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Heterologous expression of African horsesickness virus VP2 and the development of a potential diagnostic assay

by

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University of Pretoria

DECLARATION

I, Vuyokazi Epipodia Mareledwane, do hereby declare that this dissertation is my own work. It has been submitted to the University of Pretoria for the degree of Master of Science. It has not been submitted before for any degree at any other University.

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Signature Date

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LITERATURE REVIEW

1. INTRODUCTION

African horsesickness (AHS) is a seasonal, non-contagious but infectious disease that affects equids namely horses, donkeys, mules, zebras and occasionally dogs (Henning, 1949). The most susceptible of the equids are horses which are regarded as indicator species with a fatality rate above 90%, compared to zebras that do become infected, but show no symptoms (reviewed by Mellor, 1994). The virus is transmitted by insect vectors of the *Culicoides species*. In the field AHSV transmission between the vector and host occur by bites (Du Toit, 1944). The disease is endemic throughout Africa, across the sub-Sahara and has made its appearance in areas such as Spain, Morocco, Portugal, Pakistan and India (reviewed by Rodriguez *et al*., 1992, Mellor & Hamblin, 2004). AHS is designated an A-list status disease by the Office International des Epizooites (OIE).

1.1 HISTORY

The AHS virus was first recognized by Father Monclaro in 1569 during his journey to central and east Africa (Henning, 1949). In southern Africa in 1719 after the first introduction of horses, a major outbreak of AHSV occurred in which 1700 animals died. Since then outbreaks occurred regularly (1780, 1801, 1839, 1854, 1862 and 1891). The largest outbreak of the disease was recorded in 1854 -1855, resulted in 70 000 deaths, killing more than 40% of the horses in the Cape of Good Hope (Henning, 1949). During the 19th century, travelers, hunters, explorers and missionaries encountered AHS and its scourge was deeply felt since horses played a vital role for transportation purposes. (Henning, 1949). Outbreaks have decreased over the past centuries due to the introduction of

vaccines and a major decrease in the horse and zebra populations (reviewed Mellor & Hamblin, 2004).

1.2 STRUCTURE

The virus family Reoviridae is composed of nine genera namely- *Orthoreovirus, Cypovirus, Aquareovirus, Rotavirus, Coltivirus, Fijivirus, Phytoreovirus, Oryzavirus and Orbivirus.* AHSV together with 19 other virus species belong to the genus *Orbivirus*. AHSV consists of 9 recognized serotypes (Howell, 1962) and apart from serotype 4 there is relatedness between serotypes 1, 2 also 3, 7, 5, 8 and between 6 and 8. (Coetzer & Erasmus, 1994). Studies have revealed similarities in the structure and chemical composition of bluetongue virus (BTV) and African horsesickness virus (AHSV) which indicated that a close relationship between AHSV and BTV exists (Verwoerd & Huismans, 1969).

The AHS virion is an unenveloped particle, which is 70nm in diameter and made up of 3 layers the outer capsid, the core and the subcore (Els & Verwoerd, 1969). It consists of a double-stranded RNA genome that has 10 segments of which each encodes for at least one polypeptide (Howell, 1962, Verwoerd *et al*., 1972). The genome is enclosed within the core particle, which is made up of two major proteins, VP3 and VP7 and three minor proteins VP1, 4 and 6. VP7 is on the surface layer of the core particle and VP3 forms an inner shell and is a major component of structural integrity (Huismans *et al*., 1987). Like VP7 it is also highly hydrophobic in nature. Both VP3 and VP7 are highly conserved proteins (reviewed by Roy 1992). VP1 is the largest viral protein with (149 kDa) and is highly conserved (Huismans & van Dijk 1990, Urakawa *et al*., 1989). The second minor protein VP4 is a component of the subcore. The third and the last minor protein is VP6 which is encoded for by segment 9 and is a component of the mRNA complex (Wade-Evans et al., 1992, reviewed by Roy 1992).

The icosahedral core of the virion is surrounded by an outer coat composed of VP5 and VP2. These two are the least conserved proteins of the virion between virus serotypes. VP2 is the most variable protein, has neutralizing epitopes and is responsible for inducing serotype-specific antibodies. (Bremer *et al*., 1990, Huismans & Erasmus 1981, reviewed by Roy 1989, Roy 1992, Burrage *et al*., 1993).

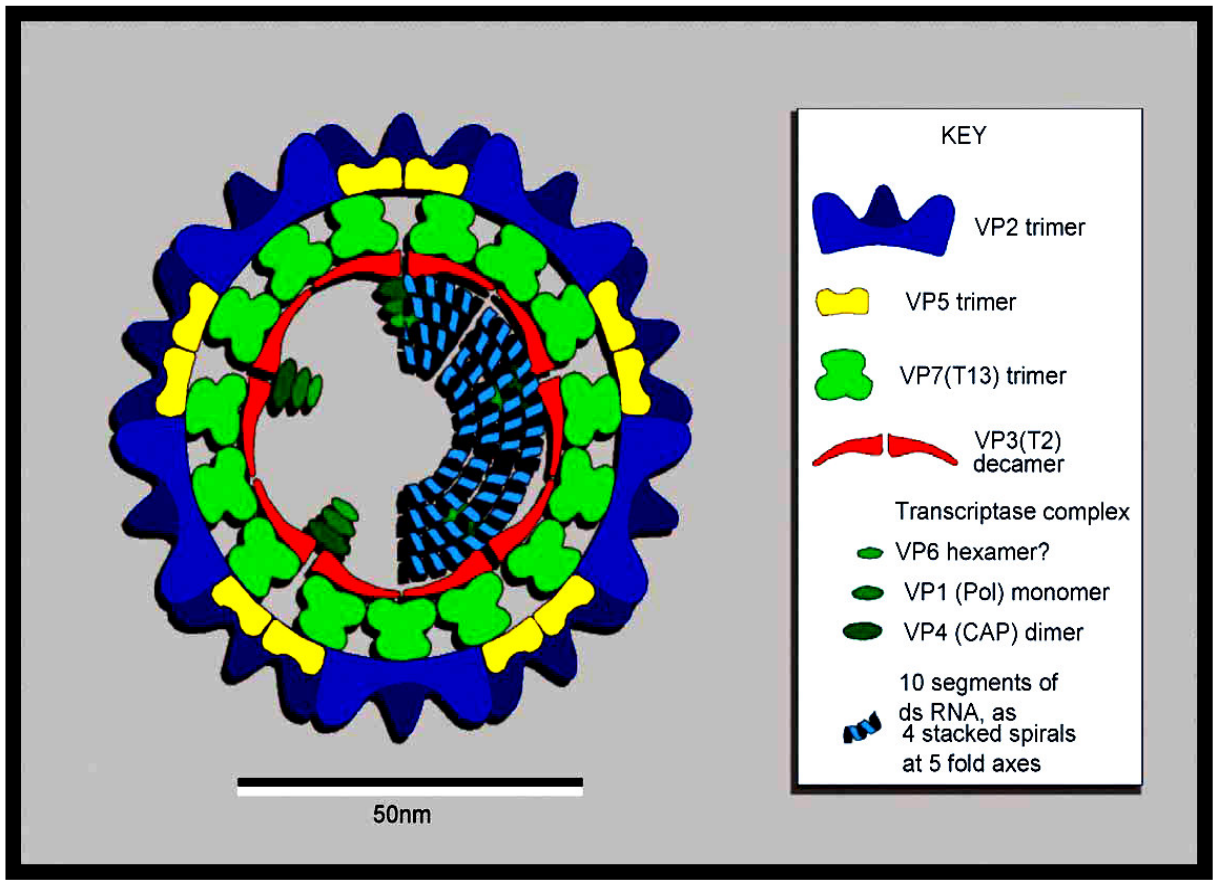


Fig. 1.1: Schematic diagram of the structure of the Bluetongue virus (BTV) particle based on structural data. (Mertens, 2004). Due to striking similarities in structure between AHS and BTV, the BTV structure has been used to illustrate the structure of AHSV particle (Verwoerd & Huismans, 1969).

1.3 HOST RANGE

Equines are the species mostly affected by AHSV. Horses are more susceptible while mules and donkeys are less susceptible with a mortality rate of 50-75%, they are also regarded as accidental hosts (Theiler, 1921). Zebras are highly resistant to the disease and have long been considered as reservoirs of AHSV. They rarely show signs of infection and are believed to play a role in the persistence of the virus in Africa (Theiler, 1921). In addition to equids, dogs are the only other species that contract the highly fatal form of the disease, but only after eating of horse meat that is contaminated (Bevan, 1911).

1.4 VECTORS

In 1944 Du Toit demonstrated the transmission of AHS by *Culicoides. Culicoides* vectors become infected by feeding from infected blood and transmit the virus to hosts through biting (Du Toit, 1944). *Culicoides imicola* is the main vector responsible for transmitting AHSV in South Africa (Venter 1997) . This species frequently occur in Africa, South-East Asia, Spain, Morocco and Portugal (Capela *et al*., 2003, Ortega *et al*., 1998). Geographical and seasonal distribution of *Culicoides imicola* is affected directly by factors such as temperature, light, rainfall and moisture. Elevated temperatures increase the metabolic activity of the vector, resulting in frequent bloodfeeding. Rainfall is considered as an important factor because it preserves the larval and pupal stages of the midges. Other factors responsible for the distribution of *C. imicola* include high rates of activity, low rates of dispersal, rapid larval movement, low adult mortality and year round breeding (Baylis *et al*., 1999, Mellor *et al*., 2000).

1.5 VACCINATION

Earlier AHS vaccines were attenuated by passage in mouse brain (Alexander *et al*., 1935). They occasionally resulted in encephalitis in horses and an accidental human aerosol infection was also reported in the 1980s (Swanepoel *et al*., 1992). This problem was solved by passaging the vaccine virus strain in cell culture (Mircharmsy & Taslimi, 1964). Onderstepoort Biological Products in South Africa supplies the vaccines in two parts: the trivalent and quadrivalent. The trivalent consists of serotypes 1, 3 and 4 while the quadrivalent consists of serotypes 2, 6, 7 and 8. Serotypes 8 and 6 provide the necessary cross protection for serotypes 5 and 9 (von Teichman *et al*., 2010)

During the period of an outbreak in Spain, Portugal and Morocco 1987-1991 an inactivated vaccine was used but after the eradication of the disease it was no longer available (Duduorget *et al*., 1992). Scalen *et al*, 2002 successfully demonstrated that a subunit vaccine of a recombinant AHSV 5 VP2, with the proper adjuvant can be used to elicit antibody production necessary for protection against AHSV serotype 5.

REFERENCES

ALEXANDER, R. A. 1935. Studies on the neurotrophic virus of horsesickness 11. Some physical and chemical properties. *Onderstepoort Journal of Veterinary Science and Animal Industry*, 4:323-348.

BAYLIS, M., MEISWENKEL, R. & VENTER, G. J. 1999. A preliminary attempt to use climate data and satellite imagery to model abundance and distribution of Culicoides Imicola in southern Africa. *Journal of the South African Veterinary Association*, 70:80-89.

BEVAN, L. E. W. 1911. The transmission of African Horsesickness to the dog by feeding. *Veterinary Journal,* 67:402-408.

BURRAGE, T.G., TREVEJO, R., STONE-MARSCHAT. M. & LAEGREID, W.W. 1993. Neutralizing epitopes of African horsesickness virus serotype 4 are located on VP2. *Virology*, 196:799-803.

BREMER, C. W., HUISMANS, H. & VAN DIJK, A. A. 1990. Characterization cloning of the AHSV. *Journal of General Virology,* 72:793-799.

CAPELA, R., PURSE, B. V., PENA, I., WITTMAN, E. J, MARGARITA, Y., CAPELA, M., ROMAO, L., MELLOR, P. S. & BAYLIS, M. 2003. Spatial distribution of *Culicoides* species in Portugal in relation to the transmission of African horsesickness and bluetongue viruses. *Medical and Veterinary Entomology*, 17:165-177.

COETZER, J. A. W. & ERASMUS, B. J. 1994. Infectious disease of the livestock with special reference to Southern Africa. Oxford University Press Cape Town. 1:460-475. ISBN 0 19 570916 0

DUDOURGET, P., PREAUD, J. M., DETRAZ, F., LACOSTE, A. C., ERASMUS, B. J. & LOBARD, M. 1992. Development, production and quality control of an industrial inactivated vaccine against horsesickness virus serotype 4 in Bluetongue, African horsesickness and related Orbivirises, CRC Press, Boca Raton pp 874-886.

DU TOIT, R. M. 1944. The transmission of Bluetongue and African horsesickness of *Culicoides*, *Onderstepoort Journal of Veterinary Science*, 19:7-16

ELS, H. J. & VERWOERD, D. W. 1969. Morphology of bluetongue virus. *Virology,* 38:213-219.

HENNING, M. W. 1949. Animal diseases in South Africa, Central News Agency, South Africa, 2nd edition. 581-602.

HOWELL, P. G. 1962. The isolation and identification of further antigenic types of AHSV. *Onderstepoort, Journal of Veterinary Research*, 29:139-142.

HUISMANS, H. & ERASMUS, B. J. 1981. Identification of the serotype specific and group specific antigens of the Bluetongue virus. *Onderstepoort Journal of Veterinary Research,* 48:51-58.

HUISMANS, H., VAN DIJK, A. A. & ELS, J. H. 1987. Uncoating of parental Bluetongue virus to core and subcore particlesin infected L cells. *Virology*, 157:180-188.

HUISMANS, H. & VAN DIJK, A. A. 1990. Bluetongue virus structural components. *Current topics in Microbiology and Immunology*, 162:21-37.

MELLOR, P. S. 1994. Epizoology and vectors of African horse sickness virus. *Comparative Immunology, Microbiology and Infectious Disease*, 17:287-296.

MELLOR, P. S., BOORMAN, J. & BAYLIS, M. 2000. *Culicoides* biting midges: their role as arbovirus vectors. *Annual Reviews of Entomology*, 45:306-340.

MELLOR, P. S. & HAMBLIN, C. 2004. African horsesickness. *Veterinary Research,* 35:445-466.

MERTERNS, P. 2004. The dsRNA viruses. Virus Research, 101:3-13.

MIRCHAMSY, H. & TASLIMI, H. 1964. Immunization against AHS with tissue culture adapted neurotrophic viruses. V*eterinary Journal*, 19:7-16.

ORTEGA, M. D., MELLOR, P. S., RAWLINGS, P. & PRO. M. J. 1998. The seasonal and geographical distribution of *Culicoides imicola*, *C. pulicaris* group and *C. obsoletus* group biting midges in central and northern Spain. *Archives Virological Supplements*, 14:85-91.

RODRIGUEZ, M., HOOGHUIS, H. & CASTANO, MA. 1992. African horsesickness in Spain. *Veterinary Microbiology*, 33:129-142.

ROY, P. 1992 Bluetongue virus proteins. *Journal of General Virology,* 73:3051-3064.

ROY, P. 1989. Bluetongue virus genetics and genome structure. *Virus Research,* 13:179-206.

SCALEN, M., PAWESKA, J. T., VERSCHOOR, J. A. & VAN DIJK, A. A. 2002. The protective efficacy of a recombinant VP2 based African horsesickness subunit vaccine candidate is determined by adjuvant. *Vaccine,* 20:1079-1088.

SWANEPOEL, R., ERASMUS, B. J., WILLIAMS, R. & TAYLOR, M. B. 1992. Encephalitis and chorioretinitis associated with neurotropic African horsesickness virus infection in laboratory workers. *South African Medical Journal,* 81:458-461.

THEILER, A. 1921 African horsesickness. *Scientific Bulletin*, 19:1-29

URAKAWA, T., RITTER, D. G. & ROY, P. 1989. Expression of the largest RNA segment and synthesis of VP1 protein of bluetongue virus in insect cells by recombinant baculovirus: association of VP1 protein with RNA polymerase activity. *Nucleic Acids Research*, 17:7395-2142.

VENTER, G. J., NEVILL, E. M. & VAN DER LINDE, T. C. 1997. Seasonal abundance and parity of stock- associated Culicoides species (Diptera:Ceratopogonidae) in different climatic regions in southern Africa in relation to their viral potential. *Onderstepoort Journal of Veterinary Research*, 64:259-271.

VERWOERD, D. W. & HUISMANS, H. 1969. On relationship between BTV and AHSV and reovirus: hybridization studies. *Onderstepoort Journal of Veterinary Research*, 36(2):175-180.

VERWOERD, D. W., ELS, H. J., DE VILLIERS, E. & HUISMANS, H. 1972. Structure of the bluetongue virus capsid. *Journal of Virology*. 10:783-794.

VON TEICHMAN, B F., DUNGU, B. & SMIT T. K. 2010. *In vivo* cross protection to AHSV serotypes 5 and 9 after vaccination with serotypes 8 and 6. *Vaccine.* 28:6505-6517.

WADE-EVANS, A. M., MERTENS P. P. C. & BELSHAM, G. J. 1992. Sequence of genome segment 9 of bluetongue virus (serotype 1, South Africa) and expression analysis demonstrating that different forms of VP6 are derived from

initiation of protein synthesis at two distinct sites. *Journal of General Virology*, 73:3023-3026.

CHAPTER 2

EXPRESSION OF THE GENOME SEGMENT ENCODING VP2 USING THE BACULOVIRUS EXPRESSION SYSTEM

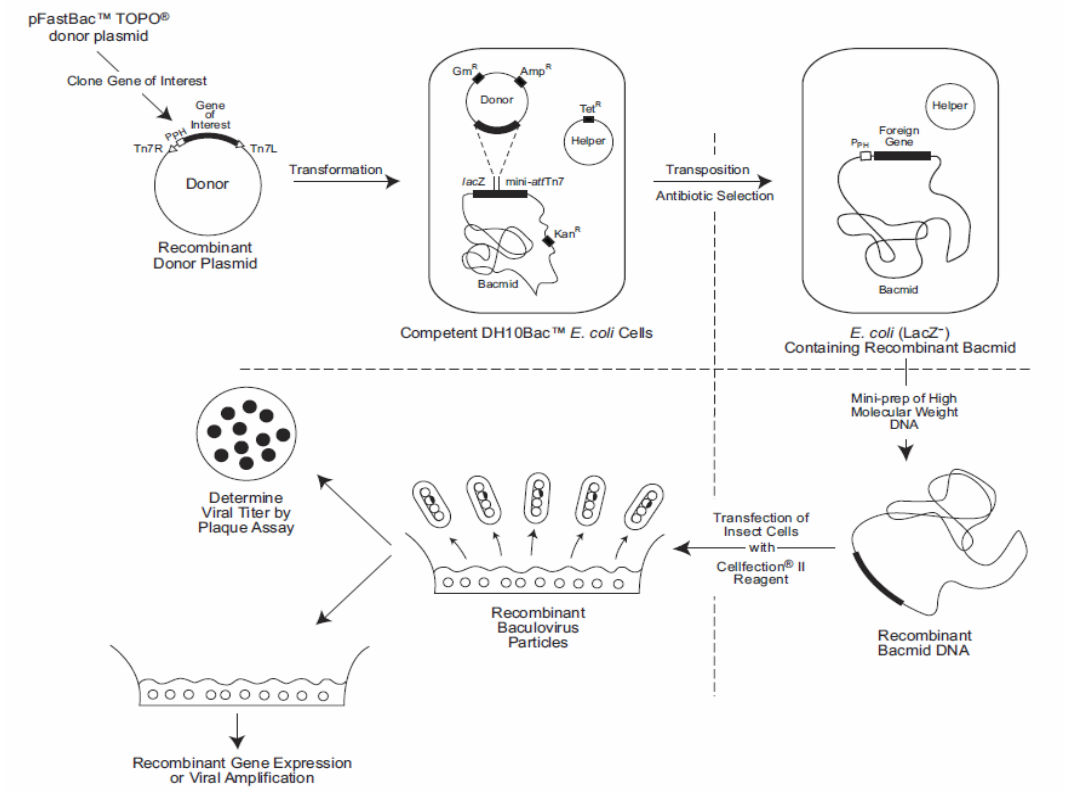
2.0 INTRODUCTION

The polyhedrin gene of the baculovirus is not essential for viral replication and is highly expressed in insect cells (Smith *et al*., 1983a). This has led to its manipulation and use for the expression of high yields of heterologous proteins (Smith *et al*., 1983b). A study by Smith and coworkers (1983b) described the expression of high levels of beta interferon using a baculovirus expression vector under the control of a polyhedron promoter. Baculovirus expression is based on the principle of the replacement of the polyhedrin gene with the gene of interest. The DNA of the baculovirus *Autographa californica* nuclear polyhedrosis (AcMNPV) is commonly used for construction of baculovirus expression vectors (Luckow & Summers, 1988, Mori *et al*., 1992). In addition to them being utilised as expression vectors, baculoviruses have been of interest in the agricultural field for biopesticides (Lu *et al*., 1996), in the biomedical field for gene therapy (Hofmann *et al*., 1998, Sandig *et al*., 1996) and as potential vaccine vectors (Abe *et al*., 2003, Scalen *et al*., 2001).

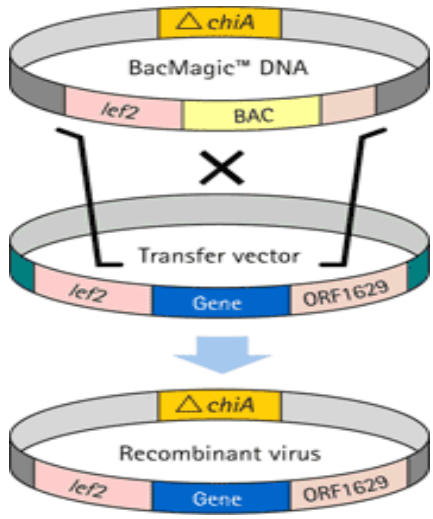
The baculovirus expression system has been used over the past few decades for the expression of African horsesickness virus (AHSV) and bluetongue virus (BTV) (from the *Reoviridae* family) proteins in insect cells (Roy *et al*., 1996, Roy *et al*., 1990). Inumaru and colleagues described the expression of recombinant baculoviruses expressing BTV serotype 17 VP3 in insect cells (Inumaru *et al*., 1987). The VP3 antigen induced antibodies in mice and rabbits. For VP3 expression, Inumaru and colleagues (1987) transfected *Spodoptera frugiperda (Sf 9*) cells with AcNMPV and plasmid pAcSI17.3. Since then, more baculovirus expression systems have been developed. Traditionally, the classical method required the co-transfection of the recombinant transfer vector together with the baculoviral DNA. This would be followed by several rounds of plaque purification

to select the recombinant among the non-recombinant wild type virus. As can be expected the process was extremely laborious and cumbersome. This technology was followed by the development of linearised baculovirus DNA, Kitts and co-workers (1990) linearised the baculoviral DNA by introducing a Bsu361 restriction enzyme site. Digestion of the baculovirus DNA disrupts the ORF 1629 required for replication. Recombinant baculoviruses were generated and the ORF 1629 was restored, white plaques were observed against blue plaques (non recombinant virus). Other technologies such as the BAC-to-BAC expression systems have been developed (Luckow *et al*., 1993) for facilitating the rapid production of recombinant proteins.

This chapter deals with the generation of recombinant baculoviruses using the BacMagic DNA and BAC-to-BAC system. pBAC transfer vectors (recombinant pBACgus-1 & -5) are transfected together with the BacMagic DNA into insect cells. These vectors provide relevant promoters and tags for purification and detection of the protein. BacMagic DNA allows expression of only recombinant baculoviruses. Replication of non-recombinants is inhibited because only homologous recombination occurs within the insect cells. When this recombination occurs, ORF 1629 which was previously deleted is restored while the sequence of interest replaces the BAC (bacterial artificial chromosome) sequence (Fig. 2.1). The BAC-to-BAC system of generating baculovirus recombinants differs from the BacMagic in that recombination occurs by site specific transposition (Luckow *et al*., 1993) between the recombinant pFastBac and the bacmid (Fig. 2.1). The recombinant pFastBac is attached to a mini-Tn7 site, and the bacmid to the mini-*att*Tn7 region and the LacZ regions. Upon successful transposition the recombinant bacmids are identified as white colonies. Another major difference between the BacMagic DNA and the BAC-to-BAC is that the BacMagic has removed the plaque purification step, which is normally referred to as laborious and time consuming (Fig. 2.1).



(a)



(b)

Fig 2.1: Diagrams representing construction of recombinant baculoviruses using the **(a)** BAC-to-BAC expression system (diagram obtained from Invitrogen manual) and **(b)** BacMagic DNA expression system (diagram obtained from Novagen manual).

The aim of this chapter is to express and compare the expression levels of AHSV VP2 gene between the pFastBac-1 and pBACgus-1 and -5. Expression of the protein of interest in pBACgus-1 is under the control of the *polh* promoter very late stage in the replication cycle. While expression of the protein of interest in pBACgus-5 is under the control of the gp64 promoter in the early and late phase of the replication cycle (as shown in Fig. 2.4). The BacMagic DNA was reported by Novagen, the manufacturer as an improvement on the traditional method, due to the removal of the time consuming plaque purification step (as mentioned in the previous paragraph). The BAC-to-BAC has been reported to be unstable as a result of accumulation of defective interfering particles (DI's) in cell culture (Piljman *et al*., 2001, Piljman *et al*., 2002 & Kool et al, 1991). DI's are viral particles that are generated in cell culture and after numerous rounds of passaging, they become prevalent.

2.1 MATERIALS AND METHODS

2.1.1 AMPLIFICATION OF THE VP2 ENCODING REGION

The AHSV 2 VP2 was amplified from AHSV 2 VP2pFastBac (the AHSV 2 VP2 was cloned by Potgieter *et al*., 2003) which is available at the Biochemistry Division at Onderstepoort Veterinary Institute. Primers (synthesized by Inqaba Biotech) with the *Xho*I and *Not*I restriction enzyme sites were designed for the amplification of VP2-gene (Fig. 2.2). The restriction enzyme sites were added on the ends of the primers to facilitate the cloning the VP2-gene into the baculovirus expression vectors. Extra nucleotides were added to the ends of the primers to ensure that the endonucleases do cut at the end of the PCR product. The sequences for both the forward (a) and reverse primers (b) respectively were as follows:

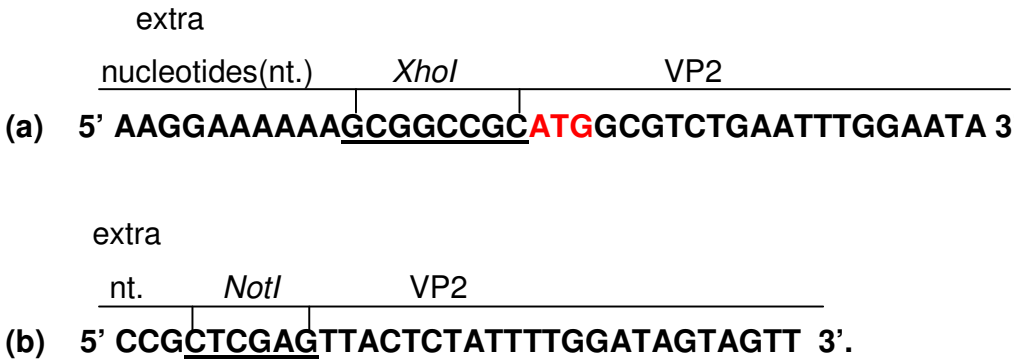


Fig. 2.2: Primers used for the amplification of AHSV 2 VP2: (a) - forward primer and (b) - reverse primer. The primers consist of restriction enzyme sites (underlined) and are flanked on the left side by extra nucleotides for efficient digestion by the endonucleases. ATG (in red) represents the start codon.

The PCR reaction was performed in a total volume of 50 µl. The PCR consisted of distilled H_2O (dH_2O), the *Ex Taq* buffer, dNTPs, the template VP2pFastBac, the enzyme *Ex Taq* (*Takara*) and primers.

Table 2.1: Components and the concentrations of the PCR

Components of the PCR	Final concentration
Buffer	1 ×
dNTPs	0.2 µM
Forward Primer	0.2 pMol
Reverse Primer	0.2 pMol
DNA Polymerase	1 U

The PCR was performed in a GeneAmp PCR System 2700 (Applied Biosystems). The reaction consisted of 30 cycles of which the first cycle was the initial denaturation step at 94°C for 2 min. Thereafter, the 29 cycles consisted of the following: the denaturation step at 94°C for 30 sec, the annealing step at 47°C for 30 sec and the initial extension step at 72°C for 1 min 30 sec. The last cycle of the PCR was the final extension step at 72°C for 5 min. The PCR products (5µl) were mixed with 1µl of the 6 × loading dye. The products were separated on a 1% TBE agarose gel containing ethidium bromide to a final concentration of 0.5 µg/ml. The gel was electrophoresed at 100 V for 1.5 hrs. The DNA bands were visualized on a UV transilluminator and the images were captured using an AutoChemi UVP BioImaging System .

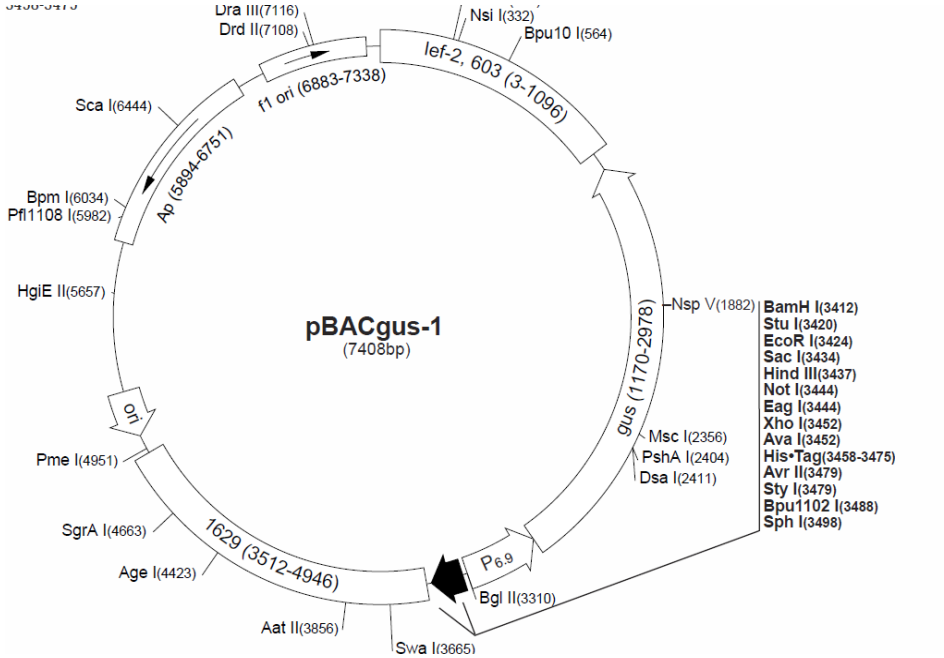
2.1.2 PURIFICATION OF THE VP2 AMPLICON

Purification of the VP2 amplicons was done using the QIAquick PCR purification kit (QIAgen). The principle of the kit is based on the binding of DNA to the silica membrane of the column, in conditions where there is a high salt concentration and correct pH. The DNA is eluted while impurities and contaminants are washed

away. The first buffer added to the PCR products is the binding buffer that corrects both the salt concentration and pH because the binding of the nucleic acid to the silica membrane only occurs under high salt concentrations. For binding, the PCR products were added to the column and centrifuged for 60 sec at 10 000 × g on a bench top microcentrifuge. The column was washed with a buffer containing ethanol, and centrifuged for 60 sec at 10 000 × g. The washing step is for the removal of unwanted impurities such as salts which also bound to the membrane. For elution of DNA, the column was placed in a clean microcentrifuge tube, a buffer containing 10 mM Tris-Cl (pH 8.0), was added and centrifuged for 1 min. A sample of the purified PCR products was separated on a 1 × TBE agarose gel and stained with 0.5 µg/ml ethidium bromide. The DNA bands were visualized on a UV transilluminator.

2.1.3 DIGESTION OF VECTORS AND VP2 AMPLICON

Baculovirus expression vectors pBACgus-1 and -5 and the purified VP2 amplicon were double digested with the restriction enzymes *Xho*I (Roche) and *Not*I (Roche) to ensure overhangs on both the insert and the vector, to create compatibility for efficient ligation. The restriction enzyme digestion for the purified VP2 amplicon was combined in a 20 µl reaction volume as follows: 5 µl VP2 amplicon, 2 µl of buffer H (Roche), 1 µl *Xho*I (Roche), 1 µl *Not*I (Roche) and 11 µl distilled water.and incubated overnight at 37°C. For restriction digestion of the vectors pBACgus-1 (and pBACgus-5), a similar reaction was carried out: 5 µl pBACgus-1 (and pBACgus-5 respectively), 2 µl of buffer H (Roche), 1 µl *Xho*I (Roche), 1 µl *Not*I (Roche) and 14 µl distilled water, combined and incubated overnight at 37°C. Buffer H was used as the optimal buffer for the restriction digestion reactions as it achieves 100% percentage activity for both the enzymes involved the double digestion (as recommended by the suppliers). The VP2 amplicon and the baculovirus expression vector were separated on a 1% agarose gel to confirm digestion as well as purification (as described under section 2.1.4).



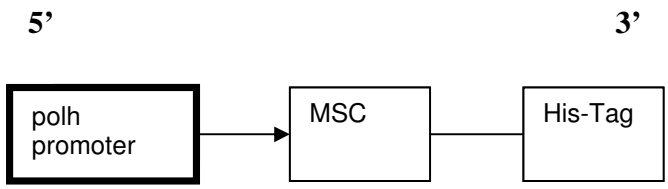
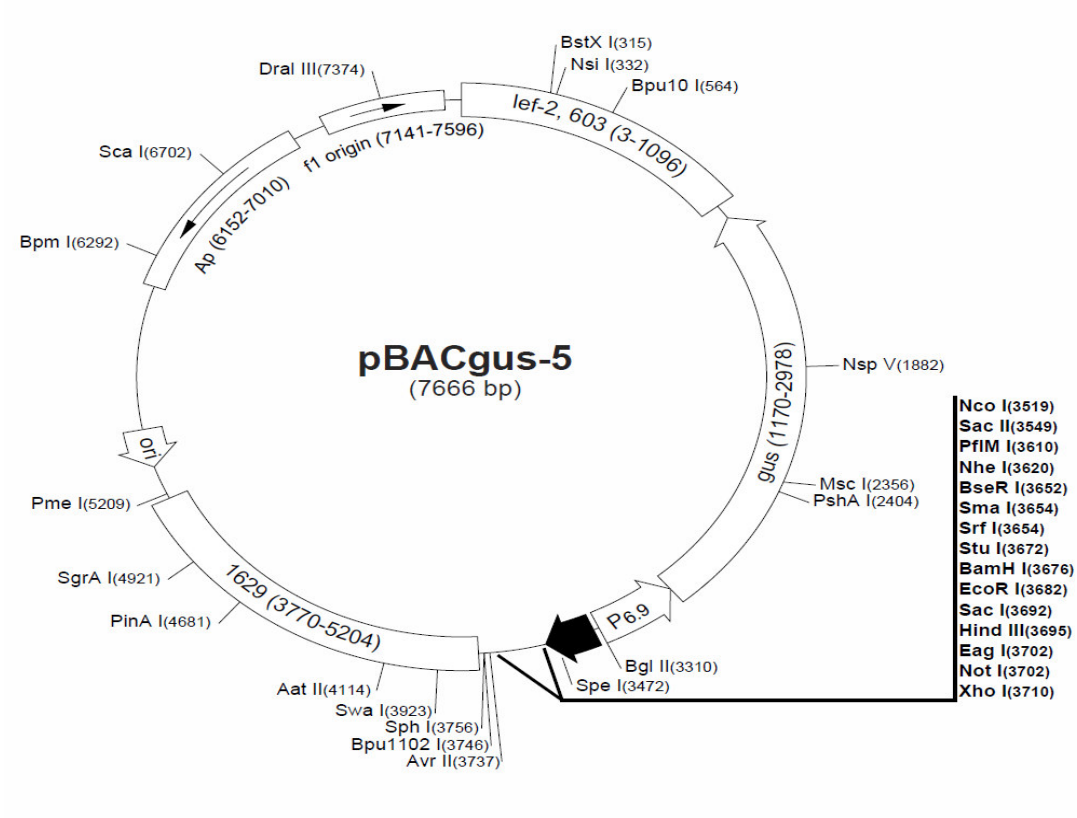


Fig 2.3 Illustration of a vector map representing pBACgus-1 (Vector map obtained from Novagen manual). The sequence representation shows the position of the *polh* promoter, MSC (multiple cloning sites) and the His-tag.



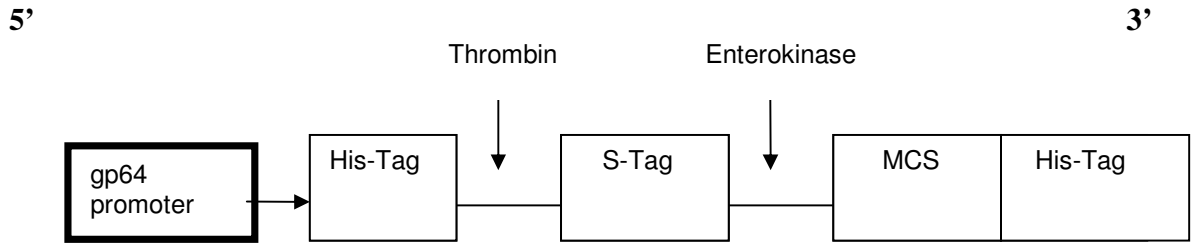


Fig. 2.4: Illustation of vector map representing pBACgus-5. (Vector maps obtained from Novagen manual). The sequence representation shows the position of the *gp64* promoter, MSC (multiple cloning sites) the His-tag, S-tag, the thrombin and enterokinase sites.

2.1.4 PURIFICATION OF DIGESTION PRODUCTS

The digestion products were cleaned using the QIAquick gel extraction kit (QIAgen). The principle of the kit is the same as 2.1.2. The digestion products (VP2 and pBACgus-1 & -5) were loaded on a 1% agarose gel and separated using a 1 × TAE buffer. The bands of interests were cut from the gel using a sharp sterile scalpel blade. The gel slices were weighed and 3 volumes of buffer QG was added for every 1 volume of the slice. To dissolve the gel slice the eppendorf tubes containing the gel slices were heated for 10 min at 50℃. After they had dissolved the colour of the mixture remained yellow, this was a confirmation that the mixture was at the correct pH for binding of DNA to the silica membrane. Isopropanol was added to improve the DNA yield and the mixture was centrifuged for 1 min at 17 900 × g in order for it to bind to the membrane. The flow-through was discarded and buffer QG was added and centrifuged for 1 min at 17 900 × g to remove any extra contaminating agarose. Buffer PE containing ethanol was added to wash away all the impurities and incubated for 5 min before centrifuging for 1 min at 17 900 × g. The DNA was eluted with 50 µl of buffer EB. The sample of the purified DNA (3 µl) was mixed with 1 µl of 6 × loading dye and run on 1% agarose gel with TBE buffer and viewed by the transilluminator with ultraviolet wavelength (280nm) .

2.1.5 LIGATION REACTION

The Rapid DNA ligation kit (Roche) was used for the ligation reaction of the insert to the vector. Sticky-end ligation reactions were performed in 20 µl reactions containing: 10 µl of the 2 × ligation buffer (Roche), 300 ng of the digested and purified vectors pBACgus-1 (or -5) respectively, 250 ng of the digested and purified VP2 insert and 10U of the T4 DNA ligase (Roche). The ligation reactions were incubated for 1 hr at room temperature. The whole 20 µl ligation reaction was transformed into the competent *E. coli* SURE (Stratagene) cells as described below.

2.1.6 TRANSFORMATION

(i) Preparation of competent cells

Competent *E. coli* cells were made by a chemical method described by Inoue *et al*. (1990). The method was found by Inoue and co-workers (1990) to be simple and affective and produced cells with high transformation efficiencies. that retain their competency during storage. The method briefly: frozen stock of *E. coli* SURE cells (Stratagene) were thawed and streaked out on an LB agar plate and incubated overnight at 37℃. Single colonies (11-12) were picked and inoculated in 250 ml SOB medium (2% bacto tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate) pH 7.0 in a 2ℓ flask and grown to OD_{600} = 0.6 at 18℃ with shaking (200-250 rpm). The culture was incubated on ice for 10 min and the cells were pelleted for 10 min for 4℃ at 4000 rpm JA-14 rotor in a Beckmann J2-21M/E centrifuge. The cells were gently resuspended in 80 ml of ice cold transformation buffer (TB) (10 mM Pipes, 55 mM $MnCl_2$**,** 15 mM $CaCl_2$, 250 mM KCl), incubated for 10 min on ice and spun down at 4000 rpm for 10 min at 4℃ in a JA-14 rotor in a Beckmann J2-21M/E centrifuge. The cell pellet was resuspended in 20 ml of TB and dimethyl sulfoxide (DMSO) to a final concentration of 7%. After incubation on ice for 10 min the cell suspension was dispensed into aliquots in 1-2 ml cryotubes and then snap frozen in liquid nitrogen. Cells were stored in liquid nitrogen for up to one year.

(ii) Transformation

Transformation of the chemically competent *E. coli* SURE cells was done as described by Inoue *et al.* (1990). The ligation reaction (20 µl) was added into 200 µl of thawed competent cells and placed in an ice bath for 30 min. The mixture was heat shocked at 42℃ for 30 sec, incubated on ice for a further 2 min. Cells were subjected to heat shock to make the inner membrane of the cell permeable

to the recombinant plasmid thus increasing the transformation efficiency. Approximately 800 µl SOC medium (2% bacto tryptone, 0.5% yeast extract, 10 mM sodium chloride, 2.5mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose) was added and the mixture was shaken for 1 hr in a 37°C incubator for the recovery of the cells. From the transformation mixture 100 µl aliqouts were plated out on LB agar containing (10 µg/ml) tetracycline and (20 µg/ml) ampicillin and incubated overnight at 37°C. The competent cells were transformed with the pUC19 plasmid in order to calculate the transformation efficiency. The transformation efficiency was determined using the formula below:

$$\textbf{Transformation efficiency (cfu/ µg)} = \frac{\text{Colony forming units}}{0.001\ \text{ng}} \times \frac{10^3\ \text{ng}}{1\ \mu\text{g}} \times \text{dilution factor}$$

2.1.7 PLASMID ISOLATION

Plasmid isolation was done as described by the QIAprep miniprep product manual (QIAgen). The method is based on the alkaline lysis of bacterial cells (Birnboim & Doly, 1979), followed by the DNA attaching to the silica under high salt conditions. The method involves three steps: the first one is the clearing of bacterial lysate, the second one is the attaching of DNA onto the QIAprep silica membrane and the last one is the washing and elution of the plasmid DNA. A single colony picked from transformation reactions was inoculated in 5 ml LB medium containing (20 µg/ml) ampicillin, (10 µg/ml) tetracycline and incubated overnight with shaking at 37°C. After the incubation the bacterial cells were harvested at 6800 × g and pellet resuspended by buffer P1 mixed with RNase A. This is followed by the addition of buffer P2 containing SDS/NaOH which is mainly for making the bacterial cell membrane porous for the release of cellular contents. The next buffer creates a high salt binding condition which results in the precipitation of chromosomal DNA, while SDS and the smaller plasmid remains in solution. The lysate was centrifuged at 17 900 × g and the

supernatant was applied to the column with the silica membrane and centrifuged for 30-60 sec to bind the plasmid DNA. For the removal of contaminants and impurities the silica membrane was washed with buffer PE containing ethanol. For elution of the DNA 50 µl of the buffer containing 10 mM Tris-Cl (pH 8.5) was applied and centrifuged for 1 min. Restriction enzyme digestions with the appropriate enzymes was performed for confirming the correct size of the recombinant plasmid and visualized after ethidium bromide staining on a UV transilluminator on a 1% agarose gel.

2.1.8 SEQUENCING OF CLONES

The full length clones VP2pBACgus-1 and -5 were sequenced by primer walking. In order to sequence the 3.2 kbp VP2 gene, six primers were designed at regular intervals of 500 base pairs. Even though these primers were based on known sequence information, a single continuous open reading frame for the AHSV 2 VP2 could not be obtained. Three additional primers (400^{th}, 600^{th}, 1200^{th}) based on the new sequence information, in the reverse orientation, were designed and this allowed a single continuous open reading frame for the AHSV 2 VP2 to be obtained for both clones. For sequencing the ends of the clones primers pBAC (reverse primer), Polh (forward primer) and Stag (forward primer) were designed (Table 2.2 and Fig. 2.5). The kit that was used for sequencing was the Big Dye Terminator v 3.1 Cycle Sequencing Kit and the sequencer the Genetic Analyser 3100 (both from Applied Biosystems). The Staden Package (Bonfield *et al*., 1995) was used for sequence assembly and analysis. The sequencing was done at the Onderstepoort Veterinary Institute sequencing lab.

Table 2.2: Sequences of primers for primer walking for VP2pBACgus-1 and VP2pBACgus-5

Nt. location that primer annealed	Sequences
500th	5' TAAGGTATCCATTTGATATAAGAT 3'
1000th	5' AACGAGGCGATGATACCACA 3'
1500th	5' TATTATGGGGCTGAGATTGAG 3'
2000th	5' GTAAAGGGAGGGGGAAGAG 3'
2500th	5' TCAATGAGTTTGAGAGGTTTAG 3'
3000th	5' CGGGAGTGGGAAATGGGC 3'
1200th	5' CAATCAACCCAATGATCAACC 3'
600th	5' TAATTGATACAACACATCTTCGT 3'
400th	5' TTGTCTCTTACAGAAAACGGTA 3'
S tag	5' AGGCAGCGGCAAAGAAACG 3'
Polh	5' CCATCTCGCAAATAAATAAAGTA 3'
pBAC	5' CTGTAAATCAACAACGCACAG 3'

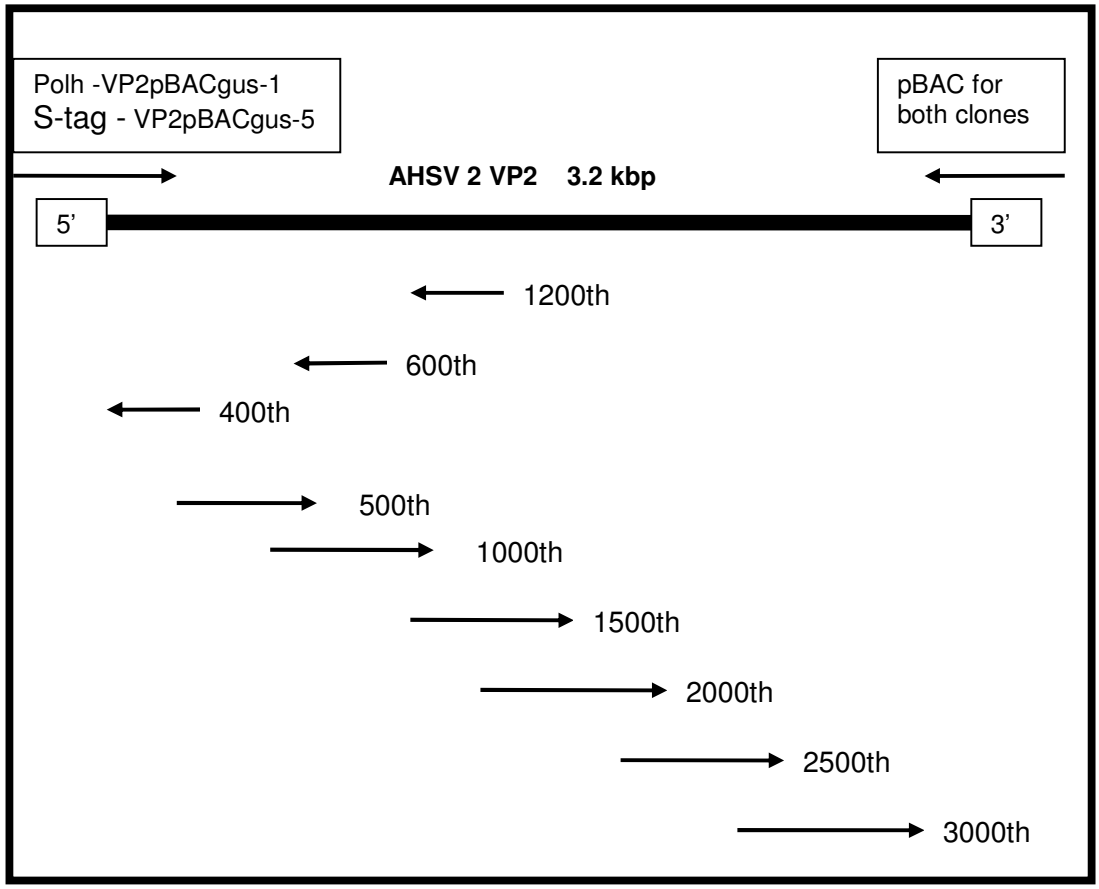


Fig. 2.5: Diagram showing primer walking for VP2pBACgus-1 and -5. The primers are indicated by the arrows, with the name indicating the annealing position on the gene and arrows direction indicating the direction of the strand synthesis

2.1.9 EXPRESSION USING THE BACMAGICSYSTEM: CELLS AND MEDIA

The *Sf9 (Spodoptera frugiperda)* cells were grown and maintained at 27℃. The media used for maintaining these cells was Grace's media (Whitehead Scientific (PTY) LTD) supplemented with 10%, fetal bovine serum (FBS) (The Scientific Group) 1% antibiotic mixture (Penicillin, Streptomycin and Amphotericin-B) (Whitehead Scientific (PTY) LTD) and 1% glutamine (200 mM stock) (Whitehead Scientific Group). In cases where the cells were grown in suspension, pluronic (Scientific Group) was added to a final concentration of 0.1% to prevent breakage of the cells. Confluent monolayers were initiated from the frozen cells

which were thawed at 37°C and grown at 27°C in 75 cm^2 tissue culture flasks to a density of 1.2 × 10^7 cells/ml. Suspension cultures were derived from these monolayers.

2.1.10 *Sf9* CELL TRANSFECTION WITH BACMAGIC

Six well plates were seeded with *Sf9* cells to a density of 1 × 10^6 cells/ml, 1 hr before use. The plates were briefly rocked side to side and back and forth to ensure even spread of the cells. During the 1 hr incubation period for attachment of the cells, the transfection mixture was prepared as follows: solution A and B in different polystyrene tubes. Solution A consisted of 83 µl serum-free Grace's medium and 6 µl FuGENE (ROCHE) was added dropwise to the mixture, vortexed for 5 sec and incubated for 5 min at room temperature. Solution B consisted of 7.5 µl BacMagic (Novagen) and 3 µl of plasmid DNA of the clones (VP2pBACgus-1 and or VP2pBACgus-5 separately). Solutions A and B were mixed, vortexed for 1 sec and incubated for 30 min at room temperature. Towards the end of transfection mixture incubation period, culture medium was removed from insect cell monolayers at a 30-60° angle so as not to disturb the monolayer. Transfection mixture (1 ml) was added per well, drop wise to the centre of the plates and was incubated 1 hr at 27°C. At the end of the incubation period, 1 ml of Grace's medium containing serum and antibiotics was added. The plates were further incubated in a plastic container with a moist paper towel, at 27°C for 4-5 days.

2.1.11 HARVESTING INFECTED CELLS AND CELL LYSIS

After the incubation period of 4-5 days the medium was removed and centrifuged 600 × g at 15°C for 2 min. X-gluc (15 µl) was added to the remaining undisturbed monolayer in the six well plates. The supernatant was stored at 4°C while the pellet was washed with 1/10 of the original volume of the culture with 1 × PBS containing 10% glycerol. The cells were re-suspended gently in lysis buffer (0.05

M Tris pH 8, 0.35 M Sodium chloride, 0.5% NP40, 0.25% deoxycholic acid to a volume of 100 ml) and incubated for 30 min. A syringe (18-21G) was used to homogenize the suspension. Approximately 10ul of the sample was mixed with 10 µl of the 2 × SDS sample buffer (62.5 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 1% ß-mercapto-ethanol, 0.005% bromophenol blue mixed with water to a final volume of 100 ml) for the SDS-PAGE analysis. The mixture was placed in boiling water bath for 5 min and sonicated for 15 min. The sample was loaded and separated on a 12% SDS-PAGE gel.

2.1.12 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Proteins were separated by the SDS polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was performed in a discontinuous buffer system (Laemmli, 1970). Glass plates assembled using spacers clamped together.

10% acrylamide separation gel was prepared as follows:

30% acrylamide/0.8% bisacrylamide	5 ml
4 X Tris Cl/SDS	3.75 ml
H_2O	6.25 ml
10% (w/v) ammonium persulfate	0.05
TEMED	0.01

The 10% acrylamide was cast into the gel mould. Sufficient space was left stacking gel. The acrylamide solution was overlaid with water-saturated butanol to prevent oxygen from diffusing into the gel and inhibiting polymerization. The gel was allowed to polymerize for 1 hr at room temperature. After polymerization the butanol overlay was poured off the gel and washed repeatedly with distilled water.

4% stacking gel was prepared as follows:

30% acrylamide/0.8% bisacrylamide	0.65 ml

4 X Tris Cl/SDS	1.25 ml
H_2O	3.05 ml
10% (w/v) ammonium persulfate	0.025 ml
TEMED (w/v)	0.005

The stacking gel was poured directly onto the surface of the polymerized separating gel. A comb was inserted into the stacking gel and was allowed to polymerize for 1 hr at room temperature. Protein samples were boiled and sonicated after the addition of the 2 × sample buffer just as mentioned above. A high molecular weight colour protein marker (15 µl) (from Bio-Rad, 250 kDa) and 13 µl of the protein sample were loaded into the wells and electrophoresed 16 hrs at 90 V and 10 mA. A 1 × TGS (0.125 mM Tris, 7.2% Glycine and 0.5% SDS) running buffer was used. The gel was stained with Fairbanks A (0.05% Coomassie Brilliant Blue, 25% isopropanol, 10% acetic acid) for 15 min and destained in a destaining solution (10% Acetic Acid) (Wong C. *et al*., 2000).

2.1.13 EXPRESSION WITH THE BAC-to-BAC: INFECTION OF INSECT CELLS

Six wells (35mm) were seeded between 80-90% confluency per well in 2 ml of media containing FBS, glutamine and antibiotics and incubated 45-60 min at room temperature. The medium was removed and 500 µl fresh medium containing 10% FBS, 1% glutamine (200 mM) and 1% the antibiotic mixture was gently added to each well, 30 µl virus supernatant (as described in 2.1.11) was added. *Sf9* cells were individually infected with recombinant baculovirus expressing VP2 AHSV serotypes 1-5. The plates were gently agitated for 1 hr at room temperature. Media (1.5 ml) containing 10% FBS, 1% glutamine (200 mM) and 1% antibiotic mixture was added and incubated at 27℃ for 48 hrs. After the incubation period the virus was removed, transferred to sterile eppendorf tube and centrifuged at 450-600 × g for 5 min, to remove debris and cells. The wells were rinsed with 1 × PBS to remove the attached cells. The attached cells were

spun down at 450-600 × g for 5 min. The pellet was resuspended in 1 × PBS. The protein sample with the sample buffer was boiled and sonicated in preparation for SDS-PAGE analysis (as described in 2.1.12).

2.1.14 GENERATING PASSAGE 2 STOCKS

Passage 2 stock of AHSV serotype 2 was generated by seeding 75 cm^2 cell culture flasks to a density of 80-90% confluency in the flask. After an hour the medium was removed and replaced with 10 ml of media containing 1% glutamine (200 mM), 1% antibiotic mixture and 10% FBS. Approximately 250 µl of the passage 1 virus was added directly to the medium and incubated with gentle agitation for 1-2 hrs. Grace's medium (10 ml) containing 1% glutamine (200 mM), 1% antibiotic mixture and 10% FBS was added and incubated for 4 days. After the incubation period the media was centrifuged at 450-600 × g for 8 min to remove cells and debris. A 0.22 µm filtrate was used for purifying the virus. The pellet was washed gently in $1/10^{th}$ of the original culture volume with 1 × PBS containing 10% glycerol. The protein sample was prepared for the SDS-PAGE analysis.

2.1.15 DETERMINING THE VIRAL TITRE OF THE PASSAGE 2 STOCK

Culture wells (35 mm) were seeded to 80-90% confluency in 2 ml of media. The cells were allowed to attach at room temperature for 45-60 min. A dilution series of the virus of 10^{-6}-10^{-11} from the passage 2 stock was prepared in Grace's medium containing 1% glutamine (20 mM), 1% antibiotic mixture and 10% FBS. The medium was removed completely from the attached cells and replaced with 1 ml of the virus dilutions. Infections were done in duplicate for each viral dilution and were gently agitated at room temperature for 2 hrs. After 2 hrs the virus dilutions were removed and immediately replaced with a cooled 2 ml 0.5% agarose overlay in the culture wells. The plates were allowed to stand on the bench top for 20-30 min and 1 ml of the Grace's media containing 1% glutamine

(20 mM), 1% antibiotic mixture and 10% FBS was added. They were placed in a large dish with a piece of wet tissue for moisture for 4 days at 27℃. Neutral red to a final concentration of 100 µg/ml in 1 ml media was added to the plates and allowed to stand for 3 hrs. The solution was then completely removed and incubated overnight at 27℃. The plagues were counted after the incubation period. The titer was determined by the following formula: total dilution factor × number of plaques.

2.2 RESULTS

2.2.1 PCR AMPLIFICATION OF THE VP2 GENOME ENCODING SEGMENT

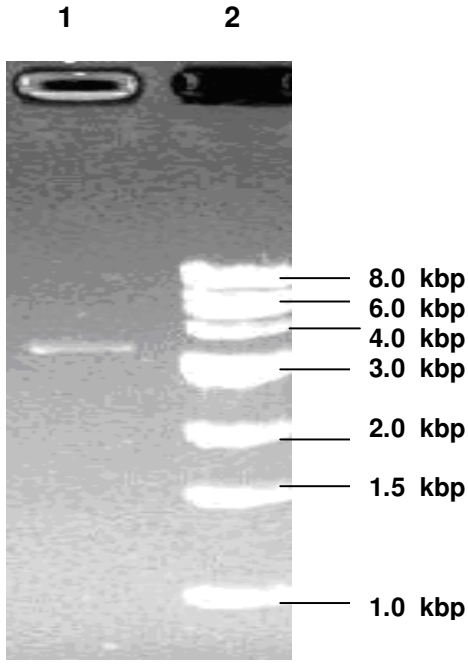


Fig. 2.6: Agarose gel electrophoretic analysis of the full length AHSV 2 VP2 amplicon. Lane 1, PCR product AHSV 2 VP2 generated (~3.0 kbp); Lane 2, molecular weight marker. The sizes of the molecular weight marker (1 kb DNA ladder, New England Biolabs) is indicated on the right side of the figure in kbp.

Primers for VP2pFastBac with *XhoI* and *NotI* sites were designed using the DNAman program (primer design illustrated on Fig. 2.2). The VP2 was amplified using a VP2pFastBac plasmid as a template. The PCR conditions (as described in the materials and method section 2.1.1) resulted in the successful amplification of the VP2 gene. This was demonstrated by the resulting VP2 amplicon with the expected size of 3.2 kbp (Fig. 2.6).

2.2.2 PURIFICATION OF THE VP2 AMPLICON

The amplicons were purified using the QIAquick kit according to the manufactures instruction (as described under section 2.1.2). The concentration of the VP2 amplicon was approximately 50-100 ng/µl in a total of 10 µl. The concentration was determined by comparing the intensity of a DNA fragment of the same size and off a known concentration with that of the unknown, purified fragment on an Ethidium bromide stained agarose gel.

2.2.3 DIGESTION OF VECTORS AND VP2 AMPLICON

Both the purified VP2 amplicon and the baculovirus plasmids were digested with *XhoI* and *NotI* (using buffer H from Roche) overnight at 37℃. This was done in order to generate sticky ends on both the baculovirus vectors and the VP2 for cloning. The VP2 amplicon and the baculovirus plasmids were separated on a 1% agarose gel to confirm if they were linearised and secondly to confirm if they are the correct and expected sizes (Fig. 2.7). VP2 was approximately 3.2 kbp and the baculovirus plasmids pBACgus-1 & 5 approximately 7.4 and 7.6 kbp respectively.

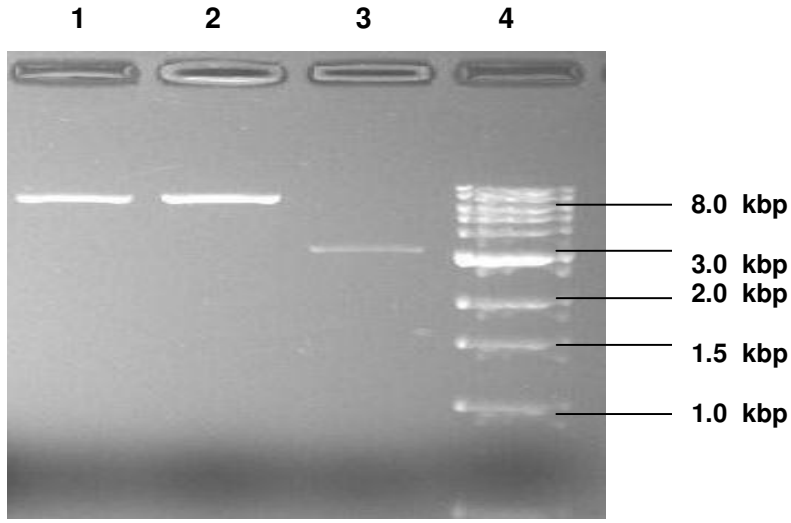


Fig. 2.7: Agarose gel electrophoretic analysis of the double digestion restriction enzyme reaction of VP2 PCR product and the pBACgus-1 and -5. Lane 1, pBACgus-1 digested with *XhoI and NotI* (7.4 kbp); Lane 2, pBACgus-5 digested with *XhoI* and *NotI* (7.6 kbp); Lane 3, AHSV-2 VP2 amplicon digested with *XhoI and NotI* (~3.0 kbp). The sizes of the molecular weight marker (1 kb DNA ladder, New England BioLabs) are indicated on the right of the figure.

2.2.4 PREPARATION OF COMPETENT CELLS, LIGATIONS AND PLASMID ISOLATIONS

Competent *E. coli* SURE cells were prepared according to materials and method section 2.1.6 (i). The resulting competent cells had a high transformation efficiency of approximately 1.18 × 10^9cfu/µg. The cells were transformed with the ligation reaction and 100 µl aliquots were plated out on LB plates with (10 µg/ml) tetracycline and (20 µg/ml) ampicillin. Approximately 10-15 colonies of similar morphology, colour and size were obtained in each plate. A single colony was picked and inoculated into 5 ml LB broth with ampicillin and tetracycline. Plasmid isolations were done as described in the materials and methods section (2.1.7). The correctness of the individual clones was confirmed by restriction enzyme digestion. In this instance the digestion was done using the *BamHI* enzyme that cut the recombinant plasmid in two places, resulting in a small DNA fragment of approximately 3 kbp and a larger DNA fragment of approximately 8 kbp (Fig. 2.8 and Fig 2.10). This confirms that the recombinant plasmids VP2pBACgus-1 & VP2pBACgus -5 were correct and in the correct orientation. Non recombinant plasmids would have resulted in incorrect sizes of the expected bands. Fig. 2.9 and 2.11 represent an illustration of the exact positions of the insert VP2 within the plasmids pBACgus-1 & -5 and also demonstrating the enzyme recognition sites used during digestion reactions. .

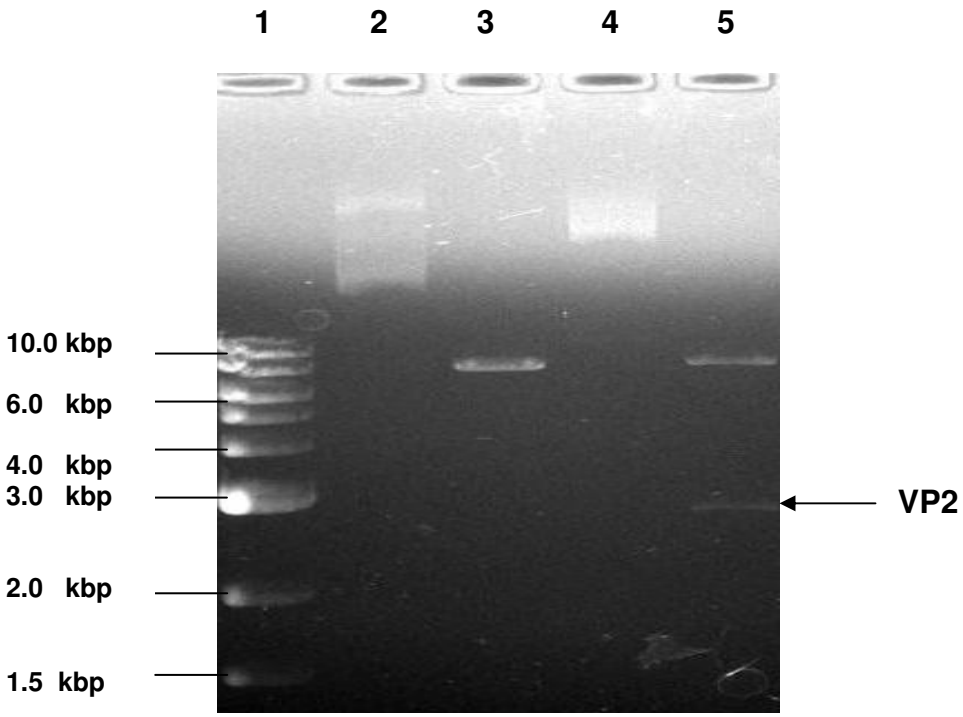


Fig. 2.8: Agarose gel electrophoretic analysis of the restriction enzyme digestion reaction of the VP2pBACgus-1 and the plasmid pBACgus-1. Lane 1, The molecular weight marker (1 kb DNA ladder, New England BioLabs) indicated; Lane 2, pBACgus-1 undigested (7.4 kbp); Lane 3, pBACgus-1 digested with *BamHI* (7.4 kbp) ; Lane 4, VP2pBACgus-1 undigested (~10 kbp); Lane 5, VP2pBACgus-1 digested with *BamHI* (the plasmid 7.4 kbp, VP2 ~3.0 kbp) . The arrow indicates a ~3.0 kbp DNA fragment that was excised from the recombinant VP2pBACgus-1 plasmid.

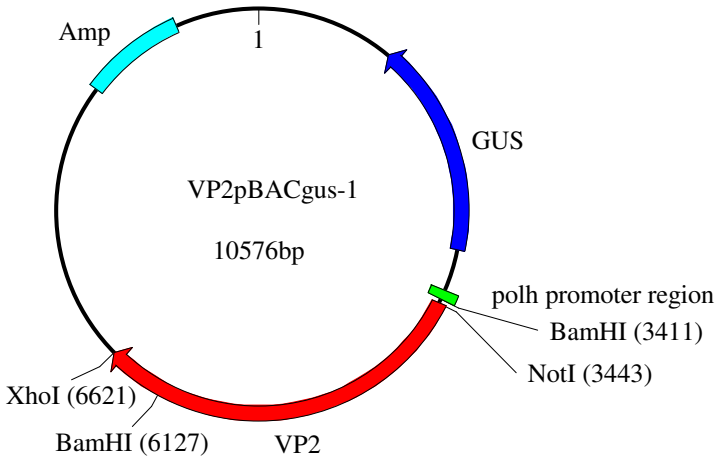


Fig. 2.9: Diagram of a plasmid map illustrating the position of the insert VP2 within VP2pBACgus-1 and the relevant enzyme recognition sites.

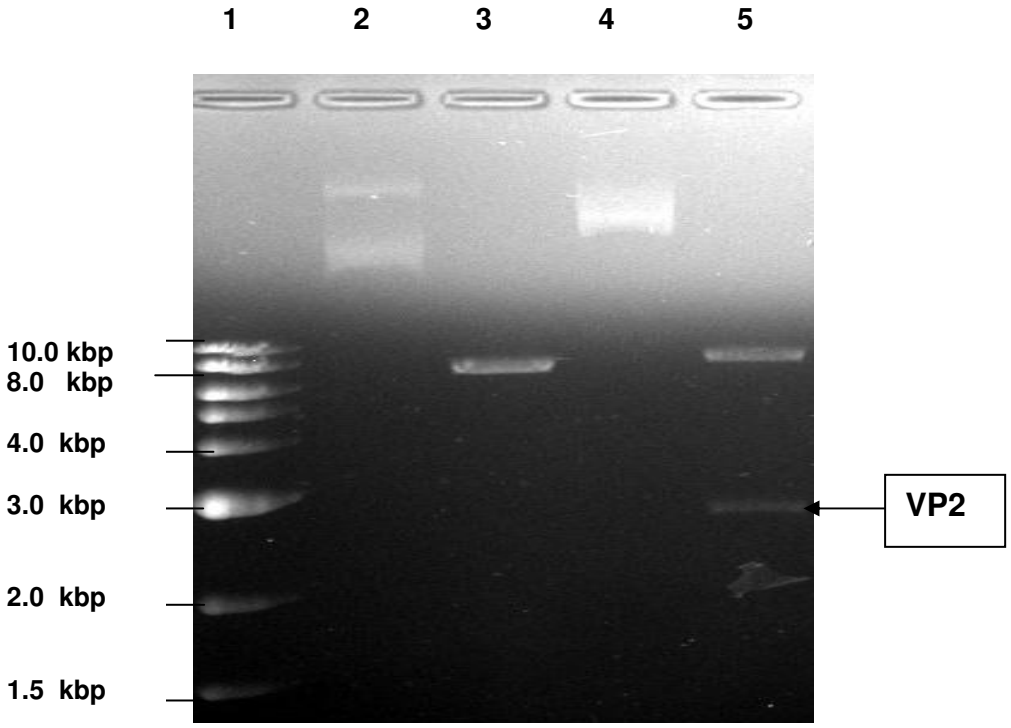


Fig. 2.10: Agarose gel electrophoretic analysis of the restriction enzyme digestion reaction of the clone VP2pBACgus-5 and the plasmid pBACgus-5. Lane 1, The molecular weight marker (1 kb DNA ladder, New England BioLabs); Lane 2, pBACgus-5 undigested (7.6 kbp); Lane 3, pBACgus-5 digested with *BamHI* (7.6 kbp); Lane 4, VP2pBACgus-5 undigested (~10 kbp) ; Lane 5, VP2pBACgus-5 digested with *BamHI* (the plasmid 7.6 kbp, VP2 ~3kbp). The arrow indicate indicate a ~3.0 kbp DNA fragment that was excised from the recombinant VP2pBACgus-5 plasmid.

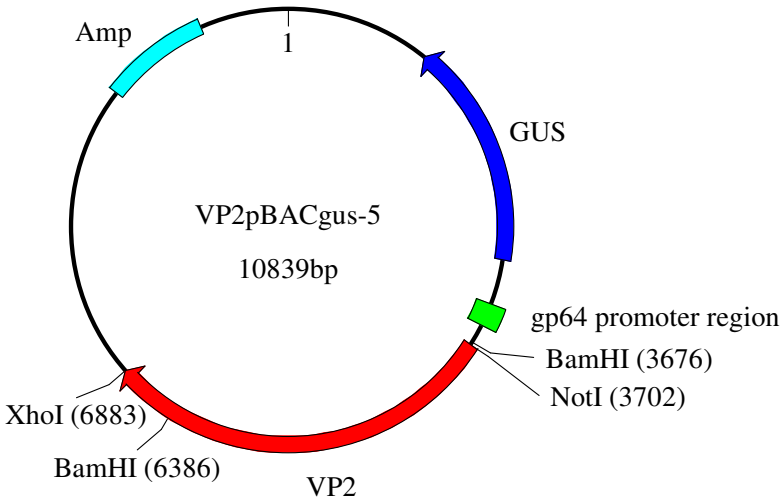


Fig. 2.11: Diagram of a plasmid map illustrating the position of the insert VP2 within VP2pBACgus-5 and the relevant enzyme recognition sites.

2.2.5 SEQUENCING OF CLONES

The polymerase enzyme is known to incorporate faulty nucleotides. Any of these mutations may result in either a different amino acid or even a stop codon. The AHSV 2 VP2 was sequenced to confirm it was in the correct open reading frame. Due to the length of the gene (approximately 3.2 kbp) primer walking was used for sequencing and the Staden Package program was used for analysis of the sequences. Initially eight primers were designed at intervals of approximately 500 bp as illustrated by Table 2.2 and Fig. 2.5. Three additional reverse primers were designed in order to form a single contig. The ends of the clones were sequenced using forward polh for VP2pBACgus-1 and forward S tag, reverse BAC for VP2pBACgus-1 (Table 2.2). The full length DNA sequence was translated into an amino acid sequence using the Staden Package program and it showed that the clones were full length and one open reading frame (Fig. 2.12 and Fig 2.13).

```
1         CCTGAATTCGAGCTCCAAGCTTGCGGCCGCATGGCGTCTGAATTTGGAATACTTTTCACC
1           P  E  F  E  L  Q  A  C  G  R  M  A  S  E  F  G  I  L  F  T

61        GAAAAGATCTATGACCAAACGTTGGAGAAAACGAATTGTGATGTGATTATCACTGAGGAG
21          E  K  I  Y  D  Q  T  L  E  K  T  N  C  D  V  I  I  T  E  E

121       AGGAAAGTGAATCGGAGAGAGGTGGAAGGAGTGCGGGGATATGTATGGGAAGAAACAAAC
41          R  K  V  N  R  R  E  V  E  G  V  R  G  Y  V  W  E  E  T  N

181       CACCGTTTTGGATTATGTGAGAATTCATTTGACGAAAAAATATCGGAAACCATGTATTGC
61          H  R  F  G  L  C  E  N  S  F  D  E  K  I  S  E  T  M  Y  C

241       CAAATTAAGTGTGAAGGTGCTTACCCGATCTTTCCACATTATATTGTTGATGCGTTGAGG
81          Q  I  K  C  E  G  A  Y  P  I  F  P  H  Y  I  V  D  A  L  R

301       TATGGTAAGATGATCGATAGAAATGATAACCAGGTTAGGGTTGATCAGGATGACAAGCGA
101         Y  G  K  M  I  D  R  N  D  N  Q  V  R  V  D  Q  D  D  K  R

361       TTGATGAAAATTAAAATTCAGCCGTATATGGGTGAAATGTATTTCTCACCCGAGAGCTAT
121         L  M  K  I  K  I  Q  P  Y  M  G  E  M  Y  F  S  P  E  S  Y

421       TCTACCGTTTTCTGTAAGAGACAAGCAGAGCTCATGTCAATTGAAGATCTAAGGTATCCA
141         S  T  V  F  C  K  R  Q  A  E  L  M  S  I  E  D  L  R  Y  P

48        TTTGATATAAGATGCGACTTCGAAGAAACTTCGTTCCAAACGAAATCCTCTTTAGATGGT
161         F  D  I  R  C  D  F  E  E  T  S  F  Q  T  K  S  S  L  D  G

541       AAGAAGCTTAGATTATTGGAAAAATGGAAACGGGCTTCGCAAGAACGCATGCACGAAGAA
181         K  K  L  R  L  L  E  K  W  K  R  A  S  Q  E  R  M  H  E  E

601       AACGACCGTGGAAAGTGTGCTGGTCACGACGAAGATGTGTTGTATCAATTAGTTAAGAAA
201         N  D  R  G  K  C  A  G  H  D  E  D  V  L  Y  Q  L  V  K  K

661       TTAAGGTATGGCCTACTGTATCCACATAGCTATACCCTTAACACGAAATATAAAATTGTC
221         L  R  Y  G  L  L  Y  P  H  S  Y  T  L  N  T  K  Y  K  I  V

721       AACCCAAGCGTTTCACAGATTAAGGACTGGCTGTTAAAAACAAGGGATGGTATTAGAGAA
241         N  P  S  V  S  Q  I  K  D  W  L  L  K  T  R  D  G  I  R  E

781       GAATCTGGAATATCAGATAAGCAGGGACCATTAGCGGAGTTGGTGAGTGCAATCAAAGAC
261         E  S  G  I  S  D  K  Q  G  P  L  A  E  L  V  S  A  I  K  D

841       GAGGAGCTAAGTCGGGGAGTAATTGAGCGGATCGTACAATATGGCTCGCAATTTAGTTCG
281         E  E  L  S  R  G  V  I  E  R  I  V  Q  Y  G  S  Q  F  S  S

901       TGTGCAGGGGAACGAGAAGATGATATCCCGATCGAAACATTGATTCGTTACTGCGACTCG
301         C  A  G  E  R  E  D  D  I  P  I  E  T  L  I  R  Y  C  D  S

961       CTGACGACATTTGTTCATCGAAAGAAGAGAGAACGAGGCGATGATACCACAGCGCGTGAC
321         L  T  T  F  V  H  R  K  K  R  E  R  G  D  D  T  T  A  R  D

1021      GTCTTCAGAAATGCCTTAGTTGGAAGTATGCCTAAAATGGACTTCAAGAATCAAATGAAG
341         V  F  R  N  A  L  V  G  S  M  P  K  M  D  F  K  N  Q  M  K

1081      ATGACAAGAGGGTGGGGCAACTATACGTTCTTTTCATACATTGATAGGTTTAGCCGGACT
361         M  T  R  G  W  G  N  Y  T  F  F  S  Y  I  D  R  F  S  R  T

1141      TACAAGATGAATATTGATCCGAATAAGGGTTTATGGAACGAACACAAACAAAAAGTGAAG
381         Y  K  M  N  I  D  P  N  K  G  L  W  N  E  H  K  Q  K  V  K
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1201    AAACAATTAGAAGAAAAACAGGAAGAGAATGGGTCTCCCATGTCCGTTCAGATCGATGGA
401       K  Q  L  E  E  K  Q  E  E  N  G  S  P  M  S  V  Q  I  D  G

1261    GTTTATATCCGAACAGATGTGCCGTATGGAACGGTTGATCATTGGGTTGATTGGGTTGTT
421       V  Y  I  R  T  D  V  P  Y  G  T  V  D  H  W  V  D  W  V  V

1321    GACACAATCATGCTTAAGGAGACTGACAAAATGATAAAGGATTACGAATTTAAGAAATTA
441       D  T  I  M  L  K  E  T  D  K  M  I  K  D  Y  E  F  K  K  L

1381    AAACGTGAAGAATTGATAGCTGGTATGAACAAGTTGGAGGATGGTTTGAGATGCATTGTT
461       K  R  E  E  L  I  A  G  M  N  K  L  E  D  G  L  R  C  I  V

1441    TATTGCTTGATTTTAGCGTTATATGATTATTATGGGGCTGAGATTGAGGGATTCAAAAAA
481       Y  C  L  I  L  A  L  Y  D  Y  Y  G  A  E  I  E  G  F  K  K

1501    GGGACAAACGCTTCATCTATAGTTGAGACTGTATCACAAATGTTCCCAAACTTCCGTGGA
501       G  T  N  A  S  S  I  V  E  T  V  S  Q  M  F  P  N  F  R  G

1561    GATATCATCGACAAATTTGGCATACAGCTGAAGGTGAAAAGGGAAGCTGAGGAGTTGTTC
521       D  I  I  D  K  F  G  I  Q  L  K  V  K  R  E  A  E  E  L  F

1621    CTTCCGAAGGATATGGTATCATCTTTTCTAGAGGATGGCGAAGAGGGATATAAATATCAA
541       L  P  K  D  M  V  S  S  F  L  E  D  G  E  E  G  Y  K  Y  Q

1681    TACGGTTGGAAAGATAATGAAGAATTAGTGGCAAGCGATTATGGTGAAATTTTGACTCAG
561       Y  G  W  K  D  N  E  E  L  V  A  S  D  Y  G  E  I  L  T  Q

1741    TCAGTACAAATTCTGTTCGAAGGATTGATGAGAGGGGAGAAGTGGACTTCGATTATAGAT
581       S  V  Q  I  L  F  E  G  L  M  R  G  E  K  W  T  S  I  I  D

1801    GATCCTCAGTCCTACTTTGAGGATGATATATTTGCGAGAAAAGCTAATAGGATGTTTTTG
601       D  P  Q  S  Y  F  E  D  D  I  F  A  R  K  A  N  R  M  F  L

1861    AGAGGCGGAGAGACGGTTGAACGGCATGTGAAATTGAAGGTTAACGCTCAGACAGAGAAC
621       R  G  G  E  T  V  E  R  H  V  K  L  K  V  N  A  Q  T  E  N

1921    GTTGAGGGCATCACATACTTTTCAAAAAGATTTGTATCTTATTGGTTCAGAATTGAACGC
641       V  E  G  I  T  Y  F  S  K  R  F  V  S  Y  W  F  R  I  E  R

1981    ACAACGCACGTAAAGGGAGGGGGAAGAGTAGATATTCGGGATCGAAAAACAGGATACCAG
661       T  T  H  V  K  G  G  G  R  V  D  I  R  D  R  K  T  G  Y  Q

2041    CAGTTCGATGTAGAAGATTTTAAACCTGCAAGTGTAGGGGAACTTGGATTTCACGCGTCA
681       Q  F  D  V  E  D  F  K  P  A  S  V  G  E  L  G  F  H  A  S

2101    ACATATATTTATCAAGATTTACTTGTGGGAGCAAATAGAGGCGAGCGTGTGAAGGACGCG
701       T  Y  I  Y  Q  D  L  L  V  G  A  N  R  G  E  R  V  K  D  A

2161    AAAGAGTTAGTGTGGATGGATTTATCTCTTACCAATTTCGGGTTTGTGCGAAGCTACAAT
721       K  E  L  V  W  M  D  L  S  L  T  N  F  G  F  V  R  S  Y  N

2221    AGATGTTGGATTGCGGCCTTTGTGGAGGCGGAGATTTCGCTCAGATTTTATCTCATAACG
741       R  C  W  I  A  A  F  V  E  A  E  I  S  L  R  F  Y  L  I  T

2281    TCGATCTTTTGTAGATATTTTACTGGCGATAGGAAGAGTTTCGCTAAGATACTAGATGGA
761       S  I  F  C  R  Y  F  T  G  D  R  K  S  F  A  K  I  L  D  G

2341    GTCAAGTCATTGAAAGAAAGATTATGGTTCCCTACCTACAAACATTACTATGTTGCCGTC
781       V  K  S  L  K  E  R  L  W  F  P  T  Y  K  H  Y  Y  V  A  V
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2401    ATTCAGAAGATTTACTCAGATGATAGGAATCTAGATAGGAATGAATTTTGTAATAGAGTC
801       I  Q  K  I  Y  S  D  D  R  N  L  D  R  N  E  F  C  N  R  V

2461    ATGAATATCATGACAAGACGGTCAGTACTCAATGAGTTTGAGAGGTTTAGAAGAGCTATT
821       M  N  I  M  T  R  R  S  V  L  N  E  F  E  R  F  R  R  A  I

2521    GAGTCGCCAAAGCTGATTGACACTTTGTCGCTTAATTTTCTCCTTTGGATAATTTTCGAA
841       E  S  P  K  L  I  D  T  L  S  L  N  F  L  L  W  I  I  F  E

2581    CAAGAGAACATAGATGTTAATTTTGCGGATAAGAGGCATCCACTATTAATATCAACGACT
861       Q  E  N  I  D  V  N  F  A  D  K  R  H  P  L  L  I  S  T  T

2641    AAAGGTTTGAGAGTAATCCCGATTGACGTTTTCAATAGTTCACTAGCTCTTTCTCCAAGT
881       K  G  L  R  V  I  P  I  D  V  F  N  S  S  L  A  L  S  P  S

2701    GGTTGGATCCCTTACGTTGAGAGGATATGTGCAGAGGCTAAGGAAAACCGTACGCTGAGT
901       G  W  I  P  Y  V  E  R  I  C  A  E  A  K  E  N  R  T  L  S

2761    TCCGATGAATTGAGAATTAAAACCTGGTTCGTAGAGTACTATCTAAATATAAACTTAGAG
921       S  D  E  L  R  I  K  T  W  F  V  E  Y  Y  L  N  I  N  L  E

2821    AGGAGGGCGGAGCCTAGGATGAGTTTCAAGAGTGAAGCGCTGATCACGTGGATCGGATCG
941       R  R  A  E  P  R  M  S  F  K  S  E  A  L  I  T  W  I  G  S

2881    AATTGTGGTGGAGTGACTGATTATGTGGTTCAGCTTTTGCCTGTTCGGAAACCGAAACCG
961       N  C  G  G  V  T  D  Y  V  V  Q  L  L  P  V  R  K  P  K  P

2941    GGCCTGCTAGTGGTTGTTTATTCGGAGGACGGGAGTGGGAAATGGGCAGAATGGGCGCTA
981       G  L  L  V  V  V  Y  S  E  D  G  S  G  K  W  A  E  W  A  L

3001    CGCGATTTTTTAGACGTTGAAGGTAGCTTAGGATTAATTTTCATAACTCGTAAAACTGTC
1001      R  D  F  L  D  V  E  G  S  L  G  L  I  F  I  T  R  K  T  V

3061    AAGAATGGGAGCGCATTGGGAGTCCGAGATTTAAAAATCTACAATCGAGGGAGAGTAGAT
1021      K  N  G  S  A  L  G  V  R  D  L  K  I  Y  N  R  G  R  V  D

3121    AGATTAGTTTTAATTTCGAGCGGCGTTTATACTTTTGGGAACAAATTTTTATTCTCGAAA
1041      R  L  V  L  I  S  S  G  V  Y  T  F  G  N  K  F  L  F  S  K

3181     CTACTATCCAAAATAGAGTAACTCGAGCACCACCACCACCACCACTAACCTAGGTA
1061      L  L  S  K  I  E  *  L  E  H  H  H  H  H  H  *  P  R
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Fig.2.12: Full length sequence and translated amino acid sequence of VP2pBACgus-1. The start codon ATG (in green) and the stop codon TAA (in green) showed the open reading frame of VP2.

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1          TCAAAAGGCCTGGATCCGAATTCGAGCTCGAAGCTTGCGGCCGCATGGCGTCTGAATTT
1           L  K  R  P  G  S  E  F  E  L  E  A  C  G  R  M  A  S  E  F

61         GGAATACTTTTCACCGAAAAGATCTATGACCAAACGTTGGAGAAAACGAATTGTGATGTG
21           G  I  L  F  T  E  K  I  Y  D  Q  T  L  E  K  T  N  C  D  V

121        ATTATCACTGAGGAGAGGAAAGTGAATCGGAGAGAGGTGGAAGGAGTGCGGGGATATGTA
41           I  I  T  E  E  R  K  V  N  R  R  E  V  E  G  V  R  G  Y  V

181        TGGGAAGAAACAAACCACCGTTTTGGATTATGTGAGAATTCATTTGACGAAAAAATATCG
61           W  E  E  T  N  H  R  F  G  L  C  E  N  S  F  D  E  K  I  S

241        GAAACCATGTATTGCCAAATTAAGTGTGAAGGTGCTTACCCGATCTTTCCACATTATATT
81           E  T  M  Y  C  Q  I  K  C  E  G  A  Y  P  I  F  P  H  Y  I

301        GTTGATGCGTTGAGGTATGGTAAGATGATCGATAGAAATGATAACCAGGTTAGGGTTGAT
101          V  D  A  L  R  Y  G  K  M  I  D  R  N  D  N  Q  V  R  V  D

361        CAGGATGACAAGCGATTGATGAAAATTAAAATTCAGCCGTATATGGGTGAAATGTATTTC
121          Q  D  D  K  R  L  M  K  I  K  I  Q  P  Y  M  G  E  M  Y  F

421        TCACCCGAGAGCTATTCTACCGTTTTCTGTAAGAGACAAGCAGAGCTCATGTCAATTGAA
141          S  P  E  S  Y  S  T  V  F  C  K  R  Q  A  E  L  M  S  I  E

481        GATCTAAGGTATCCATTTGATATAAGATGCGACTTCGAAGAAACTTCGTTCCAAACGAAA
161          D  L  R  Y  P  F  D  I  R  C  D  F  E  E  T  S  F  Q  T  K

541        TCCTCTTTAGATGGTAAGAAGCTTAGATTATTGGAAAAATGGAAACGGGCTTCGCAAGAA
181          S  S  L  D  G  K  K  L  R  L  L  E  K  W  K  R  A  S  Q  E

601        CGCATGCACGAAGAAAACGACCGTGGAAAGTGTGCTGGTCACGACGAAGATGTGTTGTAT
201          R  M  H  E  E  N  D  R  G  K  C  A  G  H  D  E  D  V  L  Y

661        CAATTAGTTAAGAAATTAAGGTATGGCCTACTGTATCCACATAGCTATACCCTTAACACG
221          Q  L  V  K  K  L  R  Y  G  L  L  Y  P  H  S  Y  T  L  N  T

721        AAATATAAAATTGTCAACCCAAGCGTTTCACAGATTAAGGACTGGCTGTTAAAAACAAGG
241          K  Y  K  I  V  N  P  S  V  S  Q  I  K  D  W  L  L  K  T  R

781        GATGGTATTAGAGAAGAATCTGGAATATCAGATAAGCAGGGACCATTAGCGGAGTTGGTG
261          D  G  I  R  E  E  S  G  I  S  D  K  Q  G  P  L  A  E  L  V

841        AGTGCAATCAAAGACGAGGAGCTAAGTCGGGGAGTAATTGAGCGGATCGTACAATATGGC
281          S  A  I  K  D  E  E  L  S  R  G  V  I  E  R  I  V  Q  Y  G

901        TCGCAATTTAGTTCGTGTGCAGGGGAACGAGAAGATGATATCCCGATCGAAACATTGATT
301          S  Q  F  S  S  C  A  G  E  R  E  D  D  I  P  I  E  T  L  I

961        CGTTACTGCGACTCGCTGACGACATTTGTTCATCGAAAGAAGAGAGAACGAGGCGATGAT
321          R  Y  C  D  S  L  T  T  F  V  H  R  K  K  R  E  R  G  D  D

1021       ACCACAGCGCGTGACGTCTTCAGAAATGCCTTAGTTGGAAGTATGCCTAAAATGGACTTC
341          T  T  A  R  D  V  F  R  N  A  L  V  G  S  M  P  K  M  D  F

1081       AAGAATCAAATGAAGATGACAAGAGGGTGGGGCAACTATACGTTCTTTTCATACATTGAT
361          K  N  Q  M  K  M  T  R  G  W  G  N  Y  T  F  F  S  Y  I  D

1141       AGGTTTAGCCGGACTTACAAGATGAATATTGATCCGAATAAGGGTTTATGGAACGAACAC
381          R  F  S  R  T  Y  K  M  N  I  D  P  N  K  G  L  W  N  E  H
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1201    AAACAAAAAGTGAAGAAACAATTAGAAGAAAAACAGGAAGAGAATGGGTCTCCCATGTCC
401       K  Q  K  V  K  K  Q  L  E  E  K  Q  E  E  N  G  S  P  M  S

1261    GTTCAGATCGATGGAGTTTATATCCGAACAGATGTGCCGTATGGAACGGTTGATCATTGG
421       V  Q  I  D  G  V  Y  I  R  T  D  V  P  Y  G  T  V  D  H  W

1321    GTTGATTGGGTTGTTGACACAATCATGCTTAAGGAGACTGACAAAATGATAAAGGATTAC
441       V  D  W  V  V  D  T  I  M  L  K  E  T  D  K  M  I  K  D  Y

1381    GAATTTAAGAAATTAAAACGTGAAGAATTGATAGCTGGTATGAACAAGTTGGAGGATGGT
461       E  F  K  K  L  K  R  E  E  L  I  A  G  M  N  K  L  E  D  G

1441    TTGAGATGCATTGTTTATTGCTTGATTTTAGCGTTATATGATTATTATGGGGCTGAGATT
481       L  R  C  I  V  Y  C  L  I  L  A  L  Y  D  Y  Y  G  A  E  I

1501    GAGGGATTCAAAAAAGGGACAAACGCTTCATCTATAGTTGAGACTGTATCACAAATGTTC
501       E  G  F  K  K  G  T  N  A  S  S  I  V  E  T  V  S  Q  M  F

1561    CCAAACTTCCGTGGAGATATCATCGACAAATTTGGCATACAGCTGAAGGTGAAAAGGGAA
521       P  N  F  R  G  D  I  I  D  K  F  G  I  Q  L  K  V  K  R  E

1621    GCTGAGGAGTTGTTCCTTCCGAAGGATATGGTATCATCTTTTCTAGAGGATGGCGAAGAG
541       A  E  E  L  F  L  P  K  D  M  V  S  S  F  L  E  D  G  E  E

1681    GGATATAAATATCAATACGGTTGGAAAGATAATGAAGAATTAGTGGCAAGCGATTATGGT
561       G  Y  K  Y  Q  Y  G  W  K  D  N  E  E  L  V  A  S  D  Y  G

1741    GAAATTTTGACTCAGTCAGTACAAATTCTGTTCGAAGGATTGATGAGAGGGGAGAAGTGG
581       E  I  L  T  Q  S  V  Q  I  L  F  E  G  L  M  R  G  E  K  W

1801    ACTTCGATTATAGATGATCCTCAGTCCTACTTTGAGGATGATATATTTGCGAGAAAAGCT
601       T  S  I  I  D  D  P  Q  S  Y  F  E  D  D  I  F  A  R  K  A

1861    AATAGGATGTTTTTGAGAGGCGGAGAGACGGTTGAACGGCATGTGAAATTGAAGGTTAAC
621       N  R  M  F  L  R  G  G  E  T  V  E  R  H  V  K  L  K  V  N

1921    GCTCAGACAGAGAACGTTGAGGGCATCACATACTTTTCAAAAAGATTTGTATCTTATTGG
641       A  Q  T  E  N  V  E  G  I  T  Y  F  S  K  R  F  V  S  Y  W

1981    TTCAGAATTGAACGCACAACGCACGTAAAGGGAGGGGGAAGAGTAGATATTCGGGATCGA
661       F  R  I  E  R  T  T  H  V  K  G  G  G  R  V  D  I  R  D  R

2041    AAAACAGGATACCAGCAGTTCGATGTAGAATATTTTAAACCTGCAAGTGTAGGGGAACTT
681       K  T  G  Y  Q  Q  F  D  V  E  Y  F  K  P  A  S  V  G  E  L

2101    GGATTTCACGCGTCAACATATATTTATCAAGATTTACTTGTGGGAGCAAATAGAGGCGAG
701       G  F  H  A  S  T  Y  I  Y  Q  D  L  L  V  G  A  N  R  G  E

2161    CGTGTGAAGGACGCGAAAGAGTTAGTGTGGATGGATTTATCTCTTACCAATTTCGGGTTT
721       R  V  K  D  A  K  E  L  V  W  M  D  L  S  L  T  N  F  G  F

2221    GTGCGAAGCTACAATAGATGTTGGATTGCGGCCTTTGTGGAGGCGGAGATTTCGCTCAGA
741       V  R  S  Y  N  R  C  W  I  A  A  F  V  E  A  E  I  S  L  R

2281    TTTTATCTCATAACGTCGATCTTTTGTAGATATTTTACTGGCGATAGGAAGAGTTTCGCT
761       F  Y  L  I  T  S  I  F  C  R  Y  F  T  G  D  R  K  S  F  A

2341    AAGATACTAGATGGAGTCAAGTCATTGAAAGAAAGATTATGGTTCCCTACCTACAAACAT
781       K  I  L  D  G  V  K  S  L  K  E  R  L  W  F  P  T  Y  K  H
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2401    TACTATGTTGCCGTCATTCAGAAGATTTACTCAGATGATAGGAATCTAGATAGGAATGAA
801       Y  Y  V  A  V  I  Q  K  I  Y  S  D  D  R  N  L  D  R  N  E

2461    TTTTGTAATAGAGTCATGAATATCATGACAAGACGGTCAGTACTCAATGAGTTTGAGAGG
821       F  C  N  R  V  M  N  I  M  T  R  R  S  V  L  N  E  F  E  R

2521    TTTAGAAGAGCTATTGAGTCGCCAAAGCTGATTGACACTTTGTCGCTTAATTTTCTCCTT
841       F  R  R  A  I  E  S  P  K  L  I  D  T  L  S  L  N  F  L  L

2581    TGGATAATTTTCGAACAAGAGAACATAGATGTTAATTTTGCGGATAAGAGGCATCCACTA
861       W  I  I  F  E  Q  E  N  I  D  V  N  F  A  D  K  R  H  P  L

2641    TTAATATCAACGACTAAAGGTTTGAGAGTAATCCCGATTGACGTTTTCAATAGTTCACTA
881       L  I  S  T  T  K  G  L  R  V  I  P  I  D  V  F  N  S  S  L

2701    GCTCTTTCTCCAAGTGGTTGGATCCCTTACGTTGAGAGGATATGTGCAGAGGCTAAGGAA
901       A  L  S  P  S  G  W  I  P  Y  V  E  R  I  C  A  E  A  K  E

2761    AACCGTACGCTGAGTTCCGATGAATTGAGAATTAAAACCTGGTTCGTAGAGTACTATCTA
921       N  R  T  L  S  S  D  E  L  R  I  K  T  W  F  V  E  Y  Y  L

2821    AATATAAACTTAGAGAGGAGGGCGGAGCCTAGGATGAGTTTCAAGAGTGAAGCGCTGATC
941       N  I  N  L  E  R  R  A  E  P  R  M  S  F  K  S  E  A  L  I

2881    ACGTGGATCGGATCGAATTGTGGTGGAGTGACTGATTATGTGGTTCAGCTTTTGCCTGTT
961       T  W  I  G  S  N  C  G  G  V  T  D  Y  V  V  Q  L  L  P  V

2941    CGGAAACCGAAACCGGGCCTGCTAGTGGTTGTTTATTCGGAGGACGGGAGTGGGAAATGG
981       R  K  P  K  P  G  L  L  V  V  V  Y  S  E  D  G  S  G  K  W

3001    GCAGAATGGGCGCTACGCGATTTTTTAGACGTTGAAGGTAGCTTAGGATTAATTTTCATA
1001      A  E  W  A  L  R  D  F  L  D  V  E  G  S  L  G  L  I  F  I

3061    ACTCGTAAAACTGTCAAGAATGGGAGCGCATTGGGAGTCCGAGATTTAAAAATCTACAAT
1021      T  R  K  T  V  K  N  G  S  A  L  G  V  R  D  L  K  I  Y  N

3121    CGAGGGAGAGTAGATAGATTAGTTTTAATTTCGAGCGGCGTTTATACTTTTGGGAACAAA
1041      R  G  R  V  D  R  L  V  L  I  S  S  G  V  Y  T  F  G  N  K

3181    TTTTTATTCTCGAAACTACTATCCAAAATAGAGTAACTCGAGCACCACCACCACCACCAC
1061      F  L  F  S  K  L  L  S  K  I  E  *  L  E  H  H  H  H  H  H
```

Fig 2.13 Full length sequence and translated amino acid sequence of VP2pBACgus-5. The start codon ATG (in green) and the stop codon TAA (in green) showed the open reading frame of VP2.

2.2.6 *Sf9* CELL TRANSFECTION USING BACMAGIC

After it was established that the cloning was a success the recombinant baculovirus plasmids were used for the transfection of *Spodoptera frugiperda (Sf 9)* cells. The *Sf9* cells were the cell line of choice as the baculovirus is easily propagated in this cell line (reviewed by Possee., 1997). After addition of X-gluc two of the six well plates turned from colourless to blue (Fig. 2.15) and this served as confirmation of recombination. The colour change is the result of the reporter gus gene encoding glucoronidase. On the SDS-PAGE no recombinant protein was observed at the expected size (Fig 2.14) of 110 kDa in lanes 3-7.

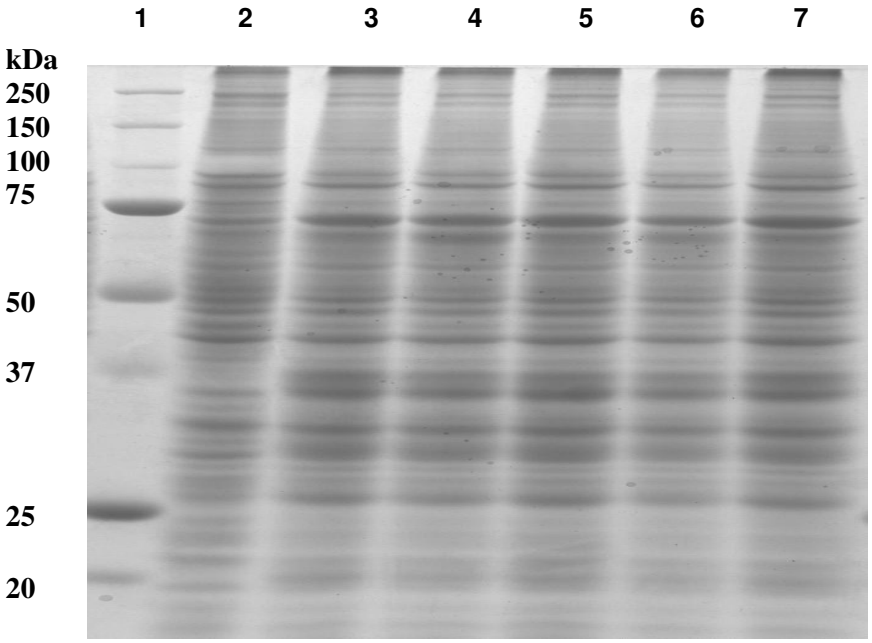


Fig. 2.14: Coomassie-stained SDS-PAGE gel of the total protein fraction of VP2BACgus-1 & -5 expressed by BacMagic DNA in *Sf9* cells. Lane 1, Molecular weight marker (in kDa); Lane 2, Total protein fraction of *Sf9* cells containing only the plasmid BACgus-1; Lane 3, Total protein fraction of recombinant cells containing VP2BACgus-1; Lane 4, Total protein fraction of recombinant cells containing VP2BACgus-1; Lane 5, Total protein fraction of *Sf9* cells containing only the plasmid BACgus-5; Lane 6, Total protein fraction of recombinant cells containing VP2BACgus-5; Lane 7, VP2BACgus-5

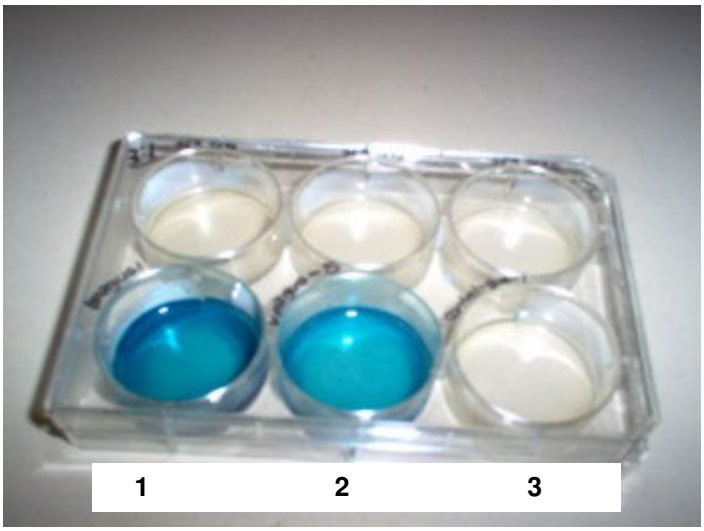


Fig. 2.15: The picture represents blue wells serving as positive identification for the generated recombinant baculoviruses and the colourless wells are the negative control containing uninfected *Sf9* cells. Lane 1, recombinant VP2BACgus-1; Lane 2, recombinant VP2BACgus-5. Lane 3, negative control containing uninfected *Sf9* cells.

2.2.7 EXPRESSION USING THE BAC-to-BAC

One of the major objectives of this chapter was to compare the expression levels between the BacMagic and the BAC-to-BAC. The BacMagic is the untested system while the BAC-to-BAC was routinely used in the Biochemistry division. The BacMagic has never been used for production of recombinant proteins the Biochemistry Division. The BacMagic was tested for its ability to produce a higher yield of recombinant protein compared to the BAC-to-BAC. Protein expression using BacMagic was unsuccessful and this led to the expression of AHS2 VP2 using the BAC-to-BAC. Monolayers of *Sf9* cells were individually infected with recombinant baculovirus expressing VP2 of serotypes AHSV 1-5 all VP2, but of major interest to this study was AHSV 2 VP2 (Fig. 2.16 lanes 6 and 7).

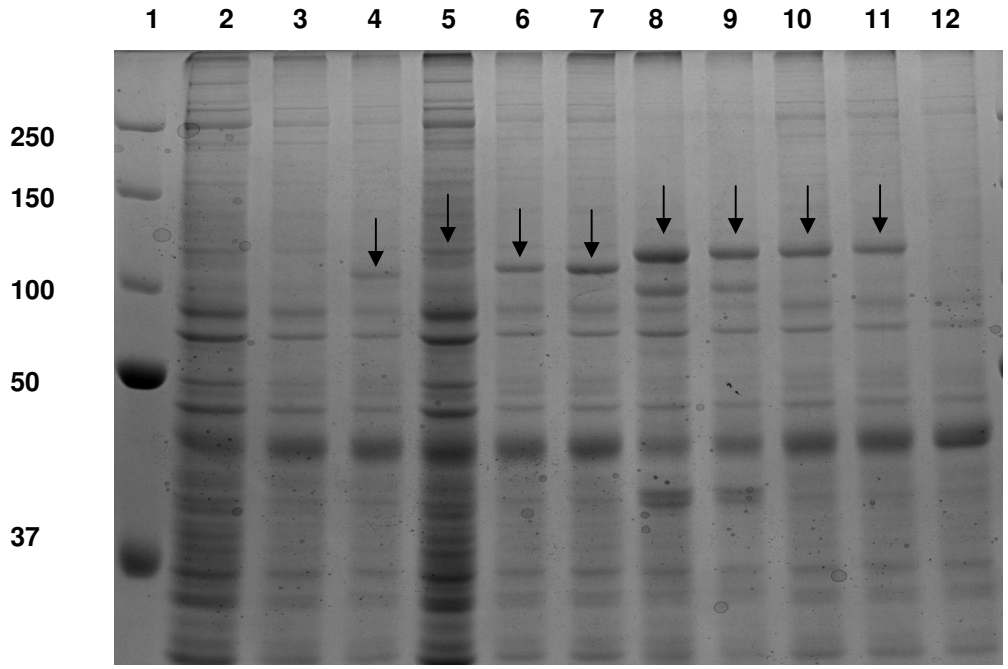


Fig. 2.16: Coomassie-stained SDS-PAGE gel of the total fraction of proteins expressed by the BAC-to-BAC in *Sf9* cells. Lane 1, Protein molecular weight marker (in kDa); Lane 2, Uninfected *Sf9* cells; Lane 3, Wild type; Lane 4, Recombinant AHSV 2 VP2; Lane 5, Recombinant AHSV 1 VP2; Lane 6, Recombinant AHSV 2 VP2; Lane 7, Recombinant AHSV 2 VP2; Lane 8, Recombinant AHSV 3 VP2; Lane 9, Recombinant AHSV 3 VP2; Lane 10, Recombinant AHSV 4 VP2; Lane 11, Recombinant AHSV 4 VP2; Lane 12, No recombinant AHSV 5 VP2 was expressed (The VP2 protein bands ~110 kDa are indicated by the arrows).

After 4-5 days of incubation the cells were lysed. The recombinant AHSV 2 VP2 protein was released from the *Sf9* cells, analysed by an SDS-PAGE gel and visualized by Coomassie-blue staining. The analysis revealed a band of approximately 110 kDa. This was an indication that recombinant VP2 gene was successfully expressed. With the BAC-to-BAC VP2 was expressed (Fig. 2.16).

2.2.8 EXPRESSION OF PASSAGE 2 RECOMBINANT VP2 GENE

Passage 2 of the baculovirus recombinants expressing VP2 was generated by infecting *Sf9* cells with passage 1 stock of the virus. The resulting presence of a band with the expected size of 110 kDa. This indicated that passage 2 of the recombinant baculovirus was expressed. (Fig. 2.15).

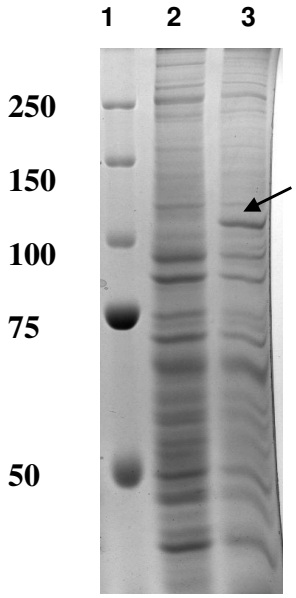


Fig. 2.17: Coomassie-stained SDS-PAGE gel of the total fraction of the protein expressed by BAC-to-BAC in *Sf9* cells. Lane 1, Protein molecular weight marker (in kDa); Lane 2, Uninfected *Sf9* cells; Lane 3, Passage 2 stock of the recombinant AHSV 2 VP2 ~110kDa (The VP2 protein band ~110kDa is indicated by the arrow).

For determining the titre of the passage 2 stock a plaque titration assay was performed. Clear regions with similar morphology, size and shape identified as plaques were observed against a red background created by the neutral red. A titre of 2.3×10^6 p.f.u/ml was obtained. While the BacMagic failed to express the recombinant protein, the BAC-to-BAC expressed it successfully but the pFastBac 1 lacks the tags needed for purification of the protein.

2.3 DISCUSSION

The baculovirus expression system is of interest to a variety of scientists for the expression of recombinant proteins. Baculovirus expression utilizes the viral life cycle by producing large amounts of the polyhedrin late in infection, this results in the survival of the virus for longer periods (reviewed Roy, 2004). The BVES express high levels of recombinant protein and because of its large genomes can accommodate large segments of foreign DNA (Fraser, 1986). The above and many other reasons have led to it being chosen as the expression system for this study. The objectives of the work presented in this chapter were to clone and express the VP2 gene of AHSV 2 serotype 2 in the pBac and pFastBac and to compare levels of expression between these two systems.

The BacMagic is the untested system in the Biochemistry Division at ARC-OVI and was evaluated for its ability to express the AHSV 2 VP2 in *Sf9* insect cells. Baculovirus expression transfer plasmids, pBACgus-1 & -5, with different promoters *polh* and *gp64* respectively, were used for the expression. The *polh* promoter is for expression very late in the replication cycle while *gp64* promoter is for expression early and late in replication. These vectors were designed for the expression of a single recombinant protein, with the expectation of a high yield of the expressed protein. The pBACgus-1 & pBACgus-5 plasmids carry the *gus* gene encoding glucoronidase which serves as a reporter to verify recombinant viruses by staining with X-gluc. Recombinant VP2pBACgus-1 and -5 clones were successfully generated and sequenced to verify the correct open reading frame of the genes. *Sf9* cells were transfected with the recombinant clones. Recombinant baculoviruses were successfully generated as verified by the reporter *gus* gene. Unfortunately, expression of recombinant VP2 protein could not be seen on a SDS-PAGE gel. No other method, such as western blotting was used to confirm this, because the pFastBac expression system in use in Biochemistry (ARC-OVI) does express the VP2 protein in high enough quantities to observe on Coomassie stained SDS-PAGE.

The VP2 protein was successfully expressed using the BAC-to-BAC expression system. Recombinant baculoviruses of four serotypes of AHSV were expressed by the BAC-to-BAC after infection of *Sf9* cells. Despite the fact that this system was able to express the recombinant VP2, there was no means to purify this protein for further use.

In summary, two BVES were evaluated for the expression of AHSV2 VP2. The BacMagic system is a versatile system that enables the user to choose from a range of promoters and tags for purification. Recombinant baculoviruses were generated but low levels of expression were found. The BAC-to-BAC expression system is more limited in the choice of promoters and no tags are available, but expressed the recombinant VP2 protein in relatively high levels. The absence of a tag-system for purification will however limit this system's usefulness.

Expression of recombinant proteins in bacteria, specifically *E. coli* has developed at a very high rate the last decade. The use of codon optimized, synthetic genes greatly enhanced the changes for successful expression of heterologous proteins in these bacterial systems. The latter option was investigated and the results are presented in Chapter 3.

REFERENCES

ABE, T., TAKAHASHI, H., HAMAZAKI, H., MIYANO-KUROSAKI, N., MATSUURA, Y. & TAKAKU, H. 2003. Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. *Journal of immunology,* 171:1133-1139.

BIRNBOIM, H. C. & DOLY, J. A. rapid alkaline extraction procedure for screening recombinant plasmid DNA. 1979. *Nucleic Acid Research*, 7:1513-1523.

BONFIELD, J. K., SMITH, K. K & STADEN, R. 1995. A new DNA sequence assembly program. *Nucleic Acid Research*, 23:4992-4999.

FRASER, M. J. 1986. Ultrastructural observations of virion maturation in Autographa californica nuclear polyhedrosis virus infected Spodoptera frugiperda cell cultures. *Journal of Ultrastructural and Molecular Structure Research*, 95:189-195.

HOFMANN, C., LEHNET, W. & STRAUSS, M. 1998. The baculovirus system for gene delivery into hepatocytes. *Gene Therapy and Molecular Biology,* 1:231-239.

INOUE, H., NOJIMA, H. & OKAYAMA, H. 1990. High efficiency transformation of *E. coli* with the plasmid. *Gene,* 96:23-28.

INUMARU, S., GHIASI, H. & ROY, P. 1987. Expression of Bluetongue virus group specific antigen VP3 in insect cells by a baculovirus vector: its use for the detection of bluetongue virus antibodies. *Journal of General Virology,* 68:1627-1635.

KITTS, P. A., AYRES, M. D. & POSSEE, R. D. 1990. Linearisation of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acid Research*, 18:5667-5672.

KOOL, M., VONCKEN, J. W., VAN LIER, F. L., TRAMPER, J. & VLAK, J. M. 1991. Detection and analysis of Autographa californica nuclear polyhedrsis virus mutants with defective interfering properties. *Virology*, 183:739-746.

LU, A., SESHAGIRI, S. & MILLER, L. K. 1996. Signal sequence and promoter effects on the efficacy of toxin-expressing baculoviruses as biopesticides. *Biological Control*, 7:320-332.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophageT4. *Nature*, 227:680-685.

LUCKOW, V. A. & SUMMERS, M. D. 1988. Trends in the development of the baculovirus expression vectors. *Biotechnology*, 6:47-55.

LUCKOW, V., LEE, S. C., BARRY, G. F. & OLINS, P. 1993. Efficient generation of infectious recombinant baculoviruses by site specific-transposition mediated insertion of the foreign genes into a baculovirus genome propagated in *E. coli. Journal of Virology*, 67:4566-4579.

MORI, H., NAKAZAWA, H., SHIRAI, N., SHIBATA, N., SUMIDA, M. & MATSUBA, F. 1992. Forein gene expression by a baculovirus vector with an expanded host range. *Journal of General Virology*, 73:1877-1880.

PILJMAN, P. J., VAN DEN BORN, E., MARTENS, D. E. & VLAK, M. J. 2001. *Autographa californica* baculoviruses with large genomic deletions are rapidly generated in infected cells. *Virology*, 283:132-138.

PILJMAN, G. P., DORTMANS, J. C. F. M., VERMEESCH, A. M. G., YANG, K., MARTENS, D. E., GOLDBACH, R. W. & VLAK, J. M. 2002. Pivotal role of the non-*hr* origin of DNA replication in the genesis of the defective interfering baculoviruses. *Journal of Virology*, 5605-5611.

POSSEE, R. D. 1997. Baculoviruses as expression vectors. *Current opinions in Biotechnology,* 8:569-572.

POTGIETER, A. C., CLOETE, M., PRETORIUS, P. J. & VAN DIJK, A. A. 2003. A first full outer capsid protein sequence data set in the *Orbivirus* genus (family *Reoviridae*): cloning, sequencing, expression and analysis of a complete set of full length outer capsid VP2 genes of the nine African horsesickness virus serotypes. *Journal of General Virology*, 84:1317-1326.

ROY, P., URAKAWA, T., VAN DIJK, A. A. & ERASMUS, B. J. 1990. Recombinant virus vaccine for Bluetongue disease in sheep. *Journal of Virology*, 64:1998-2003.

ROY, P., BISHOP, D. H. L., HOWARD, S., AITCHISON, H. & ERASMUS , B. 1996. Recombinant baculovirus synthesized African horsesickness virus (AHSV) outer capsid protein VP2 provides protection against virulent AHSV challenge. *Journal of General Virology*, 77:2053-2057.

ROY, P. 2004. Baculovirus solves a complex problem. *Nature Biotechnology*, 22:1527-1528.

SANDIG, V., HOFMANN, C., STEINERT, S., JENNINGS, G., SCHLAG, P. & STRAUSS, M. 1996. Gene transfer into hepatocytes and human liver tissue by Baculovirus vectors. *Human Gene Therapy,* 7:1937-1945.

SCANLEN, M., PAWESKA, J. A., VERSCHOOR, A. A. & VAN DIJK. 2001. The protective efficacy of a recombinant VP2-based African horsesickness subunit vaccine candidate is determined by adjuvant. *Vaccine*, 20:1079-1088.

SMITH, G. E., FRASER, M. J. & SUMMERS, M. D. 1983a. Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. *Journal of Virology*, 46:584-593.

SMITH, G. E., SUMMER, M. D. & FRASER, M. J. 1983b. Production of human β interferon in insect cells infected with a Baculovirus expression vector. *Molecular cellular biology,* 3:2156-2165.

WONG, C., SRIDHARA, S., BARDWELL, J .C. A. & JACOB, U. 2000. Heating greatly speeds Coomassie blue staining and destaining. *Biotechniques*, 28:426-432.

CHAPTER 3

EXPRESSION OF THE AHSV 4 VP2 GENE IN THE *E. coli* BACTERIAL EXPRESSION SYSTEM

3.0 INTRODUCTION

Bacterial expression systems have been used extensively for the production of recombinant proteins. *Escherichia coli* (*E. coli*) particularly has remained a powerful tool for high levels of expression of recombinant proteins. However, the success rate for expression is determined and directly influenced by factors such as the gene sequences to be expressed, toxicity of the protein, its folding capabilities and most importantly preferred codon usage of *E. coli* in respect to the gene of interest (reviewed by Makried, 1996). Codon usage in *E. coli* is one of the major factors that affect the quality and quantity of the expressed protein (Reviewed by Kane, 1995). Of the 61 codons in the *E. coli*, they are categorized into two groups the major and the rare codons (Zhang *et al*., 1991). Arginine codons AGG and AGA are identified as the rare codons, this is due to the scarcity of the tRNA's that recognize them (Ikemura, 1981, Chen & Inouye, 1994, Holm, 1986). This result in translational errors, mis-incorporation of amino acids and much worse if these codons are located nearer to the initiation codon (Kurland & Gallant, 1996, Chen & Inouye, 1994, Spanjaard *et al*., 1990). Techniques including codon optimization, stabilizing of the initiation complex, additional tRNA pools were attempted to resolve problems arising as a result of codon usage (Chen & Inouye, 1990, Reviewed by Kane, 1995, Spanjaard *et al*., 1990, Burgess-Brown *et al*., 2008).

The *E. coli* expression system lacks many of the post-translational modifications present in higher eukaryotic systems and also lacks the ability for the formation of disulfide bonds (reviewed by Makried, 1996). However, some eukaryotic proteins are glycosylated in a form that can be reproduced by *E. coli*. This was demonstrated by Fuh and co-workers (1990) who expressed a naturally glycosylated human growth hormone receptor and the protein was correctly folded and retained its biological form after expression.

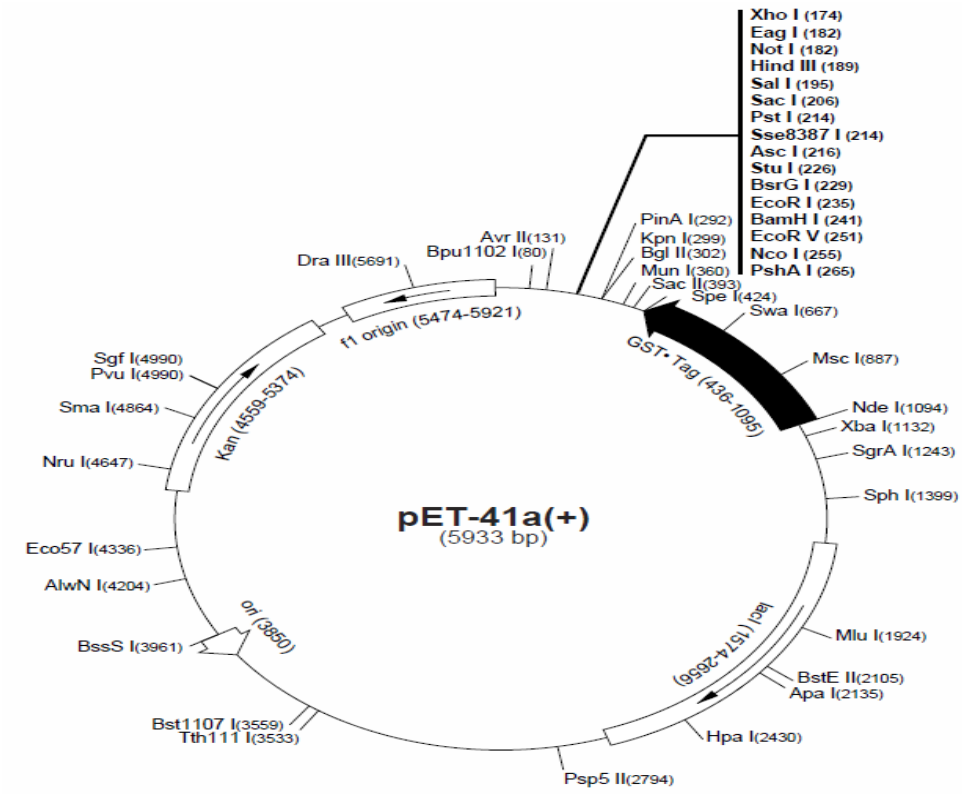
The promoters in *E. coli* expression systems are developed such that the introduction of an inducer allows the production of the desired proteins (Seo *et al*., 2009). Different promoters are induced under different conditions and factors such as low pH (Chou *et al*., 1995), anaerobiosis (Oxer *et al*., 1991) and stationary growth (Shimada *et al*., 2004) for the production of high levels of recombinant proteins.

In chapter 2, AHSV 2 VP2 was expressed using two different baculovirus expression systems. Different promoters and transfer vectors were evaluated, but none of these could express AHSV2 VP2 to the expected levels. We decided to use a completely different expression system. A codon optimized AHSV 4 VP2 gene was expressed in a bacterial system under low temperature conditions. The pET expression system was first described by Studier & Moffat (1986) and Dubendorf & Studier (1991). The expression vector chosen for this study, was the pET41(a+) (Novagen). For expression to occur the recombinant pET41(a+) is transformed into an *E. coli* host lysogenised by a DE 3 fragment. This phage fragment encodes the T7 RNA polymerase and is under the control of the *lacUV5* promoter that is IPTG (isopropyl-ß-D thiogalactopyranoside) inducible. Upon addition of IPTG in culture T7 RNA polymerase is produced, it recognizes and binds to the T7 promoter and this results in transcription.

3.1 MATERIALS AND METHODS

3.1.1 VECTOR AND OPTIMIZED GENE

The preferred host strain for this study was the *E. coli* BL21 (DE3) cells. The pET41a(+) encodes different tags for the purpose of detection and purification of the target protein. Fig 3.1 is a schematic representation of the vector that was used for cloning the gene of interest. The AHSV 4 VP2 used in this study was digested from the bacterial codon optimized VP2_pGA4 (kindly donated by Dr. A. C. Potgieter, Virology Division, ARC-OVI made by GeneArt) and the plasmid map is shown in Fig. 3.2.



5’ 3’

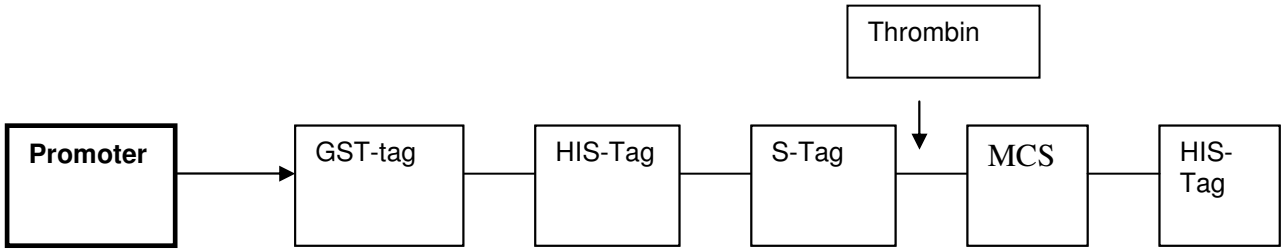


Fig. 3.1: Diagram above is a schematic representation of the plasmid pET41(a+) that was used for cloning AHSV4 VP2. The sequence representation shows the position of the tags (GST, HIS and S-tags), the multiple cloning site, T7 promoter and the thrombin cleavage site (Vector map obtained from Novagen).

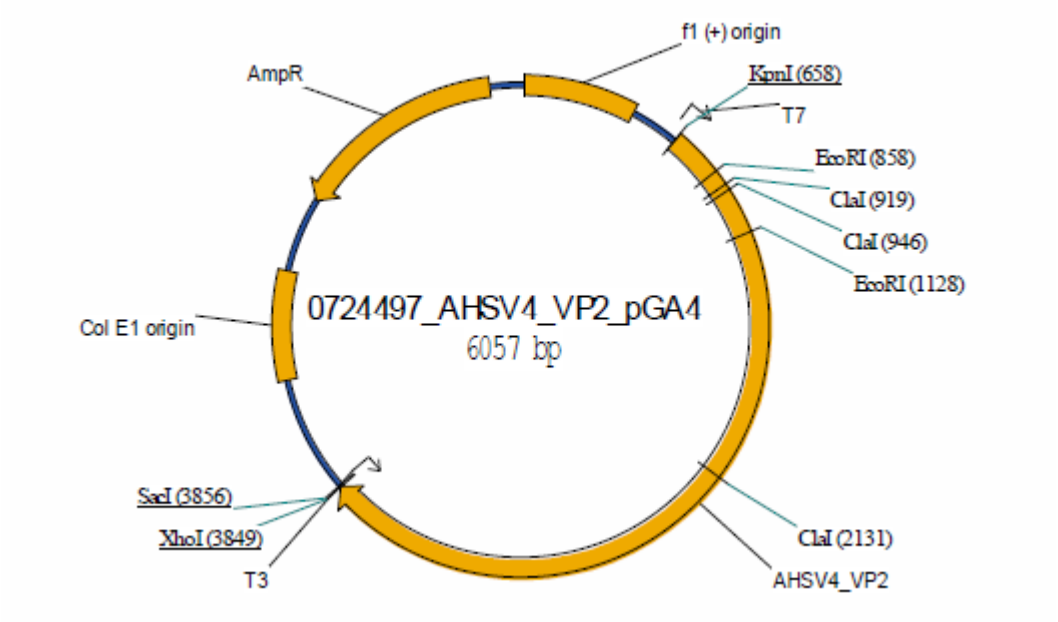


Fig. 3.2: Schematic representation of the bacterial codon optimized AHSV 4 VP2 pGA4. AHSV 4 VP2 was digested from AHSV 4 VP2 pGA4 using restriction enzymes *KpnI* and *SacI* .

3.1.2 ENZYMATIC DIGESTION OF pET 41(a) AND THE AHSV4 VP2pGA4

AHSV 4 VP2 was digested from the VP2_pGA4 using the restriction enzymes *KpnI* and *SacI*. The plasmid pET 41(a+) was also digested with the enzymes *KpnI* and *SacI*. Digestion reactions were carried out to a final volume of 10 µl for 2 hrs at 37℃. After the incubation period, the reactions were mixed with 2 µl of the loading dye and separated on a 1% TAE agarose gel. Sizes of the bands were estimated by comparison with a molecular weight size marker.

3.1.3 PURIFICATION OF THE LINEARISED INSERT AND VECTOR

The AHSV 4 VP2 and the linearised vector (pET 41a+) were gel purified from the 1% TAE agarose gel using the QIAgen MinElute kit (QIAgen). Purifications were performed according to the manufacturer's instructions (just as described in section 2.1.2). The main purpose was to remove unwanted salts, impurities and contaminants. The dissolved agarose fragments were added to the silica columns for the absorption of DNA. The DNA was bound to the column by centrifugation, with the aid of a buffer that helps DNA to bind to the silica membrane at the correct pH. The bound DNA silica columns were washed with a buffer containing ethanol for the removal of unwanted impurities. For the elution of the purified DNA from the column, 10 µl of the elution buffer containing 10 mM Tris (pH 8.5) was added. The low elution volumes (10 µl) yielded a high concentration of DNA.

3.1.4 CLONING OF GENOME SEGMENT ENCODING AHSV4 VP2 GENE INTO THE pET41(a+)

The AHSV 4 VP2 gene and vector (pET 41(a+)) were ligated using the T4 DNA ligation kit (Roche). The ligation reaction consisted of 250 ng of the insert, 150 ng of the vector, 5 U of T4 DNA ligase (Roche) and 2 µl of the 2 × ligation buffer. The ligation reaction was performed to a final volume of 10 µl and was incubated overnight at 4℃.

3.1.5 TRANSFORMATION INTO BL21 (DE3) CELLS

Competent *E. coli* BL21 (DE3) cells were prepared just as described in Chapter 2 section 2.1.6 (i) using the Inoue method (Inoue *et al*., 1990). The overnight ligation reaction was transformed into competent *E. coli* BL21 DE3 cells as described previously (Chapter 2 in section 2.1.6 ii). The transformation reaction (100 µl) was plated out on LB plates containing kanamycin (30 µg/ml) and incubated overnight at 37℃.

3.1.6 RECOMBINANT PLASMID ISOLATION

Possible positive clones were picked as single colonies and inoculated in 5 ml of LB broth containing kanamycin (30 µg/ml) and incubated overnight with shaking at 37℃. The possible recombinants were further identified and screened by plasmid isolations (described in section 2.1.7) and digestions with *KpnI* and *SacI*. Digestion products were analysed on a 1% TBE agarose gel and the sizes of the DNA fragments were compared with the molecular weight marker. The restriction enzyme digestion with the enzymes *KpnI* and *SacI* was for confirming the positive clones.

3.1.7 OPTIMISATION OF EXPRESSION FOR THE RECOMBINANT VP2pET41(a+) IN *E. COLI* BL21 (DE3) CELLS

Expression was initiated by transforming the recombinant plasmid VP2pET41(a+) into competent *E. coli* BL21 (DE) cells. The transformation mix was plated out on LB plates containing kanamycin (30 µg/ml). A starter culture was initiated by picking a single colony and inoculating it in 5 ml LB broth containing kanamycin, overnight with shaking at 37℃. Overnight culture (120 µl) was added to 30 ml of LB broth containing kanamycin (30 µg/ml), shaken at 250 rpm in an incubator at 37℃. Once the desired OD_{600nm} was obtained, the expression was induced with the required concentration of IPTG (refer Table 3.1). For optimal expression three different sets of experiments were performed with different parameters for optimizing maximum yields of the protein. The parameters tested were IPTG concentration, temperature after

induction and OD_{600nm} reading before induction. In each set of experiments one parameter varied while the other two parameters remained constant (Table 3.1).

Table 3.1: Parameters used in optimizing for maximal protein expression

Parameters	**1st Set of**	**2nd Set**	**3rd Set**
OD_{600nm}	0.4, 0.6, 0.8	0.6	0.6
Temp. (℃)	37	18, 25, 37	37
IPTG (mM)	0.6	0.6	0.4, 0.6, 0.8

Cultures induced and grown at 37℃ were incubated for 4 hrs with shaking, whereas the cultures induced at 25℃ were grown for 5 hrs with shaking and 18℃ overnight with shaking. Cell growth was monitored using the spectrophotometer at 600 nm wavelength. After the respective incubation periods with shaking had elapsed the bacterial cells were lysed (as described in section 3.1.8), separated on Criterion XT precast gel 4-12% Bis-Tris (Bio-Rad) and stained by Coomassie blue. After expression was optimized, further experiments were carried out to determine the conditions that yielded more soluble protein. The experiments considered temperatures at 37℃ and 18℃ including other parameters such as OD_{600nm} and IPTG. The protein was lysed and separated into soluble as described in 3.1.8 and insoluble fraction and results were analyzed on Criterion XT precast gel 4-12% Bis-Tris (Bio-Rad).

3.1.8 LYSIS OF THE CELLS

For lysis, cells were harvested by centrifugation at 10 000 × g for 10 min. The supernatant (medium) was removed and excess liquid was drained from the cell pellet. The weight of the pellet was determined. For every gram of wet paste 5 KU/g of Bugbuster (Novagen) was added. Benzonase nuclease enzyme (25 U) (Novagen) and 1 KU rLysozyme (Novagen) was added for every 1 ml Bugbuster. The cell suspension was incubated at room temperature for 20 min on a shaking platform. To separate the soluble from the insoluble protein fraction, the cell suspension was centrifuged 16 000 × g

for 20 min at 4℃. The soluble fraction was kept at 4℃ for the purification of the rVP2 and the insoluble fraction was kept for analysis by SDS-PAGE.

3.1.9 PURIFICATION OF rVP2

The soluble recombinant VP2 (rVP2) was purified using the both the Glutathione-S-Transferase (GST)-tag and the Histidine (His)-tag in order to compare the yield of purified soluble protein.

3.1.9 (a) GLUTATHIONE-S-TRANSFERASE-TAG

The rVP2 protein was purified using the GST-Bind kit (Novagen), according to the manufacturers instructions. A short description of the method: The GST bind resin (500 µl) was washed thoroughly with 2.5 ml of 1 × GST/bind wash buffer and centrifuged for 2 min at 1000 × g. The supernatant was removed using a sterile pipette tip. The resin was completely re-suspended in 1 volume of 1 × GST/bind wash buffer and 2.5 ml of the soluble rVP2 lysate was added. The mixture was incubated at room temperature for 30 min with gentle agitation. Thereafter it was centrifuged for 2 min at 1000 × g. The supernatant containing unbound proteins was transferred to a sterile Eppendorf tube and kept on ice for analysis using SDS-PAGE. The resin was washed by resuspending it in 10 volumes of 1 × GST/bind wash buffer, centrifuged and the supernatant removed. Bound proteins were eluted by adding 1 volume of the 1 × GST elution buffer. The mixture was centrifuged for 2 min at 1000 × g and the supernatant was removed as it contained the purified soluble protein extract (rVP2-GST).

3.1.9 (b) HISTIDINE-TAG

Protino Ni-TED 150 (Macherey-Nagel) are columns that consists of a dry silica resin charged with N^{2+} ions. In principle the polyhistidine tag has a high affinity for the N^{2+} ions. When a protein sample is added to the pre-equilibrated gravity column the histidine tag on the recombinant protein binds to the silica resin charged with N^{2+} ions. The protein of interest is eluted with

imidazole while unwanted proteins are washed away. The rVP2-GST was also purified using Protino Ni-TED 150 (Macherey-Nagel) for the purification of Polyhistidine-tagged proteins. A short description of the method is as follows: A LEW (Lysis, Equilibrium, Wash) buffer was prepared according to the manufacturer's instructions. The columns were equilibrated with 320 µl of the 1 × LEW buffer and drained by gravitation. Soluble rVP2-GST protein (6 ml) was added and allowed to drain. The column was washed twice with (320 µl) 1 × LEW buffer and allowed to drain. For elution of the protein the column was washed three times with 240 µl of the 1 × elution buffer. The eluted fraction was kept separate.

3.1.10 PROTEIN PRECIPITATION AND CONCENTRATION

Two different concentration methods were tested.

3.1.10 (A) VIVASPIN COLUMNS

Vivaspin (20) columns (Sartorius Stedin) consist of a membrane with an appropriate molecular weight cut off for the rVP2-GST are retained in while proteins with a lower molecular weight pass through the membrane. Approximately 6 ml of the soluble protein was added to the fill concentrator of Vivaspin (20) columns and centrifuged at 5000 × g for 30 min. The soluble rVP2-GST protein was recovered.

3.1.10 (B) ACETONE PRECIPITATION

Acetone was pre-cooled before use at -20℃. Approximately 4 × volumes of acetone was added to purified soluble protein and incubated overnight at -80℃. Precipitated proteins were collected by centrifuging in an ultra centrifuge SW 50.1 rotor at 31 000 × g for 30 min at 4℃. The pellet was dried using a freeze drier (Modulyo high vacuum pump) until dry. The dry pellet was re-suspended in 0.01 M STE-TX (0.0005% Triton-X-100 (v/v), 100µl of 1 × TE buffer and 0.01 M NaCl).

3.1.11 PROTEIN CONCENTRATION DETERMINATION

The protein concentration was determined using the Bicinchoninic protein assay kit (Pierce). The principle of the kit is based on colour change of the WR (Working Reagent) from clear green to purple due to reduction of Cu^{+2} to Cu^{+1} in an alkaline medium. The working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1 reagent A:B). The reaction was carried out in the ratio 1:20. For 500 µl of the working reagent 25 µl of the protein sample was added. The mixture was incubated for 30 min at 37℃. The reaction was cooled and the absorbance was measured at 562 nm with a spectrophotometer. The concentration of the protein sample was determined using a standard curve generated using BSA standards.

3.1.12 SDS-PAGE

Protein fractions were mixed with 10 µl of 2 × SDS-PAGE sample buffer, boiled for 3 min and 8 µl was loaded on a Criterion XT precast gel 4-12% Bis-Tris (BIO-RAD). The gel was run at 100V for 2 to 2.5 hrs (using the running buffer XT MOPS (BIO RAD) to a final concentration of 1 ×), stained with Coomassie Blue (as described in section 2.1.12 Chapter 2) and thereafter destained with 10% acetic acid until clear. The stained gels were documented using an Auto Chemi™ UVP BioImaging System.

3.2 RESULTS

3.2.1 CLONING OF AHSV 4 VP2

Cloning of the gene was achieved by initially linearising the insert AHSV4 VP2 and the vector pET41(a+) using the enzymes *KpnI* and *SacI*. The digestion resulted in complementary overhangs on the digestion products. The linearised vector and insert were ligated using T4 DNA ligase (Roche) and transformed into *E. coli* BL21 (DE 3) cells. Double restriction enzyme digestion reactions that were carried out after plasmid isolations verified the positive clones. The enzyme digestion resulted in two bands with the expected sizes of the insert and the vector, approximately 3.2 kbp and 5.9 kbp respectively, when viewed on a 1% TBE agarose gel (Fig. 3.4 lanes 1-6). Fig. 3.3 gives a schematic map of the recombinant AHSV4 VP2pET41(a+) plasmid.

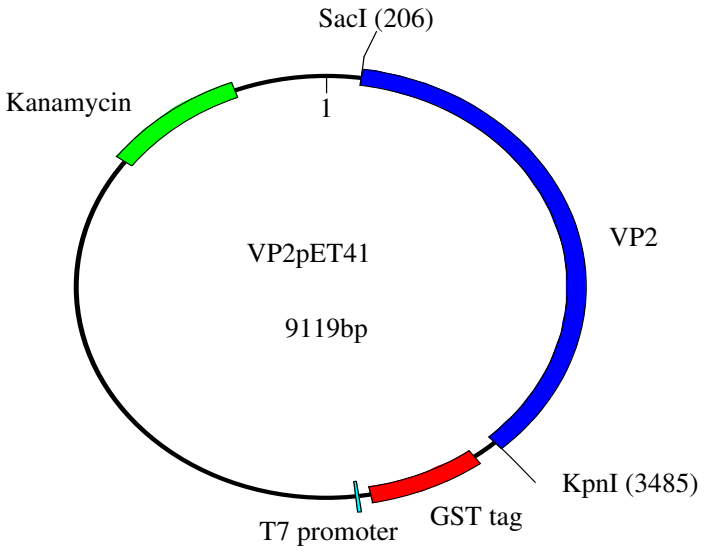


Fig. 3.3: A schematic representation of the recombinant VP2pET41(a+) plasmid with the *Sac1* and *Kpn1* restriction enzyme sites indicated.

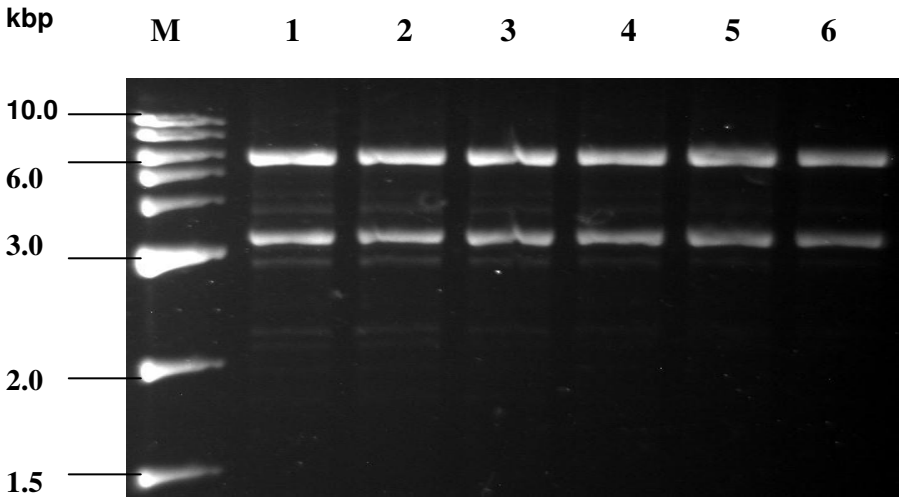


Fig. 3.4: Agarose gel electrophoretic analysis of the enzyme digestion reaction of the clones of VP2pET41 with the enzymes *KpnI* and *SacI*. Lanes 1 – 6 restriction enzyme digestion using enzymes *KpnI* and *SacI* were positive for the insert (~3.4 kbp) and the vector (~5.9 kbp). The sizes of the molecular weight marker (1 kb DNA ladder, New England Biolabs) are indicated on the left hand side of the figure.

3.2.2 EXPRESSION OF RECOMBINANT PROTEINS

E.coli BL21 (DE3) was the host strain that was used for expression of the recombinant VP2-GST protein. Optimal parameters such as induction temperature, IPTG concentration and OD_{600nm} at induction were determined for optimal expression of total protein fraction. A unique protein band of approximately 140 kDa (VP2 ~ 110 kDa and GST ~ 30 kDa) was visible and an indication of successful expression (indicated by the arrow in Fig. 3.5). Protein expression was achieved at temperatures 18 ℃ overnight (Fig. 3.5 lane 7) and 37℃ for 4 hours, and none was observed at 25℃ for 5 hours (Fig. 3.5 lane 6). It was noted that at 25℃ there was no expression of the protein, this was not investigated further as the main interest was for expression. Induction was also observed at IPTG concentrations 0.4, 0.6 and 0.8 (Fig. 3.5 lanes 2, 3, 4).

Lanes	M	1	2	3	4	5	6	7	8	9	10
IPTG (mM)			0.6	0.6	0.6	0.6	0.6	0.6	0.4	0.6	0.8
Temp(℃)			37	37	37	37	25	18	37	37	37
OD_{600nm}			0.4	0.6	0.8	0.6	0.6	0.6	0.6	0.6	0.6

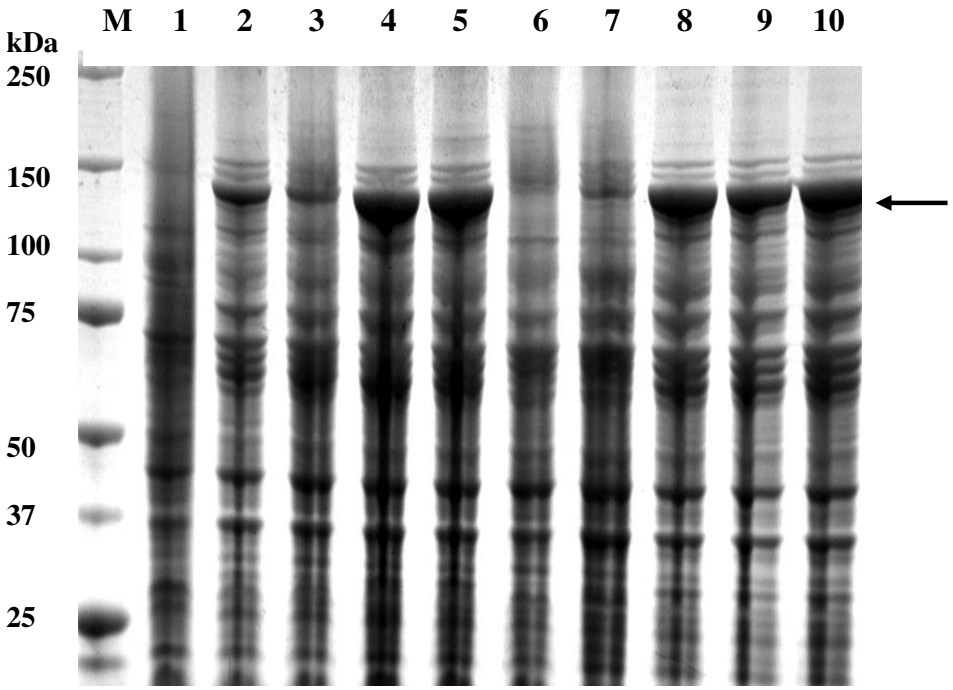


Fig. 3.5: Coomassie-stained SDS-PAGE showing optimization parameters of expression of the rVP2-GST in *E.coli* in BL21 (DE3) cells. Lane M, Protein molecular weight marker (in kDa). Lane 1, Protein fraction of *E. coli* BL21 DE3 cells containing only the plasmid (pET41a+); Lane 2, O. D. $_{600nm}$ = 0.4, Temperature 37℃, IPTG 0.6 mM, Lane 3, OD_{600nm} = 0.6, Temperature 37℃, IPTG 0.6 mM, Lane 4, OD_{600nm} = 0.8, Temperature 37℃, IPTG 0.6 mM, Lane 5, OD_{600nm} = 0.6, Temperature 37℃, IPTG 0.6 mM, Lane 6, OD_{600nm} = 0.6, Temperature 25℃, IPTG 0.6 mM , Lane 7, OD_{600nm} = 0.6, Temperature 18℃, IPTG 0.6 mM , Lane 8, OD_{600nm} = 0.6, Temperature 37℃, IPTG 0.4 mM, Lane 9, OD_{600nm} = 0.6, Temperature 37℃, IPTG 0.6 mM, Lane 10, OD_{600nm} = 0.6, Temperature 37℃, IPTG 0.8 mM. The presence of a ~140 kDa band was an indication that recombinant VP2-GST protein was expressed (~140 kDa band observed in lanes 2, 3, 4, 5, 7, 8, 9, 10).

3.2.3 DETERMINING SOLUBILITY OF THE RECOMBINANT PROTEIN

Further optimization studies were performed to determine the most suitable conditions between 18℃ and 37℃ for maximal yield of soluble rVP2-GST. Bugbuster and rLysozyme were added for lysing the cells and releasing the soluble protein. Benzonase is a nuclease enzyme that was added to reduce the viscosity of the solution, caused by the genomic DNA. Following centrifugation, the soluble fraction of the protein was separated from the insoluble fraction. *E. coli* consist of internal standard proteins approximately 40 kDa signifying that the amount of protein loaded on the SDS-PAGE was the same for all columns. The band that is 140 kDa is visible also in lane 2 (Fig. 3.6) the exact same size band appears extra pronounced in the lane 4 and this signifies the presence of VP2. Optimization results revealed that more protein was expressed at 18℃ (Fig. 3.6 lane 4) (indicated by a line) than at 37℃ (lane 7) (indicated by a line). The soluble fraction can be seen in lane 4 (Fig. 3.7) as indicated by the arrow, compared to the insoluble fraction in lane 3. Expression conditions for optimal soluble rVP2-GST were determined to be 0.6 mM IPTG for inductions of cultures grown to an OD_{600nm} at 0.6 with induction at 18℃ overnight (Fig. 3.7). The next stage was the purification of the protein.

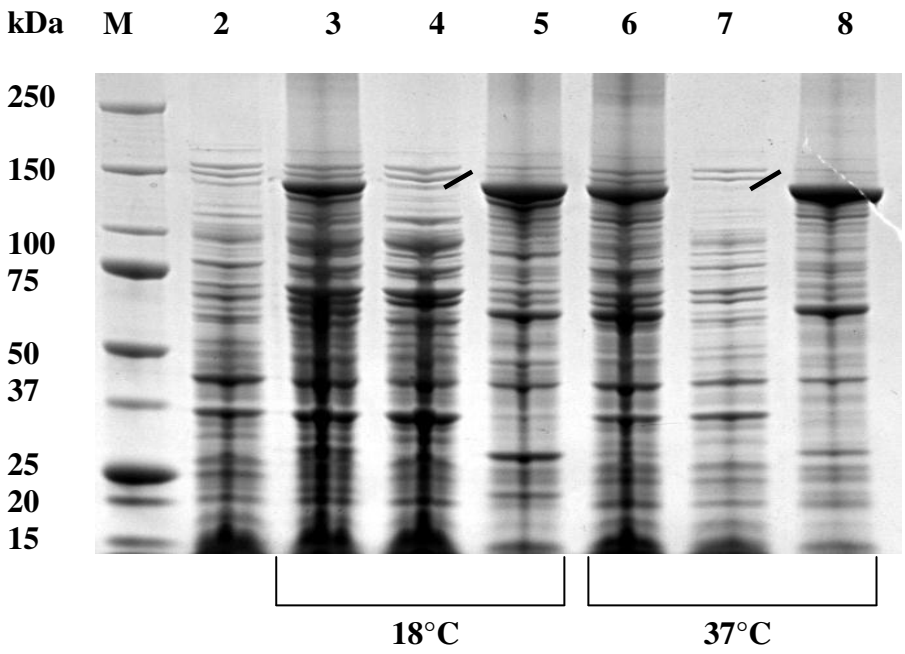


Fig. 3.6: Coomassie-stained SDS-PAGE analysis of rAHSV 4 VP2-GST expressed in *E.coli* BL21 (DE3) cells illustrating the solubility of the rVP2-GST at different induction temperatures. Lane M, Protein molecular weight marker (in kDa); Lane 2, Protein fraction of *E. coli* BL21 DE3 cells containing only the plasmid (pET41a+) ; Lane 3, Total protein fraction of recombinant cells containing recombinant VP2-GST induced at 18℃ ; Lane 4, Soluble fraction of the recombinant cells containing rVP2-GST induced at 18℃ (indicated by the line ~140kDa); Lane 5, Insoluble fraction of the recombinant cells containing rVP2-GST induced at 18℃; Lane 6, Total protein fraction of recombinant cells containing recombinant VP2-GST induced at 37℃; Lane 7, Soluble fraction of the recombinant cells containing rVP2-GST induced at 37℃ (indicated by the line ~140kDa); Lane 8, Insoluble fraction of the recombinant cells containing rVP2-GST induced at 37℃

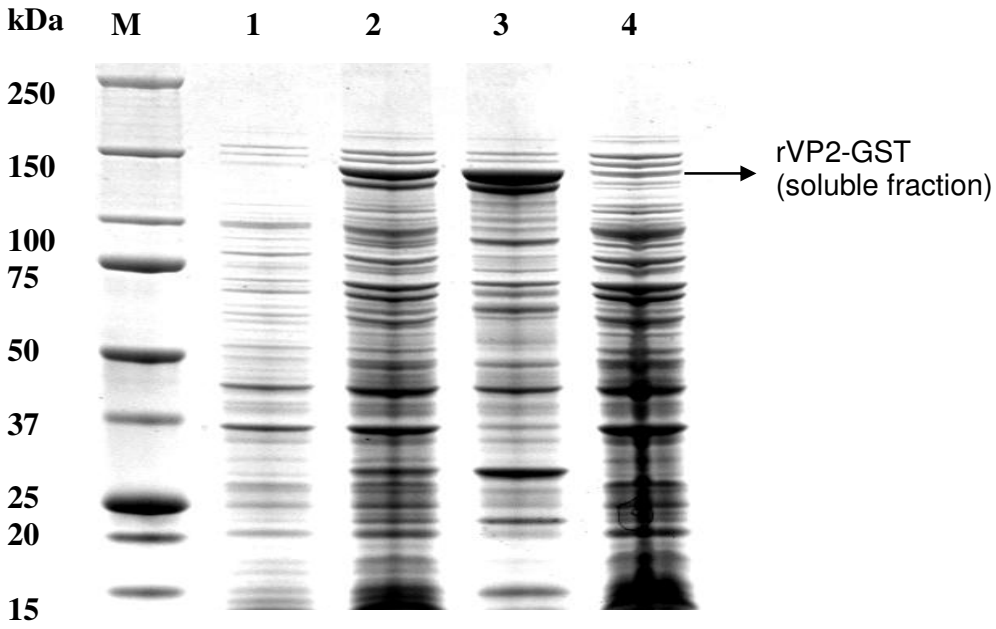


Fig. 3.7: Coomassie-stained SDS-PAGE analysis of rVP2-GST expressed *E.coli* in BL21 (DE3) cells under optimized expression conditions of 0.6 mM IPTG for inductions of cultures, grown to an OD_{600} at 0.6 with induction at 18℃ overnight. Lane M, Protein molecular weight marker (in kDa); Lane 1, Protein fraction of *E. coli* BL21 DE3 cells containing only the plasmid (pET41a+); Lane 2, Total protein fraction of recombinant cells containing recombinant VP2-GST induced at 18℃; Lane 3, Insoluble fraction of the recombinant cells containing rVP2-GST induced at 18℃; Lane 4, Soluble fraction of the recombinant cells containing rVP2-GST induced at 18℃ (indicated by the line) (rVP2-GST protein ~140kDa is indicated by the arrow)

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3.2.4 PURIFICATION AND CONCENTRATION OF THE PROTEIN

The recombinant rVP2-GST was expressed in *E coli* BL21 cells. The pET41(a) vector encodes for a His- tag, GST-tag and S-tag on the N terminal side of the AHSV 4 VP2 (Fig.3.1). Purification using Protino Ni prepacked affinity columns (Protino Ni-TED packed columns), targeting the His-tag was unsuccessful (results not shown).The recombinant protein was then purified the using the glutathione agarose (GST Bind Buffer Kit). The GST agarose was filled to its saturation point with the soluble protein and successfully purified the rVP2-GST (Fig. 3.8 lane 5). Because of the low yield of the eluted proteins acetone precipitation was used for concentrating the protein (Fig. 3.8 lane 6). The GST was the control and was purified and concentrated using acetone precipitation (Fig. 3.8 lane 9).

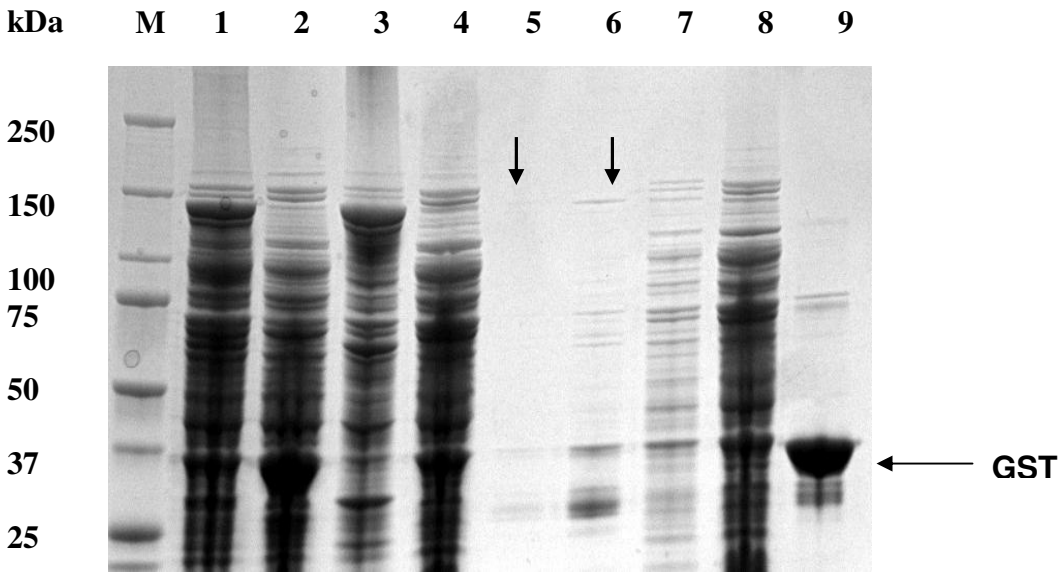

Fig. 3.8: Coomassie-stained SDS-PAGE analysis of the rVP2-GST expressed in *E.coli* BL21 (DE3) cells purified using GST agarose and concentrated using the acetone precipitation technique. Lane M, Protein molecular weight marker (in kDa); Lane 1, Total protein fraction of recombinant cells containing recombinant VP2-GST; Lane 2, Protein fraction of *E. coli* BL21 DE3 cells containing only the plasmid (pET41a+); Lane 3, Insoluble protein fraction of the recombinant cells containing rVP2-GST; Lane 4, Soluble protein fraction of the recombinant cells containing rVP2-GST (~140 kDa); Lane 5, Purified soluble rVP2-GST protein (~140 kDa); Lane 6, Acetone precipitated soluble rVP2-GST protein (~140 kDa); Lane 7, Glutathione beads containing rVP2-GST before elution of the protein; Lane 8, Unbound protein washed from the beads; Lane 9, Concentrated purified GST only (~30 kDa).

Vivaspin 20 columns and the acetone precipitation techniques were tested for the concentration of the protein. After acetone precipitation although the picture quality was low a faint band of rVP2-GST was observed, whereas with Vivaspin 20 there was no band observed. Therefore acetone precipitation was chosen because a good yield of the purified protein (Fig. 3.8 lane 6) was obtained using this technique. After the purification of the rVP2-GST other non-specific proteins were visible and this was attributed to contaminating proteins that bound to the GST agarose non-specifically.

3.3 DISCUSSION

Finding an expression host and promoter for recombinant protein expression has become a challenge for scientists in various disciplines of research. Purified heterologous proteins that are correctly folded have proven to be of great importance in aspects of research such as drug discovery, vaccines, antibody generation and so forth (Dyson *et al*., 2004). In that context, many expression systems and hosts varying from eukaryotic to prokaryotic have been exploited in order to obtain and achieve this. Among the many systems *E. coli* has been chosen as expression host, due to the fast growth of the cells, affordability of the media and the extensive knowledge on the biology and genetics of the organism (reviewed by Baneyx, 1999). However, eukaryotic proteins that are expressed in the *E. coli* are confronted with challenges varying from lack of eukaryotic chaperones to misfolding of proteins.

In this chapter AHSV4 VP2, a codon optimized gene for bacterial expression, was successfully cloned into the pET41(a+) vector under the control of a *lacUV5* promoter. Previous studies have shown that codon optimized genes have a greater potential for high levels of protein expression in heterologous host systems. A study by Zhou *et al*. (2003) reported that protein production of the codon optimized recombinant *FALVAC-1* gene increased up to three fold in *E. coli*, when compared to the non-codon optimised *FALVAC-1* gene because of improved translation. They further deduced that codon usage of the foreign gene should be similar to the host and that rare codons may affect mRNA and translation resulting in poor protein expression. A study by Chen and Inouye (1990) showed that the distance between the arginine codons and the initiation codon directly influences the rate of translation in the *E. coli*.

The induction conditions for the expression of VP2pET41(a+) were optimized by comparing different expression temperatures, cell density before induction and different IPTG concentrations . The results revealed that with the addition of 0.6 mM IPTG at OD_{600nm} 0.6 overnight at 18℃ the highest levels of soluble protein expression were achieved. Although a lot of protein was expressed at

37℃, a larger fraction of the protein was soluble at 18℃. From previous studies different workers have shown that higher temperatures have negative effect on protein folding and highly favors the formation of inclusion bodies. Niiranen and colleagues (2007) expressed the cold adapted proteins of the *Vibrio* bacterium in *E. coli*. For optimizing expression they considered factors such as temperature, expression strains and His_6, GST, MBP and NusA tags. Their findings revealed that at lower temperatures between 23℃ and 16℃, more soluble protein was obtained than at 37℃. They further concluded that low temperatures lead to a low production rate of the protein, therefore giving the protein more time for folding properly and more chaperones are available for aiding solubility. The same was proven in Eukaryotes where Li and co-workers (2001) expressed antifreeze proteins in yeast *Pichia pastoris.* Their study showed that the protein was biologically active lower temperatures of 23℃ than at 30℃.

The VP2 protein was expressed and could be seen as a unique protein band at 140 kDa. The His- and the GST-tags were used for purification purposes of the protein. Purification using the His-tag was the least effective probably because the tag is not accessible due to VP2 folding. Purification using the GST-tag yielded better results. The GST-tag is a ~30 kDa protein and the bigger tag was more accessible. This can also mean that this large tag will interfere with natural folding of the VP2. This will be evaluated in chapter 4.

The soluble fraction of the rVP2-GST was concentrated using acetone precipitation. Analysis SDS-PAGE revealed the presence of contaminating bands that could be either contaminating, non-specific proteins or over expressed molecular chaperones. Chaperones like DnaK and GroEL can be found in the cytoplasm of *E. coli.* Faint bands of the contaminants were significantly reduced with the additional wash steps before elution of the purified protein. Rial and Ceccarelli (2002) described the removal of the unwanted DnaK contamination by additional wash steps with MgATP before elution of the protein.

REFERENCES

BANEYX, F. 1999. Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology*, 10:411-421.

BURGESS-BROWN, N. A., SHARMA, S., SOBOTT, F., LOERNARZ, C., OPPERMANN, U. & GILEADI, O. 2008. Codon optimization can improve expression of human genes in *Escherichi coli. Protein Expression and Purification,* 59:94-102.

CHEN, G. T. & INOUYE, M. 1990. Suppression of the negative effect of minor arginine codons on gene expression; preferential usage of minor codons within the first 25 codons of the *Escherichi coli* genes. *Nucleic acids Research*, 18:1465-1473.

CHEN, G. T. & INOUYE, M. 1994. Role of the AGA/AGG codons, the rarest codons in global gene expression in *Escherichi coli. Genes and development,* 8:2641-2642.

CHOU, C. H., ARISTIDOU, A. A., MENG, S. Y., BENNET, G. N. & SAN K. Y. 1995. Characterization of a pH inducible promoter system for a high level expression of recombinant proteins in *Eschericia coli*. *Biotechnology & Bioengineering,* 47:186-192.

DUBENDORFF, J. W. & STUDIER, F. W. 1991. Creation of a T7 autogene. Cloning and expression of the gene for bacteriophage T7 RNA polymerase under the control of its cognate promoter. *Journal of Molecular Biology*, 219:61-68.

DYSON, M. R., SHABOLT, S. P., VINCENT, K. J., PERERA, L. K. & MCCAFFERTY, J. 2004. Production of soluble mammalian proteins in Escherichia coli: identification of protein features that correlate with successful expression. BioMed Central Biotechnology, 4:1-18

FUH, G., MULKERRING, M. G., BASS, S., MCFARLAND, N., BROCHIER, M., BOURELL, J. H., LIGHT, D. R. & WELLS, J. A. 1990. The human growth hormone receptor. *The Journal of Biological Chemistry,* 256:3111-3115.

HOLM, L. 1986. Low usage and gene expression. *Nucleic Acids Research*, 14:3075-3087.

IKEMURA, T. 1981. Correlation between the abundance of *Escherichia coli* transfer (tRNA) and the occurrence of the respective codons in its protein genes: a proposal for a synonymous choice that is optimal for the *E. coli* translational system, 151:389-409.

INOUE, H., NOJIMA, H. & OKAYAMA, H. 1990. High efficiency transformation of *E. coli* with the plasmid. *Gene,* 96:23-28.

KANE, F. J., 1995. Effects of rare codon clusters on high-level expression of heterologous proteins in *Escherichia coli*. *Current Opinions in Biotechnology*, 6:494-500.

KURLAND, C. & GALLANT, J. 1996. Errors of heterologous protein expression. *Current Opinions in Biotechnology*, 7:489-493.

LI, Z., XIONG, F., LIN, Q., D'ANJOU, M., DAUGULIS, A. J., YANG, D. S. C. & HEW, C. L. 2001. Low temperature increases the yield of biologically active herring antifreeze protein in *Pichia pastoris. Protein Expression and Purification,* 21:438-445.

MAKRIED, S. C. 1996. Strategies for achieving high-level expression of genes in *Escherichia coli. Microbiological Reviews*, 60:512-538.

NIIRANEN, L., ESPELID, S., KARLSEN, R. C., MUSTONEN, M., PAULSEN, S. M., HEIKINHEIMO, P. & WILLASSEN, N. P. 2007. Comparative expression study to increase the solubility of cold adapted *Vibrio* proteins in *Esherichia coli*. *Protein Expression and Purification*, 52:210-218.

OXER, M. D., BENTLEY, C. M., DOYLE, J. G., PEAKMAN, T. C., CHARLES, I. G. & MAKOFF, I. J. 1991. High level heterologous expression in E. coli using the anaerobically-activated *nirB* promoter. *Nucleic Acid Research*, 19:2889-2892.

RIAL, V. D. & CECCARELLI, E. A. 2002. Removal of DnaK contamination during fusion protein purifications. *Protein Expression and Purification,* 25:503-507.

SEO, J. W., HONG, W. K., RAIRAKHWADA, D., SEO, P. S., CHOI, M. H., SONG, K. B., RHEE, S. K. & KIM, C. H. 2009. The efficient plasmid vector for constitutive high-level expression of foreign genes in *Escherichia coli*. *Biotechnology Letter*, 31:877-881.

SHIMADA, T., MAKINOSHIMA, H., OGAWA, Y., MIKI, T., MAEDA, M. & ISHIHAMA, A. 2004. Classification and strength measurement of stationary phase promoters by use of a newly developed promoter cloning vector. *Journal of Bacteriology,* 186:7112-7122.

SPANJAARD, R. A., CHEN, K., WALKER, J. R. & VAN DUIN J. 1990. Frameshift suppression at tanderm AGA and AGG codons by cloned tRNA genes: assigning a codon to argU tRNA and T4 tRNA (Arg). *Nucleic Acids Research*, 18:5031-5036.

STUDIER, F. W. & MOFFAT, B. A. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. *Journal of Molecular Biology*, 189:113-130.

ZHANG, S. P., ZUBAY, G. & GOLDMAN, E. 1991. Low usage codons in *Escherichia coli*, yeast and fruit fly and primates. *Gene*, 105:61-72.

ZHOU, Z., SCHNAKE, P., XIAO, L. & LAL, A. A. 2004. Enhanced expression of a recombinant malaria candidate vaccine in *E. coli* by codon optimization. *Protein Expression & Purification*, 34:87-94.

CHAPTER 4

DEVELOPING AN ELISA USING CHICKEN IgY FOR THE DETECTION OF AHSV 4 VP2 ANTIGEN

4.0 INTRODUCTION

IgYs are immunoglobulins that are found in the egg yolk (Leslie & Clem 1969, Patterson *et al*., 1962). Earlier literature suggested that IgY was an IgG-like immunoglobulin. However, this theory was disposed of by Leslie and Clem (1969). They proposed that the chicken immunoglobulin be named IgY because of physical-chemical and antigenic properties which differentiate it from the IgG. Warr and colleagues (1995) in a review further deduced that the IgY has a molecular mass greater than the IgG. A study by Davalos-Pantoja and co-workers (2000) revealed that IgY is more hydrophobic than IgG. Also, IgY is the serum immunoglogulin of birds, whereas IgG is found only in mammals (Leslie & Clem 1969).

The use of chicken eggs for the extraction of IgY has several advantages. Firstly, economically the cost of housing chickens is low compared to other small laboratory animals such as mice and rats. Secondly, antibodies from the hen are transferred to the egg yolk (Jukes *et al*., 1934). As a result of this, IgY can easily be extracted from the yolk and there is no bleeding of animals involved, which is normally required for the preparations of serum.

The aim of this chapter was to develop a serotype specific ELISA for the detection of AHSV 4 VP2 using IgY isolated and purified from chickens injected with recombinant AHSV 4 VP2-GST. This was a proof of concept in which the serotype specificity of IgY produced against the VP2-GST of AHSV 4 will be determined. Chicken IgY has a variety of applications ranging from diagnostics to therapeutic reagents. A group specific double sandwich ELISAs was developed by Du Plessis and colleagues (1999) that exploited the IgY as a secondary antibody for the detection of VP7 of AHSV.

4.1 MATERIALS AND METHODS

4.1.1 ANIMALS

Ethical clearance was obtained from the Onderstepoort Veterinary Institute (OVI) Animal Ethics Committee for the three chickens that were used for the immunisation study. Two 6 month old specific pathogen free (SPF) white Leghorn chickens were obtained from Deltamune (Pty) Ltd (Centurion). The chickens were kept at OVI, at the Immunology and Biotechnology chicken room. The chickens were housed in individual cages (2700 cm^2 cage per chicken) and full length feeding troughs were provided. A temperature controlled room and a light control (12 hour cycle) was used. The facility was monitored twice daily for temperature recording, cleaning trays, for supplying feed and water. Nipple bottles were used for supplying water and the chickens were allowed out of their cages regularly. Chickens were fed with layer mash and once egg laying began were provided with an additive calcium phosphate. The eggs were collected, labelled and stored at 4℃. The chicken that was injected with the recombinant GST-VP2 was referred to as YB and the control chicken that was injected with the recombinant GST was referred to as YR.

4.1.2 IMMUNISATION OF CHICKENS

The first chicken was immunised in the pectoral muscles with the purified recombinant VP2-GST (YB), the second chicken served as a control chicken (YR) and was immunised only with the purified recombinant GST. The chickens were injected in the pectoral sites with 500 µl of the antigen (in PBS) containing an equal volume of the adjuvant Montanide ISA 260 (SEPPIC, France). The initial immunisation was followed by two booster injections. The primary injection consisted of the prepared purified recombinant VP2-GST (for the control a recombinant GST) to a final amount of 80 µg. A first booster was administered after 3 weeks from the initial injection to a final amount of 40 µg. The last booster was given after 7 days to a final amount of 40 µg (Du Plessis

et al., 1999). The collected eggs were labelled and kept at 4°C until the extraction of IgY.

4.1.3 ISOLATION AND PURIFICATION OF IgY ANTIBODIES FROM AN EGG YOLK

IgY isolated from eggs from chicken YR was referred to as IgY-GST and from chicken YB referred to as IgY-VP2GST. Egg yolk was prepared as described by Polson and co-workers (1985). The method briefly: Egg yolk was mixed with 4 volumes 0.1 M phosphate buffer pH 7.6, and centrifuged 20 min at 5000 × g. Pulverised PEG (polyethylene glycol 6000) was added to the supernatant and the precipitate was discarded. The mixture was allowed to stand for 10 min and centrifuged at 5000 × g for 25 min. The supernatant was discarded, the precipitate dissolved in 0.1 M phosphate buffer pH 7.6 and pulverised PEG was added. The mixture was allowed to stand for 10 min. Thereafter, it was centrifuged at 5000 × g for 25 min. The supernatant was discarded and the pellet was dissolved in 0.1 M phosphate pH 7.6 buffer. The concentration was determined on a spectrophotometer (Shimadzu UV 1800)at 280 nm. Before storage at 4°C Na-azide was added to the purified dissolved pellet to a final concentration of 0.02% for inhibiting bacterial growth. The presence of IgY in the dissolved pellet was confirmed by an ELISA.

A 96 well Polysorb microtitre plate (Denmark), was coated with 50 µl per well with IgY-GST and or IgY-VP2GST (20 µg/ml) in PBS for 2 hrs at 37°C. Control wells were coated with 50 µl volumes per well of with of 2% milk powder in PBS (MP/PBS) and incubated for 2 hrs at 37°C. All samples were tested in triplicate. Contents of the plate was discarded followed by a blocking step with 300 µl per well of MP/PBS and incubated for 40 min at 37°C. The plate was washed 3 times with PBS containing 0.05% Tween-20. Goat anti-chicken IgG conjugated to horseradish peroxidase (Serotec) diluted 1:2500 in 2% milk powder, was added in a 50 µl volume to each well and incubated for 40 min at 37°C. After the final wash, 50 µl of the chromogen [that consisted of 1 mg/ml

O-phenylenediamine dihydrochloride dissolved in 5 ml 0.1M citrate buffer (pH 4.5) containing 2.5 µl hydrogen peroxide (30% v/v)] was added to each well and incubated for 40 min 37°C. The reaction was stopped with 50 µl 2N H_2SO_4 per well and the signal determined at 492 nm on a microtitre plate reader using the BDSL Immunoscan MS (labsystems).

4.1.4 ELISA OF IgY-VP2GST AGAINST AHSV 4

For the ELISA, a Polysorb microtitre plate was coated with 50 µl of the purified recombinant AHSV 4 VP2 in PBS at a final concentration of 10 µg/ml. The plate was incubated overnight at 4°C. The liquid contents of the wells were aspirated and followed by a blocking step with 300 µl of MP/PBS for 40 min at 37°C. After blocking the liquid contents of the plate was emptied and washed with 3 times with PBS containing 0.05% Tween-20. The 50 µl IgY-VP2GST in PBS was added in each well to a final concentration of 20 µg/ml and incubated for 40 min 37°C.The plate was washed as before, 50 µl goat anti-chicken IgG conjugated to horseradish peroxidase (Serotec) diluted 1:2500 in MP/PBS was added to each well and incubated for 40 min at 37°C. After the final wash the chromogen was added, reaction stopped and the signal read (refer above 4.1.3).

4.1.5 GST PRE-ABSORPTION OF IgY-VP2GST

Three rows of wells of a microtitre plate were coated with 300 µl of the recombinant GST to a final concentration of 20 µg/ml for 1 hr. Thereafter the contents of the well was aspirated and blocked with 300 µl per well of MP/PBS. The milk powder in the first row was removed and filled with 300 µl per well of the IgY-VP2GST (30 µg/ml) and incubated for 20 min at 37°C. The milk powder was removed from the second row of wells and the antibody from the first row of wells was transferred to the second row of wells and incubated for 20 min at 37°C. The milk powder was then removed from the third row of wells and the antibody solution from the second row of wells was now transferred to the third row of wells and again incubated for 20 min at 37°C.

After this pre-absorption treatment, the antibodies were used in an ELISA described in the section below.

4.1.6 ELISA WITH PRE-ABSORBED IgY-VP2GST

An ELISA was performed for evaluating signal between the treated IgY-VP2-GST against the antigen. A microtitre plate was coated in triplicate with 50 µl of rGST (10 µg/ml) and incubated for 2 hrs at 37℃. Contents of the plate were discarded and blocked with 300 µl of the 2% milk powder. The plate was incubated for 40 min at 37℃. Thereafter the liquid in the wells was aspirated and the plate washed 3 times with PBS containing 0.5% Tween-20. 50 µl of the treated IgY-VP2GST (20 µg/ml) were added to the wells and incubated for 40 min at 37℃. After washing the plate 3 times, 50 µl of the goat anti-chicken conjugate (Serotec) were added and the plate incubated for 40 min at 37℃. After washing the plate, 50 µl of the enzyme substrate 1 mg/ml O-phenylenediamine dihydrochloride was added and colour development stopped by the 2N H_2SO_4.

4.1.7 THROMBIN CLEAVAGE OF THE GST TAG

Biotinylated thrombin (Novagen) is used for the removal of the tag from the expressed protein. The tag is cleaved from the fusion protein at a thrombin recognition site. GST tag was cleaved from rVP2-GST using biotinylated thrombin (Novagen). The recombinant protein was expressed and purified as described in the materials and methods Chapter 3 sections 3.1.7, 3.1.9 and 3.1.10. GST agarose beads (500 µl) were washed with the binding buffer twice and thereafter the purified recombinant protein (6 ml) was added and incubated for 30 min with shaking. The mixture was centrifuged at 1000 × g for 2 min. A 1 × thrombin cleavage buffer (1.5 ml) was added to the protein bound to the GST beads, the mixture was centrifuged at 500 × g for 10 min and the pellet resuspended in the 1 × thrombin cleavage buffer (500 µl). Biotinylated Thrombin (Novagen) (1U/µl) was added and incubated 2 hrs at room temperature in an orbital shaker. After the incubation period, 800 µl of Streptavidin agarose (Novagen) was added with further incubation at room

temperature for 30 min in an orbital shaker. The mixture was centrifuged at 500 × g for 5 min. The supernatant was collected as the recombinant VP2 cleaved from the tag.

4.2 RESULTS

4.2.1 IMMUNISATION OF CHICKENS

Two specific pathogen free white Leghorn chickens were used in this study (materials and methods section 4.1.1). Chicken YB was injected with the recombinant VP2-GST, chicken YR was injected with the recombinant GST and served as the control for measuring background resulting from the GST-tag and other bacterial proteins.

4.2.2 PURIFICATION OF IgY

IgY was extracted using the method described by Polson and co-workers (1985) (materials and methods section 4.1.3). ELISA was done in triplicate and the average of the readings was calculated. From the control chicken (YR) the IgY obtained produced absorbance reading of 0.6 while IgY from chicken YB yielded an absorbance reading of 0.55. MP/PBS that was used as the control yielded an absorbance of value 0.06. The recorded absorbance was proof that IgY was extracted by the Polson method.

4.2.3 SPECIFICITY OF IgY-VP2GST AND IgY-GST FOR rVP2-GST and rGST

Microtitre plates were coated with the recombinant VP2-GST while rGST coated wells were used as negative control. After the incubation, the wells were blocked, thereafter washed and dried. IgY-VP2GST was added to the wells and IgY-GST was used in the case of the negative controls. After incubation goat anti-chicken conjugate was added as secondary antibody. After which the chromogen was added to the wells. The reaction was stopped with 2N H_2SO_4 and the signal was measured.

The ELISA described above was done for evaluating the ability of the specific IgY antibodies namely IgY-VP2GST and IgY-GST, to detect recombinant antigens namely rVP2-GST and (YR) rGST. High absorbance values of 0.4 were obtained when IgY-VP2GST was tested on rGST and even higher values above 1.0 were obtained when IgY-GST was tested on rGST [Fig. 4.1(b) and absorbance values represented by table 4.1(b)]. The values of IgY-VP2GST tested on rGST-VP2 [Fig. 4.1(a) and absorbance values represented by table 4.1(a)] were lower when compared with values obtained from IgY-VP2GST tested on rGST. The results showed that a higher signal was obtained towards the tag (GST). This revealed that the GST more immunogenic than VP2.

Table 4.1(a): Absorbance values (492 nm) of the specificity of: Column 1- IgY-VP2GST tested on rVP2-GST, Column 2- IgYGST tested on rVP2-GST, Column 3- Negative MP/PBS. (A, B, C, designated on the table are replicates)

	1	2	3
A	0.152	0.295	0.05
B	0.148	0.267	0.046
C	0.139	0.271	0.048
Average	0.146	0.277	0.048

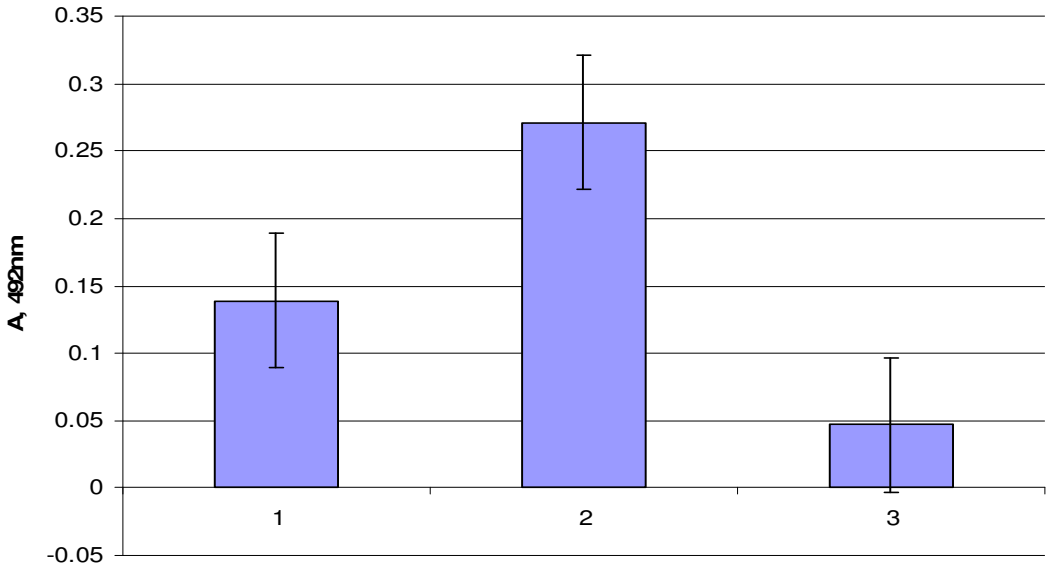

Fig. 4.1(a) Bar chart showing ELISA absorbance values illustrating the specificity of: 1- IgY-VP2GST tested on rVP2-GST, 2- IgYGST tested on rVP2-GST,3- Negative MP/PBS

Table 4.1(b): Absorbance values (492 nm) for illustrating the specificity of: Column 1- Negative MP/PBS, Column 2- IgY-VP2GST tested on rGST, Column 3- IgY-GST tested on rGST. (A, B, C, designated on the table are replicates)

	1	2	3
A	0.047	0.392	1.038
B	0.046	0.361	1.028
C	0.050	0.365	1.092
Average	0.047	0.372	1.052

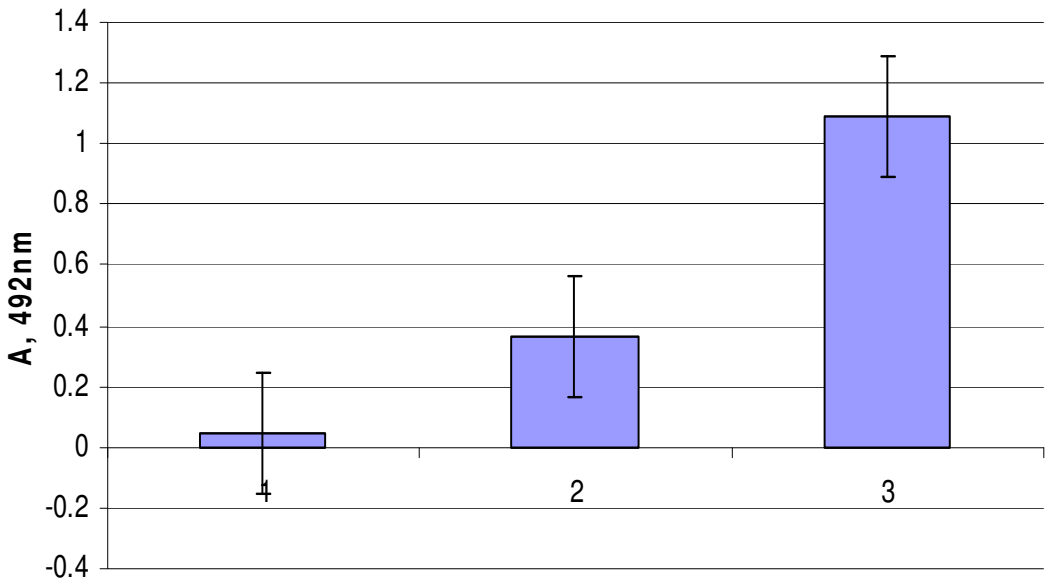

Fig. 4.1(b) Bar chart showing ELISA absorbance values for illustrating the specificity of: 1- Negative MP/PBS, 2- IgY-VP2GST tested on rGST, 3- IgY-GST tested on rGST

IgY antibodies directed towards the GST-tag after recombinant VP2GST was removed as described in section 4.1.5. The pre-absorbed IgY-VP2GST was tested for its ability to bind GST with the ELISA as described in section 4.1.6. The results (Fig. 4.2 and absorbance values represented by table 4.2) showed that the absorption on GST reduced the ELISA signal from an absorbance reading of 0.37 for the non-absorbed IgY to 0.12 for the pre-absorbed IgY. The results demonstrate that after removal of antibodies against rGST there was a significant reduction in the signal and this proves that antibodies directed against the GST tag are responsible for the high signal obtained in Fig. 4.1 (a).

Table 4.2: Absorbance values (492 nm) after pre-absorbance of GST-specific IgY from IgY-VP2GST. Column (milk powder) MP (a) Column MP (b) Column YB (a) Pre-absorbed Column YB (b). (A, B, C, designated on the table are replicates).

	MP (a)	MP (b)	YB (a)	YB (b)
A	0.048	0.054	0.125	0.5
B	0.054	0.046	0.119	0.391
C	0.047	0.039	0.117	0.231
Average	0.049	0.046	0.120	0.374

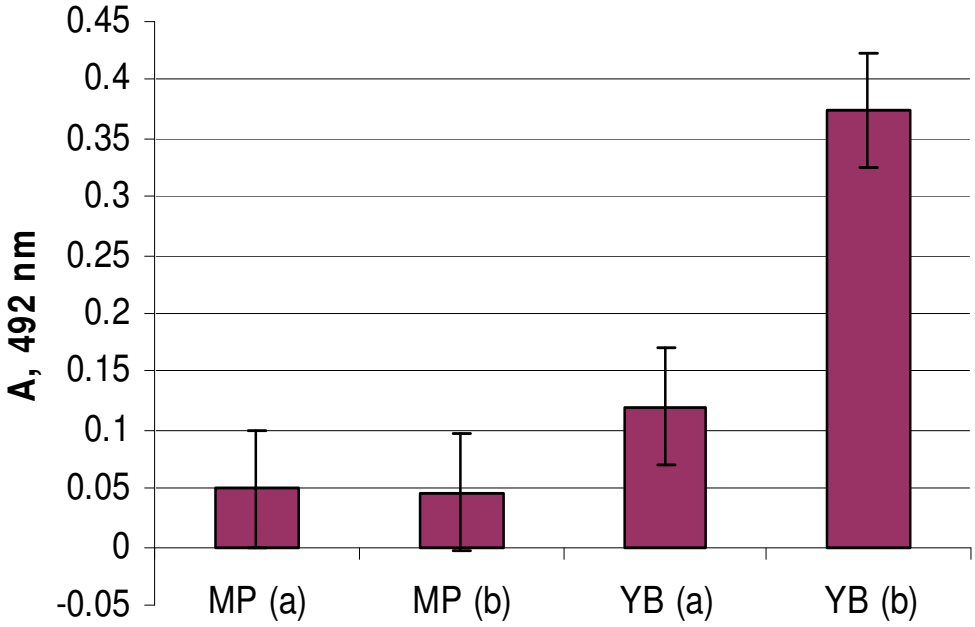

Fig. 4.2: ELISA illustrating the effect of pre-absorption of YB-IgY. (a) Milk powder, MP (b) Milk powder. YB (a) Pre-absorbed YB-IgY tested on rGST. YB (b) Non-absorbed YB-IgY tested on the rGST

4.2.4 THROMBIN CLEAVAGE OF THE TAG

Since the majority of the IgY antibodies were directed towards the GST-tag an attempt was made to remove this tag using biotinylated thrombin digestion (refer materials and method section 4.1.7).The aim was to inject the digestion product (rVP2) into chickens and isolate the resulting IgY antibodies.

Unfortunately the thrombin digestion proved unsuccessful. The starting concentration of VP2-GST was too low and resulting digested product (VP2) could never be recovered.

DISCUSSION

The aim of this chapter was to develop a serotype-specific ELISA for AHSV 4 VP2. In chapter 3 a high yield of recombinant AHSV 4 VP2-GST was obtained in an optimised bacterial expression system and subsequently purified. This purified VP2-GST was used as an antigen for the immunisation of chickens. IgY antibodies were purified from egg yolks and its specificity towards VP2 was investigated.

Further investigations were carried out using an ELISA where specificity of the IgY-VP2GST and IgY-GST were tested. A higher signal was obtained from IgY-GST control chicken tested on rGST than on IgY-VP2GST tested on rGST. This proved that the GST tag was more immunogenic than the recombinant VP2. Therefore two strategies were implemented to reduce the concentration of GST specific antibodies. The first strategy was to pre-absorp IgY-VP2GST against the immobilised rGST. Unfortunately the signal of the treated IgY-VP2GST was too low for any practical application. This prompted the second strategy which was the cleavage of the GST-tag from the protein using biotinylated thrombin with the aim of injecting the recombinant VP2 into chickens and isolating the specific IgY antibodies. This strategy was not practically applicable since the purified rAHSV 4 VP2 protein concentration levels after cleavage were too low to work with.

Several factors can contribute to the low levels of VP2 specific antibodies observed. Factors such as viral diseases affect chickens, resulting in immunomodulatory effects which impact negatively on antibody production (Schade *et al*., 1996). This was eliminated in this study because specific pathogen free hens were used for the immunisation studies. Also the possibility that too low quantities of the antigen were administered was ruled out because a study by Larsson and co-workers (1998) revealed that quantities as low as 0.1 µg of antigen was enough to elicit a good immune response in hens. Because the GST tag is relatively big (25-30 kDa), it could have interfered with the natural folding of the VP2 and the low signals meant

that the relevant immunological epitopes were not available for the necessary antigen antibody interactions, which resulted in a low absorbance values

Possible alternative strategies would be to optimise the concentrations for rVP2 without a tag, a review by Waugh (2005) suggests that the tag should be removed as it might influence the biological activity of the protein. Different species could also be tested for immunisations to determine the species that yields the best immune response against AHSV VP2. Huisman and Erasmus (1981) immunised three different species: rabbits, guinea pigs and sheep, in preparation for Bluetongue virus antibodies. They established that the immune response in rabbits is much better than the guinea pigs (Huisman *et al.*, 1979).

REFERENCES

DAVALOS-PANTOJA, L., ORTEGA-VINUESA, J. L., BASTOS-GONZALEZ, D. & HILDALGO-ALVAREZ, R. 2000. A comparative study between the adsorption of IgY and IgG on latex particles. *Journal of Biomaterials Science Polymer Edition*, 11:657-673.

DU PLESSIS, D. H., VAN WYNGAARD, W., ROMITO, M., DU PLESSIS, M. & MAREE, S. 1999. The use of chicken IgY in a double antibody sandwich ELIZA for detecting African horsesickness virus. *Journal of Veterinary Research,* 66:1-25.

HUISMANS, H., BREMER, C. W. & BARBER, T. L. 1979. The nucleic acid and proteins of epizootic haemorrhagic disease virus. *Onderstepoort Journal of Veterinary Research*, 46:95-104.

HUISMANS, H. & ERASMUS, B. J. 1981. Identification of the serotype specific and group specific antigens of the Bluetongue virus. *Onderstepoort Journal of Veterinary Research,* 48:51-58.

JUKES, T. H., FRASER, D. T. & ORR, M. D. 1933. The transmission of the Diphetheria antitoxin from hen to egg. *The Journal of Immunology*, 26:353-360.

LARSSON, A., CARLANDER, D. & WILHELMSSON, M. 1998. Antibody response in laying hens with small amounts of antigen. *Food and Agricultural Immunology*, 10:29-39.

LESLIE, G. A. & CLEM, L. W. 1969. Phylogeny of immunoglobulin structure. Immunoglobulins of the chicken. *Journal of Experimental Medicine*, 130:1337-1352.

PATTERSON, R.M YOUNGNER, J. S., WEIGLE, W. O. & DIXON, F. J. 1962. Antibody production and transfer to egg yolk in chickens. *Journal of Immunology*, 89:272-278.

POLSON, A., COETZER, T., KRUGER, J., VIN MALTZAN, E. & VAN DER MERWE, K. J. 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunological Investigations*, 14(4):323-327.

SCHADE, R., STAAK, C., HENDRIKSEN, C., ERHARD, M., HUGL, H., KOCH, G., LARSSON, A., POLLMAN, W., VAN REGENMORTEL, M., RIJKE, E., SPIELMANN, H., STEINBUSCH, H. & STRAUGHAN, D. 1996. The production of avian (egg yolk) antibodies: IgY. *ATLA*. 24:925-934.

WAUGH, D. S. 2005. Making the most of affinity tags. *Trends in Biotechnology*, 23:316-320.

WARR, G. W., MAGOR , K. E. & HIGGINS, D. A. 1995. IgY: clues to the origins of modern antibodies. *Immunology Today*, 16:392-398.

CHAPTER 5

CONCLUDING REMARKS

African horsesickness virus (AHSV) is a double-stranded RNA virus (dsRNA) responsible for a disease affecting equids of which horses are most susceptible. The disease spreads rapidly resulting in fatalities within a short period of time. Consequences of this disease affect mostly the racing industry and farmers. AHSV belongs to the Family Reoviridae, genus Orbivirus with 9 serotypes .The virus genome consist of 10 double-stranded RNA segments (Bremer, 1976) encoding seven capsid proteins and three non-structural proteins. The genome is enclosed by an outer capsid layer consisting of 2 major proteins VP2 and VP5 (Roy *et al*. 1996). VP2 is encoded by the second largest genome segment and is the most variable capsid protein. It is also the main determinant for serotype-specificity (Bremer *et al*. 1990). It has been demonstrated by Burrage et al. (1993) that there are neutralizing epitopes for AHSV located on VP2.

This was a proof of concept study to investigate the use of recombinant AHSV VP2 protein in a serotype-specific ELISA. Eukaryotic expression systems (Baculovirus) and a prokaryotic expression system (*E. coli*) were investigated for their ability to express this heterologous gene. The two baculovirus sytems were compared including combinations of tags and promoters. Previous groups (Scalen *et al*., 2001) reported up to 10% soluble expression of the native gene AHSV 5 VP2. The BAC-to-BAC system used does not include any tag fused to the heterologous gene and this makes purification of the recombinant protein difficult. The BAC-to-BAC system was used as a benchmark against which the expression of VP2 with the untested BacMagic expression system was compared. Disappointingly very low to no expression of recombinant VP2 was found.

The expression of soluble biologically active native AHSV VP2 proteins have been attempted by various groups (Scalen *et al*., 2001., Roy *et al*., 1996) and has proved to be a major obstacle for the development of subunit vaccines, as well as recombinant VP2 directed diagnostic assays. This is especially true for

expression in *E. coli* (unpublished communication by various researchers at OVI). The recombinant vectors containing the native VP2 gene tended to be very unstable and the complete or parts of VP2 gene were constantly deleted. However the development and commercial availability of synthetic genes and the ability to design these genes for optimal expression in specific hosts opened up new possibilities for VP2. A bacterial codon optimized AHSV 4 VP2 genome segment (kindly donated by Dr. A. C. Potgieter) was cloned into the pET 41(a+) vector. Every step of the expression and purification of the soluble VP2 protein was optimized. This is the first report of the successful and purification of recombinant AHSV VP2 protein in *E. coli*.

The rAHSV 4 VP2-GST was used as an antigen for immunizing hens. With the IgY antibodies obtained the development of an ELISA was investigated. The results revealed that the GST component of the protein was more immunogenic than the VP2 and an ELISA specific for AHSV 4 VP2 was not feasible. Efforts to cleave the tag were unsuccessful because rVP2 yields were too low.

In conclusion, further investigations will be required to use alternative expression systems such yeast, plants or mammalian cells to improve the yield of soluble recombinant VP2. The use of codon optimized synthetic genes with the respective expression system is highly recommended.

REFERENCES

BURRAGE, T.G., TREVEJO, R., STONE-MARSCHAT. M. & LAEGREID, W.W. 1993. Neutralizing epitopes of African horsesickness virus serotype 4 are located on VP2. *Virology*, 196:799-803.

BREMER, C. W. 1976. A gel electrophoretic study of the protein and nucleic acid components of African horsesickness virus. *Onderstepoort Journal Of Veterinary Research, 43:193-199.*

BREMER, C. W., HUISMAN, H. & VAN DIJK, A. A. 1990. Characterization cloning of the AHSV. *Journal of General Virology,* 72:793-799.

ROY, P., BISHOP, D. H. L., HOWARD, S., AITCHISON, H. & ERASMUS, B. J. 1996. Recombinant Baculovirus-synthesized African horsesickeness virus outer capsid protein VP2 provides protection against virulent AHSV challenge. *Journal of General Virology,* 77:2053-2057.

SCANLEN, M., PAWESKA, J. A., VERSCHOOR, A. A. & VAN DIJK. 2001. The protective efficacy of a recombinant VP2-based African horsesickness subunit vaccine candidate is determined by adjuvant. *Vaccine*, 20:1079-1088.