNA transcription (Aiyegbo *et al.*, 2013). Yeasts were engineered to express rotavirus VP6 protein together with the core protein (VP2) to enable the formation of double-layered particles. The choice of the yeasts was based on the fact that, although every other form of expression systems such as bacterial cells, insect cells, transgenic plants and animals have been used for the production of rotavirus sub-unit vaccine candidates, the major problem with the expression systems is the large-scale production for the insect cells, transgenic animals and plants (Engeroff & Bachmann, 2019) or poor post-translational modification of the recombinant proteins as found in the bacterial cells expression system (Roldao *et al.*, 2010; Rosano & Ceccarelli, 2014). This is more feasible in the yeasts than any of the other expression systems. Currently, there are only a few reports on the use of a yeast expression system for the production of rotavirus non-replicating, sub-unit proteins and VLPs. *S. cerevisiae* was the first yeast to be used to express rotavirus proteins that formed VLPs (Rodríguez-Limas *et al.*, 2011), followed by the use of *P. pastoris* and *H. polymorpha* to express rotavirus VP6 protein (Bredell *et al.*, 2016).

Despite its vast industrial application to produce biopharmaceutical products, yeast expression usage to produce rotavirus non-replicating vaccine candidates have been underutilised. In this study, non-*S. cerevisiae* species were explored. With the unique wide-range yeast expression system (Albertyn *et al.*, 2011), ORFs encoding rotavirus VP2 and VP6 proteins of the South African field strain RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6] were successfully expressed in engineered yeast strains. The yeast strains were obtained from the UNESCO-MIRCEN yeast culture collection at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State; a resource collection comprising over 3 000 different yeast strains suitable for screening purposes.

Previously, an attempt to express rotavirus VP2 and VP6 proteins encoded by the rotavirus VP2 and VP6 ORF in yeasts failed (Makatsa, 2015). Each of the ORFs was codon optimised for expression in yeast, specifically *A. adeninivorans, K. lactis* and *P. pastoris/H. polymorpha.* In this study, the expression vectors were manipulated to improve expression. An additional ATG upstream of the ORFs was discovered and deleted and the Kozak sequence in the expression cassette was optimised. Although the expression of both VP2 and VP6 was obtained from all three optimised ORFs (Table 4.5), co-expression of the two rotavirus capsid proteins was obtained only in the recombinant *H. polymorpha* (UOFS_Y0915). Removal of the ATG, therefore, proofed essential to ensure expression. However, modification of the Kozak

sequence did not seem to have a significant impact on improving gene expression since no proteins could be detected in a number of yeast strains screened. Transmission electron microscope analysis revealed that DLPs of about 58 nm in size was formed from the two proteins expressed by *H. polymorpha* (UOFS_Y0915). Morphologically, this DLP has a rough edge and surface structure due to the protruding VP6 trimeric unit on this structure and the size obtained was similar to those reported in the literature (Estes & Kapikian, 2007; Labbé *et al.*, 1991).

The immunogenic properties of rotavirus VP6 antigen depend on its non-glycosylated form (Soler *et al.*, 2005). To pursue this goal, putative glycosylation sites in the rotavirus VP6 ORF was sequentially mutated to prevent glycosylation when directed for secretion in the yeast. The observation of secretion of the rotavirus VP6 protein from the engineered *K. lactis* (UOFS_Y1167) was a first. This secreted protein was confirmed with an ELISA kit specifically designed to react with the rotavirus VP6 antigen (Garaicoechea *et al.*, 2008). This established a proof-of-concept, paving the way for screening of other non-*S. cerevisiae* yeasts for the extracellular secretion of rotavirus VP6 protein. The secretion obtained from the *K. lactis* (UOFS_Y1167) transformed with the modified yeast expression vector containing wild-type and mutated VP6 encoding ORFs is a milestone and called for further investigation into the structural differences in these two forms of rotavirus VP6 protein.

7.2 Future recommendations

Rotavirus multi-layered and single-layered VLPs are immunogenic and in this work, rotavirus VP6-based vaccine candidates were expressed in selected, engineered yeast strains. This was done both as intracellular co-expression with VP2 to enable DLP (a multi-layered particle) formation and as a standalone, non-glycosylated, secretory protein. The results of this study are a good foundation for further investigation into the immunogenic functions of these proteins using animal models. This should likely be preceded by structural studies of these proteins as the structure is very crucial for their biological function. With this in place, the techniques and the proof-of-concept screen developed in this study can then be expanded to large-scale production of a rotavirus VP6-based vaccine.

With the few available reports describing the use of yeast expression systems for rotavirus VLPs and sub-unit proteins (Bredell *et al.*, 2016; Rodríguez-Limas *et al.*, 2014; Rodríguez-Limas *et al.*, 2011), this study has contributed to the existing knowledge and made a novel impact by the use of a unique wide-range yeast expression vector containing two ORFs encoding the rotavirus VP2 and VP6 proteins, which have been optimised for three different yeast strains With the available results accrued in this study, a number of future directions will

be of great benefit to reach the goal of a rotavirus VP6-based vaccine. These include an investigation into the use of alternative promoters, including constitutive and inducible promoters, driving the expression of the recombinant VP2 protein. Another aspect for future investigation is the use of ribosomal binding sequence upstream of VP2 ORF (Rodríguez-Limas *et al.*, 2011) to facilitate the proportional production of the rotavirus VP2 protein needed for the formation of DLPs or perhaps co-transformation of the expression plasmids – one for VP2 expression and the other for VP6 expression. Understanding the proteolytic profile of the yeast can help with appropriate protease inhibitors, which can prevent any abrupt cleavage of the recombinant protein (truncated protein) via either lysosomal or proteasomal pathway as suspected in the *H. polymorpha* (UOFS_Y1507). Attention can also be given to screening of more yeast strains for potential secretion of non-glycosylated rotavirus VP6 protein, as well as the structural investigation of both secreted VP6 proteins (encoded by the mutated VP6 ORF and un-mutated ORF) obtained in this study to confirm that the protein is not glycosylated. A final future prospect is ultrastructural analysis and ultimately, an investigation into the immunogenic properties using animal models before clinical evaluation of the VP6-based vaccine candidates.

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Appendix A (Chapter Three)

A1. The *in-silico* maps of all the wide-range yeast expression plasmids containing single, optimised ORFs

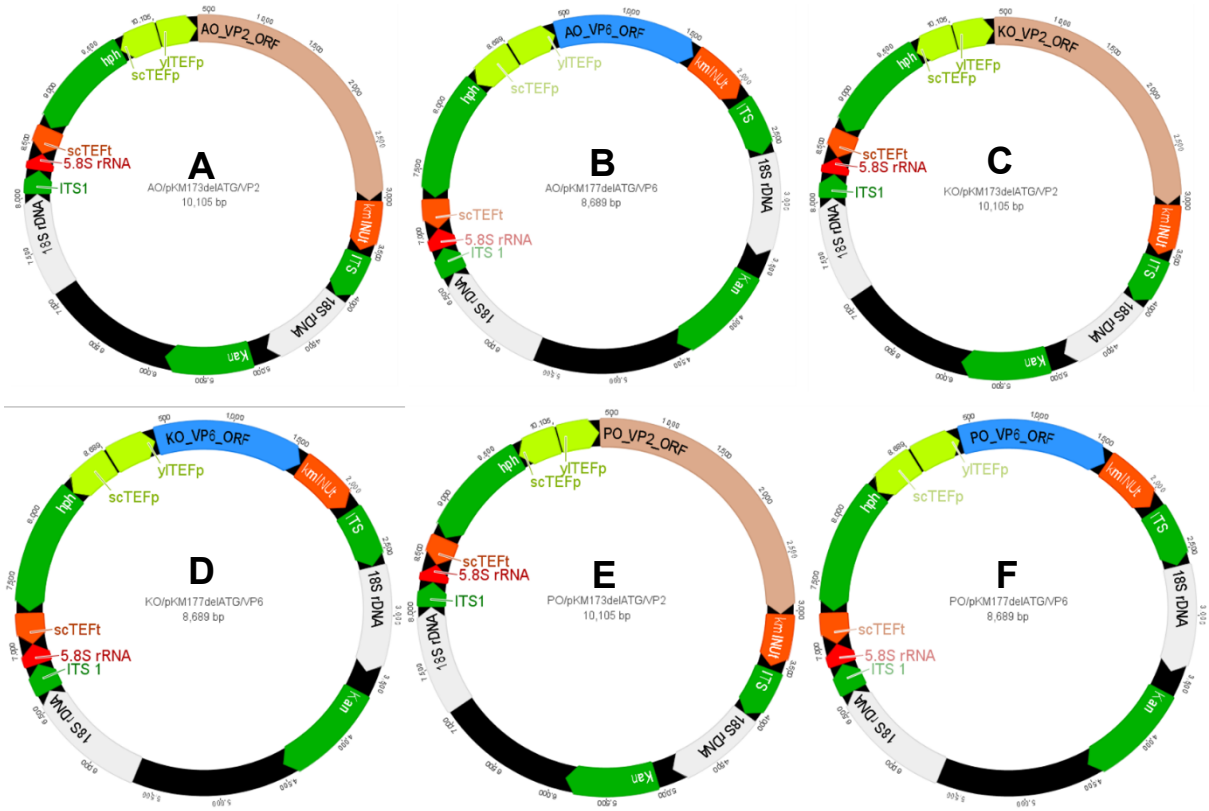


Figure A1: Wide-range yeast expression vectors containing single, optimised ORFs. (A) pKM173delATG/AOVP2 **(B)** pKM177delATG/AOVP6 **(C)** pKM173delATG/KOVP2 **(D)** pKM177delATG/KOVP6 **(E)** pKM173delATG/POVP2 **(F)** pKM177delATG/POVP6

A2. Primers

Table A1 lists the primers used for site-directed mutagenesis (-ATG- deletion and introduction of the optimised Kozak sequence) and Sanger sequencing to verify all the constructs. The annealing temperature for each PCR run was calculated from the average of the Tm of the two primers minus 5°C.

Table A1: Primers used for site-directed mutagenesis and Sanger sequencing

Primer	*Sequence (5´ to 3´)*	*Tm (°C)*	*Purpose*
pKM173delATG_F	CTCGAGATGAAGTTCGCATACT	58.0	To delete ATG from the
pKM173delATG_R	TTCGGGTGTGAGTTGACAAGG	60.5	pKM173
pKM177delATG_F	CTCGAGATGAAGTTCGCATACT	58.0	To delete ATG from the
pKM177delATG_R	TTCGGGTGTGAGTTGACAAGG	60.5	pKM177
pKMdelATGVP2KO_F	CTCGAGCATGGCTTACAGAA	57.7	To delete ATG from KOVP2
pKM173delATG_R	CCTTGTCAACTCACACCCGAA	60.5	ORF
pKMdelATGVP2PO_F	CTCGAGATGGCTTACAGAAAGA	57.6	To delete ATG from POVP2
pKM173delATG_R	CCTTGTCAACTCACACCCGAA	60.5	ORF
pKMdelATGVP6PO_F	CTCGAGATGGACGTTTTGTACTC	59.1	To delete ATG from POVP6
pKM177delATG_R	CCTTGTCAACTCACACCCGAA	60.5	ORF
PO_pKM173VP2_Koz_F	CTCACACCCGAACTCGAGATGGCTTACAGAAAGAG	71.2	To introduce Kozak motif into
PO_pKM173VP2_Koz_R	CTCTTTCTGTAAGCCATCTCGAGTTCGGGTGTGAG	71.2	the POVP2 ORF
PO_pKM177VP6_Koz_F	CTCACACCCGAACTCACCATGGACGTTTTGTACTC	71.7	To introduce Kozak motif into
PO_pKM177VP6_Koz_R	GAGTACAAAACGTCCATGGTGAGTTCGGGTGTGAG	71.7	the POVP6 ORF
VP6_Seq_AF3	TCTATGCTGGTCAAGTAGAGC	56.9	Sanger sequencing to verify 5´-
pKM_Seq_F1_Inqaba	GTCAAGACTGTCAAGGAGGG	54.7	and 3´-end ligation of the AOVP6 cassette into the dual-expression plasmid
VP6_Seq_KF3	GAGTGTTCACTGTCGCTTC	56.3	Sanger sequencing to verify 5´-
pKM_Seq_F1_Inqaba	GTCAAGACTGTCAAGGAGGG	54.7	and 3´-end ligation of the KOVP6 cassette into the dual-expression plasmid
VP6_Seq_PF3	GAGTTTTCACCGTTGCTTC	55.0	Sanger sequencing to verify 5´-
pKM_Seq_F1_Inqaba	GTCAAGACTGTCAAGGAGGG	54.7	and 3´-end ligation of the POVP6 cassette into the dual-expression plasmid
pKM_Seq_F1_Inqaba	GTCAAGACTGTCAAGGAGGG	57.5	Sanger sequencing of
pKM173/177_R	GAACAGCTAGAGTGCGTT	54.7	pKM173delATG/Koz/AOVP2 sub-cloned

F = forward; R = reversed; ORF = open reading frame; AO = *A. adeninivorans,* KO = *K. lactis* and PO = *P. pastoris/H. polymorpha* codon-optimised ORFs. All the primers were obtained from the Integrated DNA Technologies, IDT (USA) except the pKM_Seq_F1_Inqaba, which was obtained from the Inqaba Biotec (South Africa).

A3. Deletion of additional -ATG- from pKM173/KOVP2 and pKM177/POVP6 ORFs

The targeted -ATG- for pKM173/KOVP2 and pKM177/POVP6 was removed site-directed mutagenesis using primers that flank the -ATG- **(Table A1)** region to be removed **(Section 3.2.5)**.

A3.1. pKM173/KOVP2 ORF

The primer binding site flanking the -ATG- on the pKM173/KOVP2 (10 108 bp) is shown in **Figure A2**. The binding is such that the -ATG- to be deleted was excluded in the PCR amplification, followed by *DpnI* digestion, phosphorylation, ligation **(Section 3.2.5.1)** and bacterial transformation **(Section 3.2.4.1).** After transformation, nineteen of the colonies were screened for the deleted -ATG- **(Figure A3)**. Plasmids were extracted from the positive clones **(Section 3.2.4.2)** and sequenced to confirm the deleted -ATG- **(Section 3.2.4.7)**.

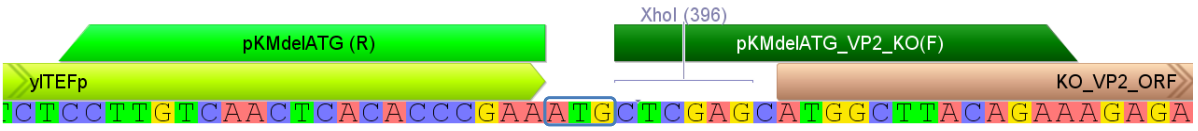


Figure A2: The primer pair flanking the -ATG- targeted for removal in the pKM173/KOVP2 (10 108 bp). PCR amplification using the primers resulted in the exclusion of the -ATG-.

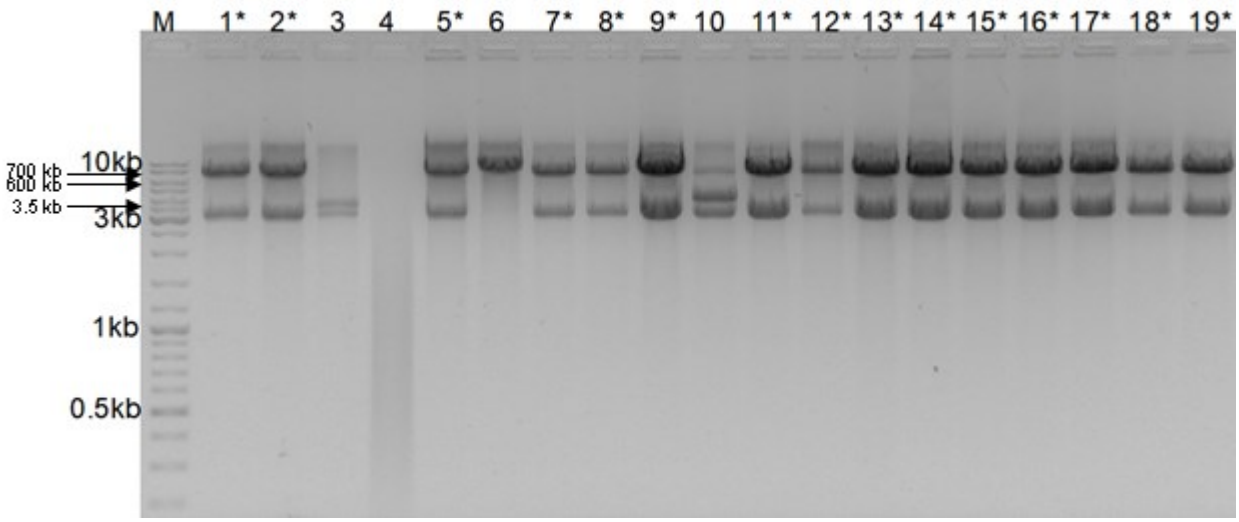


Figure A3: Agarose gel analysis of the *XhoI* and *BamHI* restriction enzyme digests of mini-prep extractions for pKM173delATG/KOVP2 (10.1kb). Expected band sizes were 7 021 bp and 3 091 bp. The clones labelled with an asterisk were sequenced to verify the deleted -ATG-. "M" is the GeneRuler™ 1 kb DNA Ladder.

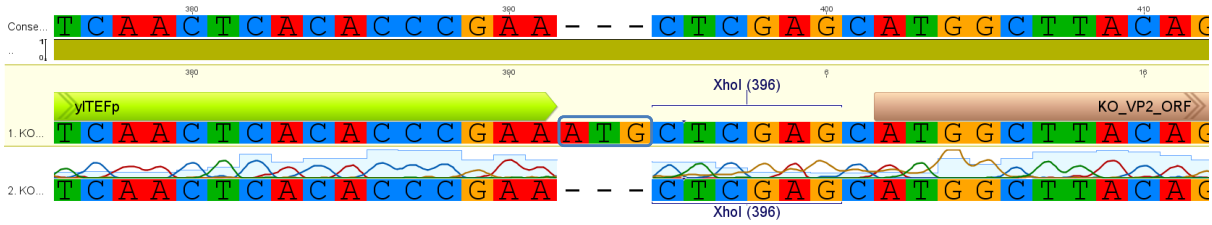


Figure A4: Alignment of the *in-silico* sequence of the pKM173/KOVP2 (middle) and Sanger sequencing result (bottom) for the pKM173delATG/KOVP2 indicating the removed -ATG- as shown in the consensus sequence (top). The *XhoI* recognition site at the ligation site between the KOVP2ORF and the 3´-end of the promoter (ylTEFp) are also shown.

A3.2. pKM177/POVP6

The primer binding site flanking the -ATG- on the pKM177/POVP6 (8 692 bp) is shown in **Figure A5.** The binding is such that the -ATG- to be deleted was excluded in PCR amplification, followed by *DpnI* digestion, phosphorylation, ligation **(Section 3.2.5.1)** and then transformed into bacteria **(Section 3.2.4.1).** After transformation, nineteen of the colonies were screened for the deleted -ATG- **(Figure A7)**. Plasmids were extracted from the positive clones **(Section 3.2.4.2)** and sequenced to confirm the deleted -ATG- **(Section 3.2.4.7)**.

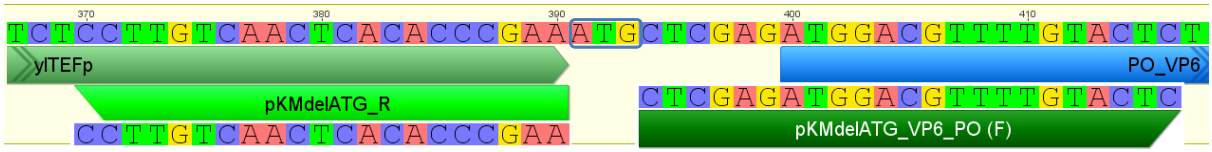


Figure A5: The primer pair flanking the -ATG- targeted for removal in the pKM173/POVP6 (8 692 bp). PCR amplification using the primers resulted in the exclusion of the -ATG-.

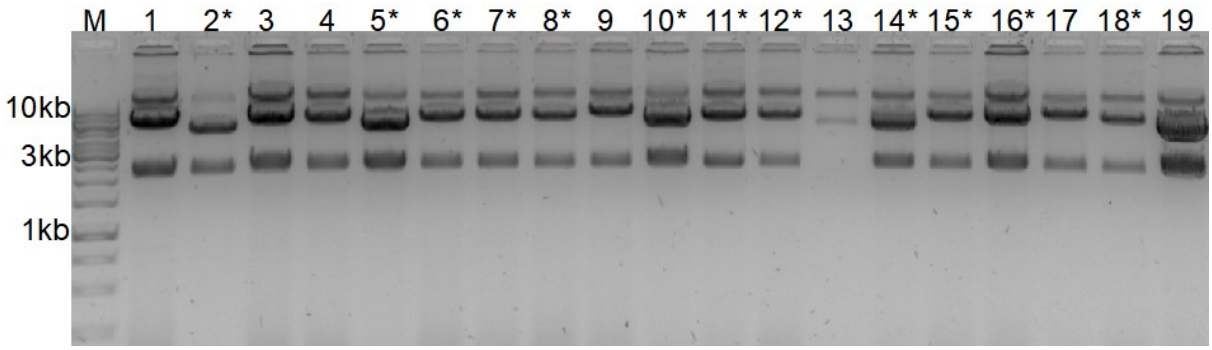


Figure A7: Agarose gel analysis of the *NotI* restriction enzyme digests of mini-prep extractions for pKM177delATG/POVP6 (8 695 bp). Expected band sizes were 6 481 bp and 2 214 bp. The clones labelled with an asterisk were sequenced to verify the deleted -ATG-. “M" is the GeneRuler™ 1 kb DNA Ladder.

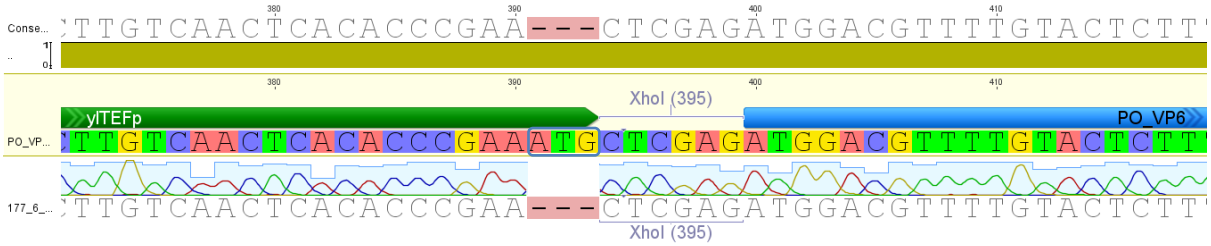


Figure A8: Alignment of the *in-silico* sequence of the pKM173/POVP2 (middle) and the Sanger sequencing result (bottom) for the pKM177delATG/POVP6 indicating the removed -ATG- as shown in the consensus sequence (top). The *XhoI* recognition site at the ligation site between the POVP2 ORF and the 3´-end of the promoter (ylTEFp) are also shown.

A4. Optimisation of Kozak consensus sequence for the expression plasmid, pKM177delATG/POVP6

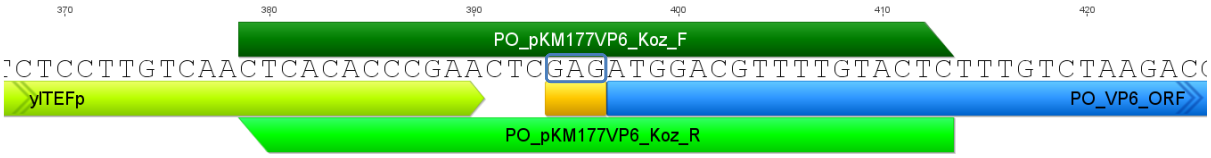


Figure A9: Overlapping primer binding site on the pKM177delATG/POVP6 ORF to introduce a Kozak motif by changing the -GAG- to -ACC- (indicated in yellow). Successful mutagenesis should remove the *XhoI* recognition site (-CTCGAG-) and introduce the *NcoI* recognition site (-CCATGG-).

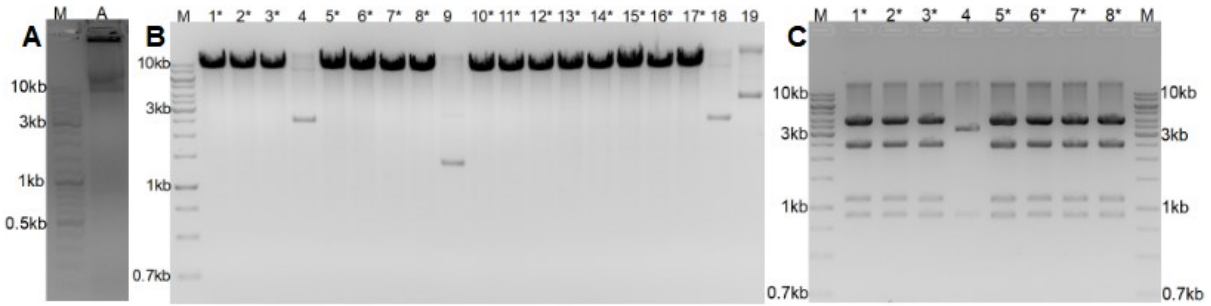


Figure A10: Overview of screening for pKM177delATG/POVP6. (A) shows the amplified plasmid after overnight digestion with *DpnI.* Expected band size was 10 108 bp. **(B)** shows the agarose gel for 19 mini-prep plasmids digested with *XhoI-Eco47III.* Introduction of the Kozak sequence altered and removed the *XhoI* recognition site, therefore digestion resulted in a linearised fragment (10 108 bp). Clones indicated by asterisks were selected for further verification with *NcoI.* **(C)** confirms the accurate introduction of the Kozak sequence into the ORF of pKM177delATG/POVP6 of all the selected clones (except clone 4) when digested with *NcoI* to yield the four expected fragments (4 077 bp, 2 557 bp, 1 144 bp and 925 bp). Clones with asterisks were sequenced. "M" is the GeneRuler™ 1 kb DNA Ladder.

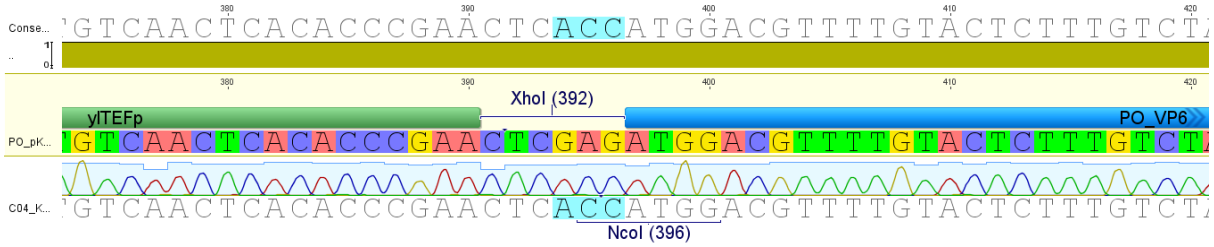


Figure A11: Alignment of the *in-silico* sequence of the pKM177delATG/POVP6 (middle) and the Sanger sequencing result (bottom) for the pKM177delATG/Koz/POVP6 indicating the introduction of the Kozak sequence -ACC- in the consensus sequence (top). The *XhoI* recognition site at the ligation site between the POVP6 ORF and the 3´-end of the promoter (ylTEFp) removed in the process while the *NcoI* recognition site was introduced.

A5. Construction of dual expression plasmids containing optimised VP2 and VP6 encoding ORFs

A5.1. KO_Dual Vector Kozak (pKM173delATG/Koz/KOVP2-6)

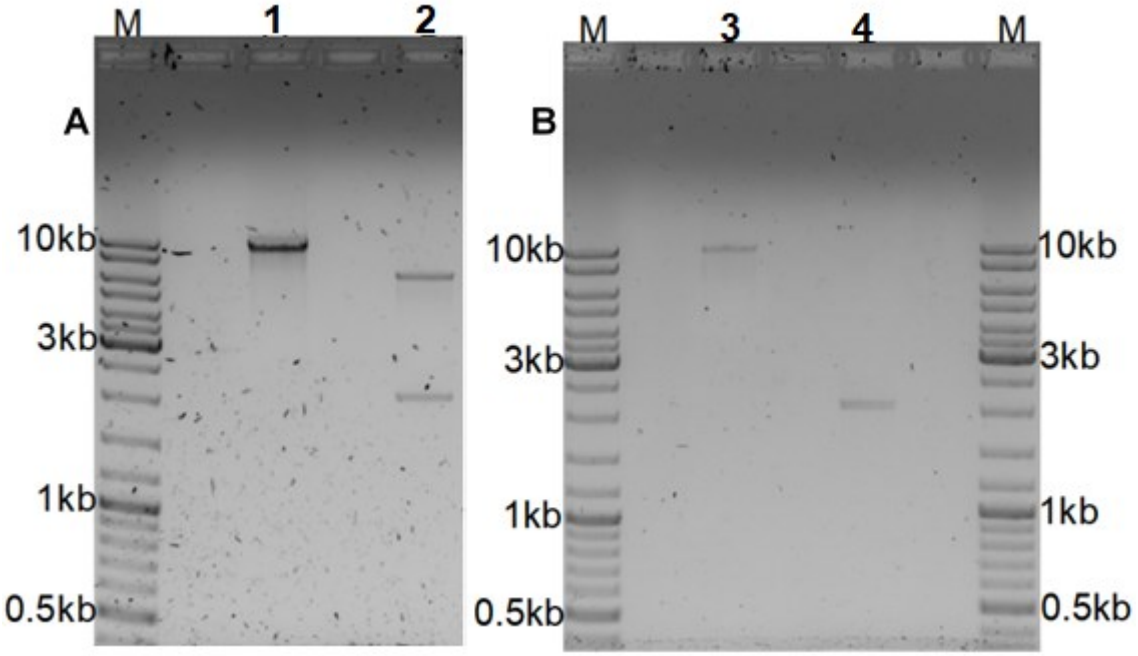


Figure A12: Agarose gel analysis of the fragments generated during the construction of the dual expression vector, pKM173delATG/Koz/KOVP2-6. (A) *I-SceI* digestion of the vector to linearise pKM173delATG/Koz/KOVP2 (10.1 kb) (lane 1) and digestion of pKM177delATG/Koz/KOVP6 to release the KOVP6 ORF-containing cassette (2.1 kb) as insert, which include its promoter (ylTEFp) and terminator (kmINUt) (lane 2). **(B)** dephosphorylated and purified pKM173delATG/KOVP2 (10.1 kb) **(Vector)** (lane 3) and purified KOVP6 ORF-containing expression cassette (2.1 kb) as **Insert** (lane 4) followed by ligation and transformation. "M" is the GeneRuler™ 1 kb DNA Ladder.

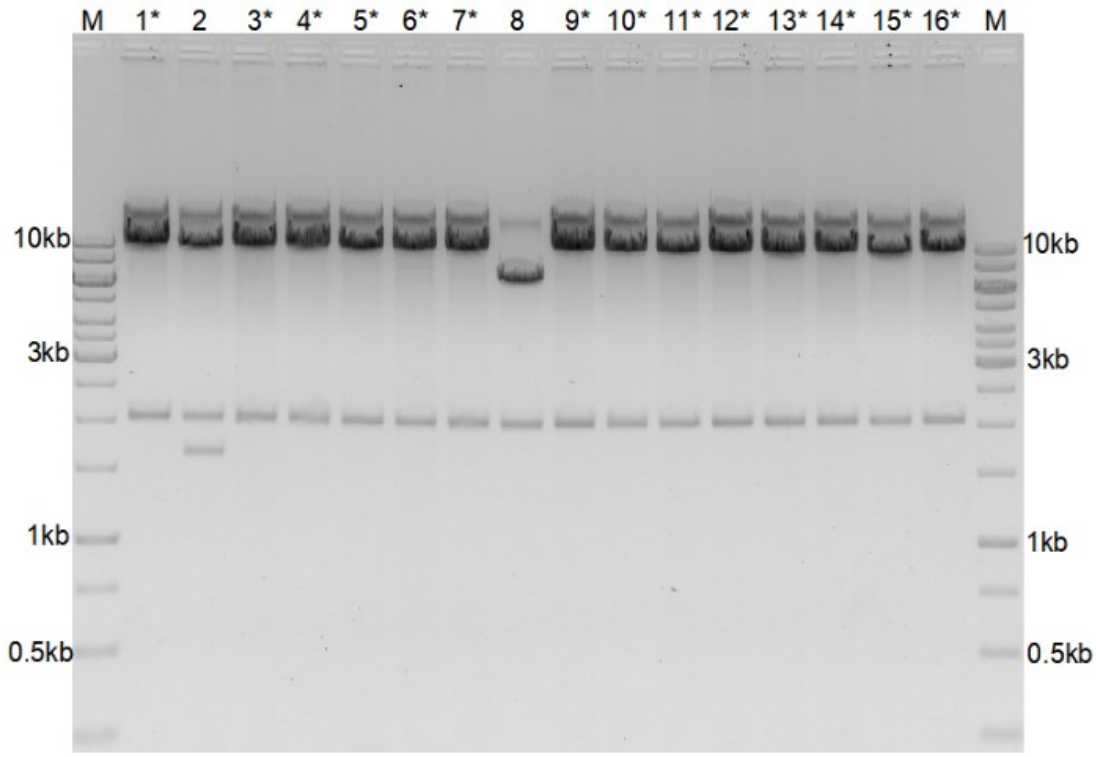


Figure A13: Agarose gel analysing the fragment generated from the *XhoI* digestion of the pKM173delATG/Koz/KOVP2-6 (KO_Dual Vector Kozak). The two expected fragments are 10 109 bp and 2 106 bp). All the colonies appeared positive (asterisks) except clones 2 and 8. "M" is the GeneRuler™ 1 kb DNA Ladder.

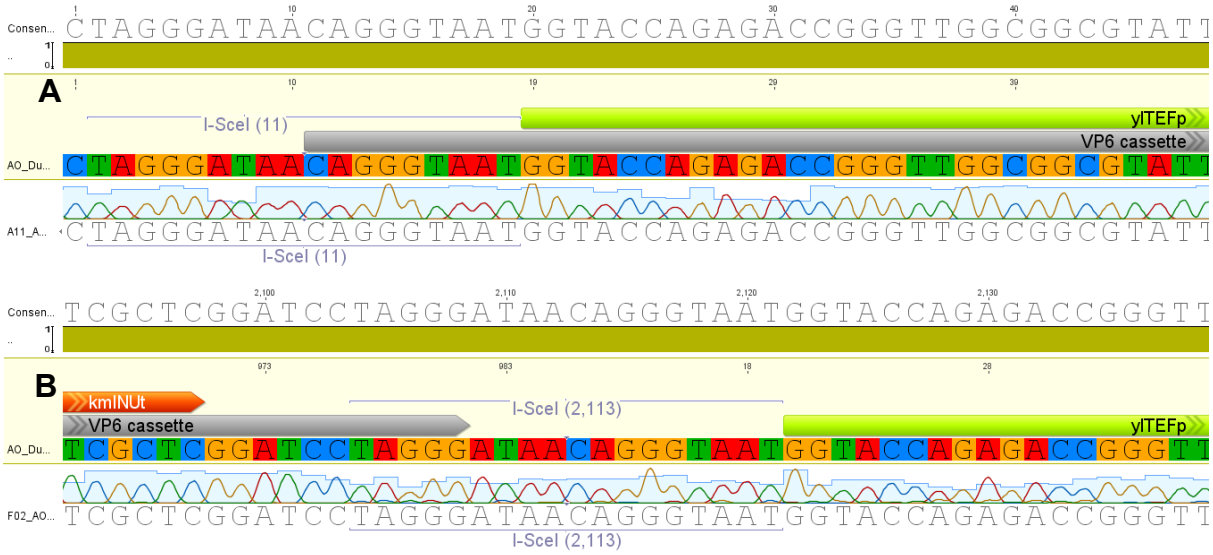


Figure A14: Sanger sequencing for the pKM173delATG/Koz/KOVP2-6 confirming the 5´-end ligation (A) and the 3´-end ligation (B) of the inserted expression cassette. All the sequenced clones matched the *in-silico* sequence (middle) and were thus confirmed to have the correct insertion of the expression cassette

A5.2. PO_Dual Vector Kozak (pKM173delATG/Koz/POVP2-6)

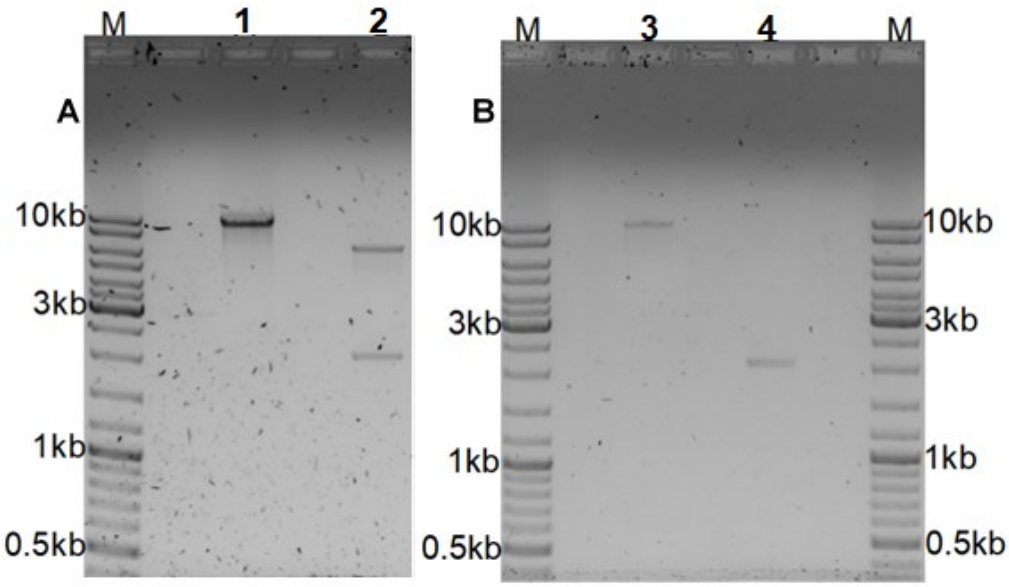


Figure A15: Agarose gel analysis of the fragments generated during the construction of the dual expression vector, pKM173delATG/Koz/POVP2-6. (A) *I-SceI* digestion of the vector to linearise pKM173delATG/Koz/POVP2 (10.1 kb) (lane 1) and digestion of pKM177delATG/Koz/POVP6 to release the POVP6 ORF-containing cassette (2.1 kb) as insert, which include its promoter (yITEFp) and terminator (kmINUt) (lane 2). **(B)** dephosphorylated and purified pKM173delATG/POVP2 (10.1 kb) **(Vector)** (lane 3) and purified KOVP6 ORF-containing expression cassette (2.1 kb) as **Insert** (lane 4) followed by ligation and transformation. "M" is the GeneRuler™ 1 kb DNA Ladder.

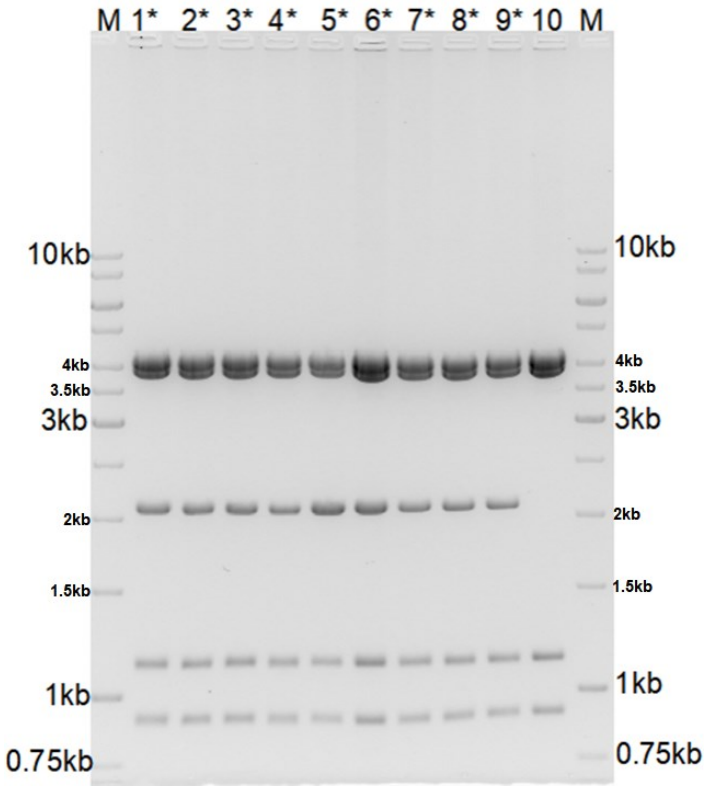


Figure A16: Agarose gel analysing the fragment generated from the *NcoI* digestion of the pKM173delATG/Koz/POVP2-6 (PO_Dual Vector Kozak). The five expected fragments are 4 077 bp, 3 974 bp, 2 101 bp, 1 144 bp and 925 bp. All the colonies appeared positive (asterisks) except clone 10. "M" is the GeneRuler™ 1 kb DNA Ladder.

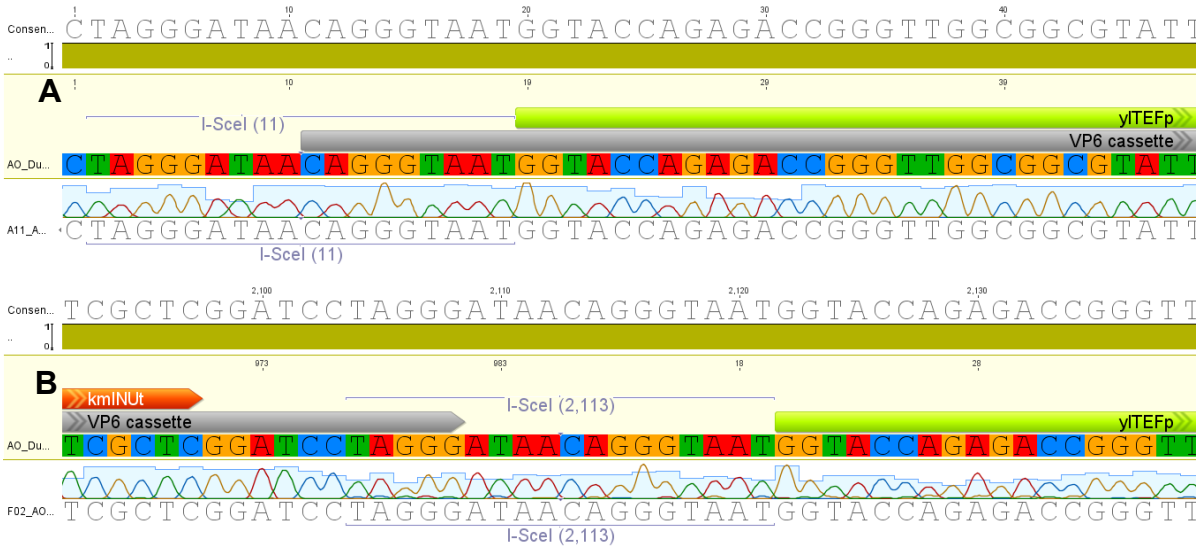


Figure A17: Sanger sequencing for the pKM173delATG/Koz/POVP2-6 confirming the 5´-end ligation (A) and the 3´-end ligation (B) of the inserted expression cassette. All the sequenced clones matched the *in-silico* sequence (middle) and were thus confirmed to have the correct insertion of the expression cassette.

Appendix B (Chapter Five)

B1. N-terminal acetylation of methionine predicted for VP6 of RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6]

NetAcet 1.0 prediction results, 1 sequence 397 amino acids

```
#  MDVLYSLSKTLKDARDKIVEGTLYSNVSDLIQQFNQMIITMNGNEFQTGG  #    50
#  IGNLPIRNWNFDFGLLGTTLLNLDANYVETARNTIDYFVDFVDNVCMDEM  #   100
#  VRESQRNGIAPQSDSLRKLSGIKFKRINFDNSSEYIENWNLQNRRQRTGF  #   150
#  TFHKPNIFPYSASFTLNRSQPAHDNLMGTMWLNAGSEIQVAGFDYSCAIN  #   200
#  APANTQQFEHIVQLRRVLTTATITLLPDAERFSFPRVINSADGATTWYFN  #   250
#  PVILRPNNVEVEFLLNGQIINTYQARFGTIVARNFDTIRLSFQLMRPPNM  #   300
#  TPSVAALFPNAQPFEHHATVGLTLKIESAVCESVLADASETMLANVTSVR  #   350
#  QEYAIPVGPVFPPGMNWTDLITNYSPSREDNLQRVFTVASIRSMLV      #   397
```

OUTCOME: Because there is no Ala (A), Gly (G), Ser (S) or Thr (T) at positions 1 – 3 of the sequence, hence, N-terminal methionine may not be acetylated.

B2. NetOGlyc 4.0 Server prediction results for potential O-glycosylation motifs in VP6 of RVA/HUMAN-wt/ZAF/GR10924/1999/G9P[6]

#seqname	source	feature	start	end	score
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SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	8	8	0.0406371
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	10	10	0.0925454
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	22	22	0.0406813
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	25	25	0.0345439
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	28	28	0.0233996
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	40	40	0.0734718
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	48	48	0.167708
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	68	68	0.0299242
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	69	69	0.0741608
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	80	80	0.0464254
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	84	84	0.0679841
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	104	104	0.343213
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	113	113	0.21066
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	115	115	0.109207
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	120	120	0.149104
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	132	132	0.0257767
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	133	133	0.0411026
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	148	148	0.230821
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	151	151	0.382204
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	161	161	0.238102
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	163	163	0.0403696
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	165	165	0.066875
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	169	169	0.154454
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	179	179	0.0114003
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	186	186	0.0221609
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	196	196	0.026663
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	205	205	0.0513118
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	219	219	0.210686
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	220	220	0.313837

#seqname	source	feature	start	end	score
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	222	222	0.128245
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	224	224	0.104196
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	233	233	0.108297
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	240	240	0.0565912
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	245	245	0.0834616
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	246	246	0.0970304
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	272	272	0.0141162
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	279	279	0.0105334
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	287	287	0.018211
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	291	291	0.0193239
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	301	301	0.345532
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	303	303	0.126232
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SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	323	323	0.0374745
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	328	328	0.0286477
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	333	333	0.0112695
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	339	339	0.0594754
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	341	341	0.0505404
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	347	347	0.310166
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SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	372	372	0.177353
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	375	375	0.334358
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	377	377	0.149303
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	387	387	0.174448
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	390	390	0.185391
SEQUENCE	netOGlyc-4.0.0.13	CARBOHYD	393	393	0.0548326

OUTCOME: From the analysis, there is no evidence of O-glycosylation because none of the predicted potential sites scored the threshold value of 0.5.

B3. Overview of the site-directed mutagenesis carried out consecutively on the potential N-glycosylation sites

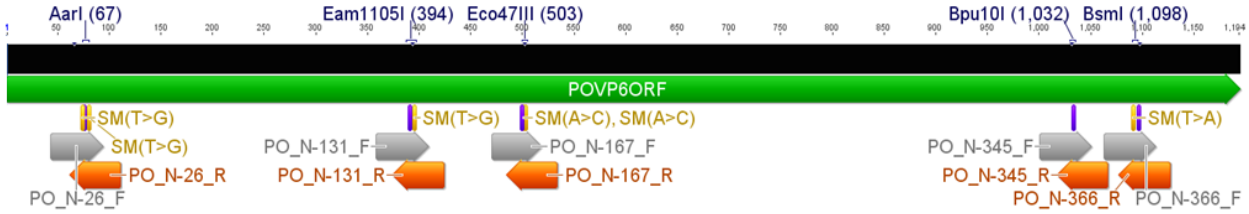


Figure B1: Overview of the site-directed mutagenesis in the *P. pastoris/H. polymorpha* codon-optimised rotavirus VP6 ORF. The primer binding site is shown where asparagine is exchanged for the glutamine. Also, silent mutation to create a new restriction site for screening on. Each of these is shown in detail in **Figure B2**.

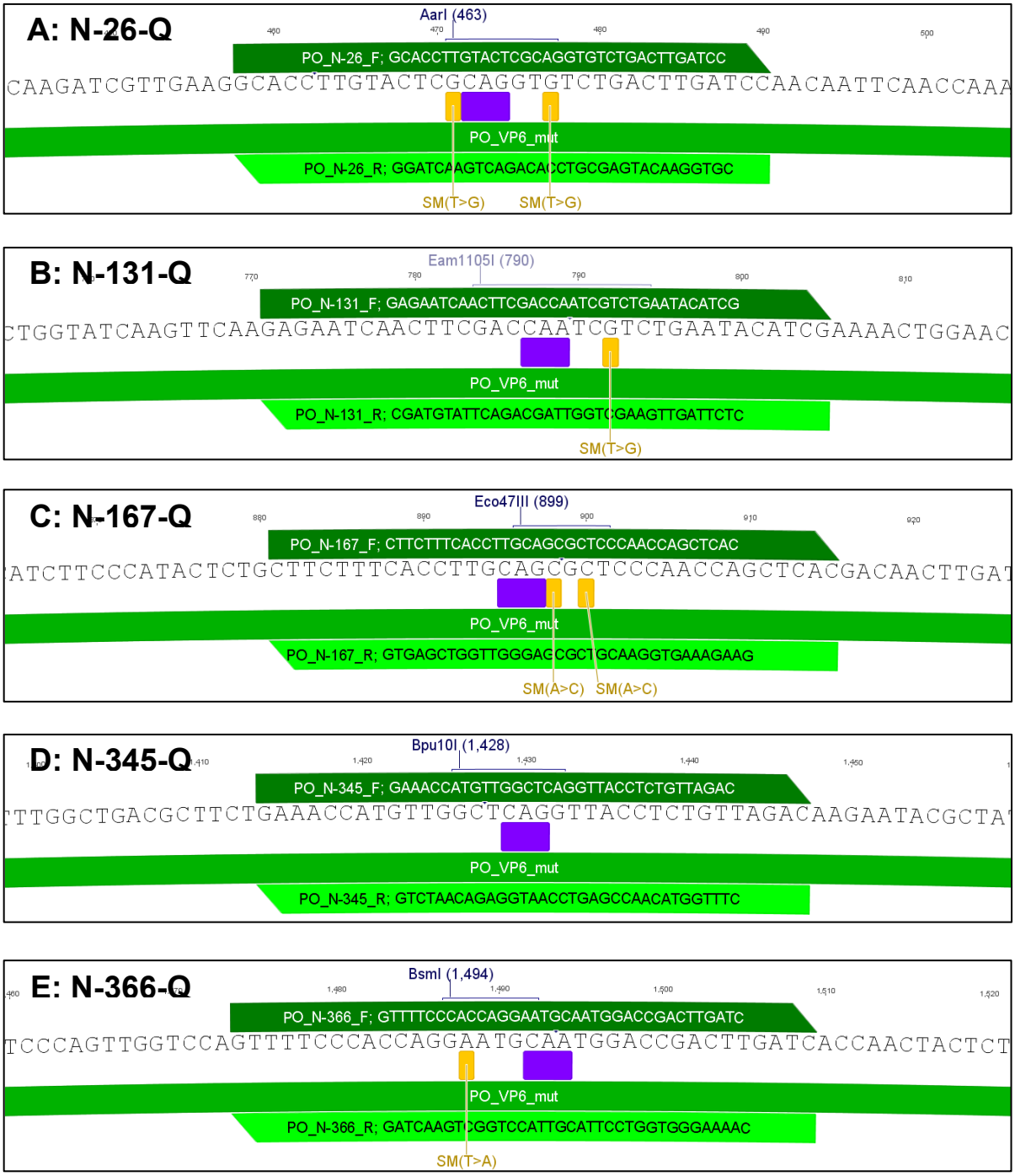


Figure B2: Primer binding sites showing nucleotides changed during site-directed mutagenesis and the introduction of silent mutations. Here, the exchanged nucleotides at each site of mutations were indicated as X > Y where Y replaced X, to create a new restriction endonuclease site. Each of the blue annotated codons is the site where asparagine (N) was changed to glutamine (Q) in the VP6 protein.

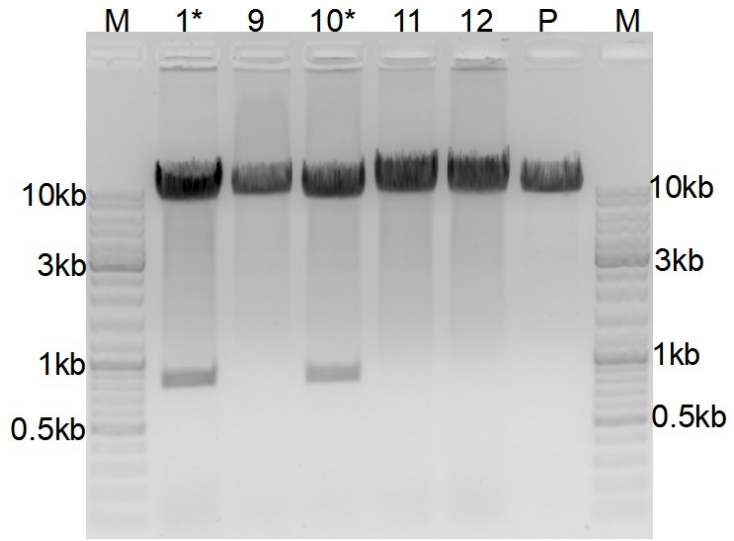


Figure B3: Site-directed mutagenesis of -AAC- to –CAA- (N-131-Q) in pKM177delATG/Koz/POVP6-N26. Selected clones were screened with *Eam1105I* and *Eco47III* restriction enzymes. Expected fragments of 7 884 bp and 805 bp were observed for clones 1 and 10. Confirmation of AAC to CAA change was performed with Sanger sequencing. "M" is the GeneRuler™ 1 kb DNA Ladder and "P" is the pKM177delATG/Koz/POVP6-N26 plasmid

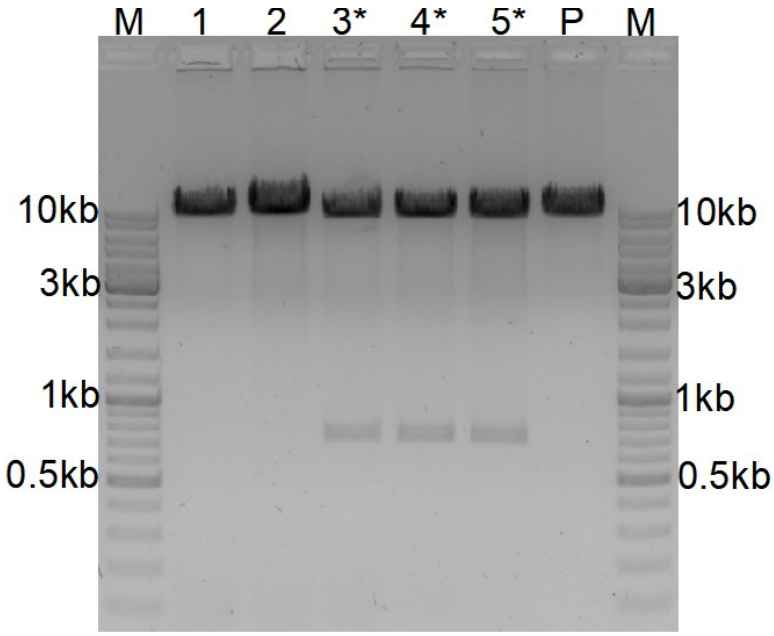


Figure B4: Site-directed mutagenesis of -AAC- to –CAG- (N-167-Q) in pKM177delATG/Koz/POVP6-N26,131. Selected clones were screened with *Eco47III* restriction enzymes. Expected fragments of 7 993 bp and 695 bp were observed for clones 3, 4 and 5. Confirmation of AAC to CAA change was performed with Sanger sequencing. "M" is the GeneRuler™ 1 kb DNA Ladder and "P" is the pKM177delATG/Koz/POVP6-N26,131 plasmid

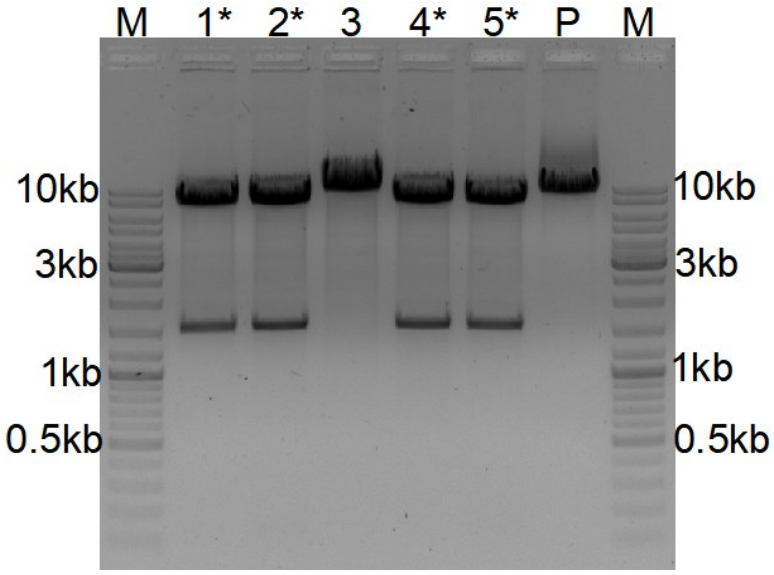


Figure B5: Site-directed mutagenesis of -AAC- to –CAG- (N-345-Q) in pKM177delATG/Koz/POVP6-N26,131,167. Selected clones were screened with *Bpu10I* restriction enzymes. Expected fragments of 7 169 bp and 1 525 bp were observed for clones 1, 2, 4 and 5. Confirmation of AAC to CAG change was performed with Sanger sequencing. "M" is the GeneRuler™ 1 kb DNA Ladder and "P" is the pKM177delATG/Koz/POVP6-N26,131,167 plasmid

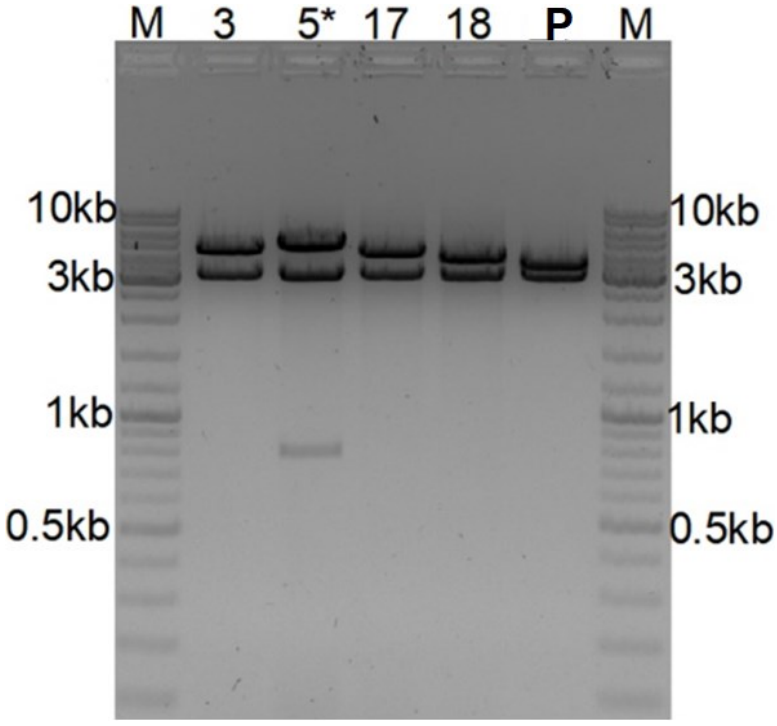


Figure B6: Site-directed mutagenesis of -AAC- to –CAA- (N-366-Q) in pKM177delATG/Koz/POVP6-N26,131,167,345. Selected clones were screened with *BsmI* restriction enzymes. Expected fragments of 4 800 bp, 3 101 bp and 792 bp were observed for clone 5 only. Confirmation of AAC to CAA change was performed with Sanger sequencing. "M" is the GeneRuler™ 1 kb DNA Ladder and "P" is the pKM177delATG/Koz/POVP6-N26,131,167,345 plasmid

Supplementary (Chapter Four)

A1. Formula, names and Cat # for all the components of the Syn-6-MES

Formula	Company	Cat #	Chemical name
$C_6H_5O_7Na_3$	Merck	1110371000	Trisodium citrate
$(NH_4)_2SO_4$	Merck	1012171000	Diammonium tetraoxosulphate VI
NaCl	Merck	1064001000	Sodium chloride
MES hydrate	Sigma	M2933	2-(N-Morpholino)ethanesulfonic acid hydrate, 4-Morpholineethanesulfonic acid
D(+) Biotin	Sigma	B4501	$C_{10}H_{16}N_2O_3S$ (Vitamin B_7 or H) 5-[(3aS,4S,6aR)-2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl]pentanoic acid
Thiamine HCl	Sigma	Y1250	$C_{12}H_{17}N_4OS^+$ (Vitamin B_1) 2-[3-[(4-amino-2-methylpyrimidin-5-yl)methyl]-4-methyl-1,3-thiazol-3-ium-5-yl]ethanol
EDTA	Merck	324503	Disodium ethylenediaminetetraacetate dihydrate
$(NH_4)_2FeSO_4.6H_2O$	Merck	1037921000	Diammonium iron (II) tetraoxosulphate VI hexahydrate
$MnSO_4.H_2O$	Merck	1059410250	Manganese II tetraoxosulphate VI monohydrate
$ZnSO_4.7H_2O$	Merck	1088830500	Zinc tetraoxosulphate VI heptahydrate
$CuSO_4.5H_2O$	Merck	1027900250	Cupper II tetraoxosulphate VI pentahydrate
$NiSO_4.6H_2O$	Merck	1067270250	Nickel II tetraoxosulphate VI hexahydrate
$CoCl.6H_2O$	Merck	1025390250	Cobalt (II) chloride hexahydrate
$Na_2MoO_4.2H_2O$	Merck	1065210250	Sodium molybdate dihydrate
KI	Merck	1050430250	Potassium iodide
H_3BO_3	Merck	1001561000	Boric acid
$CaCl_2.2H_2O$	Merck	1024651000	Calcium chloride dihydrate
$C_6H_{12}O_6$	Sigma	49159	D(+)-Glucose
$C_3H_8O_3$	Sigma	104092	Glycerol (Propane-1,2,3-triol)
$MgSO_4.7H_2O$	Merck	1058860500	Magnesium tetraoxosulphate VI heptahydrate
KCl	Merck	1049330500	Potassium chloride
$(NH_4)_2HPO_4$	Merck	1012070500	Diammonium hydrogen tetraoxophosphate V

A2. Evaluation of DLP formation in *H. polymorpha* (UOFS_Y0915)

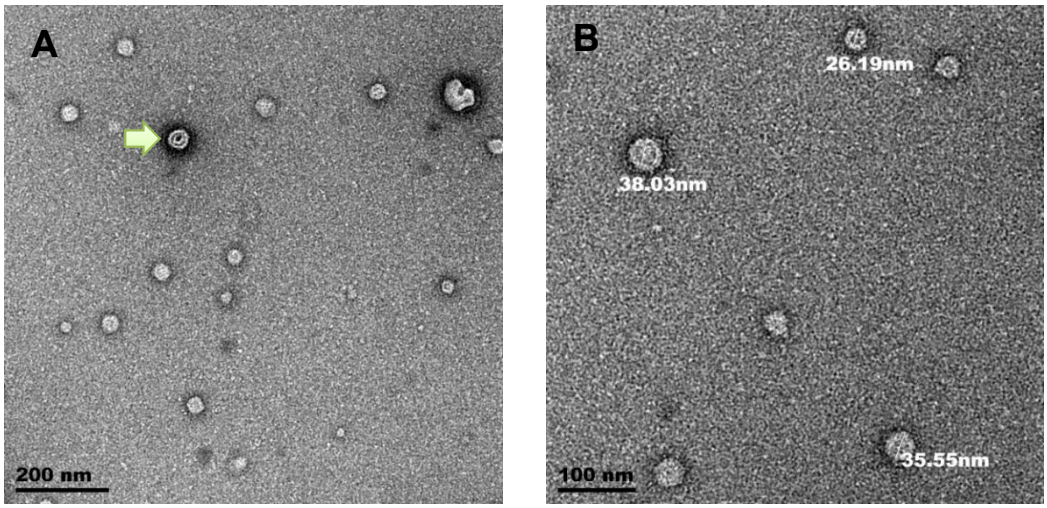


Figure A2: Ultrastructural analysis of the rotavirus DLPs. This was the first attempt to evaluate the formation of the DLPs but VP6 sphere-like particles seemed to predominate the expression.