Construction and Structural Evaluation of Viral Protein 7 of African Horse Sickness Virus as a Particulate, Multiple Peptide Vaccine Delivery System.

BY

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dedikasi kajian ini saya ingin menyerahkan kepada gia, sumber inspirasi saya.

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Summary

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For the degree MSc

The highly hydrophobic viral protein (VP) 7 of African horse sickness virus (AHSV) folds into a trimeric structure that aggregates to form flat, hexagonal crystals (Chuma et al., 1992). These crystals are composed of flat sheets of hexameric rings, similar to the rings of trimers seen in the outer core surface layer. The crystals have been shown to be highly immunogenic when used as a subunit vaccine and are able to elicit a strong immune response against subsequent viral infections (Wade-Evans *et al.*, 1997). The aim of this study is to investigate the structural constraints of using these structures as a particulate, multiple peptide vaccine delivery system.

Three hydrophilic regions at amino acid position 144, 177 and 200 on the VP7 surface of this trimeric structure were targeted for insertion of peptides and a new vector was constructed in this study with a multiple cloning site at each one of the three top domain sites. The newly constructed three-site VP7 mutant gene was expressed in the Bac- To-Bac expression system and the recombinant proteins were investigated for its solubility and crystal formation by sucrose density gradient centrifugation. The structure and stability of the modified, trimeric VP7 was confirmed and further analyzed. Scanning electron microscopy showed the formation of large structures by the trimeric modified VP7 protein units. These structures differed from the hexagonal crystals formed by unmodified VP7, resulting in rough-looking, flat circular structures attached by protein cables. The high yield of protein expression and the ease, with which these particles can be purified, makes this vector ideal for vaccine use. These protein structures also seemed to remain stable after being stored under different conditions. Studies were also conducted on the stability of these structures after sonication, enabling a range of diffirent size particles to be presented to the immune system.

The purpose for the creation of multiple cloning sites was for the vaccine to be able to accommodate and efficiently present multiple epitopes to the immune system. An investigation was launched into the effect of peptide insertion at one or more of the multiple cloning sites. The initial study included the

insertion of two small peptides from AHSV VP2 at amino acid sites 144 and 177 respectively. The size of the peptides that can be inserted is also very important in the use of virus-like particles as antigen carriers. In order to utilize the full potential of the VP7 particles as an antigen presentation system, it must be possible to accommodate large epitope-containing insertions. At the extreme, a stretch of 250 amino acids from AHSV VP2 was inserted into the 177 amino acid multiple cloning site of the three-site VP7. Structural evaluation of all these expressed proteins indicated that the structure of the VP7 subunit vaccine is stable and still retains the ability to form large aggregated structures from the trimeric units. Scanning electron microscope revealed that all these peptide-containing constructs retain approximately the same structural shape as the structures formed by the three-site VP7 mutant.

List of Abbreviations

AA	-	Amino acid
AHS	-	African Horse Sickness
AHSV	-	African Horse Sickness Virus
Amp	-	Ampicillin
ATP	-	Adenosine-5'triphosphate
BHK	-	Baby hamster kidney cells
Bp	-	Base pair
BTV	-	Bluetongue virus
BLV	-	Bovine Leukemia virus
°C	-	Degree celsius
CLP	-	Core-like particle
cm^3	-	Cubic centimeters
ddH_2O	-	Deionized distilled water
DMSO	-	Dimethyl sulfoxide
DNA	-	Deoxyribonucleic acid
dNTP	-	2'-deoxynucleoside-5'triphosphate
dsRNA	-	Double stranded ribonucleic acid
E.coli	-	*Escherichia coli*
EDTA	-	Ethylenediaminetetra-acetic acid
et al	-	et alia (and others)
Fig.	-	Figure
g	-	Gram
GP	-	Glyco protein
h	-	Hour
HbsAg	-	Hepatitis B virus surface antigen
IPTG	-	Isopropyl-β-D-thiogalactopyranoside
kDa	-	Kilodalton
KB	-	Kilobase
KV	-	Kilovolt
LB	-	Lauria-Bertani
Log	-	Logarithmic
M	-	Molar
MHC	-	Major histone compatability
min	-	Minutes
ml	-	Millilitre
mm	-	Millimeter
mM	-	Millimolar
M.O.I.	-	Multiplicity of infection
M_r	-	Molecular weight
MW	-	Molecular weight
µg	-	Microgram
µl	-	Microlitre
N	-	Normal
NaAc	-	Sodium acetate
ng	-	Nanogram
NS	-	Non-structural

PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PCR	-	Polymerase chain reaction
pfu	-	Plaque forming units
pmol	-	Picomolar
PSB	-	Protein solvent buffer
RNA	-	Ribonucleic acid
rpm	-	Revolutions per minute
SDS	-	Sodium dodecyl sulphate
sec	-	Seconds
S. E. M.	-	Scanning electron microscopy
Sf	-	*Spodoptera frugiperda*
TEMED	-	N,N,N',N'-tetramethylethelenediamide
Tet	-	Tetracycline hydrochloride
Tris	-	Tris(hydroxymethyl)-aminomethane
TSB	-	Transformation suspension buffer
U	-	Units
UHQ	-	Ultra high quality
UV	-	Ultraviolet
V	-	Volt
VP	-	Viral protein
VLP	-	Virus-like particles
v/v	-	Volume per volume
w/v	-	Weight per volume
X-gal	-	5-bromo-4chloro-3indolyl-β-D-galactopyranoside

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Chapter 1

Literature Review

1.1. Vaccines

With the increasing emergence of antibiotic resistant infectious organisms more emphasis should be placed on prevention of infection and disease via immunization and less on post-infection antibiotics. (Shearer and Clerici, 1997). The principle of vaccine development is to alter a pathogen or its toxin in such a way as to render them harmless without removing their ability to illicit a similar immune response as that of the same but unmodified pathogen. Since the antigenic determinants are still present on the vaccine, the antibodies generated by the adaptive immune response after vaccination would still be able to recognize the active pathogen during subsequent natural infection. The secondary antibody response would be mounted and the infectious particles would be brought under control faster and removed more effectively.

There are a few requirements that an ideal vaccine would have to satisfy. i) Elicitation of neutralizing antibodies that reacts with all of the pathogen's strains and subtypes. ii) Induction of strong humoral and cytotoxic immune responses. iii) Induction of immune responses that recognize latently infected cells. iv) Mimic natural infection and induce local immunity at all sites of entry into the host. vi) Safety, showing no toxic effects or possibility of reversion to wild-type infection. vii) The effect of the vaccine must be long-lasting following a single inoculation. viii) Must be economically feasible, thus must be produced cost-effectively and have long-term stability in storage, excluding the use of cold-chains.

This chapter will briefly review the principles of different vaccine approaches, their respective advantages, as well as the major problems associated with each. Also included at the end of this chapter is an introduction into African Horse Sickness Virus (AHSV) Viral Protein (VP) 7 and its possible use as a subunit antigen presentation system.

1.2. Types of Vaccines

1.2.1. Inactivated Vaccines

Inactivated vaccines are infectious agents that cannot replicate and have thus lost their infectivity through damage to their nucleic acid by various agents. The method by means of which the nucleic acid is inactivated should not affect the antigenic specificity of the protein surface of the respective organisms. Common methods for the inactivation of these pathogens include heat-inactivation, irradiation (Huter *et al.*, 2000), chemical treatment, especially with formaldehyde (Bahnemann, 1990) and ultrasonication (Melamed *et al.*, 1991). The more frequently preferred formaldehyde method of inactivation has successfully been used in several cases, including the first worldwide commercialized production of a vaccine against Hepatitis A (Andrê, 1995). It has been found that these inactivation treatments might, however, result in reducing or altering the vaccine's antigenic character and could be responsible for the loss of relevant immunogenic epitopes (Duque *et al.*, 1989; Ferguson *et al.*, 1993). A further complication occurs when it was found that certain bacteria only generate specific protective antigens on their outer membrane in specific *in vitro* growth conditions (Deneer *et al.*, 1989). Also, with pathogens that aggregate it has been found that a miniscule proportion of the infectious agents in the core of the aggregate may "survive" the inactivation treatment and produce the disease, which the vaccine is supposed to prevent (Brown, 1993).

Inactivated vaccines normally elicit a narrow immune response spectrum. Their inability to infect cells results in good humoral immunity but limited, if any, cell-mediated - or mucosal immunity. In spite of this, inactivated vaccines still is one of the two major models for vaccine success, the other being live attenuated vaccines (Hilleman, 2000).

1.2.2. Live Attenuated Vaccines

Live attenuated vaccines are infectious pathogen strains that have lost their virulence or disease causing properties. These attenuated vaccines retain their ability to infect and replicate in cells and are capable of inducing strong, long-lasting immunity, cellular and humoral, similar to that induced by the natural infection (Ruprecht, 1999). Live attenuated vaccines can be delivered via the natural route of infection, thereby providing better protection at the site of entry of the pathogen. It is important to induce mucosal immunity since most pathogenic organisms are either restricted to these areas or need

to transit them during the early steps of infection (Levine *et al.*, 1983). These vaccines, because of their replicating ability within the host, generally induce a longer duration of immunity (Ellis, 2001). However, over-attenuation will limit replication, which might result in mild, non-protective immune response.

The ability of live attenuated vaccines to infect and replicate also cause the main problem, especially if under-attenuation occurred. There exists the possibility that the pathogen might back-mutate after *in vivo* replication and revert back to the lethal wild-type strain (Appel, 1978; Minor *et al.*, 1986). This is a particular problem in immunorepressed individuals where even a weakened pathogen can cause the disease. Another disadvantage of live tissue culture grown vaccines is the risk of contaminating extraneous viruses in the tissue culture serum being used (Thornton, 1986). One popular example involved the introduction of bovine viral diarrhea into vaccines grown in bovine cells (Wensvoort *et al.*, 1988).

Despite all the disadvantages and safety concerns, the importance of live vaccines is best highlighted by the fact that ten of the seventeen licensed viral-based vaccines in the United States of America are live vaccines (Hilleman, 2000).

1.2.2.1. Biological Attenuation

Biological attenuation, though not very well understood, is frequently attained through passage through atypical host animals or cultured cells (Vaughn *et al.*, 1996). The unfavorable growth conditions, which could also be induced by mutagenic chemicals (Levine *et al.*, 1987), give rise to a random series of attenuated or mutant pathogens. These new mutant strains are constantly monitored and selected for antigenic retention and loss of virulence. A combination vaccine for African horse sickness, which contains eight of the nine distinct serotypes, was attained through biological attenuation. Three of the eight serotypes were plaque attenuated by passage in cell culture, whereas the other five strains were attenuated by serial intracerebral passage in adult mice (Taylor *et al.*, 1992).

Biological attenuation can also be achieved by selecting for mutants according to their growth properties at different temperatures (Treanor *et al.*, 2000). These cold adapted temperature-sensitive pathogens are unable to grow efficiently at elevated temperatures and are consequently less virulent than the wild-type. Also, co-infection of cells with two or more different strains of a virus with

segmented genome could lead to the production of reassortant viruses that are less virulent than the wild-type strain, which could be used as a vaccine (Rennels *et al.*, 1996). This same principle is also the cause for concern when vaccination occurs with a biologically attenuated serotype combination vaccine. Reassortment of the genomic segments may occur between the different serotypes during replication in the vaccinated host, which might result in progeny virus with novel characteristics, including new virulence characteristics (Nutall *et al.*, 1992).

1.2.2.2. Genetic Attenuation

Recent advances in recombinant DNA technology and knowledge have made it possible to attenuate pathogens through the genetic manipulation of specific genes responsible for toxicity. This method is more preferred than biological attenuation. More stability is attained in the phenotype through extensive modification or deletions to the pathogenic genome to reduce virulence. By creating multiple modifications or deletion of entire genes the possibility of back-mutation to the virulent form is decrease (Kit *et al.*, 1987).

It has been shown that attenuating genes can be in the structural (Farrell *et al.*, 1994), non-structural (Liang *et al.*, 1997) or even in the noncoding region of the pathogen's genome (Kuhn *et al.*, 1992). Generally non-essential genes are deleted that may inhibit sufficient replication of the pathogen, thereby prohibiting it from causing the disease. Viruses can also be attenuated by making recombinant chimeric viruses consisting of complementary but not fully compatible genes from related viruses (Kuhn *et al.*, 1991).

Another way to genetically attenuate pathogens would be to delete or mutate genes that are necessary for *in vivo* replication. Viruses can be rendered replication-incompetent by propagating them *in vitro* in a cell line transfected with the essential gene (Reddy *et al.*, 1999; Zakhartchouk *et al.*, 1999). When introduced into an individual, the virus would be able to infect yet not replicate inside the host since it would not have access to the essential gene (Farrel *et al.*, 1994). A variation of this approach is applied with bacterial vaccines that are engineered to be metabolite-dependent. Here the metabolite-dependent bacterial vaccine is grown *in vitro* in the presence of the required metabolite. After injection into an individual, the bacterium will continue propagating as long as the individual takes supplements of the metabolite. Once the supplementation stops, the bacteria die (Donnenberg *et al.*, 1991).

It has also been shown that the establishment of persistent infection with retroviruses can be prevented by the mutation or deletion of the integrase gene (Vogel *et al.*, 1993).

1.2.3. Nucleic Acid Vaccines

1.2.3.1. DNA Vaccines

It has been shown that naked DNA encoding vaccine antigens can be transfected *in vivo* into cells (Wolff *et al.*, 1990). The produced antigens are either secreted to elicit a humoral immune response or they can be associated on the cell surface to elicit a cellular immune response. Only small quantities of proteins being expressed are required for effective priming of the immune response. The antigens are continuously produced in the cells, even for several months after DNA immunization (Yankauckas *et al.*, 1993), which is probably responsible for inducing long-term immunity. Priming with DNA vaccines followed by boosting with the antigenic protein itself induce higher levels of protection than with either DNA immunization or protein immunization individually (Amara *et al.*, 2001)

The attractiveness of this approach is in its simplicity. Expression of the gene is coupled to a strong upstream promoter capable of expressing the gene in mammalian cells. Usually the cytomegalovirus immediate/ early promoter is used (Donnely *et al.*, 1997). The introduction of introns into the plasmid, resynthesizing genes to remove cryptic splice sites and improving codon biases have all been shown to improve the expression levels of coded antigens (Vinner *et al.*, 1999). A requirement for DNA vaccines to elicit a strong humoral immune response is the presence of unmethylated CpG sequences in the plasmid backbone. These dinucleotides induce murine B cells to proliferate and secrete immunoglobulins (Krieg *et al.*, 1995; Davis *et al.*, 1998). Adjuvants are unnecessary with DNA vaccines containing the CpG sequences.

An advantage of DNA immunization where viral antigens are concerned is that the DNA encoded antigens' post-translational modifications are exactly the same as that of a natural viral infection. Also, the viral antigens are being presented efficiently on the MHC molecules for T-cell recognition (Ulmer *et al.*, 1996) It is also possible to manipulate and shift the immune response to predominantly either a humoral or cytotoxic immunity by the co-administration of plasmid encoding cytokine (Kwissa *et al.*, 2003) or interleukin genes (Kim *et al.*, 1997). Mucosal immune responses can be generated by delivery of the DNA plasmids to the mucosal surfaces (Fynan *et al.*, 1993; Kuklin *et al.*, 1997) and it has been

reported that DNA vaccines can induce immune responses in neonates even in the presence of passive anitbodies (Lewis *et al.*, 1999).

The first study involving an African horse sickness virus DNA vaccine was reported by Romito *et al*, 1999. The outer capsid protein viral protein 2 (VP2) generates the main determinant of serotype-discriminatory neutralising-specific immune responses for African horse sickness. (Huismans *et al.*, 1987; Roy *et al.*, 1996; Scanlen *et al.*, 2002). A horse was injected with plasmid DNA containing the AHSV serotype 3 VP2 gene downstream of a cytomegalovirus immediate-early enhancer/ promoter. Viral protein 2-specific antibodies were generated but neutralisation titres were low.

Although naked DNA vaccines have been shown to function without a protective delivery system, a problem that occurs is that the majority of the plasmid is degraded before entering the nucleus of a cell and initiating transcription of the gene of interest, thereby lowering expression levels. It is possible to use, for instance, the alphavirus envelope proteins to encapsulate and deliver the nucleic acids directly to the cells (Pushko *et al.*, 1997; Schultz-Cherry *et al.*, 2000). Chemical approaches have also been tested, including using various polymers and liposomes to encapsulate the DNA (Ellis, 2001).

There are other major concerns with DNA vaccines for which there have been no solution to date. The major problem is that the plasmid DNA might integrate into the genome of a multiplying host cell, and thereby activate a host protooncogene or deactivate a suppressor gene, which could lead to cancer (Xiong *et al.*, 1997). There also exists the risk that the plasmid could induce an autoimmune response to the host cell's DNA.

1.2.3.2. RNA Vaccines

An alternative to DNA vaccination is the use of RNA vaccines. The RNA fragments are less stable *in vivo* than the DNA plasmids used for vaccination. Subsequently the expression of the antigens is short-lived and therefore, an overall less affective approach than DNA vaccination. Expression levels can be boosted through the incorporation of a gene encoding an alphaviral replicase together with the gene encoding the foreign antigen, which would enable the RNA to self-replicate in the transfected cell (Tubulekas *et al.*, 1997). This approach has been shown to generate strong immune responses (Zhou *et al.*, 1994). Like DNA vaccines, these RNA fragments can also be delivered naked or encapsulated in

either non-replicative viral particles (Zhou *et al.*, 1995) or liposomes (Martinon *et al.*, 1993; Conry *et al.*, 1995).

RNA vaccines, due to their inherent low stability, generally are problematic when prepared and administered. Despite these technical and other mentioned problems, the main attraction of RNA vaccines, unlike DNA vaccines, is their inability to integrate into the genome of the host cell and possibly cause cancer.

1.2.4. Live Recombinant Vaccine Vehicles

Non-pathogenic or attenuated microorganisms, be they viral or bacterial, can be engineered as vectors for expressing foreign antigenic polypeptides from other non-related pathogens (Yilma *et al.*, 1988). Insertion of cloned genes from pathogens into the genomes of other live organisms occurs so that during infection with the recombinant vector, the surface proteins produced by the cloned genes are presented on the infected cells by the MHC complex to the T-lymphocytes (Ellis 2001). Alternatively the expressed proteins are broken up and presented to elicit a cytotoxic T-lymphocyte response. Antibodies are produced that will neutralize the pathogen from which the surface proteins were cloned.

It is also possible to express the foreign antigen on the surface of the recombinant vector. This will have the desired effect of also stimulating the humoral immune response (Liljeqvist *et al.*, 1999). Heterologous cell-surface display in the context of live bacterial vaccines have been described for both Gram-positive (Fischetti *et al.*, 1996; Ståhl *et al.*, 1997) and Gram-negative (Georgiou *et al.*, 1993) bacteria. The heterologous use of viral systems have shown to elicit strong immune responses against the foreign antigen (Dalsgaard *et al.*, 1997) and have the advantage of targeting the recombinant viral particles to specific cells within the host (Ohno *et al.*, 1997). Another advantage of live recombinant vaccine vectors is that even though the host might already have immunity against the vector itself, a strong immune reaction can still be elicited against the added antigenic polypeptides it carries (Zakhartchouk *et al.*, 1999).

A problem that might occur is that one of these "harmless" vectors might have lethal consequences in certain individuals, especially those that are immunocompromised. This problem, however, can be overcome by the incorporation of cytokines or immunomodulatory genes into the vector (Kurilla *et al.*, 1993; Sambhi *et al.*, 1991). The expression of these genes might enhance the immune response to the

pathogen. Another solution would be the use of related viruses from other species that are replication-defective in mammals (Fries *et al.*, 1996; Somogyi *et al.*, 1993). These vectors would be able to infect mammalian cells, express the transgene that would elicit its own immune response, yet would be unable to replicate.

The use of live recombinant vehicles seems promising for vaccine purposes. One of the best examples and greatest successes of a vectored vaccine is vaccinia virus carrying the rabies virus glycoprotein gene (Brochier *et al.*, 1994). The ability of thermal stable vaccinia virus to infect foxes by oral ingestion provided the basis for a campaign to reduce rabies infection in foxes in Western Europe. The campaign started in Belgium during 1989 by distribution of vaccine immunized bait from airplanes. This strategy completely eliminated rabies virus infection from Belgium's fox population by 1993. Vaccinia virus was also used as live recombinant vehicle for the delivery of AHSV serotype 4 VP2 for immunization in horses. The immunization approach was successful in inducing a protective immune response against a challenge with a lethal dose of AHSV-4 (Stone-Marschat *et al.*, 1996).

There are, however, still several problems associated with this delivery system (Medina *et al.*, 2001). These include the possibility of reversion back to the virulent phenotype, the stability of the recombinant phenotype, horizontal gene transfer to the environment that were not apparent in the small-scale trials and a possible reduction in efficacy of the vaccine because of prior exposure to the carrier. Finally, as mentioned before, the host's genetic factors might influence the vaccine efficacy.

1.2.5. Antibody-based Vaccines

1.2.5.1. Passive Vaccination Strategies

Passive vaccination is the injection of preformed antibodies into a host to treat an infection. It is especially effective where pathogenic circulating toxins are produced and a high titre of specific antibodies is required (LeClaire *et al.*, 2002). Various expression systems can be used to produce monoclonal antibodies, including bacteria (Laden *et al.*, 2002), plants (Hiatt *et al.*, 1989; Stoger *et al.*, 2002), yeast, and mammalian cells (Little *et al.*, 2000).

The increase in knowledge concerning the genetics and structure of the immunoglobulins and advancements in recombinant DNA technology has permitted the genetic manipulation of antibody

molecules. Chimaeric antibodies can now be created to suit their respective antigens and functions (Boulianne *et al.*, 1984; Better *et al.*, 1988). Research into the use of fragments derived from antibody molecules has also advanced. It has been shown that these smaller fragments have better penetration of solid tumours and rapid clearance abilities, compared to whole antibodies (Huston *et al.*, 1993). These fragments can also be fused to other molecules, such as cytokines (Boleti *et al.*, 1995), metal-binding proteins (George *et al.*, 1995), toxins or drugs (Huston *et al.*, 1993) to expand on their medical applications.

The absence of any viral or bacterial material is a great advantage, although the expression of these antibodies causes a huge problem. The chosen expression system has to be optimized for every different antibody because of their differences in amino acid sequence (Verma *et al.*, 1998). The optimality of the expression system would also depend on the required quantity and the required purity of the final product. Another problem is that with complex antigens different epitopes maybe be instumental in stimulating various aspects of the immune response required for effective protection (Adorini *et al.*, 1979). Since monoclonal antibodies are specific only for a single specific epitope, a pathogen could easily elude an immune response. Also a mutagenic slight alterations to the specific epitope may cause the failure of the antibody.

1.2.5.2. Anti-idiotype Antibody Vaccines

Another application for antibodies in vaccination studies is the anti-idiotype antibody. These antibodies are created by using neutralizing antibodies against the virus as the immunogen in an animal host. The newly generated antibodies induced against the first antibodies (anti-idiotype antibodies) should resemble the epitope on the virus that is susceptible to the neutralizing antibody. This anti-idiotype, therefore, when used to immunize another host, should elicit neutralizing antibodies against itself and by analogy against the infectious virus itself (Dalgleish *et al.*, 1988).

Immunization with anti-idiotype antibodies has been shown to confer protection in animal disease models (Poskitt *et al.*, 1991), although the same problems as with passive vaccination strategies are found with this vaccination strategy.

1.2.6. Subunit Vaccines

1.2.6.1. Particulate and Fusion Vaccines

Subunit vaccines are based on the principle that it is possible to elicit the same immune response against specific proteins from an infectious organism, as from a natural infection. This approach focuses on the use of whole surface proteins as possible vaccines since they are the first proteins the immune system normally comes into contact with and against which antibodies are generated (Burnette, 1991). It is important to have prior knowledge of the pathogenic components that are involved in inducing a protective immunity.

Recombinant DNA technology has further broadened the potential uses of subunit vaccines. Genetic fusion of foreign antigenic regions from one organism to a subunit protein from another organism, which has suitable immunogenic properties, is now possible (Uhlén *et al.*, 1992). Fusion proteins, therefore, have the combined properties of the original gene products. This allows for the generation of immune responses against foreign antigens that are being stabilized and presented on carrier proteins. Depending on the chosen epitopes, both the T- and B-cell mediated immune responses can be generated (Löwenadler and Lycke, 1994). Care should be taken not to alter the structure of the carrier protein with the insertion of the epitope amino acids.

These proteins can be produced en mass utilizing different expression systems. The expression system for a specific protein's production will depend on the type of organism the protein was originally derived from. This is done to ensure that the protein undergoes the correct posttranslational modification (Dertzbaugh, 1998). Many techniques have been developed for the extraction and purification of fusion proteins. A good example of purifying the proteins from a solution would be affinity handles. It is possible to add affinity fusion partners, which enable purification by affinity chromatography (Nygren *et al.*, 1994). Site-specific enzymatic cleavage can remove the affinity handles after purification (Nilsson *et al.*, 1997). Some of the expressed proteins, like viral envelope glycoproteins, insert themselves into the host cell's membrane. The only way to harvest these proteins is by lysis of the cell, which prolongs and increases the cost of production. Techniques have been developed to remove the transmembrane anchors of these membrane-bound proteins so that they can be secreted into the medium without lysis of the host production cells and subsequently easily harvested from the medium (Kowalski *et al.*, 1993).

Virus-like particles (VLP) can also be used as subunit vaccine or as carriers for epitopes from other viruses (Roy, 1996). These particles are attained from protein-protein self-assembly in cells expressing the required envelope proteins (Kunkel *et al.*, 2001). The particles seem to induce a more effective immune response than denatured or soluble proteins (Netter *et al.*, 2001). The reason for this enhanced immunity might be linked to the fact that VLPs are presenting confirmation epitopes to the immune system, which mimics a natural infection.

The only subunit vaccine, which is also the only recombinant expressed vaccine of the seventeen licensed viral-based vaccines in the United States of America, is that of hepatitis B (Hilleman, 2000). Valenzuela *et al.* (1982) found that *Saccharomyces cerevisiae* expressed hepatitis B virus surface antigen (HbsAg) assembled into subviral particles similar to that of the hepatitis B virus. Since then it has been found that extensive modifications can be made to the primary structure of HBcAg without impairing particle assembly, even insertions as large as 238 amino acids (Beterams *et al.*, 2000). These highly immunogenic particles can effectively serve as carriers for the presentation of other peptide sequences.

Not only proteins that form the outer capsid or membrane of the organism can be used as subunit vaccines. Many bacteria secrete toxins that are responsible for pathogenesis and tissue damage (Shewan *et al.*, 1982). It is possible to use these extracellular toxins not only as subunit vaccines to induce effective immunity, but also as carriers for epitopes from other organisms (Rauly *et al.*, 1999).

There are several advantages to subunit vaccines. They cannot replicate and are non-infectious, nor is any inactivation agents required that might alter the immunogenicity of the protein. As mentioned before, it is possible to introduce specific T- and B-cell epitopes into the subunit proteins thereby directing and enhancing the immune response. Another advantage, when compared to inactivated vaccine systems, is the absence of irrelevant protein components, which decreases the antigenic competition in the vaccinated host.

As well as advantages, there are problems associated with the use of subunit vaccines. Envelope proteins might not induce a sufficiently strong cell-mediated response against virus infected cells. Disease might be caused by a great variety of viruses and their strains (Wareing and Tannock, 2001). Many viruses change frequently through antigenic drift (Jong *et al.*, 1999). There might be contaminants in growth media (Thornton, 1986; Wensvoort *et al.*, 1988). The major problem with subunit vaccines is the size of the antigenic insertions that can be made without the alteration of the

protein structure (Roy, 1996). It is only possible to insert small antigenic regions without disturbing the overall protein structure.

1.2.6.2. Synthetic Peptide Vaccines

Another type of subunit vaccine is the synthetic peptide vaccine. Synthetic peptide vaccines are based on the principle that it is possible to generate neutralizing antibodies against the intact virus particle using small defined peptides representative of the antigens found on the viral envelope or surface proteins (Van Regenmortel, 2001). These peptides mimic the 8 to 12 long amino acid fragments that are normally displayed on the cell surface by major histocompatibility complex (MHC) molecules for T-cell recognition (Nelson *et al.*, 1992). They are also able to generate B-cells that are less conformation-dependent and which recognize linear epitopes. Depending on the epitopes from the pathogen that are being used, either the humoral or the cellular branch of the immune system can be predominantly stimulated. It has also been found that a better and broader immune response is generated when different multiple epitopes are used as synthetic peptide vaccines (Tam, 1996). Multiple copies of a single epitope can also be synthesized, which leads to a stronger immune response subsequent to injection (Coëffier *et al.*, 2001).

These peptides are strongly immunogenic when delivery is coupled with either a strong adjuvant (Hsu *et al.*, 1996) or fused with a carrier protein (Kaumaya *et al.*, 1992). When delivered alone, however, synthetic peptides are rapidly cleared *in vivo* and are subsequently not very immunogenic (Ben-Yedidia *et al.*, 1997). Also, it has been found that synthetic peptide vaccines are less efficient at generating humoral immune responses than cellular immune responses. The reason for this is that the free flexible B-cell linear peptides raise antibodies that do not bind optimally to the sequence in the way that they do when it is present in a more rigid structure within the native protein molecule (Jemmerson, 1987; Jackson *et al.*, 2000). The synthetic peptide might also form secondary structures by means of disulfide bonds, which might lower the immunogenicity of the vaccine (Simard *et al.*, 1997). Research into the chemical stabilization of the peptides in solution is being conducted with the hope of improving the immunogenicty (Cabezas *et al.*, 2000).

Despite all the disadvantages, this method of vaccination offers the ideal in safety, specificity, quality and economy since specific synthetic peptides are generated without any pathogen material (Ertl *et al.*, 1996). Consequently there are no infectious particles still drifting around and no contamination with

harmful materials from the cells. However, it must be mentioned that these synthetic peptides are only economically feasible as long as the total length of the peptide does not exceed fifty amino acids.

1.3. African Horse Sickness Virus

African horse sickness is an infectious, but non-contagious gnat (*Culicoides* sp.) -transmitted disease, which affects species of the equidae. The disease, which is endemic to sub-Saharan Africa, causes a high mortality in horses, often exceeding 90%. It also occurs in donkeys, zebras and goats, but at a considerably lower death rate (Borden *et al.*, 1971). The causative agent is a dsRNA virus of the *Orbivirus* genus, belonging to the family *Reoviridae* and is closely related to bluetongue virus (BTV), which is the prototype virus of this genus (Basak *et al.*, 1996).

Ten dsRNA segments of different sizes compose the viral genome, three large, designated L1-L3, three medium, M4-M6, and four small, S7-S10. This genome encodes at least ten proteins, seven of which are structurally related (VP1 to VP7) and four non-structural proteins (NS1 to NS3A) (Van Staden and Huismans, 1991). The morphology of purified AHSV particles is essentially identical to that of BTV particles (Burroughs *et al.*, 1994). The seven structural proteins are organised in a two-layered capsid. The genome is packaged in a highly ordered icosahedral inner core composed of two major proteins, VP3 and VP7, and three minor structural proteins VP1, VP4, and VP6. The outer capsid, which is removed when the virus infects the cell, is composed of VP2 and VP5 (Roy *et al.*, 1994). The inner core is also able to infect certain types of cells without the aid of the outer capsid proteins (Mertens *et al.*, 1996).

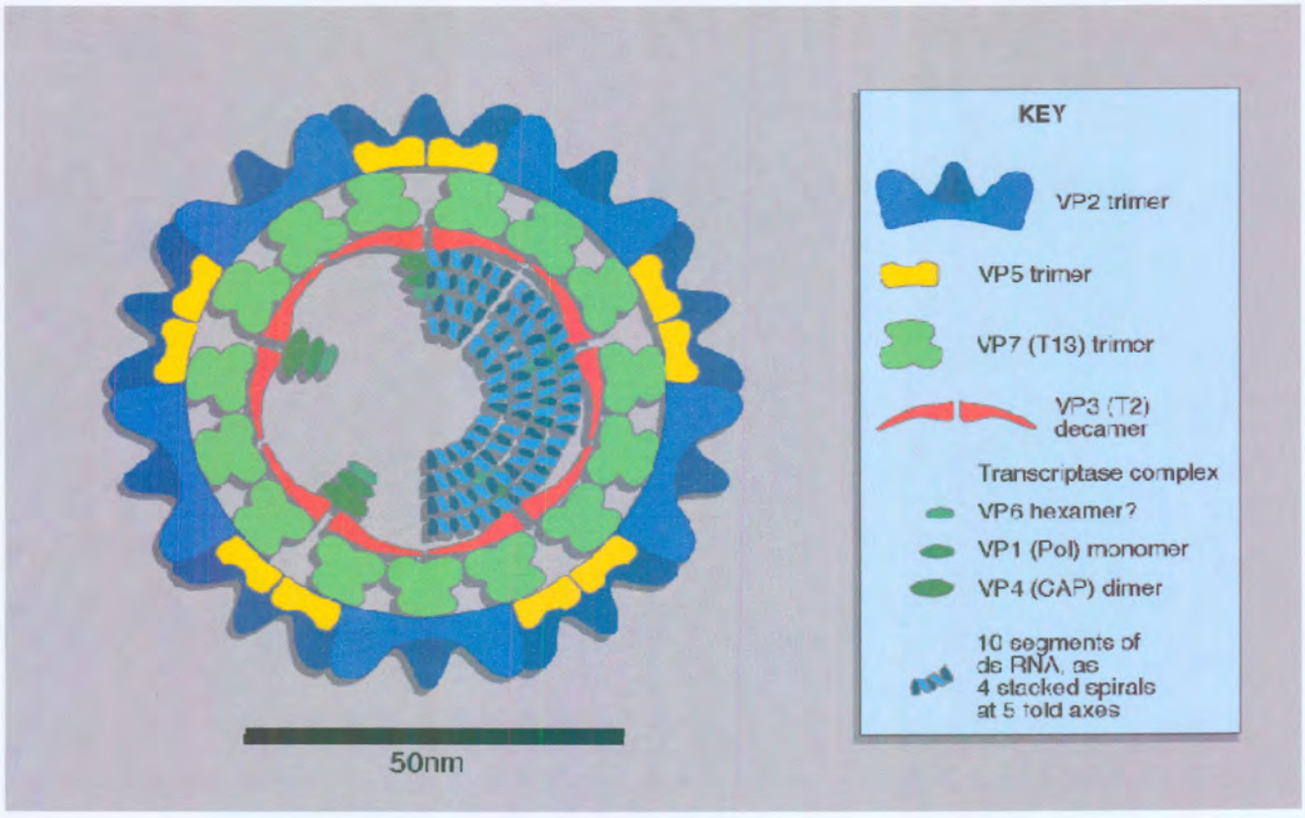


Figure 1.1. Schematic representation of the BTV particle structure. (Courtesy of P. P. C. Mertens and S. Archibald.)

1.3.1. African Horse Sickness Virus Viral Protein 7

African horse sickness virus VP7, which is coded for by the AHSV S7 dsRNA segment, has 349 amino acids. The sequence is highly hydrophobic, containing a large number of alanines, methionines, and prolines (Roy *et al.*, 1991). There are nine serotypes for AHSV and VP7 has been found to be highly conserved between these serotypes. Three VP7 monomers (M_r 38K) spontaneously organise into a trimeric structure of which the three subunits are non-covalently bound. The main outer surface components of the core particle are VP7 trimers. Two hundred and sixty of these VP7 trimers are arranged on a VP3 scaffold forming a T=13 lattice. Co-expression of the VP7 and VP3 genes of AHSV in a baculovirus expression system results in the formation of empty core-like particles (CLP) that resemble normal cores. (Maree *et al.*, 1998).

The VP7 monomer is composed of two distinct domains, a bottom and a top domain. The bottom domain (residues 1-120 and 250-349) forms a complex of hydrophobic loops and nine α-helices, five in the N-terminal portion and four in the C-terminal region. The more hydrophilic top domain

(residues 121-249) is folded into anti-parallel β-strands connected by β-turns (Basak *et al.*, 1996). It is one of the helices from the bottom domain of the VP7 trimer that forms a flat surface that interacts with the VP3 scaffold. The domains of the three monomers are twisted anti-clockwise around the three-fold axis of the trimer, allowing the top domain of one monomer to rest on the C-terminal region of the adjacent monomer within the same trimeric subunit (Grimes *et al.*, 1995). The last 16 residues at the C-terminal have been shown essential for trimer interaction (Le Blois, *et al.*, 1993). Additions of extra amino acids to the C-terminus inhibit core formation (Le Blois, *et al.*, 1993; Monastyrskaya *et al.*, 1997).

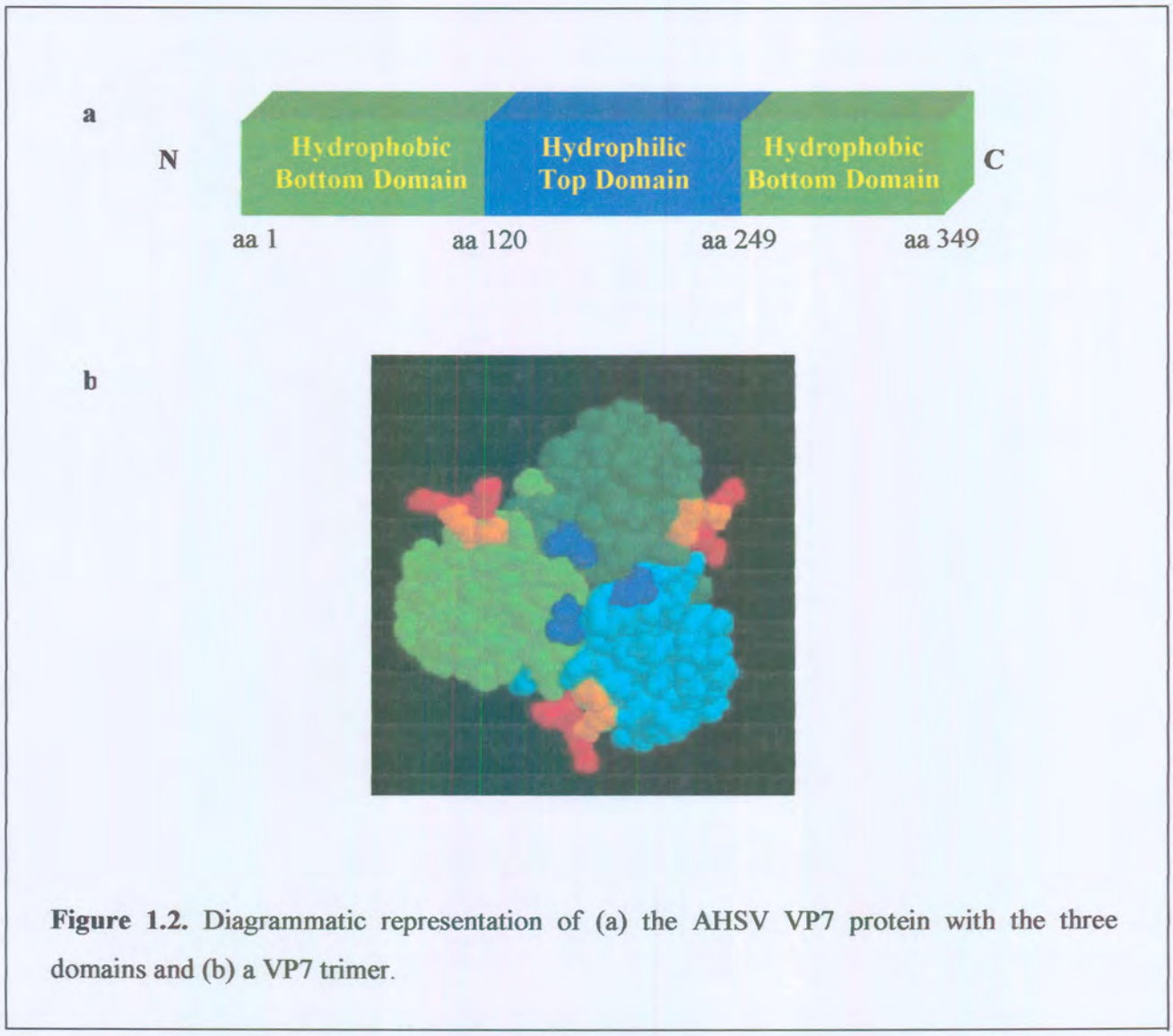


Figure 1.2. Diagrammatic representation of (a) the AHSV VP7 protein with the three domains and (b) a VP7 trimer.

As mentioned in Section 2, the virion, lacking the outer capsid, is still able to infect certain types of cells. Blue tongue virus VP7 attaches to a cell surface receptor and allows the BTV core to pass

through the cell's membrane. This is mediated by the RGD (Arg-168–Gly-169–Asp-170) motif (Grimes *et al.*, 1995). In AHSV VP7 this motif is replaced by an AGQ (Ala-167–Gly-168–Gln-169) motif, which probably does not fulfil the same function as that of the RGD motif. African horse sickness virus VP7 does however, contain a RGD motif on a highly flexible loop, amino acid segment 175 -180, at amino acid position 178 to 180. This loop is located deeper within the core than that of the easily accessible BTV motif, at the lower part of the top domain. It has been shown though that the RGD motif can still bind integrins effectively (Basak *et al.*, 1996).

A unique feature of AHSV VP7 is the ability of the VP7 trimers, when expressed in a recombinant baculovirus, to spontaneously aggregate in infected insect cells (*Spodoptera frugiperda*) into large, flat, hexagonal crystals (Chuma *et al.*, 1992; Burroughs *et al.*, 1994). This same phenomenon is found when AHSV VP7 is expressed in BHK21 cells (Wade-Evans *et al.*, 1997). Infected cells have been found to contain between one to three crystals per cell. These hexagonal crystals have an average diameter of about 6 μm and are not found when expressing BTV VP7 which is completely soluble (Oldfield *et al.*, 1990). Such large structures are unique to AHSV within the *Orbivirus* genus. Also, these structures have a highly ordered lattice that is consistent with a trimeric subunit structure and is probably due to hydrophobic interactions between the trimers (Chuma *et al.*, 1992; Basak *et al.*, 1996).

1.3.2. African Horse Sickness Virus Viral Protein 7 Vaccine

African horse sickness virus VP7 crystals have been shown to be highly immunogenic when used as a subunit vaccine and are able to elicit a strong immune response in a mouse model against subsequent viral infections (Wade-Evans *et al.*, 1997). The attained response was shown to be crystal-structure dependent since the same amount of heat denatured VP7 protein could not elicit the same level of protection. The passive transfer of antibodies from immunised mice failed to protect non-immunised mice against a subsequent viral challenge. This indicates that antibodies were not primarily responsible for the protection generated in the VP7 crystal vaccinated mice. It is possible that a protective T-cell response was generated with vaccination though further studies are required to confirm this (Wade-Evans *et al.*, 1998).

The use of BTV CLP consisting of VP3 and VP7 trimers has also been investigated as a vaccine delivery system for foreign epitopes (Roy, 1996). Chimearic VP7 particles were created by the insertion of 15aa, representing a Bovine Leukemia Virus (BLV) glyco protein (gp) 51 epitope, into the

Ala 145 site of VP7. The Ala 145 site was chosen for insertion since it forms an exposed loop on the CLP's surface. The production of stable CLPs after co-expression of the chimearic protein with VP3, indicates that the inserted epitope does not disturb the stuctural stability of the VP7 trimers. Preliminary data from subsequent immune response tests indicated that the epitope is presented effectively and could, possibly, elicit humoral immune responses.

1.4. Aims and Strategy of this Study

The aim of this study is to investigate the structural constraints associated with utilising Viral Protein 7 of African Horse Sickness Virus as a particulate, multiple peptide vaccine delivery system. To effectively fulfil the purpose of this study, the aim can be broken down into three individual parts.

1. To enable AHSV9 VP7 to act as a particulate, multiple peptide vaccine delivery system, multiple cloning sites for the insertion of peptides first have to be created. The first aim is to investigate the effect on structure and solubility of AHSV9 VP7 by the insertion of three multiple cloning sites.

Strategy:

- Introduction of site specific mutations into VP7
- Expression of newly constructed mutant genes in a baculovirus expression system
- Solubility and structural analysis of expressed proteins

2. The purpose in constructing a VP7 protein with three multiple cloning sites, is for it to be able to accommodate and efficiently present multiple epitopes to the immune system. The second aim is generated from this: investigating the effect on structure and solubility of modified AHSV9 VP7 by the insertion of peptides at two of the multiple cloning sites.

Strategy:

- Cloning of the peptides into two sites of modified AHSV VP7
- Expression of newly constructed mutant genes in a baculovirus expression system
- Solubility and structural analysis of expressed proteins

3. The major disadvantage of virus like particles as antigen carriers, is the limitation in the size of the peptides that can be inserted. In order to utilise the full potential of the VP7 particles effectively as an

antigen presentation system, an investigating was launched into the effect on structure and solubility of modified AHSV9 VP7 by the insertion of different size antigenic sequences.

Strategy:

- Cloning of the AHSV4 VP2 stretches in the 177 amino acid site of modified AHSV VP7
- Expression of newly constructed mutant genes in a baculovirus expression system
- Solubility and structural analysis of expressed proteins

Chapter 2

The Creation of the Three Site VP7 Construct and the Characterisation of the Structural Features and Stability.

2.1. Introduction

In order to utilise the VP7 crystals as a multiple antigen presentation system, areas have been identified within the VP7 sequence into which the foreign antigens can be inserted. The more hydrophilic top domain of AHSV VP7 is exposed on the surface of the assembled hexagonal crystals. This domain contains a very small number of hydrophilic regions that are potential target sites for the insertion of foreign peptides or epitopes. A particular promising site is the RGD segment contained in a highly flexible loop (amino acid position 175 to 180) in the top domain. Amino acids position 144/145 as tested by Roy, 1996 and amino acid position 200/201 (Maree, 2000 unpublished data) are both hydrophilic areas that are on exposed loops and should provide little if any structural hindrance to the VP7 trimeric structure when modifications are made. These areas should also not affect the trimer-trimer interactions that are necessary for the formation of the VP7 crystals.

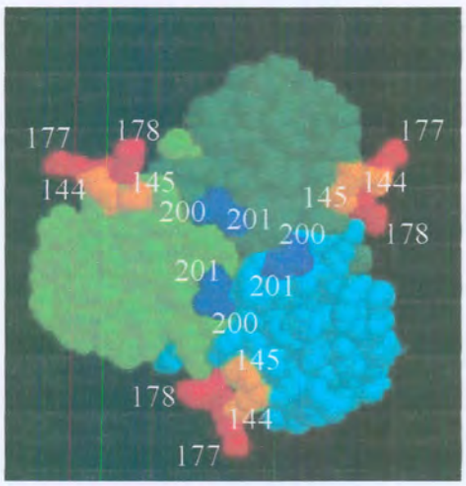


Figure 2.1. Diagrammatic representation of the accessible regions on a VP7 trimer.

A recombinant VP7 construct, VP7mt144/200 (Fig. 2.2), has already been created by the insertion of two multiple cloning sites into aa position 144/145 and 200/201 (Riley, 2003). Restriction enzyme sites *Sma*I, *Eco*RI and *Xho*I were inserted at the 144/145 site whereas restriction enzyme sites *Hind*III, *Xba*I and *Sal*I constitutes the multiple cloning site at aa position 200/201. These primarily hydrophilic insertional modifications increased the solubility of the VP7 protein and weakened the trimer-trimer interactions but did not seem to prevent crystal formation. It was, however, noted that the increase in solubility of these recombinant VP7 molecules produced crystals with a more "ruffled" appearance, instead of the smooth hexagonal crystal-like appearance as formed by wild-type VP7 (Riley, 2003).

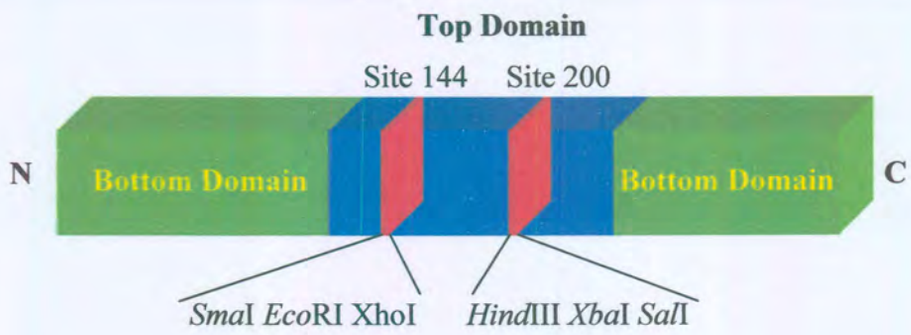


Figure 2.2. Diagrammatic representation of VP7mt144/200 (Riley, 2003)

The VP7mt144/200 construct served as template for the creation of a construct with three multiple cloning sites at aa position 144/145, 177/178 and 200/201. The extra multiple cloning site would be advantageous if this construct is to be used for a multiple antigenic subunit vaccine. Experiments were also carried out to study the effect of sonication on the crystal structure of VP7. This investigation was launched in order to find a suitable method to disrupt the aggregation of the assembled VP7 crystals. An extensional study for the possible use of recombinant VP7 as a vaccine was done to evaluate the stability of the recombinant VP7 under different storage conditions.

2.2. Materials and Methods

Materials

Life Technologies provided the oligonucleotide primers, TaKaRa Ex Taq™ polymerase, as well as the Bac-to-Bac™ baculovirus expression system and Cellfectin™. Grace's insect medium and foetal calf serum were purchased from Highveld Biological, whereas Rainbow™ protein molecular weight marker was purchased from AEC Amersham. Roche Diagnostics provided the restriction endonucleases, RNase A, T4 DNA ligase, DNA molecular weight marker II (MWII) and the High Pure™ DNA PCR Product and Plasmid Purification Kits. Perkin Elmer Biosystems ABI PRISM™ provided the Big Dye Terminator Cycle Sequencing Ready Reaction Kit. The Density Marker Beads were acquired from Pharmacia Biotechnology. The NERC Institute of Virology and Environmental Microbiology supplied the Sf9 cells. The VP7mt144/200 plasmid construct was obtained from Me. J. E. Riley. Merck and Sigma-Aldrich provided all the other chemicals.

2.2.1. Polymerase Chain Reaction

Polymerase chain reaction was used to introduce the site-specific mutation at amino acid position 177 in AHSV VP7mt144/200 (Riley, 2003). All PCR reactions were carried out using TaKaRa Ex Taq™ polymerase (Life Technologies) and consisted of 10 ng template DNA, 100 pmol of each primer (table 2.1), 3 µl 2.5 mM dNTP mix, 5 µl 10 x TaKaRa Ex Taq™ polymerase buffer (250 mM TAPS pH 9.3, 500 mM KCl, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol), 5 units (U) TaKaRa Ex Taq™ polymerase enzyme and made up to a final volume of 50 µl with ddH_2O. A GeneAmp PCR System 9600 (Perkin Elmer) was used for the PCR reactions.

The amplification conditions for both the reaction were as follows: 1 cycle at 94°C for 2 min; 25 cycles 93°C for 45 sec, 63°C for 1 min, 72°C for 2 min; 1 cycle at 72°C for 10 min.

2.2.2. Agarose Gel Electrophoresis

DNA samples were analyzed by the electrophoretic separation of DNA in agarose gels containing 0.5 µg/ml ethidium bromide. 1% - 4% agarose gels were used depending on the sizes of the fragments to be separated. TAE buffer (40 mM Tris-HCl, 20 mM NaAc, 1 mM EDTA, pH 8) was used as buffer

and in the making of the gel. The samples were mixed with a loading buffer (1% bromophenol blue, 1% xylene cyanol, 1xTAE buffer, 50% glycerol) prior to loading on the gel. Results were visualized by UV fluorescence.

Table 2.1. Primer sequences used in PCR site-directed mutagenesis of VP7mt144/200

PRIMER NAME	DIRECTED POSITION	OLIGONUCLEOTIDE SEQUENCE	Tm °C
Amplification 5' segment up to amino acid position 177			
Son2a	Nucleotides 8-25	5'CACAGATCTTTCGGTTAGGATGGACGC 3' *Bgl*II	56
7REV177RVR	Nucleotides 530-548	5'GCAGGCCTCTGCAGCCTTGGGGCTAGCAGCGC 3' *Stu*I *Pst*I	68
Amplification 3' segment from amino acid position 177			
7FOR177RVR	Nucleotides 549-567	5'GCAGGCCTGCGCGCAGGGGGGACGCAGTCATG 3' *Stu*I *BssH*II	70
VP7REVRVR	Nucleotides 1146-1167	5'CACGGTACCGTAAGTGTATTCGGTATTGAC 3' *Kpn*I	57

2.2.3. Purification of Amplified DNA Fragments

DNA fragments of interest were purified via the High Pure™ PCR Product Purification Kit (Roche Diagnostics)

2.2.4. Restriction Enzyme Digestion

The restriction endonucleases that were used in the cloning procedure during the preparation of DNA fragments or in the characterization and identification of recombinant DNA plasmids were used according to the specifications of the manufacturers (Roche Diagnostics).

2.2.5. Dephosphorylation

The digested pFastBac plasmid was dephosphoprylated by incubating the linearised DNA in a total reaction volume of 20 µl in the presence of 0.5 U of calf intestinal alkaline phosphatase and 2 U of 10 x dephosphorylation buffer (0.5 M Tris-HCl, 1 mM EDTA, pH 8.5) at 37 °C for 20 min.

2.2.6. Ligation

Sticky-blunt end ligations were carried out at 12 °C for 16 h. A high, 10:1, ratio of insert: vector molecules was used in a total volume of 20 µl, which also contained 2 U of T4 DNA ligase and 2 U of 10 x ligation buffer (660 mM Tris-HCl, 50 mM MgCl2, 10 mM dithio-erythritol, 10 mM ATP, pH 7.5).

2.2.7. Preparation of Competent *E.coli* Cells

The standard $CaCl_2$ method described by Cohen *et al* (1972) was used to prepare the *E.coli* Xl1 Blue competent cells used in all pFastBac plasmid transformations. 1 ml of an overnight Xl1 Blue culture was used to inoculate 100 ml Lauria-Bertani (LB) medium (1% NaCl (w/v), 1% tryptone (w/v), 0.5% yeast (w/v)). Cells were grown at 37°C with shaking to logarithmic (log) phase (OD_{550} = 0.5). The cells were collected by centrifugation at 5000 rpm for 5 min at 4°C. The pellet was then gently resuspended in 50 ml of freshly made ice-cold 50 mM $CaCl_2$, centrifuged again at the above-mentioned conditions and resuspended in 5ml of 50 mM $CaCl_2$. The cells were kept on ice for 1 h before sterile 15% (v/v) glycerol was added and the cells frozen away at –70°C.

2.2.8. Transformation of Competent Cells

The ligation mixture was added to 100 µl of competent cells and allowed to absorb for 30 min on ice. The mixture was then subjected to a 90 sec heat shock at 42°C and left to cool on ice for 2 min. 900 µl LB was added, followed by an incubation period of 1 h at 37°C with shaking. Aliquots of 100 µl were then plated out on LB agar plates (1.2% agar in LB medium) containing 12.5 µg/ml tetracycline hydrochloride (tet) and 100 µg/ml ampicillin (amp). Plates were then incubated overnight at 37°C.

2.2.9. Plasmid DNA Isolation and Purification

The Birnboim and Doly (1979) alkaline lysis method was used for the isolation of plasmid DNA. Selected colonies from the agar plates were inoculated into 5ml of LB medium, containing the appropriate antibiotics (amp and tet), and grown for 16 h at 37°C with shaking. 3ml of each culture was harvested by bench top centrifugation at 15000 rpm for 1 min after which the pellets were resuspended in 100µl of solution 1 (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8) and incubated at room temperature for 5 min. Cell lysis and chromosomal DNA denaturation were achieved by adding 200µl of freshly prepared alkaline-SDS buffer (0.2N NaOH, 1% SDS), followed by a 5 min incubation on ice. 150 µl cold 3 M NaAc (pH 4.8) was added resulting in the reannealing of the plasmid DNA and the precipitation of the genomic DNA, proteins and high molecular weight RNA. After a 10 min incubation period on ice, the supernatant, containing the plasmid DNA, was collected after centrifugation at 15000 rpm for 10 min at 4°C. Two volumes of 96% ethanol was added to the supernatants and incubated at –20°C for 30 min to precipitate the plasmid DNA. This was followed by 10 min centrifugation at 15000 rpm and subsequent washing of the DNA pellet with 80% ethanol. The clean DNA pellet was resuspended in UHQ.

2.2.10. Nucleotide Sequence Determination

Automated nucleotide sequence determination was done using the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer). Reactions consisted of 250-500 ng double-stranded plasmid template DNA, 3.2 pmol primer, 4 µl Terminator Ready Reaction Mix and were made up to a total volume of 10 µl with ddH_20. The subsequent PCR reaction was done in a GeneAmp PCR System 9600 (Perkin Elmer) with heated lid and consisted of 25 cycles under the following conditions: 10 sec at 96°C, 45 sec at 50°C and 4 min at °C.

Ethanol precipitation was done to remove unincorporated dye terminators and salts. A pre-made precipitation mix (50 µl 99.9% EtOH, 10 µl ddH_2O and 2 µl 3 M NaAc pH 4.8) was added to the 10 µl sequence reaction and incubated on ice for 10 min. This was followed by a 20 min centrifugation step at 13200 rpm. The pellet was washed with 250 µl 70% EtOH, 25°C, centrifuged again for 15 min at 13200 rpm and dried. Samples were then resuspended in 3 µl sequencing loading buffer (5:1 ration of deionised formamide to 25 mM EDTA pH 8 containing 50 mg/ml dextran blue) before denaturation at 95°C for 2 min, followed by cooling on ice. Sample analyses was done on a ABI PRISM 377

sequencer by loading 1.5 µl of the prepared reactions on a 4% denaturing polyacrylamide gel and running the gel for 7 h at 1.6 kV. Sequences were analysed by using the ABI PRISM Sequencing Analysis™ and Sequence Navigator™ programs.

2.2.11. Hydropathy Predictions

The ANTHEPROT package (Geourjon *et al.*, 1991; Geourjon and Deleage, 1995) and the Hopp and Woods predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983) were used to calculate the hydropathy plots of the VP7 mutants.

2.2.12. Cells and Media

Spodoptera frugiperda cell clone Sf9 was used to propagate wild type and recombinant baculoviruses. The cells were grown in suspension culture consisting of Grace's medium supplemented with 10% foetal calf serum and antibiotics (penicillin, streptomycin, fungizone) at 27°C.

2.2.13. Transposition

In order to transpose the newly synthesized VP7 recombinants, the recombinant pFastBacs were transformed into *E.coli* DH10Bac™ cells (Life Technologies). These cells contain the bacmid and the helper plasmid (Bac-to-Bac™ Expression system Manual) necessary for the transposition reaction to occur. The DMSO method described by Chung and Miller (1988) was used to make the *E.coli* DH10Bac cells competent. 100 ml LB medium was inoculated using 1 ml from a 5 ml overnight culture of DH10bac cells. This new culture was grown to early log phase (OD_{550} = 0.5). Cells were collected by centrifugation at 5000 rpm for 5 min at 4°C. The pellet was then gently resuspended in 10 ml of ice-cold TSB (0.5% NaCl (w/v), 1.6% tryptone (w/v), 1% yeast (w/v), 10% PEG (w/v), 5% DMSO (v/v), 10 mM $MgCl_2$, 10 mM $MgSO_4$) and incubated for 20 min on ice before transposition.

Recombinant pFastBac plasmid DNA was added to 200 µl of the competent DH10Bac cells and incubated on ice for 30 min. TSBG (TSB plus 20 mM glucose) was added followed by an incubation period at 37°C for 4 h and plating out on agar plates containing Kanamycin (50 µg/ml), Gentamycin (7 µg/ml), Tetracycline (10 µg/ml), isopropyl-β-D-thiogalactopyranoside (IPTG, 40 µg/ml) and 5-bromo-4chloro-3indolyl-β-D-galactopyranoside (X-gal, 300 µg/ml). The plates were incubated for 48 h after which the white colonies were inoculated into 5ml LB medium.

2.2.14. Isolation of Composite Bacmid DNA

The isolation of composite bacmid DNA proceeded according to the Baculovirus Expression System Manual (Life Technologies). The cells were collected from the overnight cultures by centrifugation and resuspended in Solution1. This was followed by the addition of alkaline-SDS buffer and incubation at room temperature for 5 min. Cold 2.5 M NaAc, pH 5.5, was added and samples were left on ice for 10 min. After centrifugation the DNA was precipitated with isopropanol and incubated on ice for 10 min. The DNA pellet was washed with 80% ethanol and air-dried for 10 min, before being dissolved in sterile UHQ.

2.2.15. Transfection

Six well (35 mm) tissue culture plates were seeded with Sf9 cells at $1.0x10^6$ cells per well in 2 ml Graces medium with antibiotics, after having been grown in a 75 ml suspension culture. The cells were allowed to seed for 1 h at room temperature. For each transfection, a solution was made by the addition of the bacmid minipreparation DNA to Graces medium, containing no antibiotics or serum. A second separate solution was prepared by the addition of 6µl of Cellfectin™ reagent (Life Technologies) to Graces medium, also containing no antibiotics or serum. The two solutions were combined and incubated at room temperature for a 45 min time period. Each seeded well was washed twice with 2 ml of Graces medium containing no serum or antibiotics. Graces medium (without serum or antibiotics) was added to the combined solutions A and B to a final volume of 1 ml. The wash medium was removed from the wells and the cells were overlaid with the lipid-DNA complexes formed within the combined solution A and B. The six well plates were incubated for a period at of 5 h at 27°C, followed by the removal of the transfection mixture and the addition of Graces medium containing serum and antibiotics. The cells were then incubated in a moist environment at 27°C for a further 96 h. The medium containing the recombinant virus was removed and stored at 4°C until further use.

2.2.16. Large-Scale Recombinant Protein Expression and Isolation

75 cm^3 Tissue culture flasks were seeded at $1.0x10^7$ with *S. frugiperda* cells before being infected with the appropriate recombinant baculovirus at a M.O.I. of 5 -10 pfu/cell. After a 72 h incubation period, the infected cells were harvested by centrifugation at 3000 rpm for 5 min at 4°C. The pellet was washed with 1 x phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM

$Na_2HPO_4.2H_2O$, 1.4 mM KH2 PO_4, pH 7.3), resuspended in 1 ml TNN lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5% Nonidet P-40) and incubated for 30 min on ice.

2.2.17. SDS-Polyacrylamide Gel Electrophoresis

A 15% SDS-PAGE gels were made as described by Laemmli (1970). The separating gels consisted of 0.375M Tris-HCl pH 8.8, 0.1% SDS, 30% acrylamide and 0.8% bisacrylamide. Polymerisation of the gels occurred by the addition of 0.008% (v/v) tetra-methyl-ethylene-diamine (TEMED) and 0.08% (w/v) ammonium peroxysulfate. The stacking gel contained the same except for 0.125 M Tris-HCl pH 6.8 and 0.1% SDS. TGS buffer (0.025 M Tris-HCl pH 8.3, 0.192 M glycine, 0.1% SDS) was used for the protein electrophoresis which occurred using a Vertical Slab Gel SE 400 unit (Hoefer Scientific Instruments). The harvested Sf9 cells containing the protein samples were added to equal volumes of 2 x protein solvent buffer ((PSB) 0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol) and denatured for 3 minutes at 95°C, before loading on the gel. The gel was run for a period of 16 – 20 h at 120V.

2.2.18. Coomassie Brilliant Blue Staining

After electrophoresis, the protein bands were visualised, using Coomassie brilliant blue stain (0.125% Coomassie brilliant blue, 50% methanol) for a period of 20 minutes. Progressive destaining occurred by placing the stained gel in a solution containing 5% ethanol and 5% acetic acid until only the minimum background remained. The gel was then stored in H_20.

2.2.19. Sucrose Gradient Studies

One fifth to half of the whole cell lysates resuspended in TNN lysis buffer (Section 2.2.16.) were loaded onto a 50%-70% discontinuous sucrose gradient (sucrose w/v, 50 mM Tris-HCl, 150 mM NaCl, pH 8). The samples were centrifuged at 12000 rpm for 75 min at 4°C, with a Sorvall AH-650 rotor. Fractions, consisting of 20 drops per fraction, were collected from the bottom of the gradient, resulting in 11 - 12 fractions depending on the temperature of the sucrose. The pellet was resuspended in 200 µl PBS. Protein content was precipitated from a sample consisting of 200 µl sucrose fractions by the addition 1200 µl PBS, followed by 45 min of centrifugation at 5000 rpm at 4°C. Quantification of the protein content was done by the Sigma Gel™ software program (Jandel Scientific) which measure the relative band intensities on a SDS-PAGE gel.

2.2.20. Density Analysis

The approximate densities of the proteins were calculated using Density Marker Beads (Pharmacia Biotechnology). 500 µl of the whole cell lysate was loaded onto a 50%-70% discontinuous sucrose gradient. Another 50%-70% discontinuous sucrose gradient was loaded with Density Marker Beads before undergoing isopycnic centrifugation: 40000 rpm for 18 h at 4°C, using a Beckmann SW55 TI rotor. The sucrose gradient containing the lysate was fractionated and analysed as described in section 2.2.19. The gradient containing the Density Marker Beads was used as density measurement reference.

2.2.21. Sonication

Protein aggregation disruption was studied with a Sonifier® Cell Disrupter B-30 unit (Branson Sonic Power Company). The sucrose gradient aggregated proteins in the pellet of a preceding sucrose gradient, suspended in PBS, were mostly subjected to 25 pulses of 20% pulsed duty cycle at 4000 Hz with a 1 min cooling-down period after every 5 pulses. The samples were then subjected to further solubility studies (2.2.19) before being analysed on a SDS-PAGE gel (2.2.17).

2.2.22. Trimerization Assay

Trimerization assays were conducted by first purifying the proteins according to the method described in section 2.2.19. The second and third fractions were pooled before undergoing 25 pulse sonication. The sonicated, unboiled protein samples were mixed with 10% glycerol and analysed on a 10% SDS-PAGE gel (2.2.17).

2.2.23. Storage Stability Studies

Sucrose gradient purified proteins, suspended in ddH_2O, were subjected to different storage conditions. This included flash-freezing the samples in liquid N_2, overnight storage at 4°C, and freeze drying the samples in a Freezemobile Twin 6 (Virtis) until. Flash-freezed samples were defrosted on ice and freeze-dried samples were re-hydrated by the addition of ddH_20 before subsequent solubility studies (2.2.19) were performed. This was followed by analysis on a SDS-PAGE gel (2.2.17).

The second PCR reaction amplified the second segment, consisting of the nucleotides from amino acid position 177 up to the 3' end of the VP7mt144/200 construct template. In this reaction primers amplified the segment with the addition of *Stu*I and *Bss*HI sites to the 5' end and a *Kpn*I site to the 3' end of the segment (Table 2.1.). The amplified segment, as well as the pFastBac-VP7mt144/177 construct, underwent separate *Stu*I and *Kpn*I digestions. The cleaned PCR product was then ligated into the prepared pFastBac-VP7mt144/177 construct to create the final three site AHSV VP7 construct; VP7mt144/177/200, size 5995 bp.

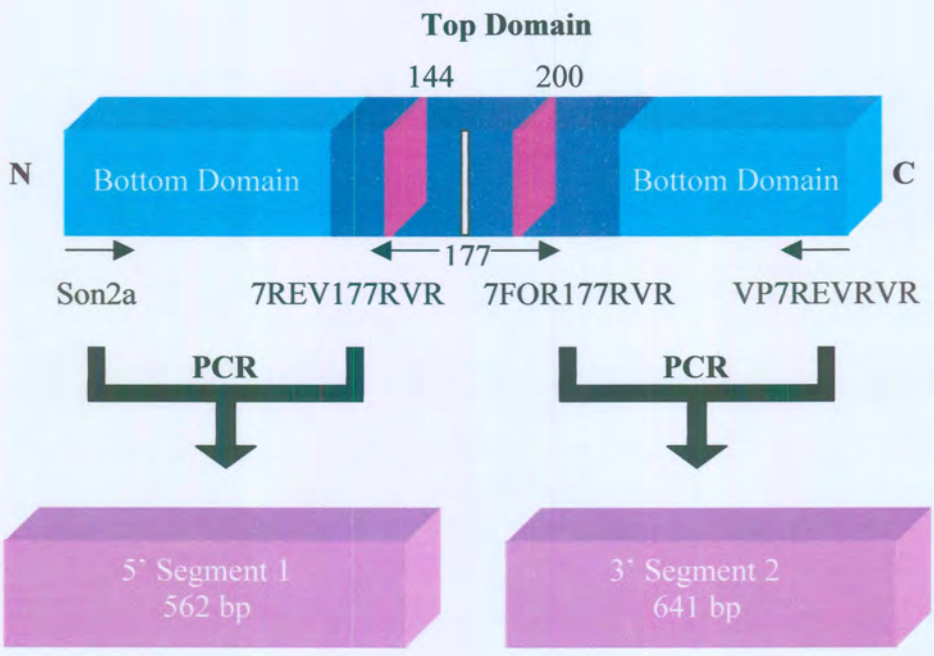


Figure 2.3. Primer binding positions and PCR amplification of the two segments cloned into pFastBac (Fig. 2.4).

2.2.24. Scanning Electron Microscopy (S. E. M.)

Purified recombinant VP7 crystals were resuspended and left for 15 min in a fixative solution (0.15 M Na_2HPO_4, 0.1% gluteraldehyde) before being filtered through a 0.22 µm nylon filter. Three subsequent washing steps with 0.075 M Na_2HPO_4 followed with a 10 min waiting step between each. The crystal covered filters were then dehydrated for 15 min consecutively in 50%, 70%, 90% and three times in 100% ethanol. Critical point drying with liquid CO_2 further dehydrated the samples before being placed on an aluminium stub and spatter coated with gold-beladium in a S. E. M. autocoating unit E5200 (Polaron Equipment Ltd.). Samples were then viewed in a Jeol 840 S. E. M.

2.3. Results

The recombinant VP7 construct, VP7mt144/200 (Riley 2003), as provided by Me. J. E. Riley, served as template for the creation of a three site construct, designated VP7mt144/177/200. The site between amino acid 177 and 178 of AHSV-9 was chosen as a target for the insertion of restriction endonuclease sites, since, as stated before, this is in the middle of a the highly flexible RGD containing loop. Restriction endonuclease sites *Pst*I (codes for Leu and Gln), *Stu*I (codes for Arg and Pro) and *Bss*HI (codes for Ala and Arg) were inserted. Fifty percent of the inserted amino acids into the hydrophilic loop are hydrophobic and could possibly affect the structure of the loop and subsequently the trimer-forming abilities of these recombinant VP7 molecules.

2.3.1. Construction of Cloning Site 177

A two-step cloning procedure, as summarised in figures 2.3. and 2.4., was required for the creation of the VP7mt144/177/200 construct. In the first step a PCR reaction was used to amplify the first segment that stretches from the 5' start of the gene up to amino acid position 177, using the VP7mt144/200 construct as template. The primers were designed to amplify the fragment adding a 5' *Bgl*II overhang and a 3' *Pst*I and *Stu*I overhang, as shown in Table 2.1. The obtained PCR product was subjected to a double separate digestion with the *Bgl*I and *Stu*I restriction endonucleases and was cleaned before being cloned directly into the prepared pFastBac plasmid vector, between the *Bam*HI and *Stu*I sites. The product's *Bgl*II[!] site is an isoschizomere of pFastBac's *Bam*HI site, thus binding to it and simultaneously destroying both the sites. This intermediate construct, size 5335 bp, was designated pFastBac-VP7mt144/177.

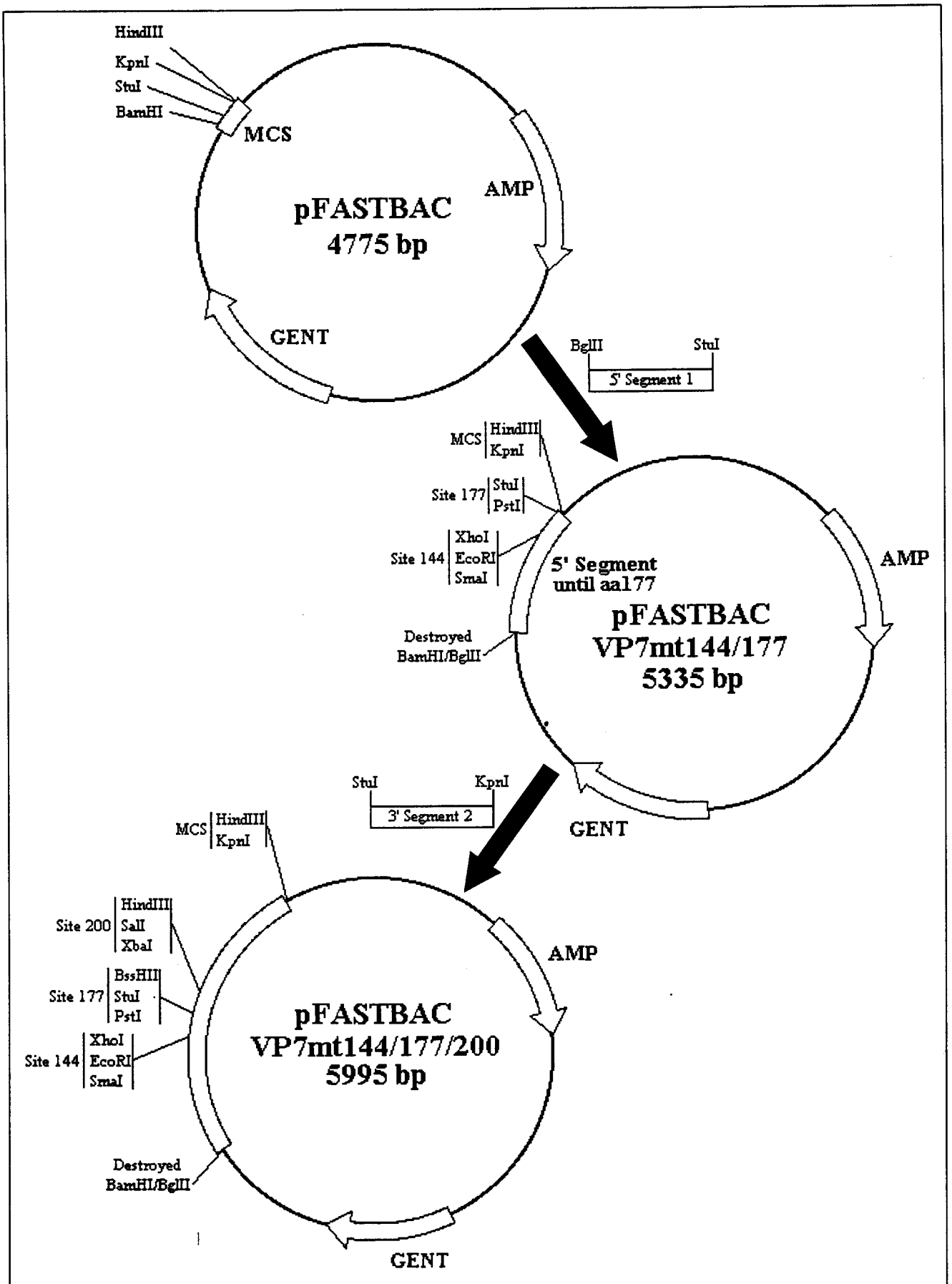


Figure 2.4. Construction of pFastBac-VP7mt144/177/200

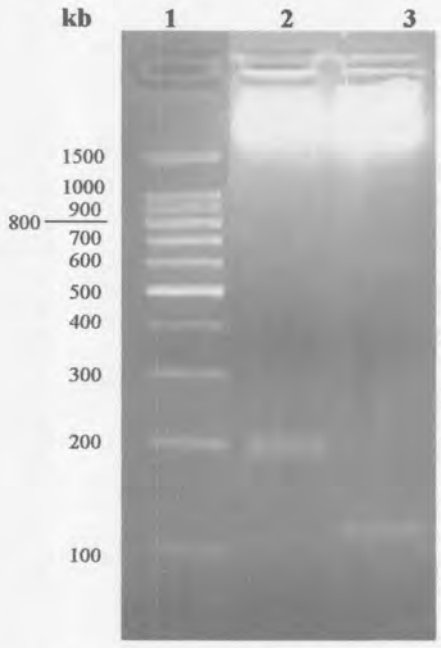


Figure 2.5. 4% Agarose gel electrophoresis of VP7mt144/177/200 digestions. Lane 1 represents the 100 bp DNA Ladder. Lane 2 contains VP7mt144/177/200 digested with *Eco*RI and *Xba*I give rise to linear plasmid DNA and a 204 bp fragment. Lane 3 contains VP7mt144/177/200 digested with *Eco*RI and *Pst*I, resulting in linear plasmid DNA and a 123 bp fragment.

2.3.2. Nucleotide Sequence Determination and Hydropathy Predictions

The primers listed in table 2.1. were used during automated DNA sequencing (section 2.2.10) to determine the nucleotide sequence of the newly constructed VP7mt144/177/200. Overlapping sequences were generated from which the complete gene sequence could be deduced. The sequencing not only confirmed the insertion of the of the 18 nucleotides constituting the three restriction endonuclease sites between amino acid position 177 and 178, but also showed the presence of three restriction endonuclease sites at both amino acid site 144 and 200, acquired from the VP7mt144/200 construct (Appendix A).

The derived sequence also supported the accuracy with which the PCR reaction was performed. No new mutations were generated with the amplification of the two fragments from the VP7mt144/200 template. It should, however, be noted that during the construction of the VP7mt144/200 construct a mutation at nucleotide position 30/31 occurred. This resulted in a CG sequence being switched to a GC

sequence and a subsequent change in amino acid 5 from wild-type arginine to alanine. As discussed in Riley 2003, this mutation should have little to no affect on the protein's structural formation.

The sequences were aligned and compared using ClustalX version 1.81 (Higgins and Sharp, 1988; Higgins *et al.*, 1996), shown in Appendix A. Subsequently, the obtained sequence was further analysed using the ANTHEPROT package (Geourjon *et al.*, 1991; Geourjon and Deleage, 1995) to calculate the hydropathy plots using the Hopp and Woods predictive method (Fig. 2.6) (Hopp and Woods, 1981; Hopp and Woods, 1983). The hydrophilic character of each amino acid is based on the property of its side chain to be more soluble in water than in an apolar solvent. As can be seen in figure 2.6 (a), VP7 is a highly hydrophobic protein. There are only a few hydrophilic regions, which are exposed on the surface of the folded VP7 trimeric structures and on the surface of the hexagonal crystals. The insertion of six amino acids, leu-gln-arg-pro-ala-arg, at the 177/178 amino acid site significantly increased the hydrophilicity of this particular region. This is expected since when added together, the residual hydrophilic character of the six amino acids equals +2.5 (Table 2.2). It should be noted the increase in hydrophilicity is located at the C-terminal of the insert, the contribution of the strong hydrophilic arginines. An even larger increase in hydrophilicity is shown at the 200/201 amino acid site. Only two of the inserted amino acids (leu and val) are hydrophobic and the hydrophilicity is more evenly spread over the region, more efficiently cancelling the effect of these two hydrophobic amino acids. There is, however, an overall slight increase in hydrophobicity at the 144/145 site. This effect is mostly generated by the amino acids at the N-terminal of the insert. The overall impact these changes have on the structure of the protein could, at this stage, not be predicted.

Table 2.2. Hydrophilic character of the amino acids added to VP7 (Hopp and Woods, 1981; Hopp and Woods, 1983).

Site 144		Site 177		Site 200	
Amino Acid	**Hydrophilic Character**	**Amino Acid**	**Hydrophilic Character**	**Amino Acid**	**Hydrophilic Character**
5'		**5'**		**5'**	
Pro	-1.4	Leu	-1.8	Lys	+3.0
Gly	0	Gln	+0.2	Leu	-1.8
Glu	+2.5	Arg	+3.0	Ser	+0.3
Phe	-2.5	Pro	-1.4	Arg	+3.0
Leu	-1.8	Ala	-0.5	Val	-1.5
Glu	+2.5	Arg	+3.0	Asp	+2.5
3'		**3'**		**3'**	
Total	**-0.7**	**Total**	**+2.5**	**Total**	**+5.5**

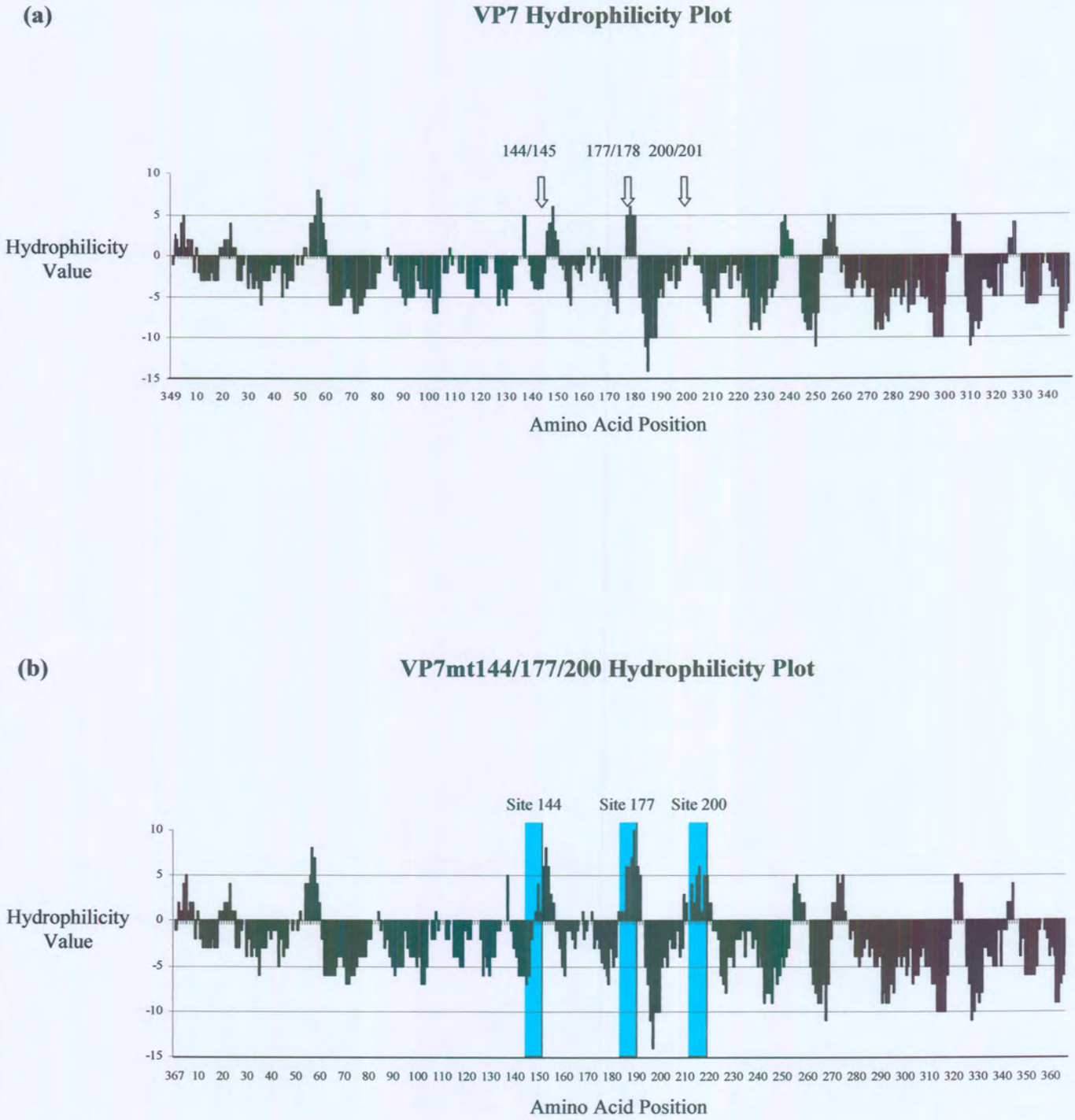


Figure 2.6. Hydrophilicity Plots of (a) VP7 and (b) VP7mt144/177/200 according to the Hopp and Woods Predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983.).

2.3.3. Baculovirus Expression

The Bac-to-Bac™ expression system was used for the expression of the all the proteins. *E.coli* $DH_{10}Bac^{TM}$ cells, containing the baculovirus genome, were transformed with the recombinant pFastBac transfer vector containing the recombinant VP7 genes (2.2.13). During the four-hour growth period, transpositon of the recombinant gene of interest occurred from the pFastBac plasmid into the genome of the baculovirus. The cells were plated out on enriched agar plates containing X-gal and IPTG, which enabled blue/white selection of positively transposed colonies containing recombinant bacmid DNA. The recombinant bacmid DNA was subsequently isolated (2.2.14) and transfected into Sf9 insect cells (2.2.15) for the generation of recombinant baculovirus expressing the gene of interest. Recombinant baculovirus was recovered and used to prepare viral stocks for subsequent viral infections, which occurred at a M.O.I. of 5-10 pfu/cell.

Expression of the proteins of interest was verified by means of SDS-PAGE gel electrophoresis (2.2.17). The effect of the 18 amino acid size increase of the expressed recombinant protein (39 kDa) can be clearly noted when compared with wild-type VP7 (37 kDa) on a 15% SDS-PAGE gel (Fig. 2.7). No significant changes to the expression levels, which might have been caused by the site directed mutations in the recombinant protein, can be detected.

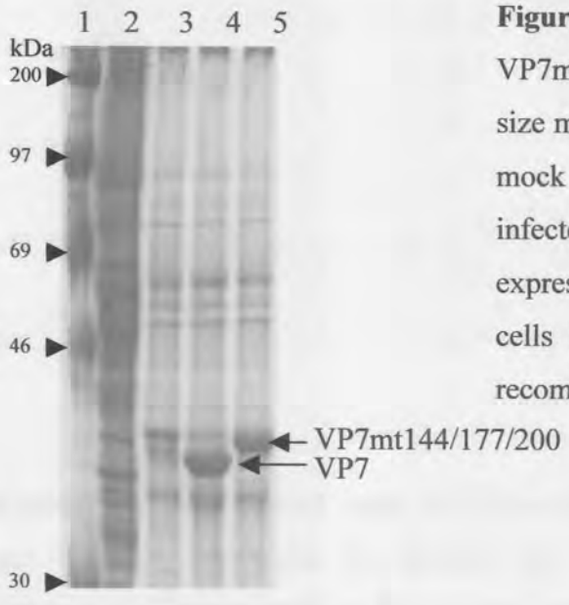


Figure 2.7. A 15% SDS-PAGE analysis of VP7mt144/177/200. Lane 1 represents the protein size marker. Lane 2 and 3 contain proteins from a mock infection and wild-type baculovirus-infected cells respectively. Lane 4 contains expressed VP7 and lane 5 contains the lysate of cells infected with baculovirus expressing the recombinant VP7mt144/177/200.

2.3.4. Solubility and the Effect of Sonication on VP7 Aggregation

Solubility studies were performed to investigate whether the hydrophilic changes in the modified VP7mt144/177/200 mutant, as discussed in 2.3.2., indeed has any significant contribution to the destabilisation of its crystal forming abilities. To study the effect the modification had on solubility, Sf9 cells were infected respectively with recombinant baculovirus stocks expressing VP7 and VP7mt144/177/200. After a 72 h incubation period, post-infected cells were harvested and lysed. Whole cell lysates were loaded on a 50%-70% discontinuous sucrose gradient and centrifuged as described in 2.2.19. Fractions were collected from the bottom of the gradient, resulting in 11 - 12 fractions. All particulate protein structures were selected and collected by means of the centrifugation conditions described in section 2.2.19, ensuring purity of the VP7 recombinant proteins, if not quantity. A sample of each fraction was SDS-PAGE gel (2.2.19). The relative protein band intensities on the SDS-PAGE gels were measured and quantified as percentages of the total specified protein content across all the fractions by the Sigma Gel™ software program (Jandel Scientific). The experiments were repeated several times to exclude inherent possibilities of variation.

Figure 2.10 shows the different protein particle distribution of the VP7 and the VP7mt144/177/200 constructs. The majority of the VP7 and VP7mt144/177/200 proteins are recovered from the pellet of the sucrose gradient. It should be noted though that approximately 56% of the VP7mt144/177/200 proteins (Fig. 2.10 b) are located in the pellet, whereas only approximately 42% of the VP7 proteins (Fig. 2.10 a) are located in the pellet. The free VP7 crystal portion peak in the second fraction from the bottom of the sucrose gradient, with decreased amounts of smaller particulate structures in the middle section and finally increasing amounts of the smallest particles, possibly soluble free VP7 trimers, in the top part of the gradient. The free VP7mt144/177/200 protein particles, however, only peak in the third fraction from the bottom of the sucrose gradient, with a decreasing amount of smaller particulate structures in the middle section of the gradient. There is no real indication of soluble, small particles or free trimers in the hydrophilic top part of the VP7mt144/177/200 sucrose gradient.

VP7mt144/177/200 proteins were also subjected to isopycnic centrifugation along with Density Marker Beads (2.2.20). The results, as presented in figure 2.9, clearly show that the VP7mt144/177/200 proteins actually occur in the first three fractions of the 50%-70% sucrose gradient when density centrifugation, and not size selection, is applied. The proteins in these three fractions, when compared to the Density Marker Beads, correspond to an approximate buoyant density of 1.108 g/ml to 1.133 g/ml.

The pellets of the sucrose gradients from both the constructs were subjected to sonication. This was aimed at disrupting the purified aggregates in the pellet and increasing the yield of purified protein that might be used for vaccination purposes (2.2.21). The samples underwent 25 pulses of sonication before, again, being loaded on a 50%-70% discontinuous sucrose gradient for further solubility studies (2.2.19). The attained results were compared with the construct's sucrose gradient in the absence of sonication. As can be seen in figure 2.11 a, VP7 pellet sonication disrupts the aggregation between the crystals, resulting in an overall increase, when compared to unsonicated VP7, in the percentage proteins contained in each fraction. An intense peak of protein crystals is noted in the third fraction from the bottom. This fraction seems to constitute approximately 44.8% of the total amount of proteins that were loaded on the gradient, whereas this fraction only constitutes approximately 12% of the total protein content in the absence of sonication. There is a rapid decline in the percentage protein located in the fourth fraction. A final increase in the ninth to eleventh fractions, peaking at fraction nine, illustrates the presence of soluble free and aggregated trimeric VP7 structures in the soluble fraction of the sucrose gradient. The pellet of this sonicated VP7 sample still contains approximately 5% of the total protein start content.

The results attained from VP7mt144/177/200 in the absence of sonication compared to sonicated VP7mt144/177/200 are illustrated in figure 2.11 b. Sonicated Vp7mt144/177/200 protein particles have the same sedimentation profile as unsonicated VP7mt144/177/200, although the percentage particles in each fraction is higher in the absence of a pellet. Also, there is an increase in smaller particles when sonicated, as illustrated by the presence of the proteins in fractions eight and nine. This tendency to revert from large aggregated protein masses to smaller particles was further investigated by the sonication of additional samples of VP7mt144/177/200 at more than 25 pulses. Figure 2.12 shows the results of stability tests that were done on VP7mt144/177/200 after no sonication, 25 pulses, 50 pulses, 100 pulses and finally, exhaustive sonication (approximately 1900 pulses). A clear shift from large particulate structures at low sonication to more soluble small particles or trimeric structures at high sonication can be observed.

2.3.5. Trimerization Assays

A trimerization assay to confirm the formation of trimeric structure by VP7mt144/177/200 was performed similar to that described by Limn *et al.*, 2000. Large particulate structures were sonicated to reduce the size of the assembled particles. These unboiled samples were loaded unto a 10% SDS

PAGE gel for analysis (Section 2.2.22). It can be seen that there is a greater tendency by VP7mt144/177/200 to form free monomers (39 kDa), even when non-denaturing conditions are being applied (Fig. 2.8). An extremely faint band of 117 kDa can be detected indicating the presence of the trimeric VP7mt144/177/200.

2.3.6. Storage Stability Studies

Cold-chain storage of vaccines cannot always be maintained, since it is expensive and not very practical, especially in third world countries. It is thus important to test the effects different storage conditions have on the stability of VP7mt144/177/200, if it is to be used as a subunit vaccine. The second and the third fractions of VP7mt144/177/200 sucrose gradients were pooled and subjected to three different storage methods. This included flash-freezing the samples in liquid N_2, overnight storage at 4°C, and freeze drying the samples (2.2.23). After subsequent sample preparation, the samples were subjected to sucrose gradient sedimentation analyses (2.2.19). The results, in figure 2.13, were compared to control VP7mt144/177/200 proteins that were fractionated on sucrose gradients directly after cell lysis. At the bottom of the gradient, the stored samples have the same protein distribution in each fraction as the control, but only at much lower levels. Towards the top of the gradient, the stored samples have a higher percentage of small particles in the soluble fraction than the control. The most conspicuous result is the high levels of VP7mt144/177/200 protein that was found in the pellet of the stored samples. Where the control have approximately 56% of the total protein amount in the pellet, the stored samples, that were taken from the second and third fractions of a sucrose gradient, have higher portions of their total protein content in the pellet. The freeze-dried sample has approximately 53% in the pellet, the flash-freezed sample has approximately 68% in the pellet, with approximately 80% of the sample that was left at 4°C occurring in the pellet.

2.3.7. Scanning Electron Microscopy (S.E.M.)

Protein samples collected from the second and third fractions were pooled, fixed, mounted and spatter coated with gold-beladium particles for investigation under the S.E.M. Unmodified VP7 protein crystals were used as control. The wild-type VP7 crystals appeared exactly as first described by Chuma *et al.*, 1992 (Fig. 2.14 a and b). They are flat, smooth, hexagonal structures with a mean size of approximately 8 μm in diameter. The structures formed by VP7mt144/177/200 proteins seem to be concentration dependent. The structures formed by a low concentration of the recombinant VP7mt144/177/200 protein have a rough, flat and an almost circular appearance (Fig. 2.14 c and d).

The units are approximately the same size as the 8 μm crystals formed by wild-type VP7. When high concentrations of VP7mt144/177/200 proteins are viewed, the units seem decompressed and have a mean size of approximately 10 μm in diameter (Fig. 2.14 e). The units are connected with web-like tentacles/ cables (Fig. 2.14 f). This study was extended by using VP7mt144/177/200 freeze dried samples for S.E.M. study (Fig. 2.15 a, b and c). The units from this sample had the same structure and texture as high concentration samples that did not undergo the drying process

A further study was conducted by S.E.M. examination of sonicated wild-type VP7 samples (Fig. 2.15 e and f) and sonicated VP7mt144/177/200 samples (Fig. 2.15 d). Twenty-five pulses of sonication reduced the VP7 crystals to large fragments of the hexagonal crystals, with chance whole hexagonal crystal units still to be found. The same amount of sonication pulses on the VP7mt144/177/200 samples reduced the VP7mt144/177/200 large units (as described above) to small fragments of nondescript shape and a mean size of approximately 1 μm. No web-like tentacles/ cables were observed between the small fragments.

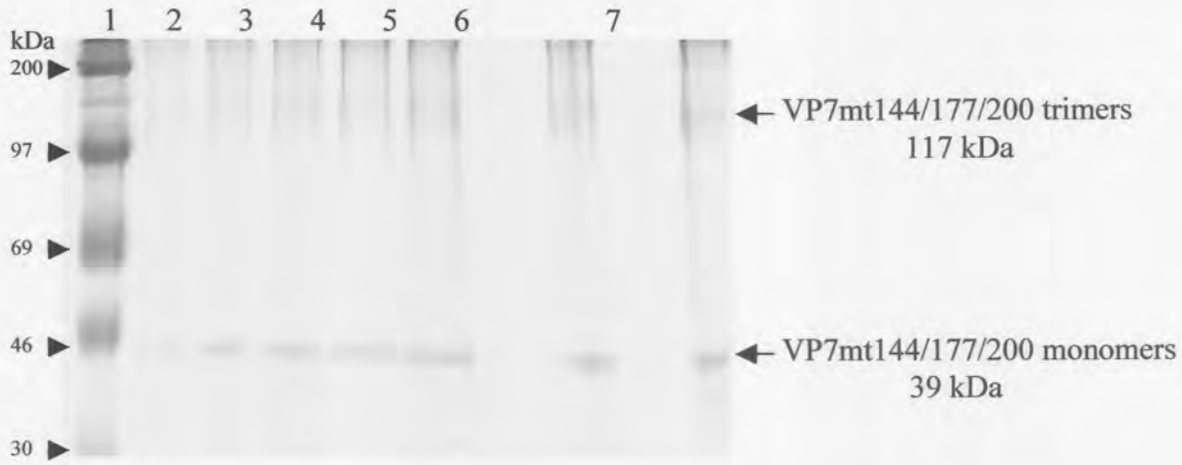


Figure 2.8. Trimerization assay of VP7mt144/177/200 analysed on a 10% SDS-PAGE gel. Lane 1 represents the protein size marker. Lane 2 to 8 contain purified VP7mt144/177/200 proteins that underwent 25 pulse sonification before being loaded unboiled onto the gel

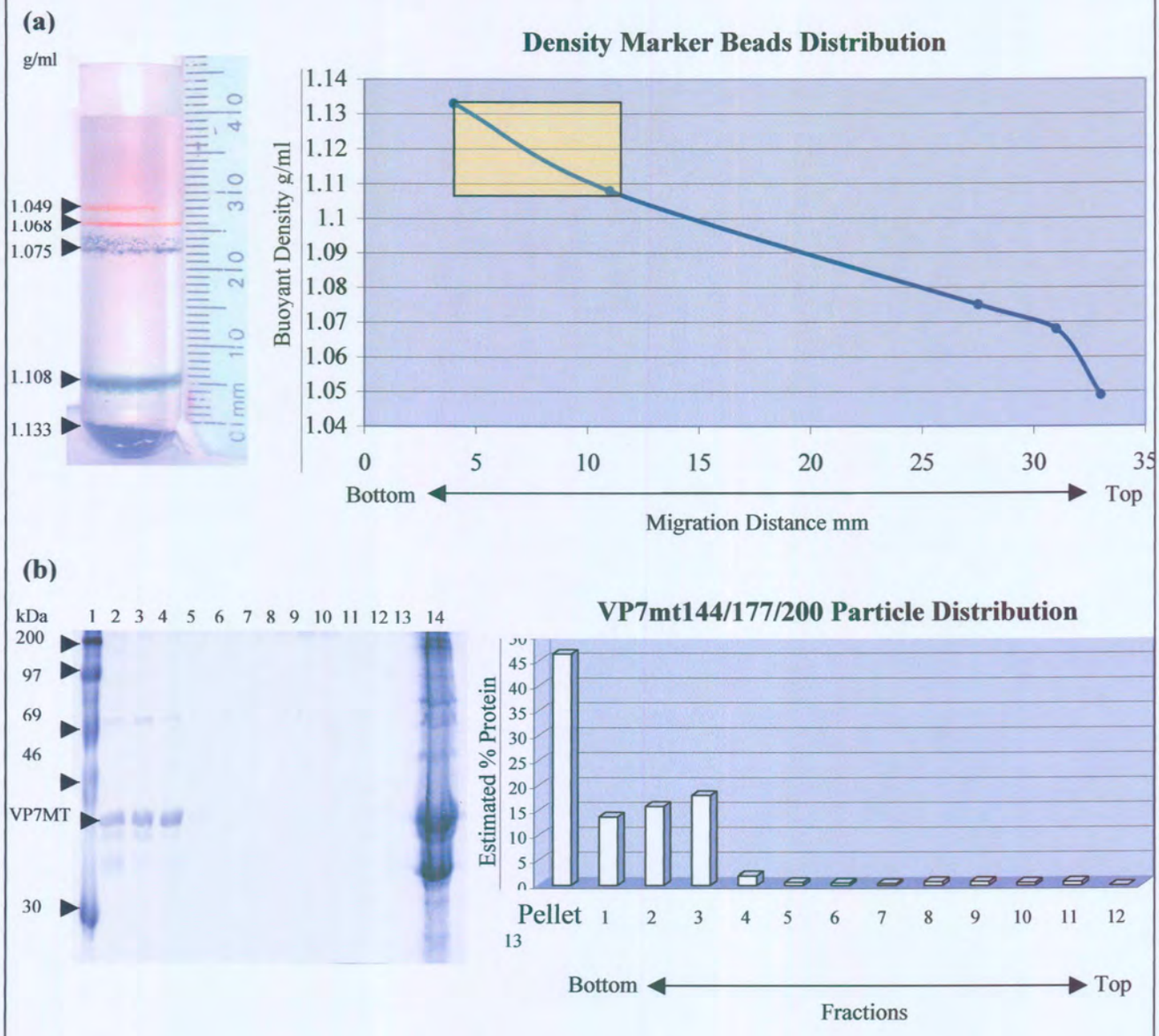


Figure 2.9. Density Marker Beads (a) and protein distribution of VP7mt144/177/200 (b) on a 50%-70% sucrose density gradient using a Beckmann SW55 TI rotor at 40000 rpm for 18 hours at 4°C. The VP7mt144/177/200 protein distribution was analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as taped from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

Measurements of the Density Marker Beads' locations were taken and converted into a graph (a). The highlighted yellow area indicates the approximate density of the VP7mt144/177/200 structures.

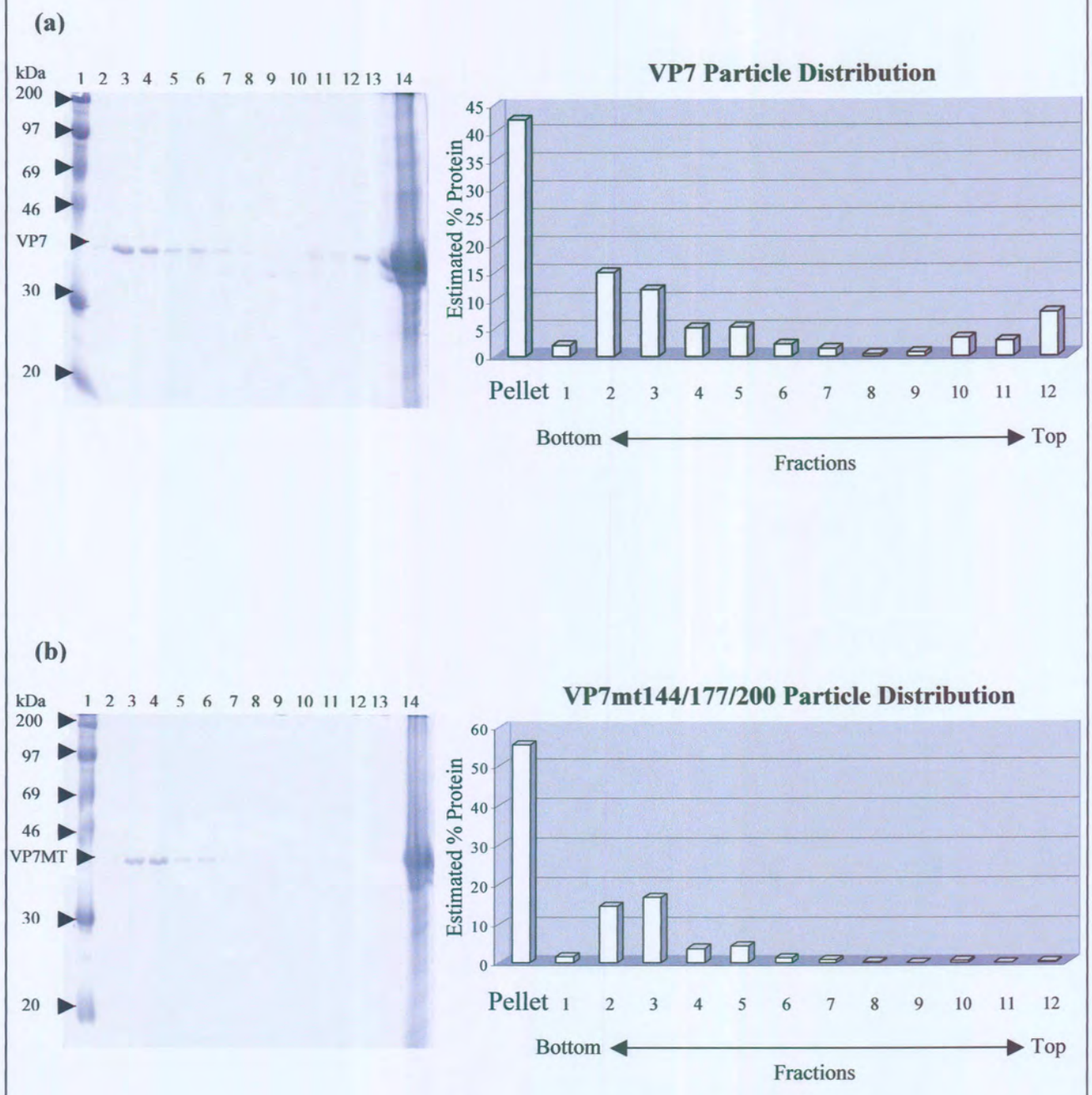


Figure 2.10 (a), (b). Protein distribution of VP7 (a) and VP7mt144/177/200 (b) on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

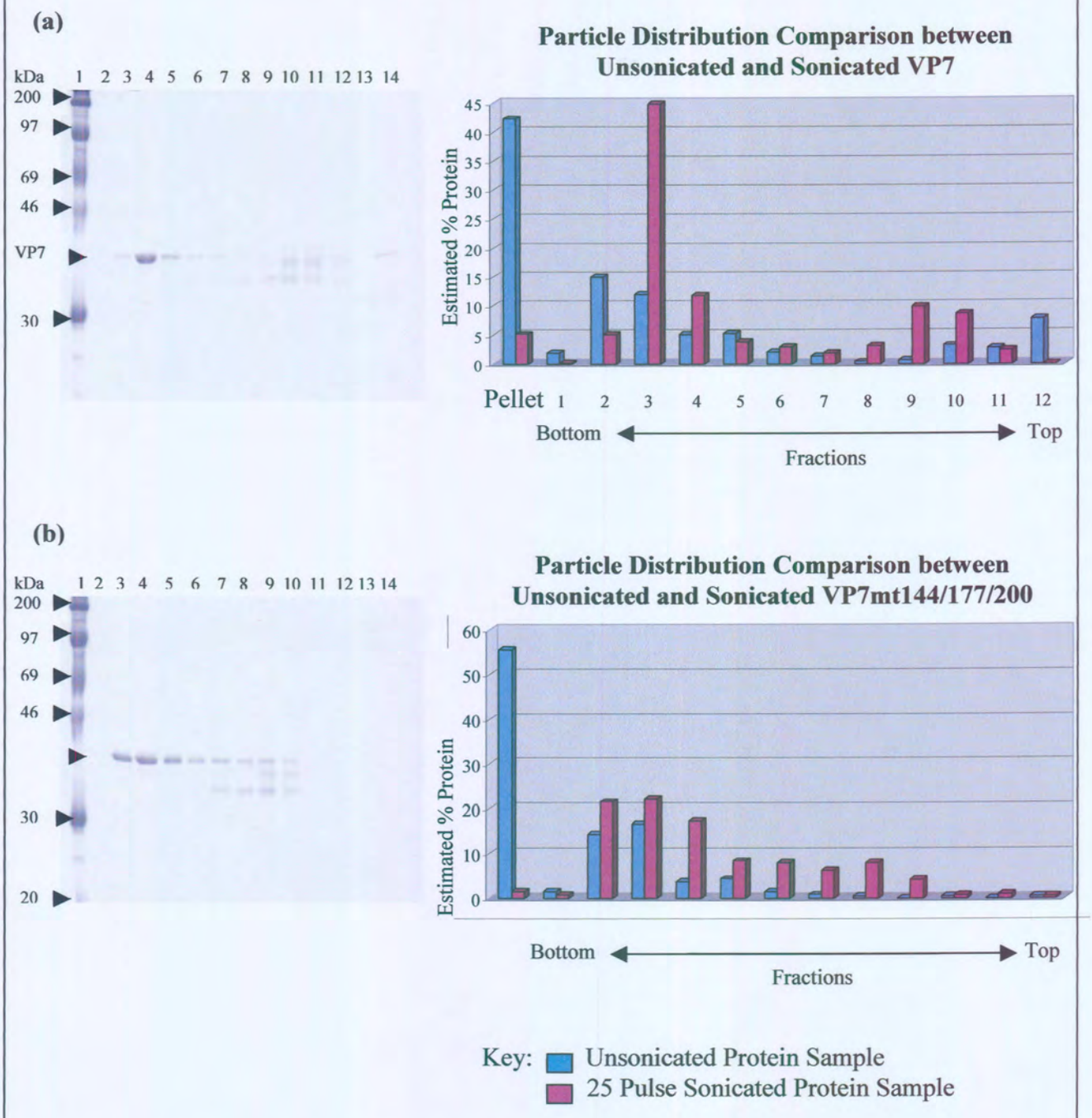


Figure 2.11 (a), (b). Protein distribution of 25 pulse sonicated VP7 (a) and sonicated VP7mt144/177/200 (b) each with a respective unsonicated control. Proteins were centrifuged on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

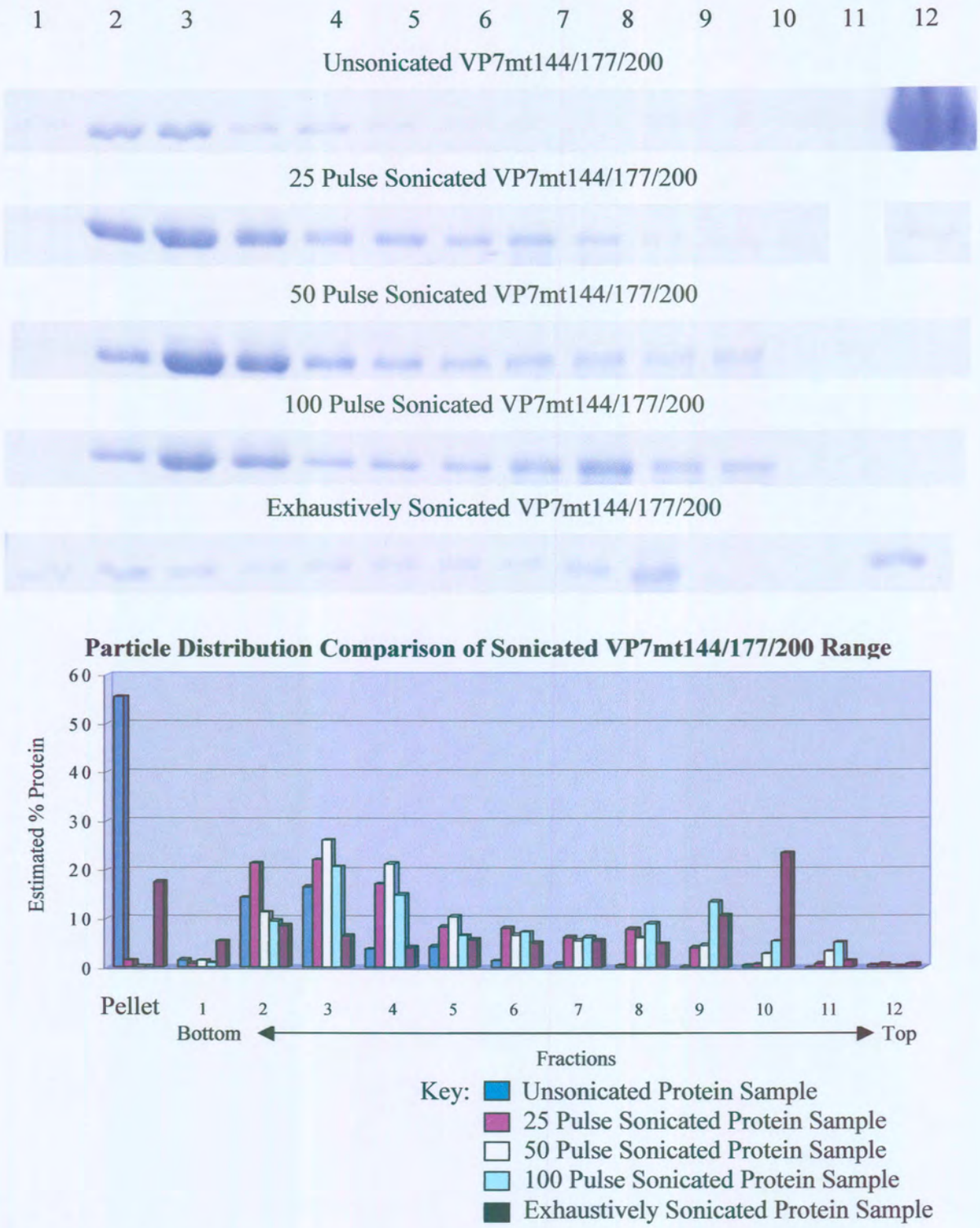


Figure 2.12. Protein distribution of sonication range of VP7mt144/177/200 pellets. Samples were centrifuged on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lanes 1-12 contain the fractions as collected from the bottom to the top of the gradients.

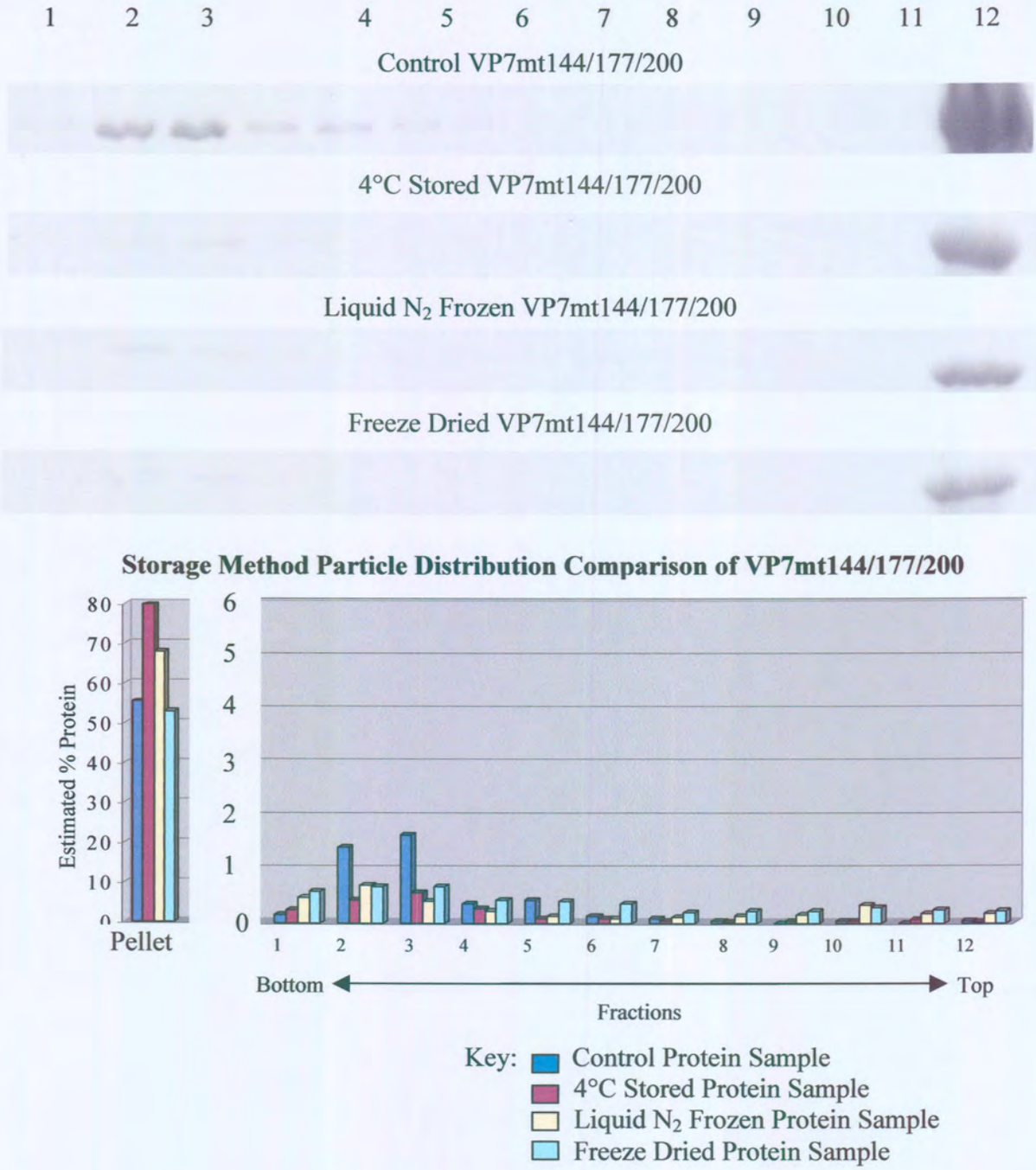


Figure 2.13. Protein distribution of sucrose gradient purified, unsonicated VP7mt144/177/200 samples stored at different conditions. Samples were centrifuged on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lanes 1-12 contain the fractions as collected from the bottom to the top of the gradients.

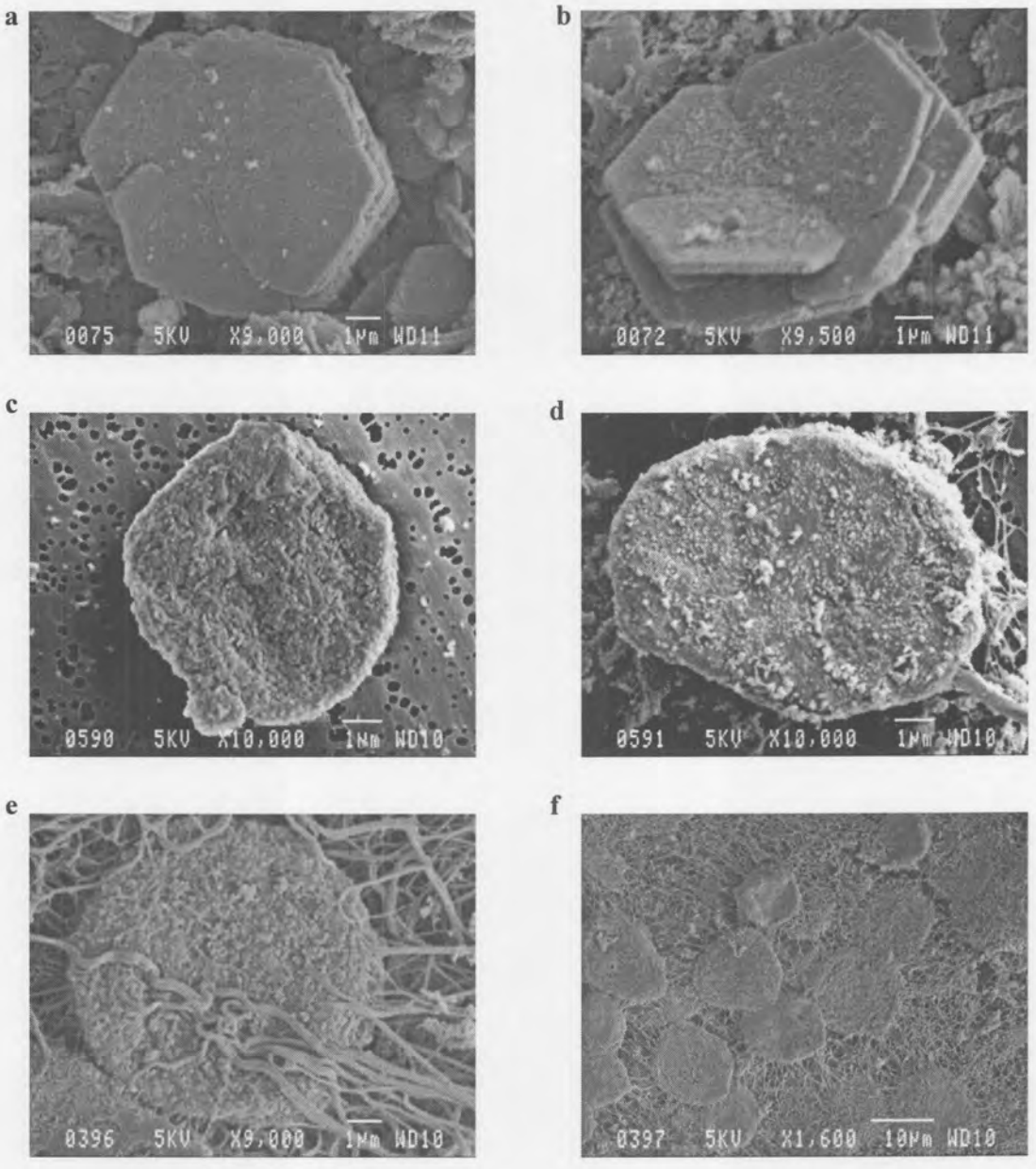


Figure 2.14. Scanning Electron Microscopy photographs depicting the structures formed by the different protein constructs. (a) and (b) show the hexagonal crystals formed by wild-type VP7. The structures formed by VP7mt144/177/200 at low concentrations are shown in figure (c) and (d), whereas (e) depicts a structure at high concentration, with a wide-angle view depicted in (f).

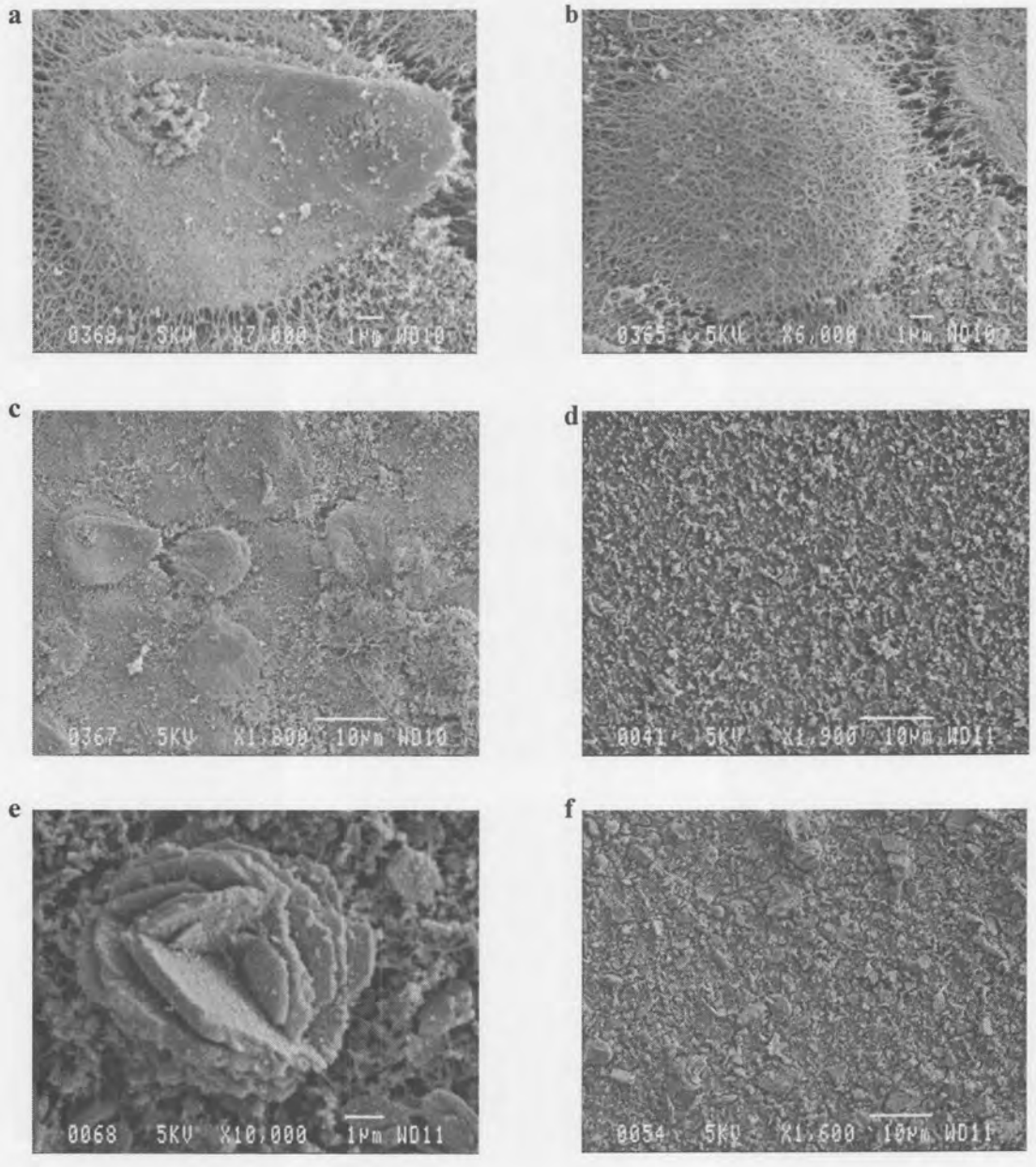


Figure 2.15. Scanning Electron Microscopy photographs depicting the structures formed by the different protein constructs under different conditions. (a) and (b) show VP7mt144/177/200 structures that had been freeze dried, with a wide-angle view depicted in (c). A wide-angle view of sonicated VP7mt144/177/200 is shown in (d). (e) depicts remaining fragments of VP7 crystals after sonication, with a wide-angle view shown in (f)

2.4. Discussion

African horse sickness virus VP7 monomers are highly hydrophobic and three of these monomers tend to spontaneously organise into trimeric structures through non-covalent interactions. These VP7 trimers, when expressed in a recombinant baculovirus, spontaneously assemble in infected insect cells into large, flat, hexagonal crystals (Chuma *et al.*, 1992; Burroughs *et al.*, 1994). These crystalline structures have a highly ordered lattice that is consistent with a trimeric subunit structure. The more hydrophilic BTV VP7, on the other hand, is completely soluble and does not form these crystal structures (Oldfield *et al.*, 1990). Thus it was deduced that the ability of AHSV VP7 trimers to assemble into crystalline structures could probably be ascribed to hydrophobic interactions between the trimers. Therefore, modifications that affect the solubility of VP7, might influence the formation of the particulate structures.

The creation of cloning sites in VP7 protein added, in total, 6 amino acid insertions at each of the three respective sites. These changes in the construction of VP7mt144/177/200 contributed to the overall hydrophilicity of the protein, the impact of which could not be determined at the early stages before solubility studies. The initial solubility studies made a comparison between the solubility of wild-type VP7 and the recombinant VP7mt144/177/200 construct (Fig. 2.10). The VP7 structures, present in the second fraction from the bottom of the gradient, seemed to be larger and more compact than the structures formed by VP7mt144/177/200, which occurred in the third fraction from the bottom. The absence of small particles in the soluble top portion of the VP7mt144/177/200 gradient indicated an increased tendency towards aggregation between the assembled particles. This was further supported by a significant increase in the percentage aggregated proteins in the pellet of the VP7mt144/177/200 sucrose gradient, when compared to VP7. The most likely possible explanation for this phenomena is that due to the structural stress caused by increased hydrophilicity at three key sites in the top domain of the trimers, the hydrophobic regions in the trimers are more exposed. This causes the trimers and the larger structures formed by them, to aggregate more. The results of the storage stability studies, which will be discussed later in this section, embrace this explanation. As mentioned in 2.3.6., the most conspicuous result was the high levels of VP7mt144/177/200 protein that was found in the pellet. The protein samples used in this particular study were pooled from the second and third fractions, thus did not occur initially in the pellet. Extremely high levels of protein aggregation can be correlated with the extend of time that the samples were left in solution. The freeze dried sample had the shortest

period of time for aggregation, therefore the lowest amount of aggregated proteins. The flash-freezed sample had a longer period for aggregation as defrosting occurred. The sample with the highest amount of aggregated proteins, is the sample that was left to stand overnight at 4°C.

Further studies were conducted on the aggregated proteins found in the pellets of the sucrose gradients. Sonication of the pellets showed that VP7 proteins form stable crystals with a distinct size and density, as illustrated by the presence of approximately 44.8% of the proteins in a single fraction (Fig 2.11 a). This observation was further supported by almost no significant increase in the intermediate particulate structures found in the middle fractions, except for an increase in the proportion of small particulate structures at the top of the gradient. The sonication product of the VP7mt144/177/200 construct resembles the right side of a Gaussian distribution curve. Structures of varying size and density are found with an increase in the soluble trimer proportion with longer sonication periods. This not only indicates instability in the VP7mt144/177/200 construct that is not found in the wild-type VP7, but also that no distinct units are formed anymore.

Scanning electron microscopy confirmed the solubility studies. The VP7mt144/177/200 structures are not smaller than crystals that are formed by wild-type VP7, as thought earlier. The VP7mt144/177/200 structures vary in shape and size, which might account for the difference in distribution during rate zonal centrifugation; than the smooth, flat hexagonal VP7 crystals moving faster through the gradient and reaching density equilibrium sooner. Higher concentrations of VP7mt144/177/200 protein units are connected with web-like tentacles/ cables. These connecting web-like structures are probably the products of aggregation between the units. It also seem that the units themselves are composed of these web-like tentacles/ cables - contradictory to the smooth, hexagonal crystals produced by wild-type VP7 and the structures formed with low concentrations of VP7mt144/177/200. This might be due to aggregational stress that is exerted on these structures, thus physically pulling them apart. All these changes can be ascribed to the changes in solubility, leading to structural stress and the changing of the trimeric unit.

Meyer, 2002, also conducted research on the presentation of neutralising epitopes on the surface of AHSV VP7. A VP7 construct was created with a single multiple cloning site at amino acid position 177. A triple repeat of a single epitope was inserted into this multiple cloning site. In total, twenty-two amino acids were inserted into position 177 with a net hydrophilic character of +8.8 (Hopp and Woods, 1981; Hopp and Woods, 1983). In these studies no distortion of the hexagonal crystalline structure was observed. These results differ from those observed with the VP7mt144/177/200

construct, which only has 18 additional amino acids with a net hydrophilic character of 7.3, as shown in table 2.2 (Hopp and Woods, 1981; Hopp and Woods, 1983). This is probably due to the more "spread-out" distribution of the insertions in the VP7mt144/177/200 construct, in contradiction to the single positional insertion made Meyer.

It is important for vaccination purposes to study the effect of different storage methods on the stability of the vaccine. Not one of the storage methods had a direct effect on the structure of the proteins. It would seem that freeze drying, with the least amount of aggregation, is the best storage method. Studies under the S.E.M. confirmed the absence of any changes to the overall structure of the freeze dried proteins. The only foreseeable problem is the aggregation of the proteins. Sonication might be a solution to the aggregation problem since both the particulate and the individual trimers have potential as a vaccine delivery system. It might even be more advantageous to use fragments and single trimer units since only T-cell mediated immunity was generated and no antibody-based humoral immune response by the injection of whole 6 μm VP7 crystals (Wade-Evans *et al.*, 1998). A possible explanation for the exclusive generation of T-cell mediated immunity might be that the large crystals are taken up by antigen-presenting cells in the body, which breaks down the crystals and only present the epitope regions to the T-helper cells.

Sonication, however, will not stop re-aggregation from occurring. Further studies might have to be conducted in the use of non-polar chemicals for dissolving the dried proteins in before injection.

Chapter 3

Structural Effects caused by Inserting Small Hydrophilic Peptides into Multiple Sites on African Horse Sickness Virus Viral Protein 7.

3.1. Introduction

The purpose in constructing a VP7 protein with multiple cloning sites is for it to be able to accommodate and efficiently present multiple epitopes to the immune system. The advantage of a presenting epitopes at different positions would be that there is less attachment competition for immune reaction generation against these epitopes than there is against epitopes that are too closely located. As shown in chapter 2, the creation of VP7mt144/177/200 by the insertion of three multiple cloning sites, had an effect on the crystal formation abilities but did not totally destroy the protein's trimer formation abilities. To test the ability of VP7mt144/177/200 to accommodate epitopes at multiple sites and retain its structure, neutralising epitopes from AHSV serotype 4 Viral Protein 2 was utilised.

African horse sickness virus VP2 is one of the two proteins that constitute the outer layer of the virion, the other being VP5 (Roy *et al.*, 1994). This hydrophilic surface protein facilitates entry into mammalian cells during infection (Mertens *et al.*, 1996). Viral protein 2 proteins maintain strong structural similarities between serotypes but display the highest level of sequence variation of all the viral-encoded proteins (Fukusho *et al.*, 1987; Vreede and Huismans, 1994). The high variability and the hydrophilic nature displayed by certain regions of VP2, suggests that these regions are exposed to immunological pressure and may contain epitopes that are serotype specific (Venter *et al.*, 2000). It has also been shown that VP2 is the main determinant of serotype-discriminatory neutralising-specific immune response (Huismans *et al.*, 1987; Roy *et al.*, 1996; Scanlen *et al.*, 2002). Due to this, VP2 neutralising domains and epitopes has been a main subject of investigation.

The neutralising epitopes for AHSV VP2 serotype 4 were defined by Martínez-Torrecuadrada *et al.*, 2001. Two neutralising epitopes, 'a' (19 amino acids) and 'b' (24 amino acids), were identified between amino acid residues 321 - 339, and 377 - 400, respectively. It was shown that a combination of these two epitopes produced a much more effective neutralising response than the individualistic epitopes. The region also corresponds to the domain in which VP2 epitopes were identified on VP2 of

AHSV-3 by means of phage display libraries (Bentley *et al.*, 2000) and a region of importance identified on VP2 of AHSV-9 (Venter *et al.*, 2000). Based on this, it was decided to insert these two epitopes, 'a' at amino acid site 177 and 'b' at amino acid site 144. In total, three new constructs were synthesised; one containing the 'a' epitope, the other containing the 'b' epitope and the last construct containing both the epitopes at the given sites.

3.2. Materials and Methods

Materials

Reagents were obtained from the suppliers listed in section 2.2.

3.2.1. Annealing of Oligonucleotide Primers

The commercially synthesized oligonucleotide primers (Life Technologies) were resuspended in ddH_2O to a final concentration of 100 pmol/µl. Oligonucleotides for 'a' and 'b' were annealed in separate reactions consisting of the following: 200 pmol of each primer and 10x annealing buffer (100 nM Tris-HCl pH 7.5, 1 M NaCl, 10 mM EDTA) in a reaction volume of 10 /µl. The reaction mix was denatured at 92°C for 10 min followed by an annealing step at 65°C for one hour. Reactions were allowed to cool slowly at room temperature for one hour before being frozen away at -20°C until further use.

3.2.2. Cloning of Epitopes into the Modified VP7mt144/177/200 Construct and Expression of the Recombinant Proteins

Cloning of the 'a' and 'b' VP2 epitopes into pFastBac-VP7mt144/177/200 construct was achieved by methods described in chapter 2, with one variation. The ligation of the annealed oligonucleotides to the digested and purified pFastBAC-VP7mt144/177/200 proceeded at 16 °C for 16 h. A 10:1, ratio of insert: vector molecules was again applied in the ligation mixture.

Ligation was followed by transformation of the DNA into competent *E.coli* Xl1 Blue cells, which yielded colonies containing the recombinant plasmids. After DNA analyses of samples by electrophoretic separation on 4% agarose gels, the purified DNA of the two single epitope recombinant constructs were sequenced. The three recombinant pFastBac-containing-VP7-mutants were transformed into competent *E.coli* DH_{10}Bac™ cells where transposition of the relevant genes occurred

to form composite bacmid DNA for use during transfection and the creation of the recombinant viruses. Protein expression was analyzed on a 15% SDS-PAGE gels before solubility and sonication essays were conducted. The protein morphology of constructs were further analyzed and viewed under the S.E.M.

Table 3.1. Oligonucleotide sequence of the VP2 epitopes.

OLIGONUCLEOTIDE SEQUENCE
19 amino acid Epitope 'a'
VP27A4F 5' P-TAAGAAGAAAGAAGAGGGTGAGGATGATACTGCTCGACAGGAGATAAGAAAAGCATGGCTGCAGG 3' *Nsi*I K K K E E G E D D T A R Q E I R K A W *Pst*I *BssH*II
VP27A4R 5' P-CGCGCCTGCAGCCATGCTTTTCTTATCTCCTGTCGAGCAGTATCATCCTCACCCTCTTCTTTCTTCTTATGCA 3' *BssH*II *Pst*I *Nsi*I
24 amino acid Epitope 'b'
VP242BF 5' P-AATTCCTCGAGGTAGACGTTGATCCAAATAAGGGTAAGTGGAAAGAACATATAAAAGAGGTAACCGAAAAATTA EcoRI XhoI V D V D P N K G K W K E H I K E V T E K L AAGAAAGCGG 3' K K A SalI
VP242BR 5' P-TCGACCGCTTTCAATAATTTTTCGGTTACCTCTTTTATATGTTCTTTCCACTTACCCTTATTTGGATCAACGTCTAC SalI CTCGAGG 3' XhoI EcoRI

3.3. Results

3.3.1. Epitope Insertion

The complimentary oligonucleotides for the neutralising epitopes of AHSV VP2 serotype 4, 'a' (19 aa) and 'b' (24 aa), were annealed and inserted respectively into amino acid site 177 and 144 of the pFastBac-VP7mt144/177/200 plasmid vector, creating VP7mt144/177-A/200 and VP7mt144-B/177/200. The recombinant construct containing the 'b' epitope at the 144 amino acid site, again underwent the cloning process as described above, this time inserting the 'a' epitope at the 177 amino acid site, creating VP7mt144-B/177-A/200 (Fig. 3.1.). The forward oligonucleotide sequence (Table 3.1.) for the 'a' epitope (VP27A4F) included a 5'-T overhang, whereas the complementary reverse oligonucleotide sequence (VP27A4R) contained a 3'-ATGCA overhang. These regions code for a digested *Nsi*I restriction endonuclease site, an isoschizomere of *Pst*I, meant to destroy the *Pst*I site of pFastBac-VP7mt144/177/200. Also included in these complementary oligonucleotide sequences for the 'a' epitope, was a new PstI endonuclease site next to a 3' overhang coding for *BssHII*, which binds to the *BssHII* digested pFastBac-VP7mt144/177/200. The oligonucleotide sequences for the 'b' epitope has the same type of overhangs, including 3' nucleotides for *Sal*I, an isoschizomere of *Xho*I, and an additional *Xho*I next to an overhang for EcoRI at the 5' end of forward of the sequence.

3.3.2. Nucleotide Sequence Determination and Hydropathy Predictions

The primers listed in table 2.1. were used during automated DNA sequencing (section 2.2.10) to determine the nucleotide sequence of the newly constructed VP7mt144/177-A/200, VP7mt144-B/177/200 and VP7mt144-B/177-A/200. The complete sequences, which confirmed the presence of the inserted epitopes into the constructs, were attained from overlapping sequences.

Nucleotide sequence determination did indicate two mutations in both the VP7mt144-B/177/200 (Appendix B-2) and VP7mt144-B/177-A/200 (Appendix B-3) constructs. The same two new mutations are present in both these constructs. This is probably due to the fact that VP7mt144-B/177/200 was used to create VP7mt144-B/177-A/200. At nucleotide position 514 and 515, two adenosines were substituted with two thymidines. This mutation occurs in the oligonucleotide sequence for the 'b' epitope and initially occurred in the synthesized oligonucleotide. Both of these changes occur in one codon, substituting a lysine for a leucine. This creates a drop in the

hydrophilicity value at this amino acid position from +3.0 to -1.8, according to the Hopp and Woods predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983). This small change in hydrophylicity could possibly have drastic effects on the stability of the structure, although, at this stage, it was unsure. It would also have had an effect on the generated immune response, if these constructs were to be used for immune binding studies.

Sequence comparisons and alignments were done using ClustalX version 1.81 (Higgins and Sharp, 1988; Higgins *et al.*, 1996) (Appendix B). Subsequent hydropathy analyses of the total recombinant structures were done (Fig. 3.2.) using the Hopp and Woods predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983) from the ANTHEPROT package (Geourjon *et al.*, 1991; Geourjon and Deleage, 1995).

Table 3.2. Hydrophilic characters the AHSV-4 epitopes (Hopp and Woods, 1981; Hopp and Woods, 1983).

Insert	**Amino Acid Amount**	**Hydrophilic Character of Insert**	
		Total	**Average**
Epitope 'a'	19	+158	+8.3
Epitope 'b'	24	+131	+5.5

As can be seen in table 3.2 and figure 3.2., both the epitopes are extremely hydrophilic. This is expected since, being presented on the surface of the AHSV particles, the are both in direct contact with the hydrophilic infected environment. The shorter nineteen amino acid 'a' epitope, with a hydrophilic character of +158, is far more hydrophilic than the twenty-four amino acid 'b' epitope, which only has a hydrophilic total of +131. The effect of these extreme hydrophilic insertions on the structure of VP7mt144/177/200 could at this stage not be predicted.

3.3.3. Baculovirus Expression

The Bac-to-Bac™ expression system was used to express the three recombinant pFastBac constructs. *E.coli* DH_{10}Bac™ cells were transformed with the pFastBac transfer vectors containing the constructs, where transposition of the recombinant VP7 genes occurred into the baculovirus genomes (2.2.13) Recombinant baculovirus genomes were selected for and the DNA isolated (2.2.14) before being transfected into Sf9 insect cells (2.2.15). The newly generated recombinant baculoviruses were then used in subsequent infections before protein expression analyses by SDS-PAGE gel electrophoresis (2.2.17)

The gel analysis (Fig. 3.3.) confirmed the expression of VP7mt144/177-A/200 (41 kDa), VP7mt144-B/177/200 (42 kDa) and VP7mt144-B/177-A/200 (44 kDa). VP7mt144/177-A/200 and VP7mt144-B/177-A/200 have much lower protein expression levels than VP7mt144/177/200 and VP7mt144-B/177/200. The 41 kDa VP7mt144/177-A/200 seems to have a much slower electrophoretic mobility rate than the 42 kDa VP7mt144-B/177/200, causing it to appear higher on the SDS-PAGE gel analysis. Another interesting phenomenon is that all three the new recombinants form double protein bands.

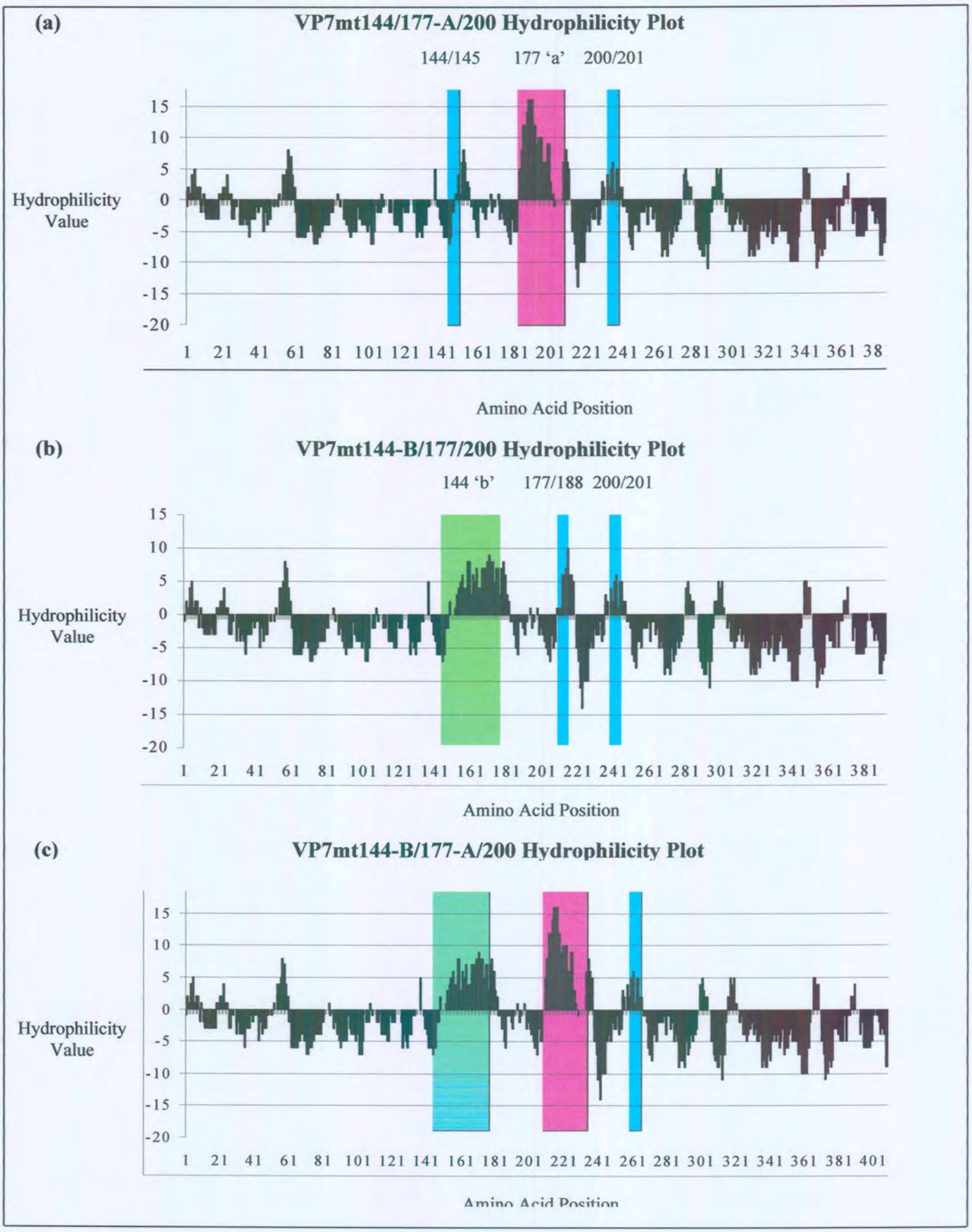


Figure 3.2. Hydrophilicity Plots of (a) VP7mt144/177-A/200, (b) VP7mt144-B/177/200 and (c) VP7mt144-B/177-A/200 according to the Hopp and Woods Predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983)

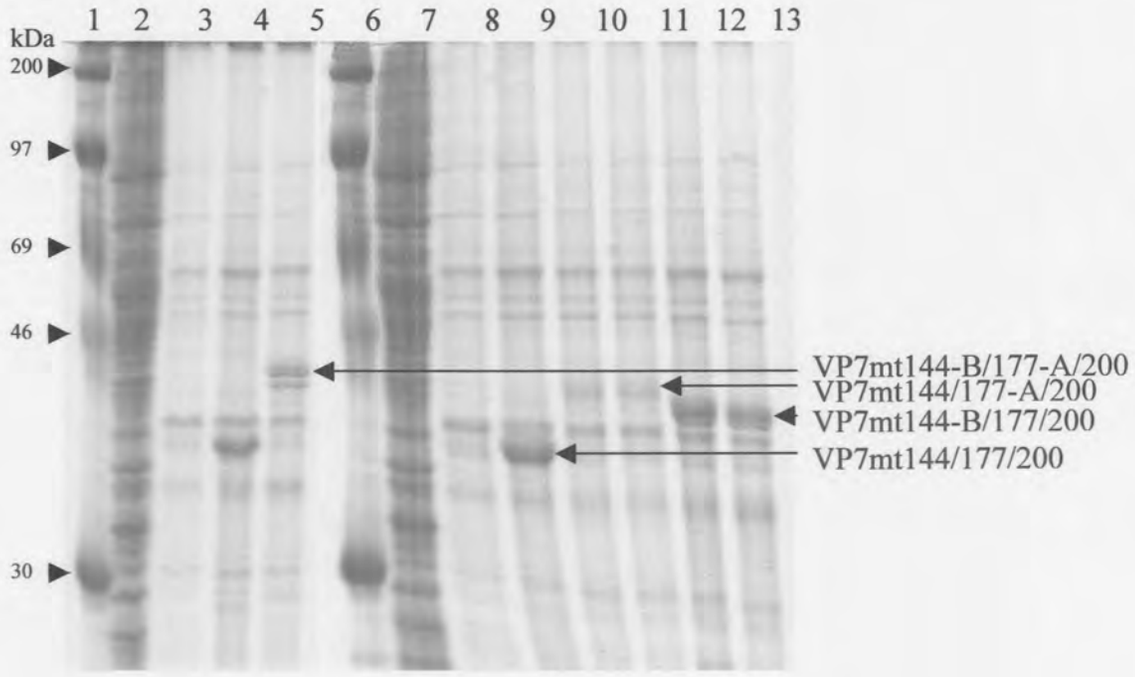


Figure 3.3. Analysis of the newly created epitope mutants compared to VP7mt144/177/200 on a 15% SDS-PAGE gel. Lanes 1 and 6 represent the protein size marker. Lanes 2 and 7 contain proteins from a mock infection, whereas lanes 3 and 8 contain wild-type baculovirus-infected cells lysates. Lanes 4 and 9 contain expressed VP7mt144/177/200 (39 kDa). Lane 5 contains the 44 kDa VP7mt144-B/177-A/200 protein. Lanes 10 and 11 contain the 41 kDa VP7mt144/177-A/200 protein, whereas lanes 12 and 13 contain the expressed VP7mt144-B/177/200 protein (42 kDa).

3.3.4. Purification of Recombinant VP7 Particles on Sucrose Gradients

Changes in solubility and stability that might have been caused by the insertion of the two epitopes at amino acid position 144 and 177 of VP7mt144/177/200 were studied. VP7mt144/177-A/200, VP7mt144-B/177/200 and VP7mt144-B/177-A/200 were expressed in Sf9 cells by recombinant baculoviruses containing the respective genes. Cells were lysed 72 h post-infection, and the

recombinant VP7 proteins were harvested and purified by rate zonal centrifugation on 50%-70% discontinuous sucrose gradients. SDS-PAGE gels were used to analyse the proteins in the fractions of the sucrose gradients and the intensities were quantified using Sigma Gel™ software program (Jandel Scientific). VP7mt144/177/200 was used as a standard control during all of these studies. Sonication studies were also done, for not only is it a method to remove protein aggregation, but it also serves as another stability study when compared to control sonicated VP7mt144/177/200.

Figure 3.4. depicts the comparative protein particle distribution of VP7mt144/177-A/200. Of the unsonicated VP7mt144/177-A/200 particulate distribution (Fig. 3.4.a), 83% of the protein occurs in the pellet of the gradient. This is 28% more than the percentage of VP7mt144/177/200 proteins in the pellet. The large particulate VP7mt144/177-A/200 structures in the first three fractions, yield approximately the same amount of proteins as the small particulate structures found in the last three fractions (7.6%). This is in contradiction with VP7mt144/177/200 where all the structures formed by VP7mt144/177/200 and not aggregated in the pellet, are large and occur in the bottom fractions of the sucrose gradients. The VP7mt144/177/200 construct forms no small particulate structures, which might be free soluble trimers, in the last fraction like VP7mt144/177-A/200. The sonicated products of the two constructs resemble each other except that VP7mt144/177-A/200 seems to form a more defined size structure after sonication than VP7mt144/177/200, as is indicated by the peak in the third fraction.

VP7mt144-B/177/200, which is longer yet less hydrophilic than VP7mt144/177-A/200, seems to be more soluble than with far less aggregation when compared to VP7mt144/177/200 (Fig. 3.5.). Only 17% of this protein occur in the pellet of the gradient. Most of the proteins for this construct (52%) occur in the first four fractions of the gradient, whereas the same fractions for VP7mt144/177/200 contain only 36.4% of the total amount. VP7mt144-B/177/200 also has a higher amount of small particles in the last four soluble fractions of the gradient (19.1%). The sonication product resembles that of VP7mt144/177-A/200 with the same, but higher, distinct peak in the third fraction from the bottom of the gradient. This is indicative of a size formation preference displayed by the construct for smaller particulate structures with a roughly constant size.

The simultaneous effect of inserting both the epitopes into VP7mt144/177/200 can be seen when analyzing the solubility of VP7mt144-B/177-A/200 (Fig. 3.6.). The protein particulate distribution of unsonicated VP7mt144-B/177-A/200 closely resembles that of VP7mt144/177/200. The pellet containing the aggregated proteins as well as the insoluble bottom fractions contain approximately the

same protein levels. The particulate protein distribution of VP7mt144-B/177-A/200, however, contains 5% small particles in the last three soluble fractions compared to only 1% as formed by VP7mt144/177/200. Sonication of the VP7mt144-B/177-A/200 results in the same protein particle distribution as VP7mt144/177-A/200 and VP7mt144-B/177/200.

3.3.6. Scanning Electron Microscopy (S.E.M.)

The second, third and fourth fractions of the protein samples were pooled and prepared for S.E.M study. The same structures as formed by VP7mt144/177/200 (Fig 2.13.c and d) are also being formed by VP7mt144/177-A/200 (Fig. 3.7.a. and b.), VP7mt144-B/177/200 (Fig. 3.7.c. and d.) and VP7mt144-B/177-A/200 (Fig. 3.7.e. and f.). Most of the structures have approximately the same size with a rough, flat and almost circular appearance. Web-like tentacles/ cables can also be observed where clusters of particulate structures occur on the grid (Fig. 3.7.d. and f.).

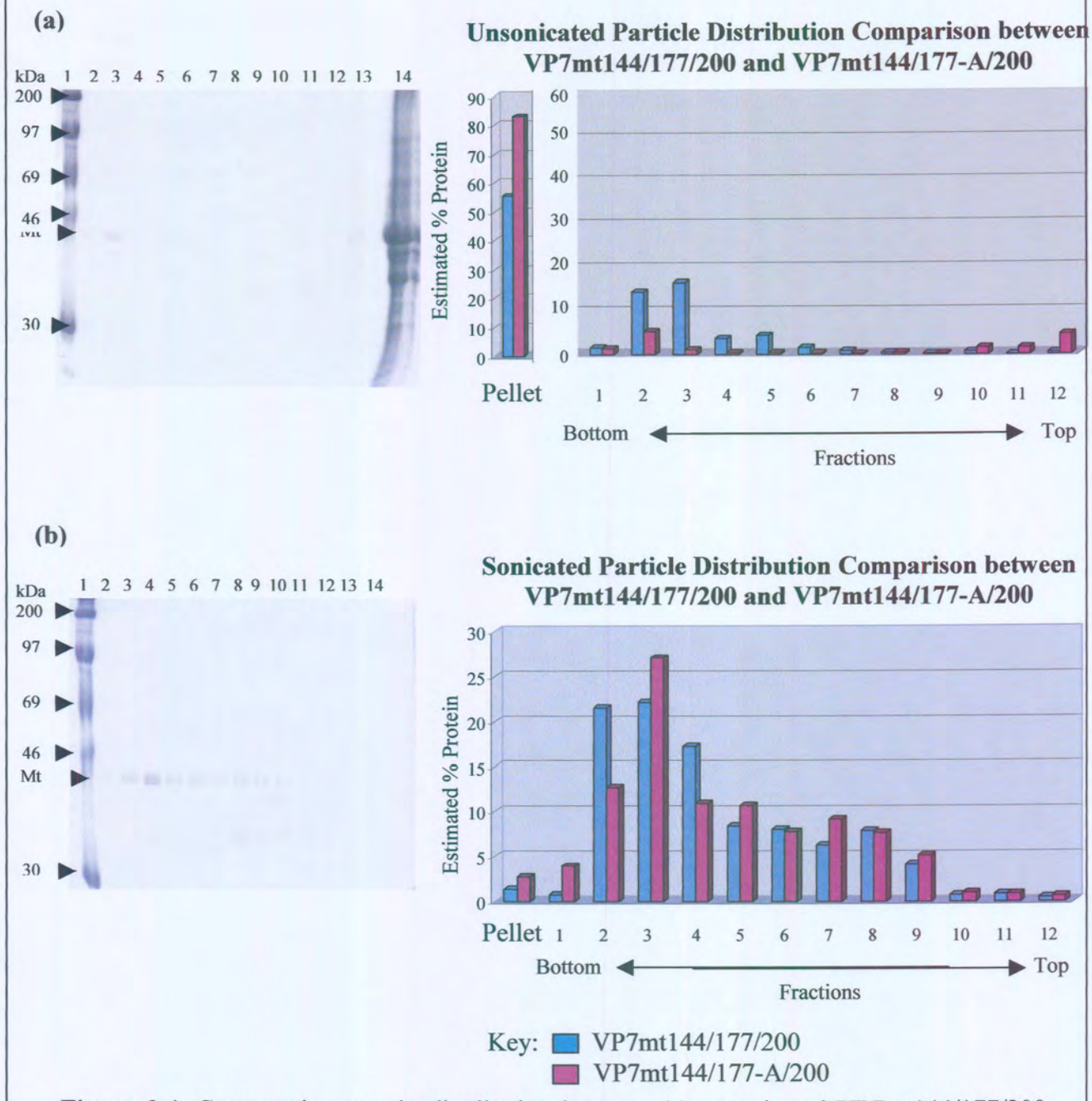
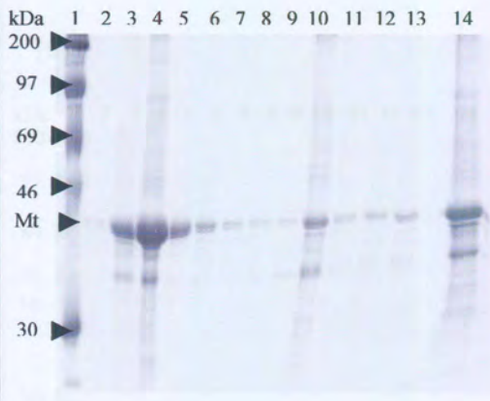
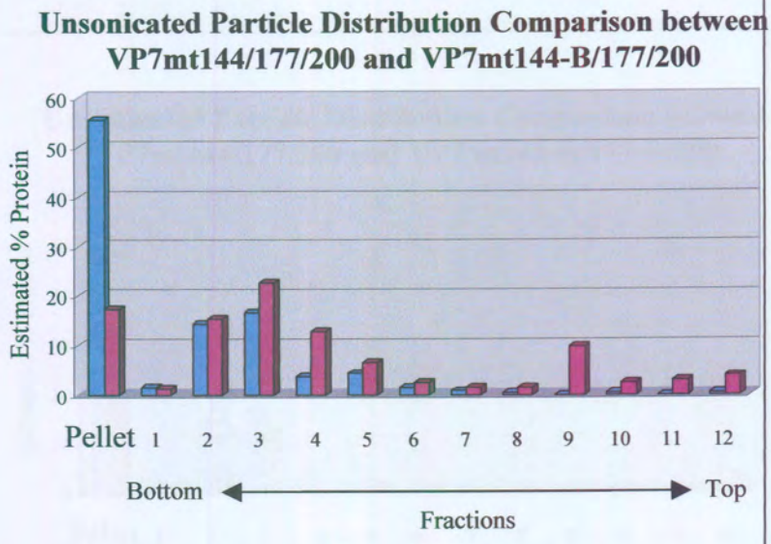


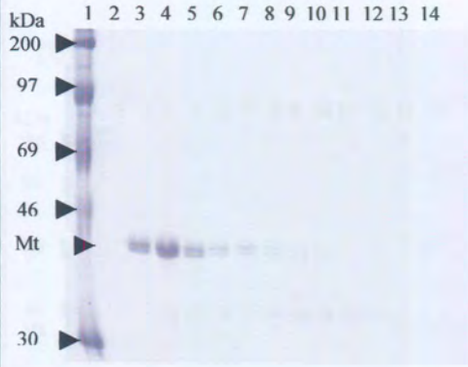
Figure 3.4. Comparative protein distribution between (a) unsonicated VP7mt144/177/200 and VP7mt144/177-A/200. (b) Represents a comparative protein distribution between 25 pulse sonicated pellets of VP7mt144/177/200 and VP7mt144/177-A/200. A Sorvall AH-650 rotor was used at 12000 rpm for 75 min at 4°C to centrifuge the proteins on a 50%-70% sucrose gradient. SDS-PAGE electrophoresis was used to analyze the collected fractions and quantification of the fractional distribution was done using the Sigma Gel™ analysis program. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

(a)





(b)



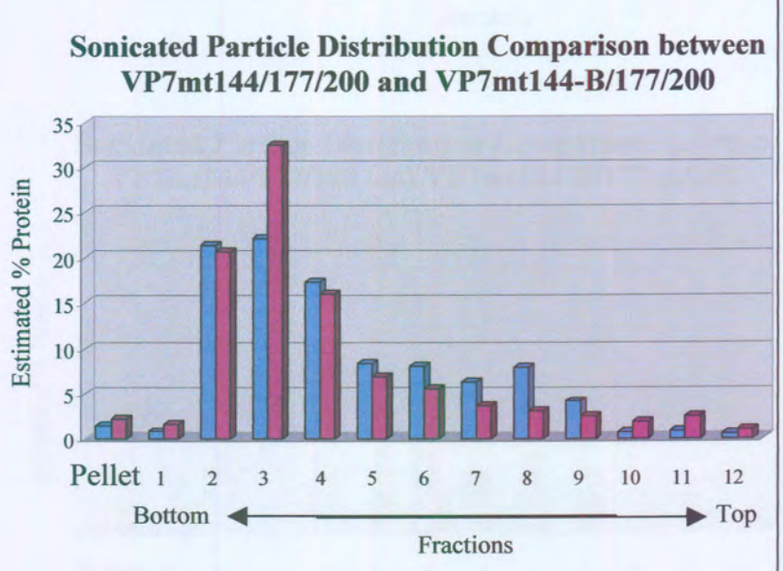


Figure 3.5. Comparative protein distribution between (a) unsonicated VP7mt144/177/200 and VP7mt144/177-B/200. (b) Represents a comparative protein distribution between 25 pulse sonicated pellets of VP7mt144/177/200 and VP7mt144/177-B/200. Centrifugation of the proteins proceeded on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C. This was followed by SDS-PAGE electrophoresis analyses, quantification by the Sigma Gel™ analysis program and conversion to the shown graphic form. Lane 1 on the SDS-PAGE gel contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

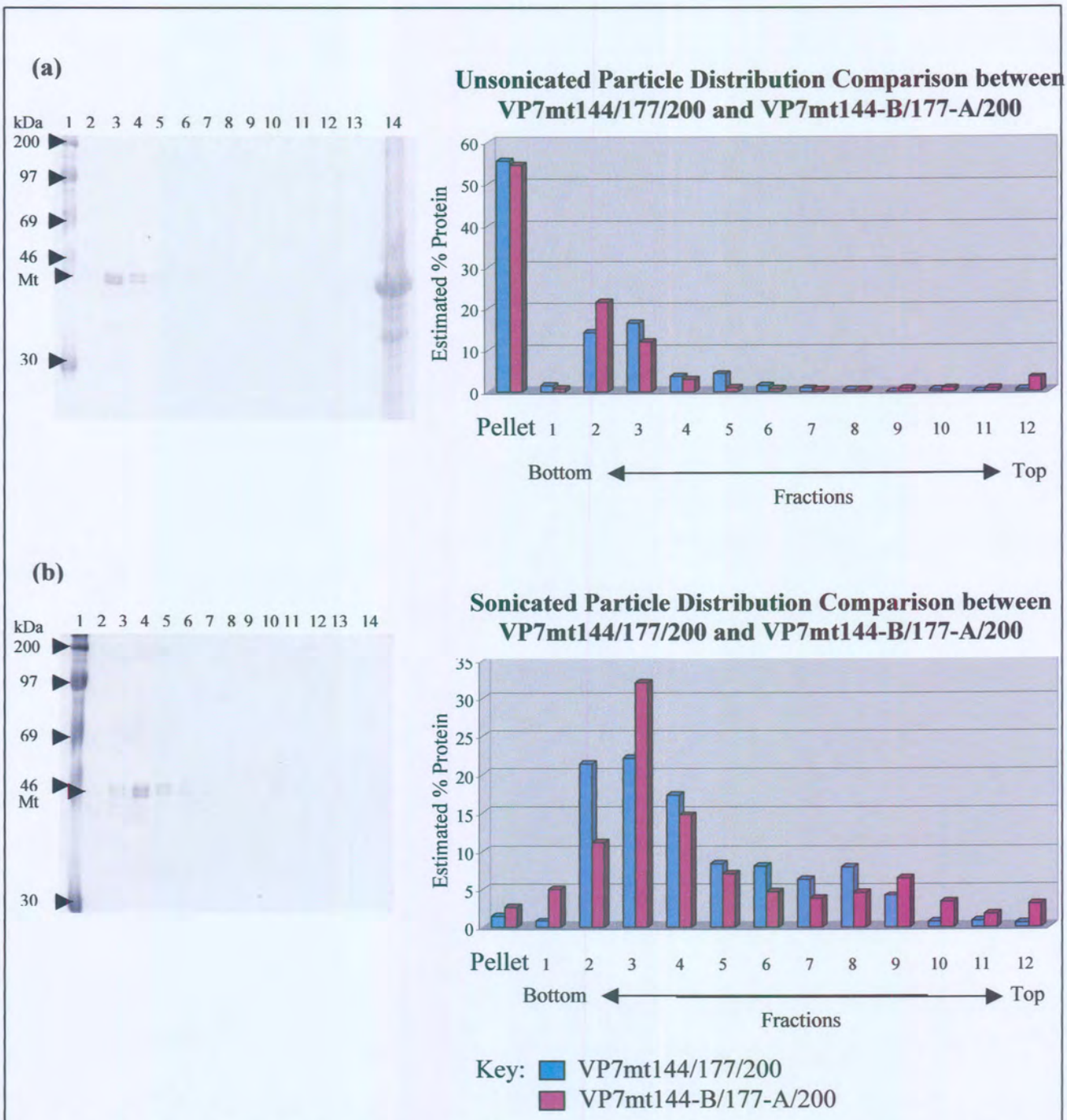


Figure 3.6. Comparison of protein fractional distribution between (a) unsonicated VP7mt144/177/200 and VP7mt144-B/177-A/200 and (b) 25 pulse sonicated pellets of VP7mt144/177/200 and VP7mt144-B/177-A/200. Proteins were centrifuged on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker. Lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients, whereas lane 14 represents the protein pellet of the gradient.

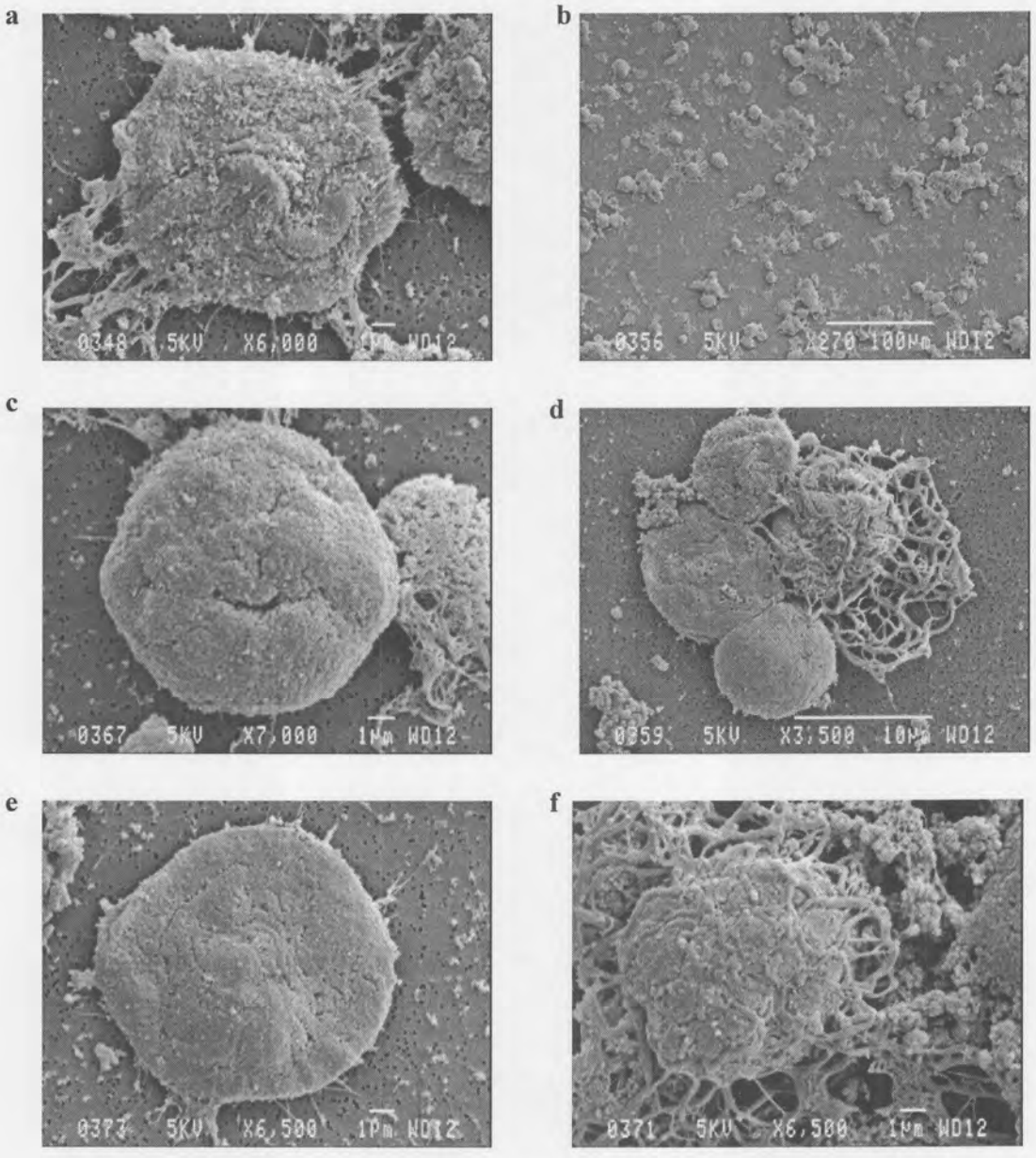


Figure 3.7. Scanning Electron Microscopy photographs depicting the structures formed by the different protein constructs: (a) VP7mt144/177-A/200 and a wide-angle view in (b). (c) Depicts VP7mt144-B/177/200 with a wide-angle view in (d). (e) And (f) are structures formed by VP7mt144-B/177-A/200.

3.4. Discussion

The possibility to efficiently present different epitope-containing peptides at multiple sites on a single subunit vaccine has definite advantages. The major advantage being that each epitope is individually presented to the immune system without the inhibition that another epitope, located next to the first, might cause. It also presents the possibility of presenting different peptides that could provide protection against different diseases or different serotypes of the same virus on a single subunit vaccine. As can be seen in chapter two, the insertion of three multiple cloning sites into VP7 resulted in morphological changes on the crystal structures formed by VP7. The insertion of twenty-two amino acids into modified VP7 containing only a single multiple cloning site at amino acid position 177 had almost no morphological effects on the crystal structures formed by VP7 (Meyer, 2002). This prompted a study into the effect of inserting larger regions into at least two of the three multiple cloning sites in VP7mt44/177/200.

The two neutralising epitopes of AHSV-4 VP2, 'a' and 'b', were chosen for insertion at amino acid site 177 and 144 respectively. The nineteen amino acid 'a' epitope is more hydrophilic than the twenty-four amino acid 'b' epitope (Table 3.2), the effect of which could not be determined until after solubility studies were conducted. The two constructs with the single epitope insertions, VP7mt144/177-A/200 and VP7mt144-B/177/200, not only served as controls for the double construct, VP7mt144-B/177-A/200. They also functioned as studies into the effects caused by larger amino acid single insertions into VP7mt144/177/200 at the two respective multiple cloning sites.

The insertion of the hydrophilic 'a' epitope at position 177 of VP7mt144/177/200 had a definite change on the solubility of the structure (Fig. 3.4.). There was a large increase in aggregation of the assembled protein units and an increase in the smaller particles, possibly free soluble trimers. This is possibly due to the tearing open of the close-knit hydrophobic structural units by the hydrophilic insertions. More hydrophobic regions are exposed to the surface of a structural unit, causing the units to aggregate. The increase in free trimers could be a direct effect of the increase in solubility by the addition of the extremely hydrophilic 'a' epitope.

The insertion of the 'b' epitope into position 144 of VP7mt144/177/200 to create VP7mt144-B/177/200 lowered the aggregation of the structural units normally formed by VP7mt144/177/200

(Fig. 3.5.). The effect on the overall structure an insert has is not only dependent on the characteristics of the insert but also the position the insertion is made. The effect that this twenty-four amino acid hydrophilic insert had on the structure of VP7mt144/177/200 might be because of both the insert position and the characteristics of the insert. The aggregational tendency of the units was lowered probably because of a tightening of the structure around possible hydrophobic regions. The insert also increased the overall hydrophilicity of the protein unit, as can be seen in the decrease in aggregation and the greater amounts of smaller particles that occur in the top of the sucrose gradients

The construct containing both the epitopes, VP7mt144-B/177-A/200, has an intermediate aggregational tendency of the particle structures when compared to the two previously discussed constructs (Fig. 3.6.). The structural effect caused by the insertion of the 'a' epitope at site 177 of VP7mt144/177/200 is neutralised by the insertion of the 'b' epitope at the 144 amino acid site. This results in VP7mt144-B/177-A/200's solubility being almost the same as the protein fractional distribution of VP7mt144/177/200. The insertion of the peptides resulted in a reduction in the size of the particles as seen by the increase in small particles found at in the top fractions of the gradients.

As can be seen from the sonication studies, the structural units can be freed from the aggregation resulting in units with a distinct size. The administered sonication pulses were not enough to reduce the structures to free trimers in the soluble fractions of the gradient. The electron microscopy studies (Fig. 3.7.) supported the evidence pointing to retention of the structure formed by VP7mt144/177/200, by all three the new constructs.

Chapter 4

The Effect of Various Lengths of Viral Protein 2 of African Horse Sickness Virus on Modified Viral Protein 7.

4.1. Introduction

The major disadvantage of virus like particles as antigen carriers, is the limitation in the size of the peptides that can be inserted. In order to utilise the full potential of the VP7 particles effectively as an antigen presentation system; the structure must be able to accommodate large epitope-containing insertions. As shown in chapter 2, the insertion of three multiple cloning sites in VP7mt144/177/200 had an effect on the morphology and appearance of the VP7 crystalline particles but it did not appear to prevent the protein from assembling into particles that could be purified on sucrose gradients. These findings were the same for the small peptides that were inserted into the 144 and 177 amino acid sites of VP7mt144/177/200. It is therefore assumed that the proteins' ability to assemble into trimers have not been destroyed in any of the cases. To further investigate the ability of VP7 to accommodate the insertion of a large range of hydrophilic amino acid peptides we inserted a range of peptides of diffirent sizes in amino acid site 177 of the vector. These peptides overlapped the previously characterised neutralising epitopes on major outer capsid protein VP2 of AHSV serotype 4. The nature of VP2 neutralising domains and epitopes makes it a suitable candidate for investigating the ability of VP7 to accommodate a range of hydrophilic peptides of various sizes.

It is also important to construct a range of different sequence inserts since it has been found that subunit vaccines are dependant on the characteristics of the insert. In other words, the subunit vaccines could retain structure with large inserts but would lose structure with a much smaller but different insert. This was best illustrated by the research done on non-structural protein 1 (NS1) of BTV. This protein, which forms tubules when expressed in insect cells, has been investigated as a possible subunit epitope presentation vaccine. Extension mutants of foreign antigenic sequences involving up to 16 amino acids added to the C terminus of NS1 were shown to form tubules, although an extension of 19 amino acids inhibited tubule formation (Monastryrskaya *et al.*, 1995). Later it was found that the 64 kDa NS1 was still able to form the tubules even after being fused to a green fluorescent protein with an approximate size of 31 kDa (Ghosh *et al.*, 2002).

4.2. Materials and Methods

Materials

Dr. M van Niekerk provided the TOPO vector containing AHSV-4 VP2. Other reagents were obtained from the suppliers described in section 2.2.

4.2.1. Polymerase Chain Reaction

Polymerase chain reaction was used to amplify the VP2 fragments from the obtained VP2-containing TOPO vector. Primers, containing the necessary attached restriction endonuclease sites (Table 4.1), were designed to anneal at specific position on the VP2 template, which would produce the required lengths of amplified fragments. All PCR reactions were carried out using reagents described in section 2.2.1

The amplification conditions for all four reactions were as follows: 1 cycle at 94°C for 2 min; 25 cycles 93°C for 45 sec, 62°C for 1 min, 72°C for 2 min; 1 cycle at 72°C for 10 min.

Table 4.1. Primer sequences used in PCR of the VP2 fragments.

PRIMER NAME	DIRECTED POSITION	OLIGONUCLEOTIDE SEQUENCE	Tm °C
Different Forward Primers			
VP100F	Nucleotides 931-960	5' CACCTGCAGTTTGATTTTTTGACAACATTCGTTCATGCG 3' *Pst*I	59
VP150F	Nucleotides 781-814	5' CACCTGCAGAAAGGTCCACTGAATGACTTACGAGTTAAAATTG 3' *Pst*I	61
VP200F	Nucleotides 631-660	5' CACCTGCAGAAGCTGAGATTTGGAATGATGTACCCACAC 3' *Pst*I	63
VP250F	Nucleotides 481-510	5' CACCTGCAGGAGAGTAAGAGAAAAGCAATCCTTGATCAG 3' *Pst*I	62
Single Reverse Primer			

VP2EPIR	Nucleotides 1206-1230	5' GTTGCGCGCTTGGCATGGTTGTCCTCCATTTTCG 3' *BssH*II	63

4.2.2. Cloning of VP2 Regions into the Modified VP7mt144/177/200 Construct and Expression of the Recombinant Proteins

Cloning of the VP2 amplified fragments into pFastBac-VP7mt144/177/200 construct was achieved by methods described in chapter 2, with one variation. The ligation of the separate fragments to the digested and purified pFastBAC-VP7mt144/177/200 proceeded at 16 °C for 16 h. A 3:1, ratio of insert: vector molecules was used in the ligation mixture.

Subsequent transformation yielded colonies containing the recombinant plasmids. DNA samples were analyzed by the electrophoretic separation of DNA on 4% agarose gels. The purified DNA of the four recombinant constructs was sequenced before being used in a transposition reaction to form composite bacmid DNA for use during transfection and the creation of the recombinant viruses. Protein expression was analyzed on a 15% SDS-PAGE gels before solubility and sonication essays were conducted. The protein morphology of constructs were further analyzed and viewed under the S.E.M.

4.3. Results

The TOPO vector containing VP2 of AHSV-4, as provided by Dr. M van Niekerk, served as template for the amplification of the four inserts. All of these inserts, the 100 aa, 150 aa, 200 aa and the 250 aa amplified regions, contained both the neutralising epitopes for AHSV VP2 serotype 4. It was thought that any effect such large inserts might have on the structure of the VP7mt144/177/200 construct, would be more subdued if the inserts were cloned into the centrally located 177 amino acid site. This multiple cloning site was constructed in the middle of a highly flexible RGD containing loop. Also, further support for this choice came from the research conducted by Meyer, 2002, as mentioned in section 2.4., which showed that AHSV VP7 was still able to form crystals when a twenty-two amonio acid insert had been made at amino acid position 177.

4.3.1. Synthesis of the Size-Constructs

The cloning procedure of the four inserts is outlined in figures 4.1 and 4.2 and were all individualistically done in one step. The primers (Table 4.1.) used for the amplification of the four fragments added a 5' *Pst*I restriction enzyme site and a 3' *Bss*HII site to the fragments. The four obtained PCR products, 318 bp, 468 bp, 618 bp and 768 bp, were subjected to a double separate digestion with the *Pst*I and *Bss*HII restriction endonucleases and purified via the High Pure™ PCR Product Purification Kit (Roche Diagnostics). The fragments were then separately cloned into the *Pst*I and *Bss*HII sites of the digested and cleaned pFastBac-VP7mt144/177/200 plasmid vector. This gave rise to four new constructs: VP7mt144/177-100/200, VP7mt144/177-150/200, VP7mt144/177-200/200, VP7mt144/177-250/200. The four constructs were digested with *Eco*RI and *Xba*I (Fig. 4.3.) to confirm the insertion of the fragments before nucleotide sequence determination.

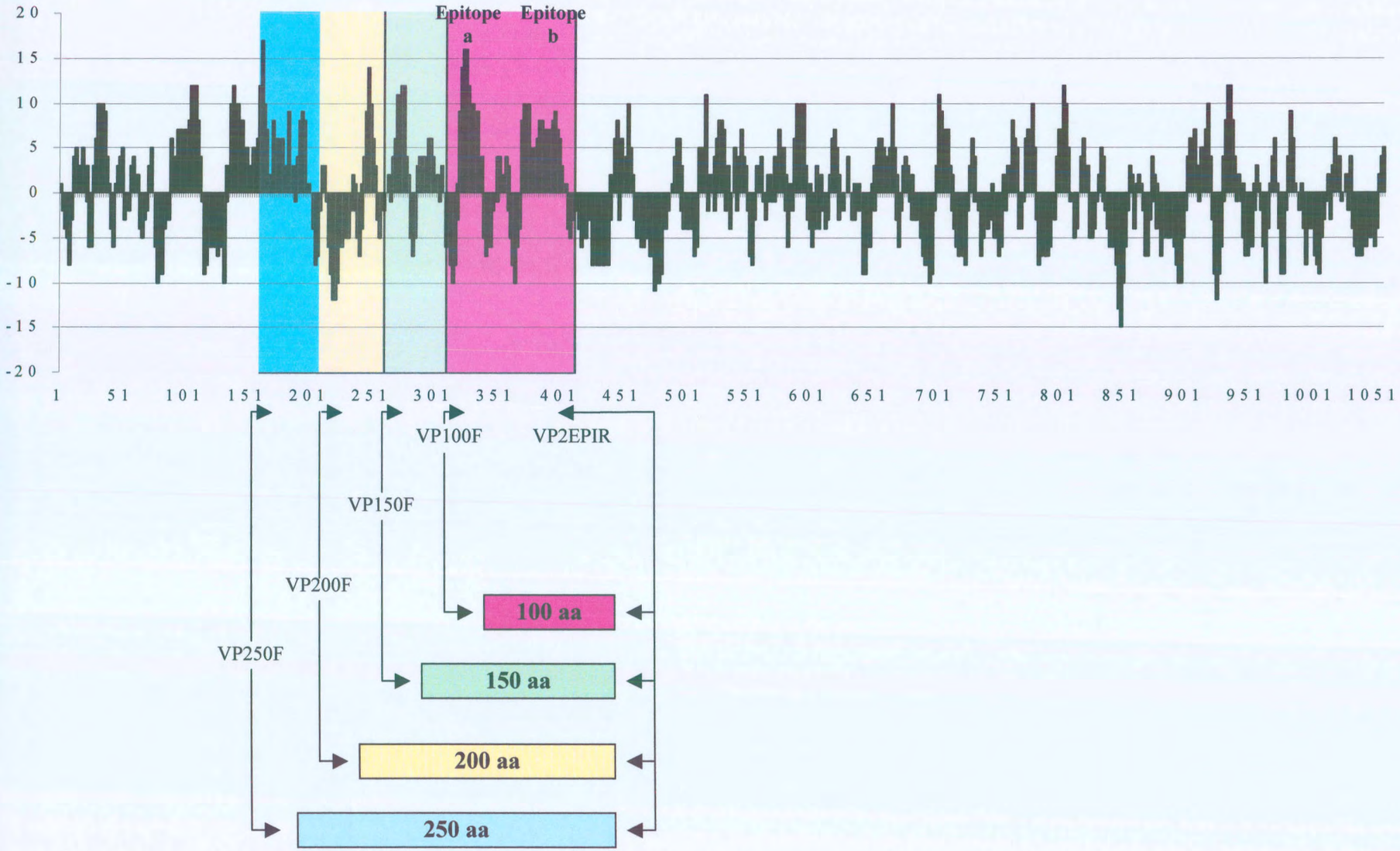


Figure 4.1. Hydrophilicity Plot of AHSV VP2 (Hopp amd Woods, 1981; Hopp and Woods, 1983). Indicated are the PCR primer binding positions and the amplified regions, as well as the positions of the a and b epitopes of VP2.

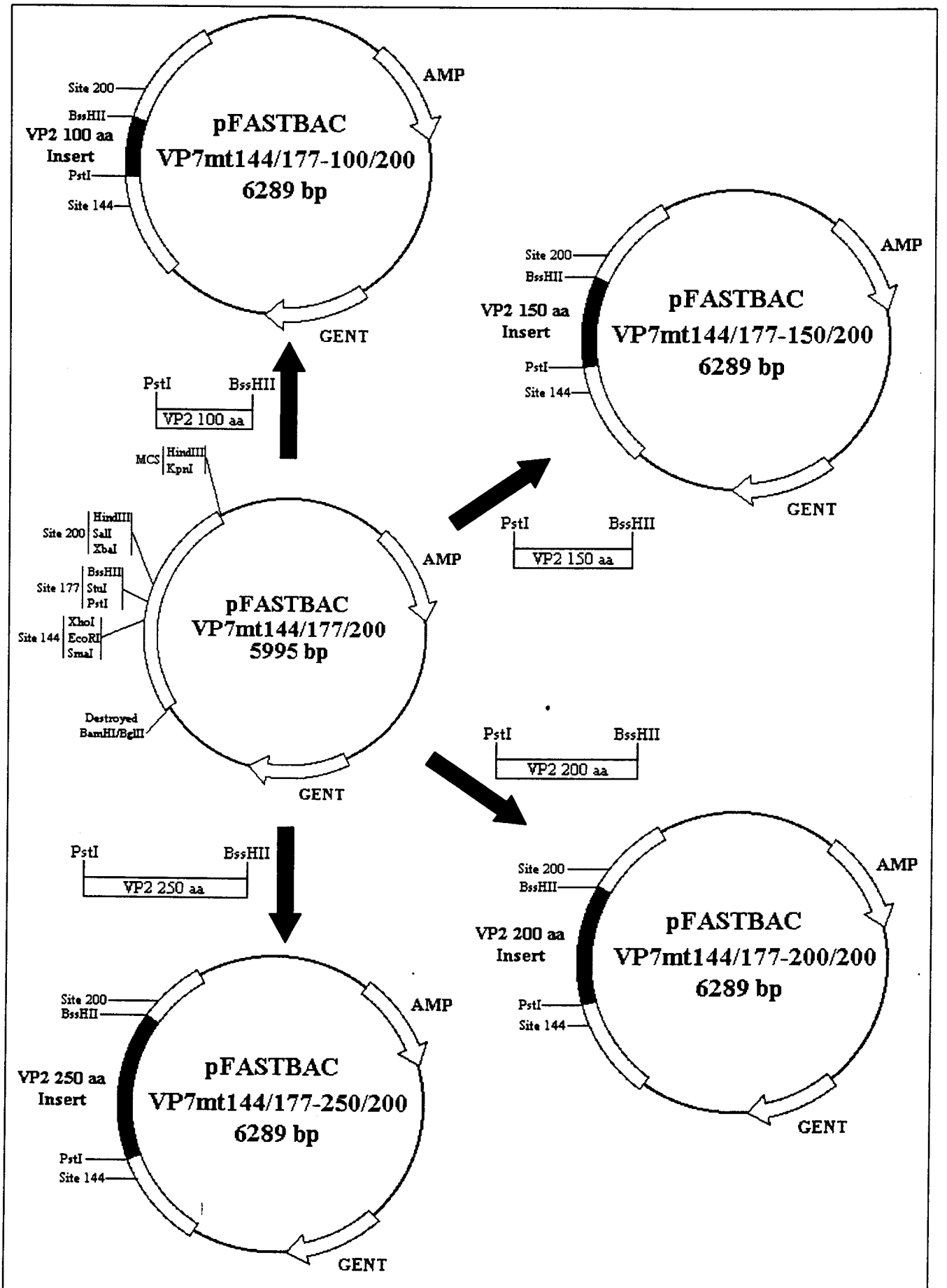


Figure 4.2. Insertion of different size fragments into the 177-site of pFastBac-VP7mt144/177/200

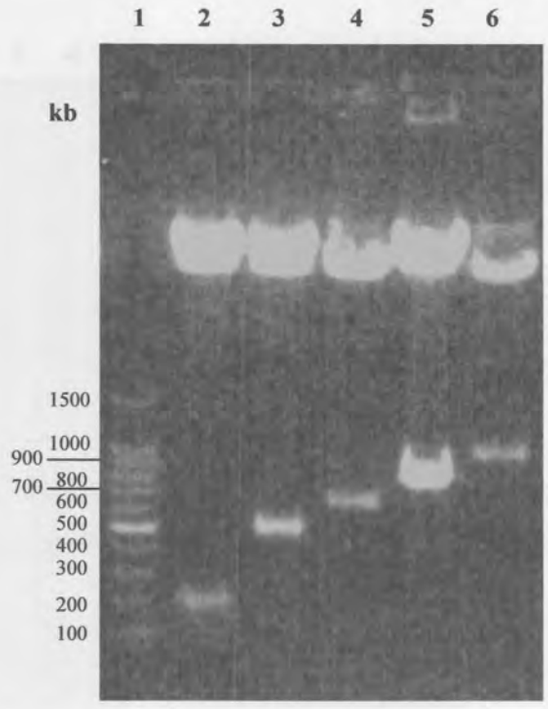


Figure 4.3. 4% Agarose gel electrophoresis of VP7mt144/177/200-insertion mutant digestions. Lane 1 represents the 100 bp DNA Ladder. Lane 2 contains VP7mt144/177/200 digested with *Eco*RI and *Xba*I give rise to linear plasmid DNA and a 204 bp fragment. Lanes 3 to 6 contain VP7mt144/177-100/200, VP7mt144/177-150/200, VP7mt144/177-200/200 and VP7mt144/177-250/200, respectively, digested with *Eco*RI and *Xba*I, which resulted in linear plasmid DNA and 498 bp, 648 bp, 798 bp and 948 bp respective size fragments.

4.3.2. Nucleotide Sequence Determination and Hydropathy Predictions

The primers listed in table 2.1. and table 4.1. were used during automated DNA sequencing (section 2.2.10) to determine the nucleotide sequence of the newly constructed VP7mt144/177-100/200, VP7mt144/177-150/200, VP7mt144/177-200/200, and VP7mt144/177-250/200. Overlapping sequences for each of the four constructs were generated from which the complete gene sequences

could be deduced. In each of the four cases, the sequencing confirmed the presence of the VP2 insert at the 177 position of the VP7mt144/177/200 construct (Appendix C).

No new mutations were generated by the PCR reactions during the synthesis of the inserts, except in VP7mt144/177-100/200. At nucleotide position 557, a guanidine was changed to a thymidine. This was due to a mistake that was made in the VP100F primer because of a faulty template sequence. The substitution resulted in a subsequent change in amino acid 186 from a cysteine to a phenylalanine. This creates a drop in hydrophilicity at that amino acid position from -1.0 to -2.5. This appears to be a rather insignificant change as compared to the hundred foreign amino acids, each with its own charge, that have been inserted into the VP7mt144/177/200 construct.

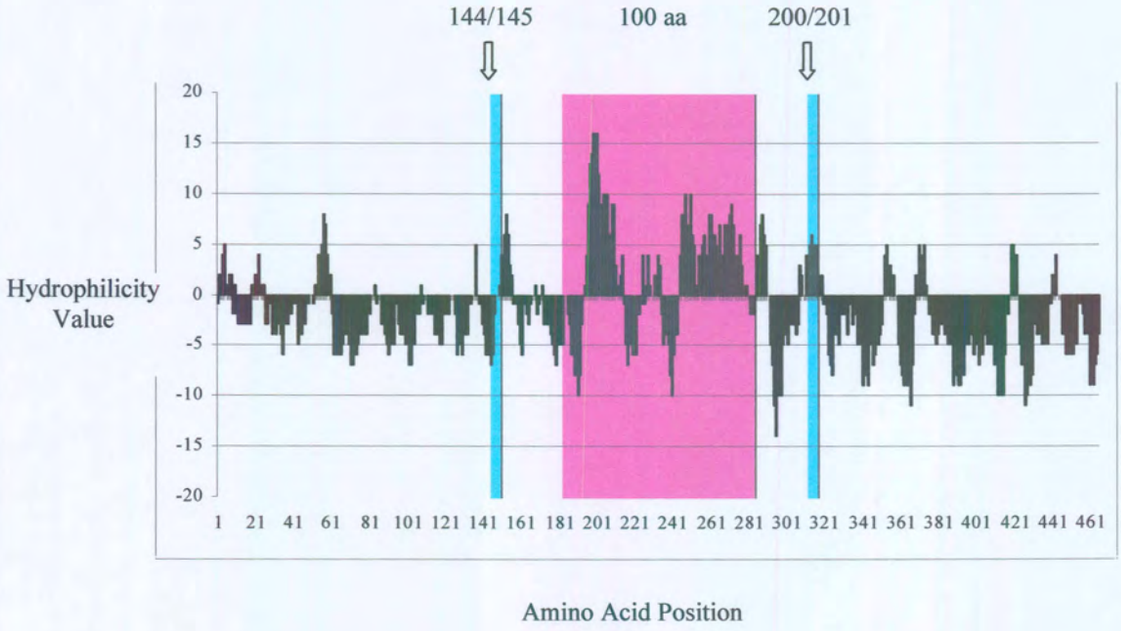
ClustalX version 1.81 (Higgins and Sharp, 1988; Higgins *et al.*, 1996) was used to do the sequence comparisons and alignments (Appendix C.). Subsequent hydropathy analyses were done (Fig. 4.4.) using the Hopp and Woods predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983) from the ANTHEPROT package (Geourjon *et al.*, 1991; Geourjon and Deleage, 1995).

Table 4.2. Hydrophilic character AHSV-4 VP2 amplified regions (Hopp and Woods, 1981; Hopp and Woods, 1983).

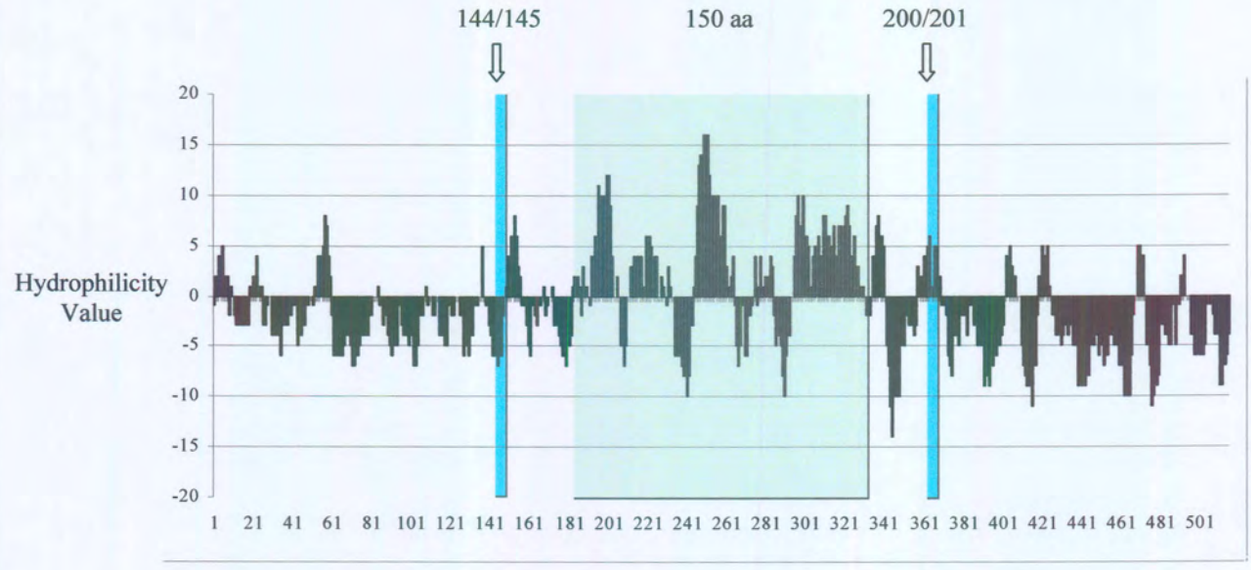
Insert	Hydrophilic Character of Insert	
	Total	**Average**
VP2 100 aa	+228	+2.28
VP2 150 aa	+366	+2.44
VP2 200 aa	+279	+1.395
VP2 250 aa	+425	+1.7

Each of the inserts has a unique overall hydrophilic character, as shown in Table 3.2. All four of the inserts are overall hydrophilic in nature. The degree of hydrophilicity increases with the size of the insert with the exception of the 200 aa insert which is less hydrophilic than the 150 aa insert. This is due to the fact that the 200 aa insert has a large number of hydrophobic regions in the last 50 aa (Appendix C-4), which lowers the average hydrophilic character for the 200 aa and 250 aa inserts. The hydrophilic domains of the inserts are presumed to contain a number of the neutralising epitopes of AHSV VP2 serotype 4.

(a) **VP7mt144/177-100/200 Hydrophilicity Plot**



(b) **VP7mt144/177-150/200 Hydrophilicity Plot**



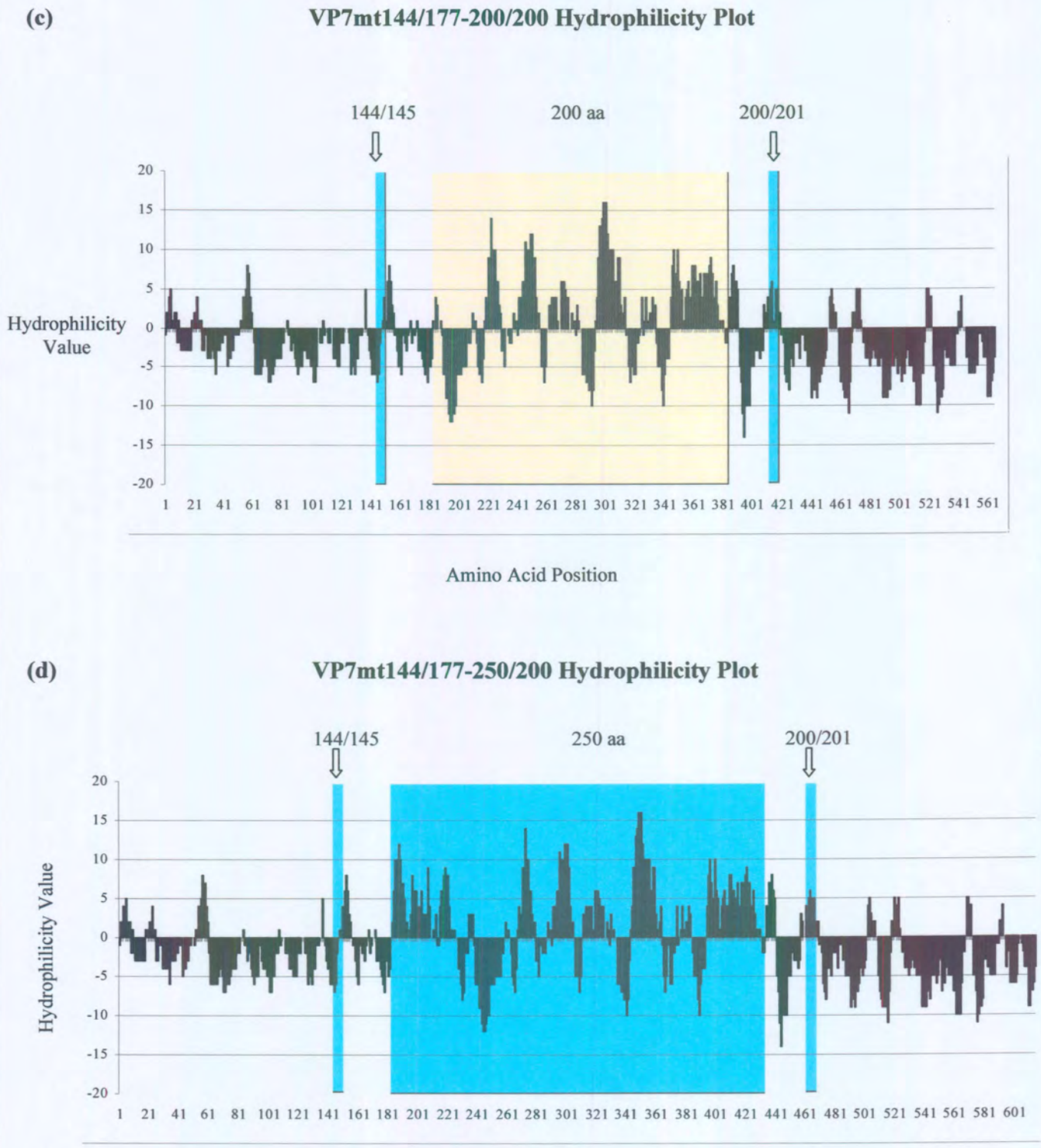


Figure 4.4. Hydrophilicity Plots of (a) VP7mt144/177-100/200, (b) VP7mt144/177-150/200, (c) VP7mt144/177-200/200 and (d) VP7mt144/177-250/200 according to the Hopp and Woods Predictive method (Hopp and Woods, 1981; Hopp and Woods, 1983).

4.3.3. Baculovirus Expression

The four constructs were expressed using the Bac-to-Bac™ expression system. The pFastBac transfer vectors containing the recombinant constructs were transformed in the *E.coli* DH_{10}Bac™ cells, which contain the baculovirus genome. Transposition of the mutant VP7-VP2 genes occurred into the baculovirus genomes (2.2.13), which were later selected for and isolated (2.2.14). Sf9 insect cells were transfected with the recombinant bacmid DNA (2.2.15) followed by infections with the newly generated recombinant baculoviruses.

SDS-PAGE gel electrophoresis (2.2.17) was used to verify protein expression. The gel analysis (Fig. 3.5.) clearly indicates the expression of VP7mt144/177-100/200 (50 kDa), VP7mt144/177-150/200 (56 kDa), VP7mt144/177-200/200 (62 kDa) and VP7mt144/177-250/200 (68 kDa). Although there is a marked decrease in protein expression of the four new recombinant proteins are compared to the expression levels attained by VP7mt144/177/200, the VP7 recombinants are nevertheless still expressed in relatively large amounts. It seems as if the recombinant with the 250 aa insert is expressed better than the three other recombinants, although this might only be an isolated incidence.

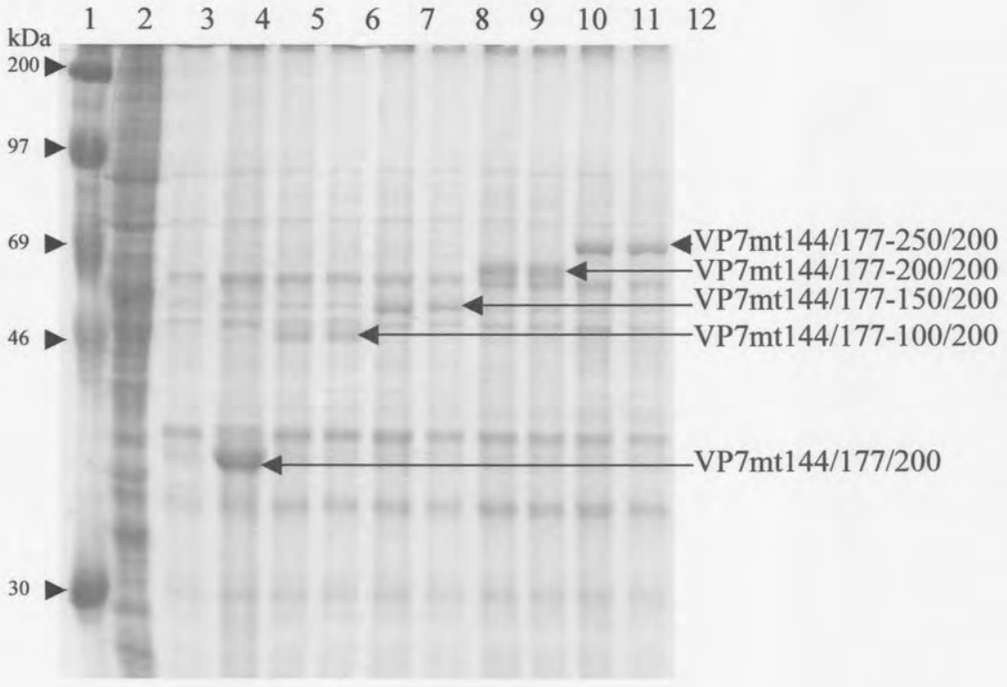


Figure 4.5. A 15% SDS-PAGE analysis of VP7mt144/177/200-insertion mutants' expression. Lane 1 represents the protein size marker. Lane 2 and 3 contain proteins from a mock infection and wild-type baculovirus-infected cells respectively. Lane 4 contains expressed VP7mt144/177/200 (39 kDa). Lanes 5 and 6 contain the 50 kDa VP7mt144/177-100/200 protein, whereas lanes 7 and 8 contain the 56 kDa VP7mt144/177-150/200 protein. Lanes 9 and 10 contain the VP7mt144/177-200/200 protein (62 kDa) and, cell lysates of cells infected with the VP7mt144/177-250/200 construct (68 kDa), are shown in lanes 11 and 12.

4.3.4. Purification of VP7 Particles on Sucrose Gradients

Sucrose gradient analysis was carried out to investigate whether the insertions that have been made, affected the formation, sedimentation and purification of the structure formed by the modified VP7mt144/177/200 mutant. Sf9 cells were infected with the recombinant baculoviruses expressing the VP7mt144/177-100/200, VP7mt144/177-150/200, VP7mt144/177-200/200 and VP7mt144/177-250/200 constructs. The proteins were harvested after 72 h by cell lysis and purification by rate zonal centrifugation on a 50%-70% discontinuous sucrose gradient. The Sigma Gel™ software program (Jandel Scientific) was again used to measure and quantify each fraction's relative protein band intensity on the SDS-PAGE gels. The experiments were repeated several times to exclude inherent possibilities of variation.

The solubility studies of the four recombinant constructs were all compared to that of VP7mt144/177/200. Further comparisons were done by sonication of the pellets of the four constructs after sucrose gradient analyses. These sonication studies were carried out to disrupt the large aggregates in the pellet and to determine if different constructs differed with respect to sonication, reflecting differences in stability. Figure 4.6. shows the comparative protein distribution of VP7mt144/177-100/200. The unsonicated protein distribution resembles the distribution formed by the VP7mt144/177/200 construct. The aggregated percentage proteins in the pellet are approximately the same although the distribution of VP7mt144/177-100/200's proteins in the first five fractions is considerably lower. VP7mt144/177/200, however, has a much lower percentage of small particles in the last three fractions of the gradient than VP7mt144/177-100/200. Whereas VP7mt144/177/200 almost forms no small particles, VP7mt144/177-100/200 has approximately 22% of these protein structures. These small particles could possibly be free soluble trimers. This could point to instability caused by the insertion, with a shift from large particulate structures to smaller, perhaps trimeric, structures.

The sucrose gradient sedimentation profile of VP7mt144/177-150/200 differs from that of VP7mt144/177/200 (Fig. 4.7.). Approximately only half of the VP7mt144/177-150/200 proteins occur in the sucrose gradient pellet, when compared to VP7mt144/177/200, which indicates a decline in aggregation between the particulate structures. Most of the VP7mt144/177-150/200 proteins occur in the first four fractions of the sucrose gradient with approximately 17% in the last three soluble fractions. This same protein fractional distribution as displayed by VP7mt144/177-150/200 is followed by VP7mt144/177-250/200 (Fig. 4.9.).

Figure 4.8. shows the protein distribution of VP7mt144/177-200/200 compared to VP7mt144/177/200. It was found that 85% of the VP7mt144/177-200/200 protein is present in the aggregated structures found in the pellet of the gradient, compared to only 56% for VP7mt144/177/200. It is also seen that only about 8% of VP7mt144/177-200/200 occur in the last three fractions as smaller particles and only 6% in the first three fraction of the gradient. This elevated aggregation is probably caused by the elevated hydrophobicity of the insertion.

VP7mt144/177-200/200 and VP7mt144/177-150/200 have similar protein fractionation profiles after sonication. These profiles resemble that of VP7mt144/177/200, except that the percentage proteins in the bottom of the gradient are a little less whereas the percentage proteins in the top of the gradient are increased. This increase indicates a greater tendency of these two constructs to form much smaller particle structures. This might be instability caused by the insertions. Whereas the third and fourth fractions of sonicated VP7mt144/177/200 are approximately the same, VP7mt144/177-100/200's third fraction is less than half of its fourth fraction. This is indicative of a slight shift in the particulate size. The rest of the protein particle distribution attained by VP7mt144/177-100/200 follow the same particle distribution as that of VP7mt144/177-150/200 and VP7mt144/177-200/200. Sonicated VP7mt144/177-250/200 also shares this protein distribution except for a peak in the first and eighth fractions. It is the eighth fraction that is most notable, reaching a peak with approximately triple the percentage proteins than any of the other constructs. A definite preference for smaller particulate structures is shown by the VP7mt144/177-250/200 construct, although larger particulate structures are still possible.

4.3.6. Scanning Electron Microscopy (S.E.M.)

Protein samples for S.E.M. studies were collected from the second, third and fourth fractions. These pooled fractions were then fixed, mounted and spatter coated with gold-beladium particles. The structures formed by the all four of the new constructs (Fig 4.10) resemble the structures formed by the VP7mt144/177/200 proteins (Fig 2.13.c and d). Most of the structures have the same 8 μm rough, flat and almost circular appearance and share the same surface texture. When high concentration of the proteins are mounted, aggregation, in the form of web-like tentacles/ cables, can be observed between the units.

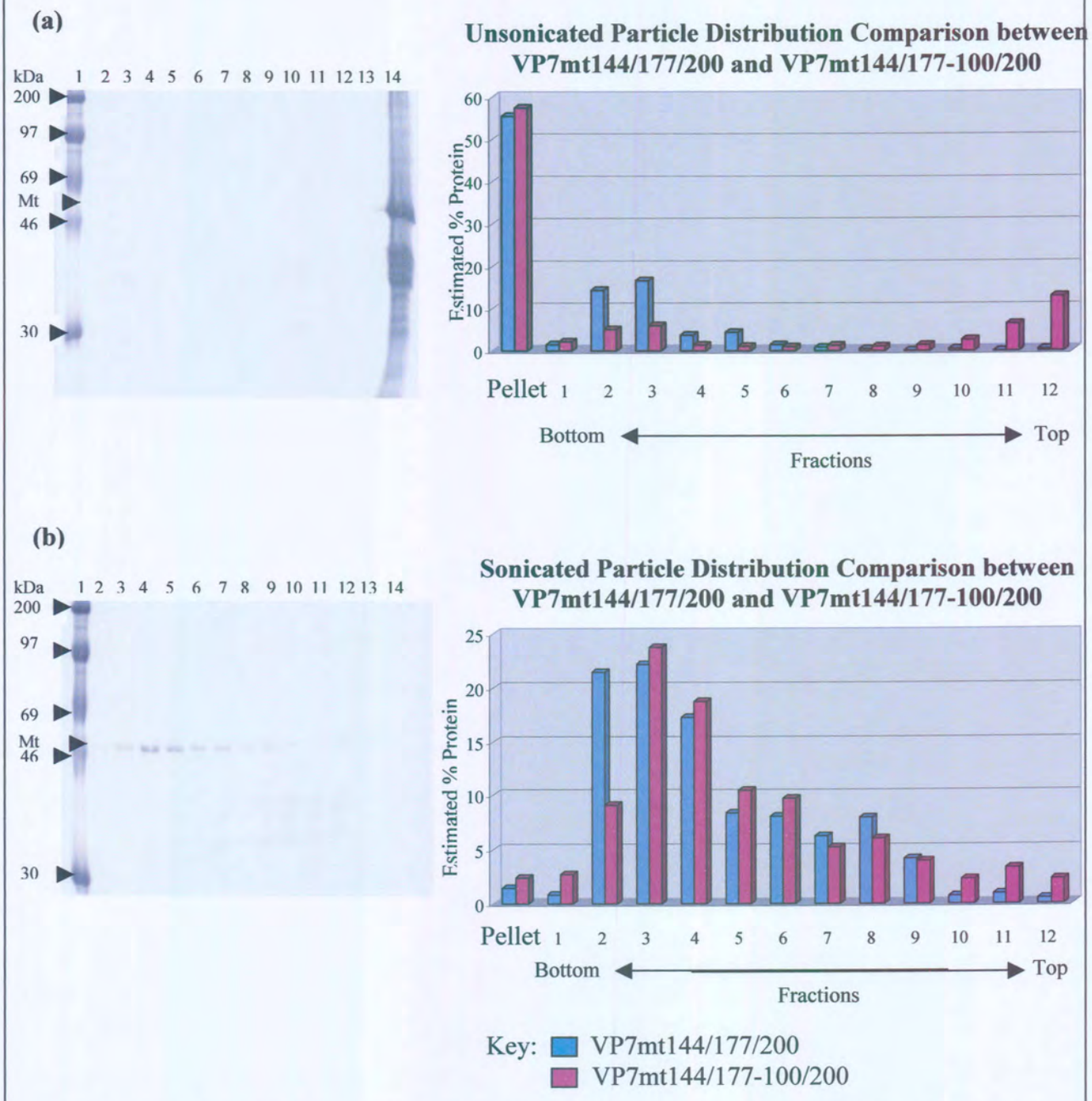


Figure 4.6. Comparative protein distribution between (a) unsonicated VP7mt144/177/200 and VP7mt144/177-100/200. (b) Represents a comparative protein distribution between 25 pulse sonicated pellets of VP7mt144/177/200 and VP7mt144/177-100/200. Proteins were centrifuged on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

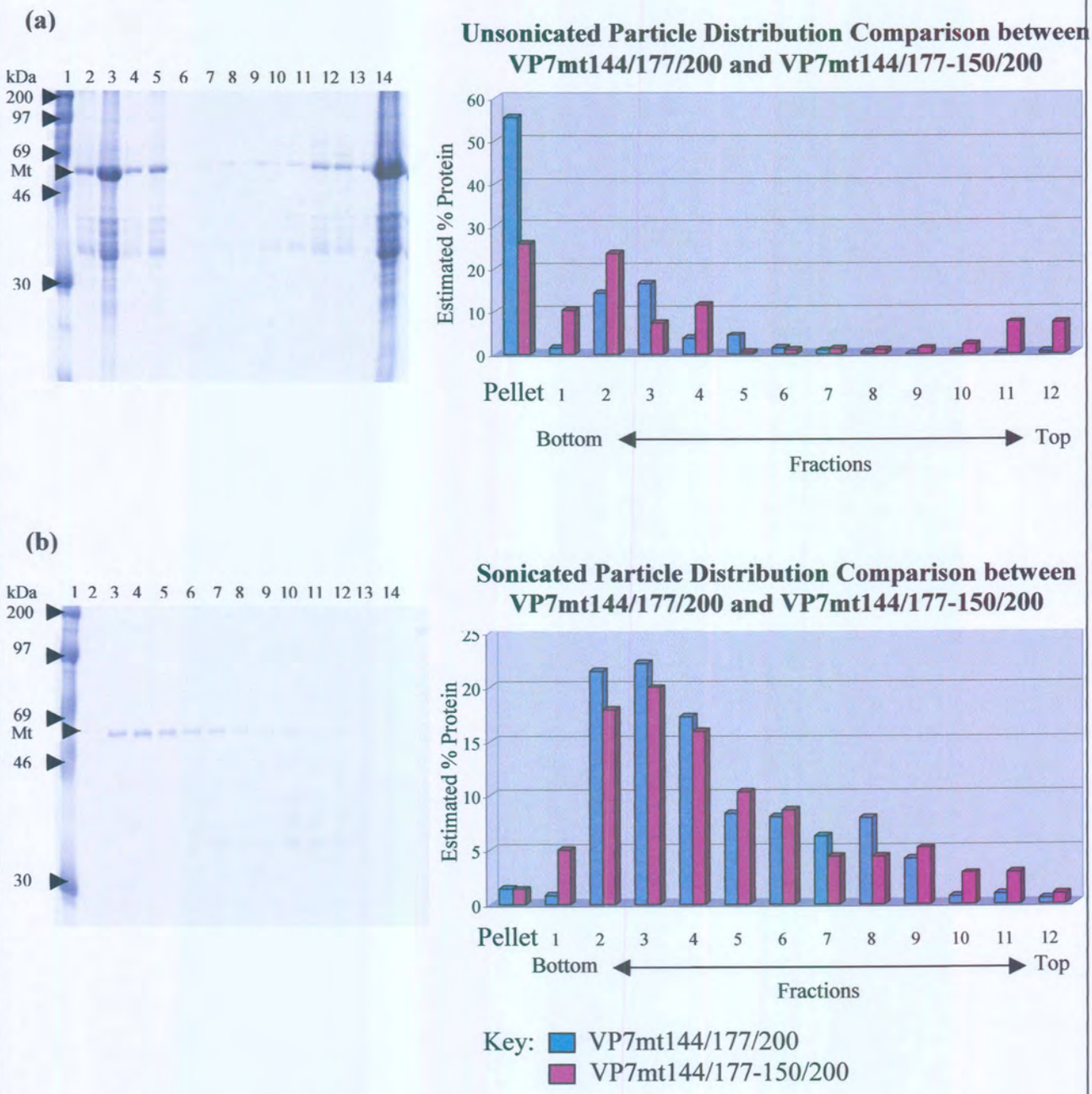


Figure 4.7. Unsonicared (a) and 25 pulse sonicated (b) protein distribution comparison between VP7mt144/177/200 and VP7mt144/177-150/200. Proteins were purified on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, before analysis by SDS-PAGE electrophoresis. Sigma Gel™ analysis program was used to quantify the protein amounts before conversion to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

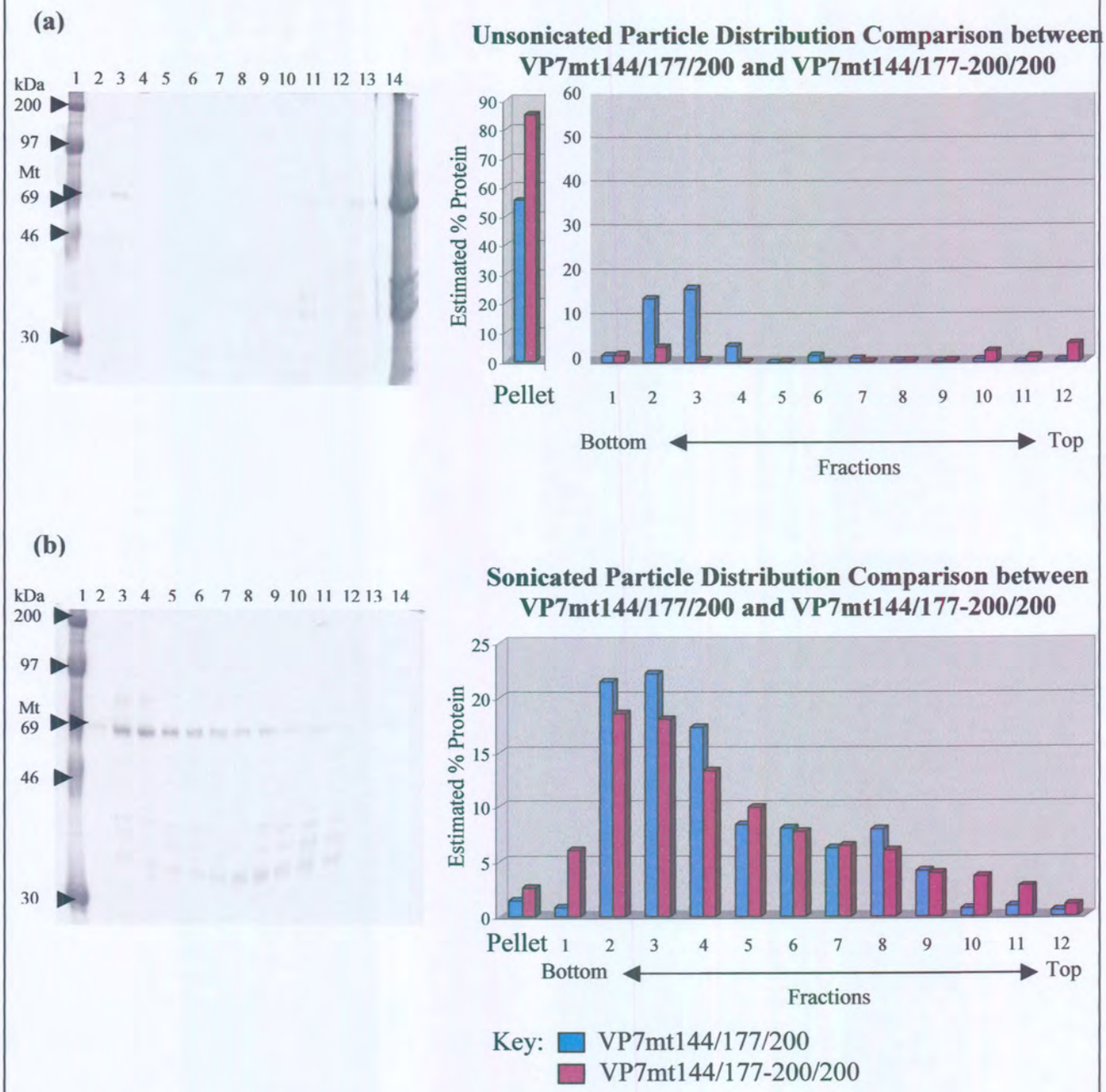


Figure 4.8. (a) Comparison in protein distribution between unsonicated VP7mt144/177/200 and VP7mt144/177-200/200. (b) Comparison in protein distribution between 25 pulse sonicated pellets of VP7mt144/177/200 and VP7mt144/177-200/200. A Sorvall AH-650 rotor was used to centrifuge proteins on a 50%-70% sucrose gradient at 12000 rpm for 75 min at 4°C. Fractions were analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

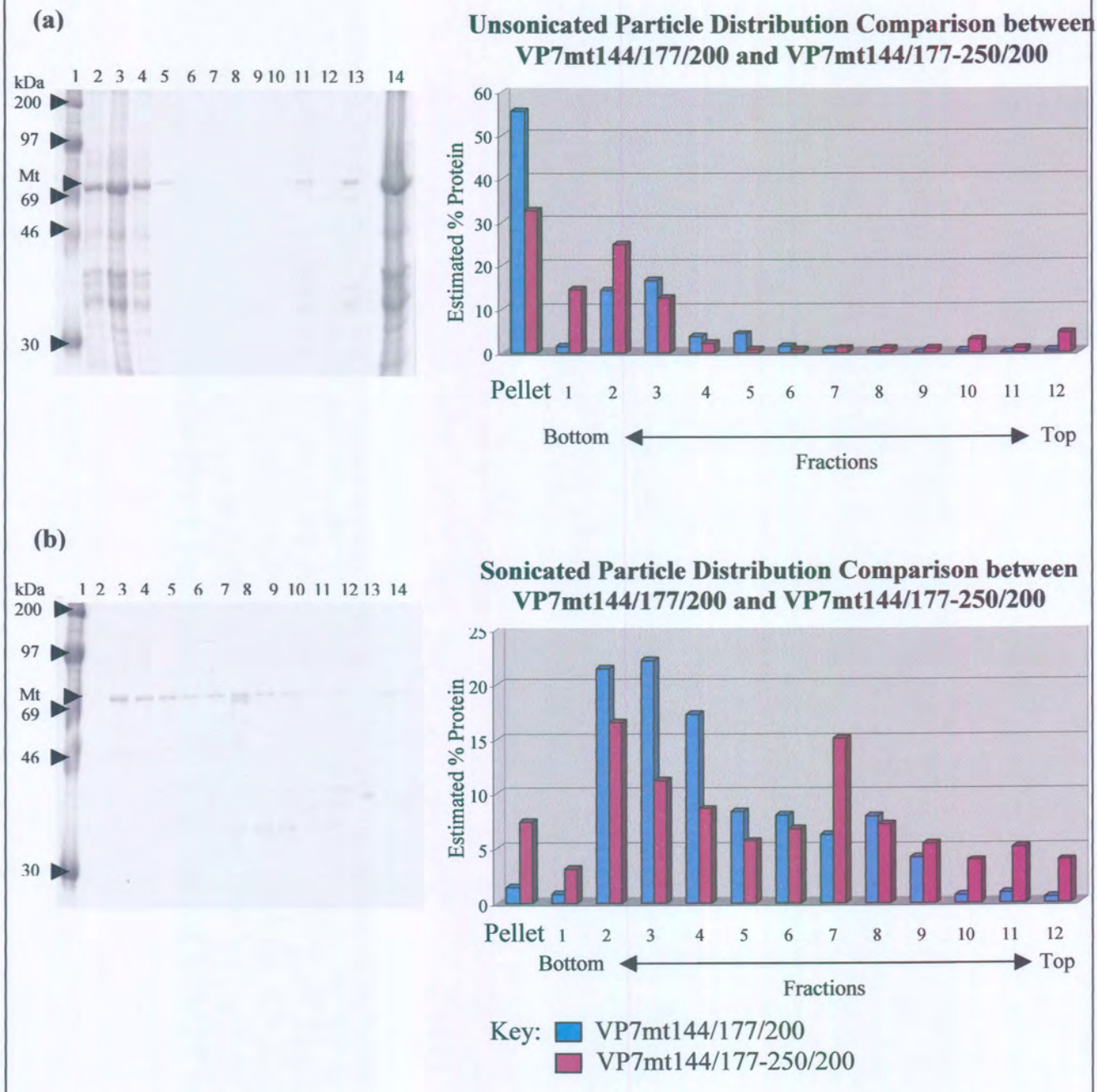


Figure 4.9. (a) Comparison between protein distributions of unsonicated VP7mt144/177/200 and VP7mt144/177-250/200. (b) Represents a comparative protein distribution between a 25 pulse sonicated pellets of VP7mt144/177/200 and VP7mt144/177-250/200. Proteins were centrifuged on a 50%-70% sucrose gradient using a Sorvall AH-650 rotor at 12000 rpm for 75 min at 4°C, analyzed by SDS-PAGE electrophoresis, quantified by the Sigma Gel™ analysis program and converted to the shown graphic form. On the SDS-PAGE gels, lane 1 contains the protein size marker, whereas lanes 2-13 contain the fractions as collected from the bottom to the top of the gradients. Lane 14 represents the protein pellet of the gradient.

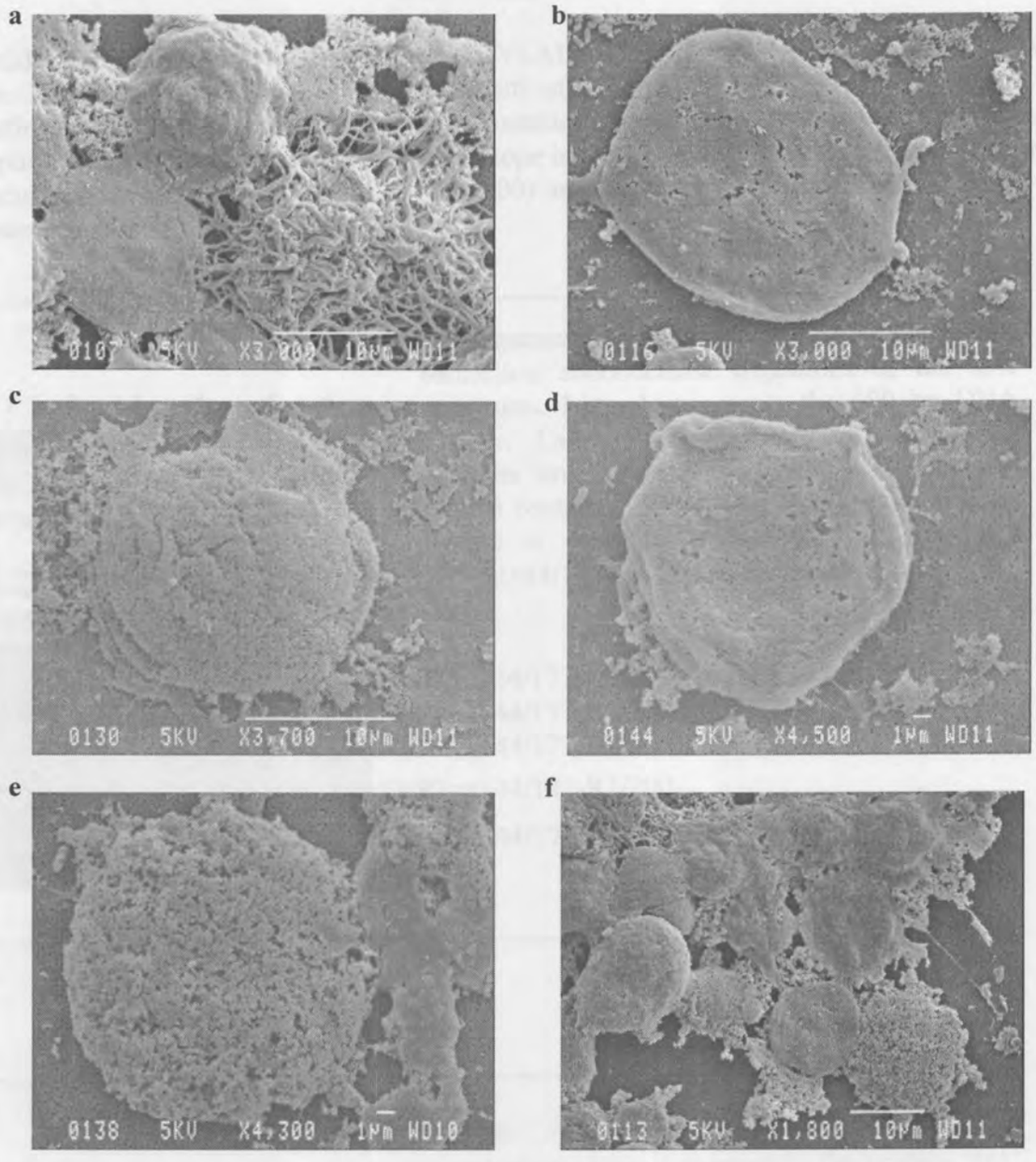


Figure 4.10. Scanning Electron Microscopy photographs depicting the structures formed by the different protein constructs: (a) VP7mt144/177-100/200 (b) VP7mt144/177-150/200, (c) VP7mt144/177-200/200, (d) and (e) VP7mt144/177-250/200. (f) Depicts a wide-angle view of the aggregated structures attained at high concentrations.

4.4. Discussion

One of the greatest challenges to subunit vaccines is the possible size of the inserts that can be made into the subunit protein without causing it to lose structure. The structure must be able to accommodate large epitope-containing insertions to increase the usefulness of the vaccine. The structure should also be able to accommodate insertions with different hydrophilic characteristics. As mentioned in chapter 1, the only licensed subunit vaccine in the United States of America is the hepatitis B virus surface antigen, which assembles into subviral particles similar to that of hepatitis B. The largest insertion that has been made into these particles without the loss of the structure, was 238 amino acids (Beterams *et al.*, 2000). The creation of VP7mt144/177/200 by the insertion of three multiple cloning sites, had an effect on the crystal formation abilities. These insertions did not, however, destroy the protein's trimer formation abilities or the trimers' ability to aggregate in organised structural units similar to the hexagonal crystals formed by wild type VP7.

To test VP7mt144/177/200's ability to function efficiently as a subunit vaccine, large insertion with different hydrophilic characteristics were made. The insertions cloned into the 177 aa site of VP7mt144/177/200 includes a 100 aa, 150 aa, 200 aa and 250 aa regions of AHSV-4 VP2. These changes to VP7mt144/177/200 contributed to the overall hydrophilicity of the protein (Table 4.2), the impact of which could not be determined at the early stages before solubility studies.

The 100 aa insertion into VP7mt144/177/200 to create VP7mt144/177-100/200 added a net hydrophilic character of +228 to the construct. This added hydrophilicity shifted the solubility of the construct, making the construct more soluble; possibly resulting in the increase of smaller particulate structures that was observed. This is supported by the results in figure 4.6.b., where the sonicated fractions containing the largest percentages proteins are fractions three and four for VP7mt144/177-100/200, whereas it is fractions two and three for VP7mt144/177/200. Also observed, was an approximate 22% increase in the percentage in the top of the gradient during the VP7mt144/177-100/200 solubility study (Fig. 4.6.a.).

The insertion of 150 aa domain into VP7mt144/177/200 had a different reaction on the solubility of VP7mt144/177/200 than the 100 aa insertion. The increased hydrophilic character of +366 lowered the aggregation ability of the protein structures. Less than half of the proteins that occur in the pellet of VP7mt144/177/200, is present in the VP7mt144/177-150/200 construct's pellet (Fig. 4.7.a). The rest of the proteins are distributed in the fractions with an increase of 16% small particles at the top of the

gradient, possibly free trimeric structures. The effect of the hydrophilic character is best illustrated by the 200 aa insertion. As mentioned before, the 200 aa amplified region is very hydrophobic in the last 50 aa. This region lowers the hydrophilic character of the insert to a net of +279, which is more than 100 aa insertion but far less than hydrophilic character of the 150 aa insertion. The effect can clearly be seen in figure 4.8.a. where 85% of the VP7mt144/177-200/200 proteins are aggregated in the pellet of the sucrose gradient. Speculations that this might be a result of an increase in size are abandoned when examining the solubility profile of VP7mt144/177-250/200 (Fig. 4.9.). Here, as in the 150 aa insertion, a very hydrophilic addition (+425) was made to the VP7mt144/177/200 construct. The solubility profile of VP7mt144/177-250/200 (Fig. 4.9.a.) is almost the same as the profile of VP7mt144/177-150/200. The only difference is seen when studying the sonication results of the structures. VP7mt144/177-250/200 forms a unique peak in the seventh fraction from the bottom of the sucrose gradient. This indicates that a distinct size structure is attained during sonication of the VP7mt144/177-250/200 aggregated proteins from the pellet and an instability in the larger particles.

The ability of the trimers to associate into large structures was confirmed by scanning electron microscopy. All four of the recombinant constructs formed structures that resembled the structures formed by VP7mt144/177/200. This indicates that the structures formed by VP7mt144/177/200 are able to absorb any structural stress if large aa regions with different hydrophilic characteristics are inserted into the 177 aa site.

Chapter 5

Concluding Remarks

The use of AHSV VP7 as a subunit vaccine has come a long way since the idea's first conception by Wade-Evans *et al.*, 1997. What first attracted attention to this protein was its ability to spontaneously form crystals when expressed in recombinant baculovirus infected cells (Chuma *et al.*, 1992). Large quantities of these crystals can easily be extracted from the cells and purified. Later they were shown to be immunogenic, enough for the generation of protective immune responses (Wade-Evans *et al.*, 1997; Wade-Evans *et al.*, 1998). These and other findings justified further investigation into and possible development of a subunit vaccine system using AHSV VP7.

This study was initiated by the creation of a VP7 construct capable of presenting epitopes at multiple sites on the surface of its structure. Multiple cloning sites were created by the insertion of six extra amino acids at the position 144, 177 and 200. These modifications caused an increase in the solubility of the protein but did, however, not eliminate the modified VP7 protein from forming trimers and assembling into distinct flat particles that can be purified easily on sucrose gradients. Large structures, not too different from the hexagonal crystals observed with wildtype VP7, were observed when the particles were analysed by electron microscopy. Also illustrated by these studies were the effect that aggregation has on the structure of these particles. With the storage studies that were conducted on VP7mt144/177/200, it was found that the increased aggregation of this protein would prove troublesome for any storage technique being applied. Sonication reduced the aggregation but this was only a temporary relief. As found by Wade-Evans *et al.*, 1997, whole crystals primarily elicit a protective cell-mediated T-cell immune response with vaccination. This is probably because the large 6 µm crystals are absorbed into antigen presenting cells by means of phagocytosis, where they are broken down to short peptides and presented on the MHC complex to the immune system. Sonication of the structures before immunisation might prove advantageous since this will reduce the size of the injected particles and could lead to humoral immune response generation in addition to the cell-mediated immunity. The easily attained high yield (approximately 0.043 ng/ cell) and purity of the VP7mt144/177/200 protein contributes greatly to its potential use as a subunit vaccine. The high yield is, of course, dependent on the health of the Sf9 cells being used, the viral stocks and other conditions.

Subsequent structural studies were conducted by inserting epitopes into at least two of the three newly created sites. The effect that the use of different sites has on the structure was best illustrated by the

creation of the VP7mt144/177-A/200 and the VP7mt144-B/177/200 constructs. The insertion of the hydrophilic VP2 'a' epitope into position 177 of VP7mt144/177/200 resulted in an increase in solubility but also an increase in aggregation. This was in contradiction to the results attained by the insertion of the hydrophilic VP2 'b' epitope at amino acid position 144 of VP7mt144/177/200, which also resulted in an increased solubility but a decrease in aggregation. These results were confirmed by the creation of a construct containing both the epitopes at their respective sites, VP7mt144-B/177-A/200. This construct had intermediate aggregational tendencies, the same level as that of the original VP7mt144/177/200 construct. All three constructs seemed to form the same size structures when aggregation was removed by sonication. The formation of particulate structures was confirmed by subsequent electron microscopy studies.

As mentioned, a major disadvantage of virus like particles as antigen carriers, is the limitation of their insertion capacity. Studies were conducted into the size of the possible inserts that can be made into the VP7mt144/177/200 construct without causing it to lose its particulate structure. A maximum of 250 amino acids from VP2 was inserted into VP7mt144/177/200 without an alteration to the particulate structures still being formed. These experiments also illustrated the effect that the hydrophilic characters of the inserts have on the overall structure of VP7mt144/177/200. These size experiments are, however, incomplete and future investigations should include studies into the effect of large insertions into the other two sites on the VP7mt144/177/200 construct. Also, the effect of large amino acid insertions into all three the multiple cloning sites at once.

Other future investigations that should be conducted to further the development and potential of VP7 as a vaccine delivery system, include the following:

- This study lacked investigations into the actual immune response generated by these constructs. These studies would indicate whether or not the inserted epitopes are presented efficiently to the immune system. Also, comparative studies should be done concerning the generated protective immune responses between whole particulate injection and the sonicated product injection.

- Another relevant area of investigation should be conducted on the use of non-polar chemical solvents for dissolving the aggregated proteins before injection.

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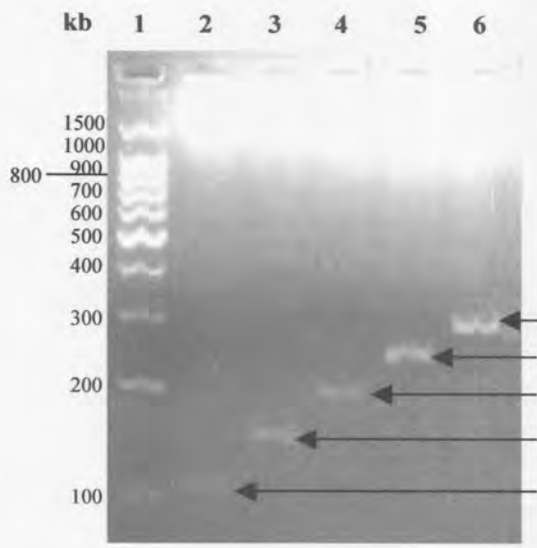
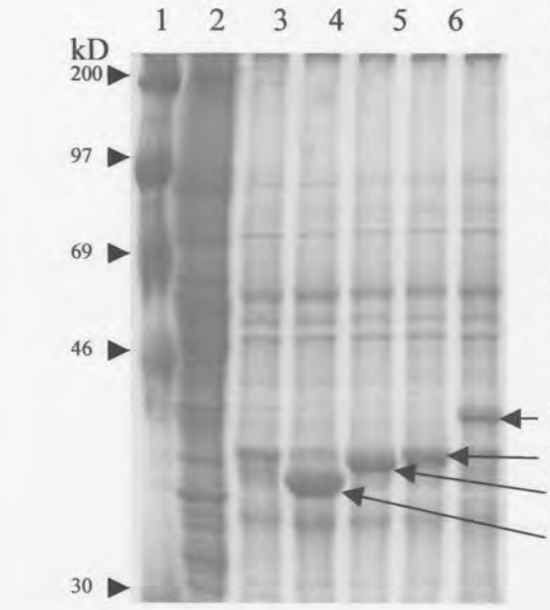
Addendum

Oligonucleotides coding for the twelve amino acid RVLAIERYLKDL epitope of glycoprotein 120 of HIV subtype C was cloned into the 177 amino acid site of VP7mt144/177/200. A single epitope was inserted creating VP7mt144/177-R1/200. This new construct was subjected to subsequent insertions of the same peptide to create up to four copies of the epitope in the original VP7mt144/177/200 construct. The constructs containing one (VP7mt144/177-R1/200) and four (VP7mt144/177-R4/200) copies of the insert were expressed in Sf9 cells.



4% Agarose gel electrophoresis of *Pst*I and *Xho*I restriction endonuclease digestions of the new constructs. Lane 1 represents the 100 bp DNA Ladder. Lane 2 contains VP7mt144/177/200, whereas lane 3 contains VP7mt144/177-R1/200. Lane 4 contains VP7mt144/177-R2/200 and lanes 5 and 6 contain VP7mt144/177-R3/200 and VP7mt144/177-R4/200 respectively.



A 15% SDS-PAGE analysis of the new recombinants. Lane 1 represents the protein size marker. Lane 2 and 3 contain proteins from a mock infection and wild-type baculovirus-infected cells respectively. Lane 4 contains expressed VP7 and lane 5 contains the lysate of cells infected with baculovirus expressing the recombinant VP7mt144/177/200. Lane 6 contains VP7mt144/177-R1/200 and lane 8 contains VP7mt144/177-R4/200

Nucleotide sequence of VP7mt144/177-R1/200 (1134 bp)

ATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGATGCGAG
AGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTATAATGGTTTAA
CAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAAATGTTTTTTATGTGT
ACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATTTCACCAGATTATGATCAAGCG
TTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATC
GTTAGAATAACGGGTCAGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGG
AGCGGTTGAGGTGCAACAATCTGGCAGATATTACGTACCG**CCCGGGGAATTCCTCGAG**CAAGGT
CGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGG
ACAGGTCAATGCGCTGCTAGCCCCAAGG**CTGCA*TAGAGTCTTGGCTATAGAGAGATACCTAAAGGA***
***TCTGCAG*GCGCGC**AGGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTG
TGATCCTCAAGGTGCG**AAGCTTTCTAGAGTCGAC**TCACTTGAGAGCGCTCCAGGAACTTTTGTCA
CCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATG
TTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATA
GATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCA
CTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTT
TCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGC
GGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCT
TGTGCTAGCAGCCTACCAC

Amino acid sequence of VP7mt144/177-R1/200 (378 amino acids)

MDAIRARALSVVRACVTVTDARVSLDPGVMETLGIAINRYNGLTNHSVSMR
PQTQAERNEMFFMCTDMVLAALNVQIGNISPDYDQALATVGALATTEIPYN
VQAMNDIVRITGQMQTFGPSKVQTGPYAGAVEVQQSGRYYVPPGEFLEQGR
TRGGYINSNIAEVCMDAGAAGQVNALLAPRLH**RVLAIERYLKDL**QARRGDA
VMIYFVWRPLRIFCDPQGAKLSRVDSLESAPGTFVTVDGVNVAAGDVVAWN
TIAPVNVGNPGARRSILQFEVLWYTSLDRSLDTVPELAPTLTRCYAYVSPT
WHALRAVIFQQMNMQPINPPIFPPTERNEIVAYLLVASLADVYAALRPDFR
MNGVVAPVGQINRALVLAAYH

Nucleotide sequence of VP7mt144/177-R4/200 (1251 bp)

ATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCATGTGTCACAGTGACAGATGCGAG
AGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGATTGCAATCAATAGGTATAATGGTTTAA
CAAATCATTCGGTATCGATGAGGCCACAAACCCAAGCAGAACGAAATGAAATGTTTTTTATGTGT
ACTGATATGGTTTTAGCGGCGCTGAACGTCCAAATTGGGAATATTTCACCAGATTATGATCAAGCG
TTGGCAACTGTGGGAGCTCTCGCAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATC
GTTAGAATAACGGGTCAGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGG
AGCGGTTGAGGTGCAACAATCTGGCAGATATTACGTACCG**CCCGGGGAATTCCTCGAG**CAAGGT
CGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGG
ACAGGTCAATGCGCTGCTAGCCCCAAGG**CTGCA*TAGAGTCTTGGCTATAGAGAGATACCTAAAGGA***
TCTGCATAGAGTCTTGGCTATAGAGAGATACCTAAAGGATCTGCATAGAGTCTTGGCTATAGAGAGA
***TACCTAAAGGATCTGCATAGAGTCTTGGCTATAGAGAGATACCTAAAGGATCTGCAG*GCGCGC**AGG
GGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCG
AAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAA
TGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGC
ACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACACGGT

TCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACG
CGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAG
GAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGA
TTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTA
CCAC

Amino acid sequence of VP7mt144/177-R4/200 (417 amino acids)

MDAIRARALSVVRACVTVTDARVSLDPGVMETLGIAINRYNGLTNHSVSMRPQTQAERNEMFFMCTD
MVLAALNVQIGNISPDYDQALATVGALATTEIPYNVQAMNDIVRITGQMQTFGPSKVQTGPYAGAVE
VQQSGRYYVPPGEFLEQGRTRGGYINSNIAEVCMDAGAAGQVNALLAPRLH**RVLAIERYLKDLHRV**
LAIERYLKDLHRVLAIERYLKDLHRVLAIERYLKDLQARRGDAVMIYFVWRPLRIFCDPQGAKLSR
VDSLESAPGTFVTVDGVNVAAGDVVAWNTIAPVNVGNPGARRSILQFEVLWYTSLDRSLDTVPELAPT
LTRCYAYVSPTWHALRAVIFQQMNMQPINPPIFPPTERNEIVAYLLVASLADVYAALRPDFRMNGVVA
PVGQINRALVLAAYH

Appendices

Appendix A. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7 with VP7mt144/177/200.

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VP7              GTTTAAATTCGGTTAGGATGGACGCGATACGAGCAAGAGCCTTGTCCGTTGTACGGGCA   42
                           M  D  A  I  R  A  R  A  L  S  V  V  R  A      14
mt144/177/200    GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCA   42
                           M  D  A  I  A  A  R  A  L  S  V  V  R  A      14
                 ****************************  ****************************

VP7              TGTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGG  102
                 C  V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G   34
mt144/177/200    TGTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGG  102
                 C  V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G   34
                 ************************************************************

VP7              ATTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACC  162
                 I  A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T   54
mt144/177/200    ATTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACC  162
                 I  A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T   54
                 ************************************************************

VP7              CAAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAAC  222
                 Q  A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N   74
mt144/177/200    CAAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAAC  222
                 Q  A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N   74
                 ************************************************************

VP7              GTCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTC  282
                 V  Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L   94
mt144/177/200    GTCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTC  282
                 V  Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L   94
                 ************************************************************

VP7              GCAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGT  342
                 A  T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  114
mt144/177/200    GCAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGT  342
                 A  T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  114
                 ************************************************************

VP7              CAGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAG  402
                 Q  M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  134
mt144/177/200    CAGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAG  402
                 Q  M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  134
                 ************************************************************

VP7              GTGCAACAATCTGGCAGATATTACGTACCG----------------CAAGGTCGAACG  444
                 V  Q  Q  S  G  R  Y  Y  V  P                   Q  G  R  T  148
mt144/177/200    GTGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACG  462
                 V  Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  154
                 ******************************                  ************

VP7              CGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGA  504
                 R  G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  168
mt144/177/200    CGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGA  522
                 R  G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  174
                 ************************************************************
```

```
VP7              CAGGTCAATGCGCTGCTAGCCCCAAGG------------------AGGGGGGACGCAGTC  556
                 Q  V  N  A  L  L  A  P  R                    R  G  D  A  V   182
mt144/177/200    CAGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAGGCCTGCGCGCAGGGGGGACGCAGTC  582
                 Q  V  N  A  L  L  A  P  R  L  Q  R  P  A  R  R  G  D  A  V   194
                 ***************************                 ***************

VP7              ATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCG------  610
                 M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A         200
mt144/177/200    ATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGAAGCTT  642
                 M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  K  L   214
                 ************************************************************

VP7              ------------TCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAAT  658
                             S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N   216
mt144/177/200    TCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAAT  702
                 S  R  V  D  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N   234
                             ********************************************

VP7              GTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCT  718
                 V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P   236
mt144/177/200    GTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCT  762
                 V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P   254
                 ************************************************************

VP7              GGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCG  778
                 G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S   256
mt144/177/200    GGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCG  822
                 G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S   274
                 ************************************************************

VP7              CTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCC  798
                 L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P   276
mt144/177/200    CTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCC  882
                 L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P   294
                 ************************************************************

VP7              ACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCG  858
                 T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P   296
mt144/177/200    ACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCG  942
                 T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P   314
                 ************************************************************

VP7              CCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTA  918
                 P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L   316
mt144/177/200    CCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTA 1002
                 P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L   334
                 ************************************************************

VP7              GCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTA  978
                 A  D  V  Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V   336
mt144/177/200    GCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTA 1062
                 A  D  V  Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V   354
                 ************************************************************

VP7              GGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACG 1020
                 G  Q  I  N  R  A  L  V  L  A  A  Y  H  Stop                  349
mt144/177/200    GGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACG 1104
                 G  Q  I  N  R  A  L  V  L  A  A  Y  H  Stop                  367
                 ************************************************************
```

Appendix B-1. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7mt144/177/200 with VPmt144/177-A/200.

```
mt144/177/200     GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                                M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
mt144/177-A/200   GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                                M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
                  ************************************************************

mt144/177/200     GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
mt144/177-A/200   GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
                  ************************************************************

mt144/177/200     TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
mt144/177-A/200   TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
                  ************************************************************

mt144/177/200     AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
mt144/177-A/200   AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
                  ************************************************************

mt144/177/200     TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
mt144/177-A/200   TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
                  ************************************************************

mt144/177/200     CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
mt144/177-A/200   CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
                  ************************************************************

mt144/177/200     AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
mt144/177-A/200   AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
                  ************************************************************

mt144/177/200     TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                    Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
mt144/177-A/200   TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                    Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
                  ************************************************************

mt144/177/200     GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                    G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
mt144/177-A/200   GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                    G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
                  ************************************************************

mt144/177/200     AGGTCAATGCGCTGCTAGCCCCAAGG--------------------------------  561
                    V  N  A  L  L  A  P  R                                     187
```

```
mt144/177-A/200 AGGTCAATGCGCTGCTAGCCCCAAGGCTGCATAAGAAGAAAGAAGAGGGTGAGGATGATA  583
                  V  N  A  L  L  A  P  R  L  H  K  K  K  E  E  G  E  D  D  T  195
                ************************

mt144/177/200   ----------------------------CTGCAGGCGCGCAGGGGGGGACGCAGTCATGA  592
                                             L  Q  A  R  R  G  D  A  V  M  I  198
mt144/177-A/200 CTGCTCGACAGGAGATAAGAAAAGCATGGCTGCAGGCGCGCAGGGGGGACGCAGTCATGA  643
                  A  R  Q  E  I  R  K  A  W  L  Q  A  R  R  G  D  A  V  M  I  215
                                             ********************************

mt144/177/200   TCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGAAGCTTTCTA  612
                  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  K  L  S  R  218
mt144/177-A/200 TCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGAAGCTTTCTA  703
                  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  K  L  S  R  235
                ************************************************************

mt144/177/200   GAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTG  672
                  V  D  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  238
mt144/177-A/200 GAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTG  763
                  V  D  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  255
                ************************************************************

mt144/177/200   CAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGG  732
                  A  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  258
mt144/177-A/200 CAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGG  823
                  A  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  275
                ************************************************************

mt144/177/200   CACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAG  792
                  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  278
mt144/177-A/200 CACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAG  883
                  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  295
                ************************************************************

mt144/177/200   ACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTT  852
                  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  298
mt144/177-A/200 ACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTT  943
                  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  315
                ************************************************************

mt144/177/200   GGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGA  912
                  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  318
mt144/177-A/200 GGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGA  1003
                  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  335
                ************************************************************

mt144/177/200   TTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTG  972
                  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  338
mt144/177-A/200 TTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTG  1063
                  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  355
                ************************************************************

mt144/177/200   ATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCC  1032
                  V  Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  358
mt144/177-A/200 ATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCC  1123
                  V  Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  375
                ************************************************************

mt144/177/200   AGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCA  1070
                  I  N  R  A  L  V  L  A  A  Y  H  Stop                        369
mt144/177-A/200 AGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCA  1161
                  I  N  R  A  L  V  L  A  A  Y  H  Stop                        386
                ************************************************************
```

Appendix B-2. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7mt144/177/200 with VP7mt144-B/177/200.

```
mt144/177/200     GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                            M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
mt144-B/177/200   GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                            M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
                  ************************************************************

mt144/177/200     GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
mt144-B/177/200   GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
                  ************************************************************

mt144/177/200     TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
mt144-B/177/200   TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
                  ************************************************************

mt144/177/200     AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
mt144-B/177/200   AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
                  ************************************************************

mt144/177/200     TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
mt144-B/177/200   TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
                  ************************************************************

mt144/177/200     CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
mt144-B/177/200   CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
                  ************************************************************

mt144/177/200     AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
mt144-B/177/200   AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
                  ************************************************************

mt144/177/200     TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAG----------  450
                    Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E                150
mt144-B/177/200   TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGGTAGACGTTGATC  463
                    Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  V  D  V  D  P  155
                  *********************************************

mt144/177/200     ----------------------------------------------------------  450
                                                                               150
mt144-B/177/200   CAAATAAGGGTAAGTGGAAAGAACATATAAAAGAGGTAACCGAAAAATTATTGAAAGCGG  523
                    N  K  G  K  W  K  E  H  I  K  E  V  T  E  K  L  L  K  A  V  175

mt144/177/200     -----CAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGG  505
                      E  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V  C  M  D  170
```

```
mt144-B/177/200 TCGAGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGG  583
                  E  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V  C  M  D  195
                     ************************************************************

mt144/177/200   ATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAGGCCTGCGC  565
                  A  G  A  A  G  Q  V  N  A  L  L  A  P  R  L  Q  R  P  A  R  190
mt144-B/177/200 ATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAGGCCTGCGC  643
                  A  G  A  A  G  Q  V  N  A  L  L  A  P  R  L  Q  R  P  A  R  215
                ************************************************************

mt144/177/200   GCAGGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATC  625
                  R  G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  210
mt144-B/177/200 GCAGGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATC  703
                  R  G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  235
                ************************************************************

mt144/177/200   CTCAAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCA  685
                  Q  G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  V  T  230
mt144-B/177/200 CTCAAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCA  763
                  Q  G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  V  T  255
                ************************************************************

mt144/177/200   CCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAG  745
                  V  D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  250
mt144-B/177/200 CCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAG  823
                  V  D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  275
                ************************************************************

mt144/177/200   TGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATA  805
                  N  V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  270
mt144-B/177/200 TGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATA  883
                  N  V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  295
                ************************************************************
                                                .

mt144/177/200   CGTCCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTT  865
                  S  L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  290
mt144-B/177/200 CGTCCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTT  943
                  S  L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  315
                ************************************************************

mt144/177/200   ATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATA  925
                  A  Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  310
mt144-B/177/200 ATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATA 1003
                  A  Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  335
                ************************************************************
                .                                                           .
mt144/177/200   TGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATC  985
                  Q  P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  330
mt144-B/177/200 TGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATC 1063
                  Q  P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  355
                ************************************************************

mt144/177/200   TATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATG 1045
                  L  V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  N  G  350
mt144-B/177/200 TATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATG 1123
                  L  V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  N  G  375
                ************************************************************
                |

mt144/177/200   GTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGT 1104
                  V  V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  Stop  368
mt144-B/177/200 GTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGT 1182
                  V  V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  Stop  393
                ************************************************************
```

Appendix B-3. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7mt144/177/200 with VP7mt144-B/177-A/200.

```
mt144/177/200    GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                           M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
mt144-B/177-A/   GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                           M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
                 ************************************************************

mt144/177/200    GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                   V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
mt144-B/177-A/   GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                   V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
                 ************************************************************

mt144/177/200    TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                   A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
mt144-B/177-A/   TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                   A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
                 ************************************************************

mt144/177/200    AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                   A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
mt144-B/177-A/   AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                   A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
                 ************************************************************

mt144/177/200    TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                   Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
mt144-B/177-A/   TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                   Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
                 ************************************************************

mt144/177/200    CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                   T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
mt144-B/177-A/   CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                   T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
                 ************************************************************

mt144/177/200    AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                   M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
mt144-B/177-A/   AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                   M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
                 ************************************************************

mt144/177/200    TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAG----------  450
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E                150
mt144-B/177-A/   TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGGTAGACGTTGATC  463
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  V  D  V  D  P  155
                 *********************************************

mt144/177/200    ----------------------------------------------------------  450
                                                                              150
mt144-B/177-A/   CAAATAAGGGTAAGTGGAAAGAACATATAAAAGAGGTAACCGAAAAATTATTGAAAGCGG  523
                   N  K  G  K  W  K  E  H  I  K  E  V  T  E  K  L  L  K  A  V  175

mt144/177/200    -----CAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGG  505
                   E  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V  C  M  D  170
```

```
mt144-B/177-A/   TCGAGCAAGGTCGAACGCGTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGG  583
                   E  Q  G  R  T  R  G  G  Y  I  N  S  N  I  A  E  V  C  M  D  195
                      **************************************************

mt144/177/200    ATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGG----------------  549
                   A  G  A  A  G  Q  V  N  A  L  L  A  P  R                    184
mt144-B/177-A/   ATGCAGGTGCTGCGGGACAGGTCAATGCGCTGCTAGCCCCAAGGCTGCATAAGAAGAAAG  643
                   A  G  A  A  G  Q  V  N  A  L  L  A  P  R  L  H  K  K  K  E  215
                 *******************************************

mt144/177/200    ------------------------------------------------CTGCAGGCGCGCA  562
                                                                   L  Q  A  R  R  189
mt144-B/177-A/   AAGAGGGTGAGGATGATACTGCTCGACAGGAGATAAGAAAAGCATGGCTGCAGGCGCGCA  703
                   E  G  E  D  D  T  A  R  Q  E  I  R  K  A  W  L  Q  A  R  R  235
                                                               **************

mt144/177/200    GGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTC  622
                   G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  209
mt144-B/177-A/   GGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTC  763
                   G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  D  P  Q  255
                 ************************************************************

mt144/177/200    AAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCG  682
                   G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  V  T  V  229
mt144-B/177-A/   AAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCG  823
                   G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  V  T  V  275
                 ************************************************************

mt144/177/200    TTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGA  742
                   D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  N  249
mt144-B/177-A/   TTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGA  883
                   D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  P  V  N  295
                 ************************************************************

mt144/177/200    ATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGT  802
                   V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  269
mt144-B/177-A/   ATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGT  943
                   V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  Y  T  S  315
                 ************************************************************

mt144/177/200    CCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATG  862
                   L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  289
mt144-B/177-A/   CCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATG  1003
                   L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  C  Y  A  335
                 ************************************************************

mt144/177/200    CGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGC  922
                   Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  309
mt144-B/177-A/   CGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGC  1063
                   Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  N  M  Q  355
                 ************************************************************

mt144/177/200    AGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTAT  982
                   P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  329
mt144-B/177-A/   AGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTAT  1123
                   P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  Y  L  L  375
                 ************************************************************

mt144/177/200    TAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTG  1042
                   V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  N  G  V  349
mt144-B/177-A/   TAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTG  1183
                   V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  N  G  V  395
                 ************************************************************
```

```
mt144/177/200     TTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGC  1102
                    V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  Stop     366
mt144-B/177-A/    TTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGC  1239
                    V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  Stop     412
                  ************************************************************
```

Appendix C-1. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7mt144/177/200 with VP7mt144/177-100/200.

```
mt144/177/200    GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                              M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
mtVP7/VP2-100    GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                              M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
                 ************************************************************

mt144/177/200    GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                   V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
mtVP7/VP2-100    GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                   V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
                 ************************************************************

mt144/177/200    TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                   A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
mtVP7/VP2-100    TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                   A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
                 ************************************************************
                                                    .

mt144/177/200    AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                   A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
mtVP7/VP2-100    AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                   A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
                 ************************************************************

mt144/177/200    TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                   Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
mtVP7/VP2-100    TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                   Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
                 ************************************************************

mt144/177/200    CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                   T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
mtVP7/VP2-100    CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                   T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
                 ************************************************************

mt144/177/200    AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
```

```
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
mtVP7/VP2-100       AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
                    ************************************************************

mt144/177/200       TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                    Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
mtVP7/VP2-100       TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                    Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
                    ************************************************************

mt144/177/200       GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                    G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
mtVP7/VP2-100       GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                    G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
                    ************************************************************

mt144/177/200       AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAGGCCT----------------------  561
                    V  N  A  L  L  A  P  R  L  Q  R  P                          187
mtVP7/VP2-100       AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGTTTGATTTTTTGACAACATTCGTTCATG  583
                    V  N  A  L  L  A  P  R  L  Q  F  D  F  L  T  T  F  V  H  A  195
                    ********************************

mt144/177/200       ------------------------------------------------------------  561
                                                                                  187
mtVP7/VP2-100       CGAAGAAGAAAGAAGAGGGTGAGGATGATACTGCTCGACAGGAGATAAGAAAAGCATGGG  643
                    K  K  K  E  E  G  E  D  D  T  A  R  Q  E  I  R  K  A  W  V  215

mt144/177/200       ------------------------------------------------------------  561
                                                                                  187
mtVP7/VP2-100       TTAAGGGGATGCCTTATATGGATTTCTCAAAACCGATGAAAATCACGCGTGGATTCAACA  703
                    K  G  M  P  Y  M  D  F  S  K  P  M  K  I  T  R  G  F  N  R  235

mt144/177/200       ------------------------------------------------------------  561
                                                                                  187
mtVP7/VP2-100       GAAATATGCTTTTCCTTGCGGCGCTCGATTCATTCAGAAAGAGGAACGGTGTAGATGTTG  763
                    N  M  L  F  L  A  A  L  D  S  F  R  K  R  N  G  V  D  V  D  255

mt144/177/200       ------------------------------------------------------------  561
```

```
                                                                                                  187
mtVP7/VP2-100   ATCCGAATAAGGGTAAGTGGAAAGAACATATAAAGGAGGTAACCGAAAAATTGAAGAAAG                    823
                  P  N  K  G  K  W  K  E  H  I  K  E  V  T  E  K  L  K  K A                    275

mt144/177/200   ----------------------------------GCGCGCAGGGGGGACGCAGTCATGATCT                    589
                                                   A  R  R  G  D  A  V  M  I  Y                  197
mtVP7/VP2-100   CGCAAACCGAAAATGGAGGACAACCATGCCAAGCGCGCAGGGGGGACGCAGTCATGATCT                    883
                  Q  T  E  N  G  G  Q  P  C  Q  A  R  R  G  D  A  V  M  I  Y                    295
                                                  ****************************

mt144/177/200   ATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGAAGCTTTCTAGAG                    649
                  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  K  L  S  R  V                    117
mtVP7/VP2-100   ATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGAAGCTTTCTAGAG                    943
                  F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  K  L  S  R  V                    315
                ************************************************************

mt144/177/200   TCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCAG                    709
                  D  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  A                    137
mtVP7/VP2-100   TCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCAG                   1003
                  D  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  A                    335
                ************************************************************
                                                  .

mt144/177/200   CTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCAC                    769
                  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  R                    157
mtVP7/VP2-100   CTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCAC                   1063
                  G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  R                    355
                ************************************************************

mt144/177/200   GCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACA                    829
                  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T                    177
mtVP7/VP2-100   GCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACA                   1123
                  R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T                    375
                ************************************************************

mt144/177/200   CGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGC                    889
                  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H                    197
mtVP7/VP2-100   CGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGC                   1183
                  V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H                    395
                ************************************************************

mt144/177/200   ACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTT                    949
```

```
                A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  217
mtVP7/VP2-100   ACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTT  1243
                A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  415
                ************************************************************

mt144/177/200   TTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATG  1009
                P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  237
mtVP7/VP2-100   TTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATG  1303
                P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  435
                ************************************************************

mt144/177/200   TGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGA  1069
                Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  257
mtVP7/VP2-100   TGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGA  1363
                Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  455
                ************************************************************

mt144/177/200   TTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCACCG  1104
                N  R  A  L  V  L  A  A  Y  H  Stop                           267
mtVP7/VP2-100   TTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCACCG  1398
                N  R  A  L  V  L  A  A  Y  H  Stop                           465
                ************************************************************
```

Appendix C-2. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7mt144/177/200 with VP7mt144/177-150/200.

```
mt144/177/200     GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                              M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
mtVP7/VP2-150     GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                              M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
                  ************************************************************

mt144/177/200     GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
mtVP7/VP2-150     GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
                  ************************************************************

mt144/177/200     TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
mtVP7/VP2-150     TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
                  ************************************************************

mt144/177/200     AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
mtVP7/VP2-150     AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
                  ************************************************************

mt144/177/200     TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
mtVP7/VP2-150     TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
                  ************************************************************

mt144/177/200     CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
mtVP7/VP2-150     CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
                  ************************************************************

mt144/177/200     AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
```

```
mtVP7/VP2-150    AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                   M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
                 ************************************************************

mt144/177/200    TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
mtVP7/VP2-150    TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
                 ************************************************************

mt144/177/200    GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                   G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
mtVP7/VP2-150    GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                   G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
                 ************************************************************

mt144/177/200    AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAGGCCT----------------------  561
                   V  N  A  L  L  A  P  R  L  Q  R  P                         187
mtVP7/VP2-150    AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAAAGGTCCACTGAATGACTTACGAGTTA  583
                   D  A  K  A  S  A  M  Y  S  G  K  G  P  L  N  D  L  R  V  K  195
                 ********************************

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-150    AAATTGAGCGGGATGATTTATCTCGAGAGACAATTATTCAGATCATTGAGTACGGTAAGA  643
                   I  E  R  D  D  L  S  R  E  T  I  I  Q  I  I  E  Y  G  K  K  215

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-150    AATTTAATTCATCAGCAGGTGATAAGCAGGGGAACATTTCAATTGAAAAATTGGTAGAGT  703
                   F  N  S  S  A  G  D  K  Q  G  N  I  S  I  E  K  L  V  E  Y  235

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-150    ATTGTGATTTTTTGACAACATTCGTTCATGCGAAGAAGAAAGAAGAGGGTGAGGATGATA  763
                   C  D  F  L  T  T  F  V  H  A  K  K  K  E  E  G  E  D  D  T  255

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
```

```
mtVP7/VP2-150   CTGCTCGACAGGAGATAAGAAAAGCATGGGTTAAGGGGATGCCTTATATGGATTTCTCAA  823
                  A  R  Q  E  I  R  K  A  W  V  K  G  M  P  Y  M  D  F  S  K  275

mt144/177/200   ----------------------------------------------------------  561
                                                                             187
mtVP7/VP2-150   AACCGATGAAAATCACGCGTGGATTCAACAGAAATATGCTTTTCCTTGCGGCGCTCGATT  883
                  P  M  K  I  T  R  G  F  N  R  N  M  L  F  L  A  A  L  D  S  295

mt144/177/200   ----------------------------------------------------------  561
                                                                             187
mtVP7/VP2-150   CATTCAGAAAGAGGAACGGTGTAGATGTTGATCCGAATAAGGGTAAGTGGAAAGAACATA  943
                  F  R  K  R  N  G  V  D  V  D  P  N  K  G  K  W  K  E  H  I  315

mt144/177/200   ----------------------------------------------------------  561
                                                                             187
mtVP7/VP2-150   TAAAGGAGGTAACCGAAAAATTGAAGAAAGCGCAAACCGAAAATGGAGGACAACCATGCC  1003
                  K  E  V  T  E  K  L  K  K  A  Q  T  E  N  G  G  Q  P  C  Q  335

mt144/177/200   --GCGCGCAGGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTT  619
                  A  R  R  G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  207
mtVP7/VP2-150   AAGCGCGCAGGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTT  1063
                  A  R  R  G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  355
                  ************************************************************

mt144/177/200   GTGATCCTCAAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTT  679
                  D  P  Q  G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  227
mtVP7/VP2-150   GTGATCCTCAAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTT  1123
                  D  P  Q  G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  375
                ************************************************************

mt144/177/200   TTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTG  739
                  V  T  V  D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  247
mtVP7/VP2-150   TTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTG  1183
                  V  T  V  D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  395
                ************************************************************

mt144/177/200   CACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTAT  799
                  P  V  N  V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  267
```

```
mtVP7/VP2-150    CACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTAT  1243
                   P  V  N  V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  415
                 ************************************************************

mt144/177/200    GGTATACGTCCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAA  859
                   Y  T  S  L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  287
mtVP7/VP2-150    GGTATACGTCCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAA  1303
                   Y  T  S  L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  435
                 ************************************************************

mt144/177/200    GATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGA  919
                   C  Y  A  Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  307
mtVP7/VP2-150    GATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGA  1363
                   C  Y  A  Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  455
                 ************************************************************

mt144/177/200    TGAATATGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTG  979
                   N  M  Q  P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  327
mtVP7/VP2-150    TGAATATGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTG  1423
                   N  M  Q  P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  475
                 ************************************************************

mt144/177/200    CGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAA  1039
                   Y  L  L  V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  347
mtVP7/VP2-150    CGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAA  1483
                   Y  L  L  V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  495
                 ************************************************************

mt144/177/200    TGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACC  1099
                   N  G  V  V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  367
mtVP7/VP2-150    TGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACC  1543
                   N  G  V  V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  515
                 ************************************************************

mt144/177/200    ACTAGTGGCTGCGGTGTTGCACGGTCACCGCTTTCATTAGTGTCGCGTCGGTTCTTATGC  1104
                   Stop
mtVP7/VP2-150    ACTAGTGGCTGCGGTGTTGCACGGTCACCGCTTTCATTAGTGTCGCGTCGGTTCTTATGA  1548
                   Stop
                 ************************************************************
```

Appendix C-3. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7mt144/177/200 with VP7mt144/177-200/200.

```
mt144/177/200     GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                              M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
mtVP7/VP2-200     GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                              M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
                  ************************************************************

mt144/177/200     GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
mtVP7/VP2-200     GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                    V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
                  ************************************************************

mt144/177/200     TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
mtVP7/VP2-200     TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                    A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
                  ************************************************************

mt144/177/200     AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
mtVP7/VP2-200     AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                    A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
                  ************************************************************

mt144/177/200     TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
mtVP7/VP2-200     TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                    Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
                  ************************************************************

mt144/177/200     CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
mtVP7/VP2-200     CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                    T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
                  ************************************************************

mt144/177/200     AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                    M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
```

```
mtVP7/VP2-200    AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                   M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
                 ************************************************************

mt144/177/200    TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
mtVP7/VP2-200    TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
                 ************************************************************

mt144/177/200    GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                   G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
mtVP7/VP2-200    GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                   G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
                 ************************************************************

mt144/177/200    AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAGGCCT----------------------  561
                   V  N  A  L  L  A  P  R  L  Q  R  P                         187
mtVP7/VP2-200    AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAAGCTGAGATTTGGAATGATGTACCCAC  583
                   V  N  A  L  L  A  P  R  L  Q  K  L  R  F  G  M  M  Y  P  H  195
                 *********************************

mt144/177/200    ------------------------------.---------------------------  561
                                                                              187
mtVP7/VP2-200    ACTATTATGTTTTGCATAGTGATTACTGTATTGTACCAAATAAGGGGGGAACTAGTATTG  643
                   Y  Y  V  L  H  S  D  Y  C  I  V  P  N  K  G  G  T  S  I  G  215

mt144/177/200    ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-200    GATCATGGCATATAAGAAAACGTACTGAGGGTGATGCGAAAGCTTCTGCTATGTATTCTG  703
                   S  W  H  I  R  K  R  T  E  G  D  A  K  A  S  A  M  Y  S  G  235

mt144/177/200    ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-200    GAAAAGGTCCACTGAATGACTTACGAGTTAAAATTGAGCGGGATGATTTATCTCGAGAGA  763
                   K  G  P  L  N  D  L  R  V  K  I  E  R  D  D  L  S  R  E  T  255

mt144/177/200    ------------------------------------------------------------  561
                                                                              187
```

```
mtVP7/VP2-200    CAATTATTCAGATCATTGAGTACGGTAAGAAATTTAATTCATCAGCAGGTGATAAGCAGG  823
                   I  I  Q  I  I  E  Y  G  K  K  F  N  S  S  A  G  D  K  Q  G  275

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-200    GGAACATTTCAATTGAAAAATTGGTAGAGTATTGTGATTTTTTGACAACATTCGTTCATG  883
                   N  I  S  I  E  K  L  V  E  Y  C  D  F  L  T  T  F  V  H  A  295

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-200    CGAAGAAGAAAGAAGAGGGTGAGGATGATACTGCTCGACAGGAGATAAGAAAAGCATGGG  943
                   K  K  K  E  E  G  E  D  D  T  A  R  Q  E  I  R  K  A  W  V  315

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-200    TTAAGGGGATGCCTTATATGGATTTCTCAAAACCGATGAAAATCACGCGTGGATTCAACA  1003
                   K  G  M  P  Y  M  D  F  S  K  P  M  K  I  T  R  G  F  N  R  335

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-200    GAAATATGCTTTTCCTTGCGGCGCTCGATTCATTCAGAAAGAGGAACGGTGTAGATGTTG  1063
                   N  M  L  F  L  A  A  L  D  S  F  R  K  R  N  G  V  D  V  D  355

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-200    ATCCGAATAAGGGTAAGTGGAAAGAACATATAAAGGAGGTAACCGAAAAATTGAAGAAAG  1123
                   P  N  K  G  K  W  K  E  H  I  K  E  V  T  E  K  L  K  K  A  375

mt144/177/200    ----------------------------GCGCGCAGGGGGGACGCAGTCATGATCT  589
                                              A  R  R  G  D  A  V  M  I  Y  197
mtVP7/VP2-200    CGCAAACCGAAAATGGAGGACAACCATGCCAAGCGCGCAGGGGGGACGCAGTCATGATCT  1183
                   Q  T  E  N  G  G  Q  P  C  Q  A  R  R  G  D  A  V  M  I  Y  395
                 *************************************************************

mt144/177/200    ATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGAAGCTTTCTAGAG  649
                   F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  K  L  S  R  V  217
```

```
mtVP7/VP2-200    ATTTCGTTTGGAGACCGTTGCGTATATTTTGTGATCCTCAAGGTGCGAAGCTTTCTAGAG  1243
                   F  V  W  R  P  L  R  I  F  C  D  P  Q  G  A  K  L  S  R  V  415
                 ************************************************************

mt144/177/200    TCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCAG  709
                   D  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  A  237
mtVP7/VP2-200    TCGACTCACTTGAGAGCGCTCCAGGAACTTTTGTCACCGTTGATGGAGTAAATGTTGCAG  1303
                   D  S  L  E  S  A  P  G  T  F  V  T  V  D  G  V  N  V  A  A  435
                 ************************************************************

mt144/177/200    CTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCAC  769
                   G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  R  257
mtVP7/VP2-200    CTGGAGATGTCGTCGCATGGAATACTATTGCACCAGTGAATGTTGGAAATCCTGGGGCAC  1363
                   G  D  V  V  A  W  N  T  I  A  P  V  N  V  G  N  P  G  A  R  455
                 ************************************************************

mt144/177/200    GCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACA  829
                   R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T  277
mtVP7/VP2-200    GCAGATCAATTTTACAGTTTGAAGTGTTATGGTATACGTCCTTGGATAGATCGCTAGACA  1423
                   R  S  I  L  Q  F  E  V  L  W  Y  T  S  L  D  R  S  L  D  T  475
                 ************************************************************

mt144/177/200    CGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGC  889
                   V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H  297
mtVP7/VP2-200    CGGTTCCGGAATTGGCTCCAACGCTCACAAGATGTTATGCGTATGTCTCTCCCACTTGGC  1483
                   V  P  E  L  A  P  T  L  T  R  C  Y  A  Y  V  S  P  T  W  H  495
                 ************************************************************

mt144/177/200    ACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTT  949
                   A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  317
mtVP7/VP2-200    ACGCATTACGCGCTGTCATTTTTCAGCAGATGAATATGCAGCCTATTAATCCGCCGATTT  1543
                   A  L  R  A  V  I  F  Q  Q  M  N  M  Q  P  I  N  P  P  I  F  515
                 ************************************************************

mt144/177/200    TTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATG  1009
                   P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  337
mtVP7/VP2-200    TTCCACCGACTGAAAGGAATGAAATTGTTGCGTATCTATTAGTAGCTTCTTTAGCTGATG  1603
                   P  P  T  E  R  N  E  I  V  A  Y  L  L  V  A  S  L  A  D  V  535
                 ************************************************************

mt144/177/200    TGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGA  1069
                   Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  357
```

```
mtVP7/VP2-200    TGTATGCGGCTTTGAGACCAGATTTCAGAATGAATGGTGTTGTCGCGCCAGTAGGCCAGA  1663
                   Y  A  A  L  R  P  D  F  R  M  N  G  V  V  A  P  V  G  Q  I  555
                 ************************************************************

mt144/177/200    TTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCACCG  1104
                   N  R  A  L  V  L  A  A  Y  H  Stop                          367
mtVP7/VP2-200    TTAACAGAGCTCTTGTGCTAGCAGCCTACCACTAGTGGCTGCGGTGTTGCACGGTCACCG  1698
                   N  R  A  L  V  L  A  A  Y  H  Stop                          565
                 ************************************************************
```

Appendix C-4. CLUSTAL X Nucleotide and amino acid sequence alignment of VP7mt144/177/200 with VP7mt144/177-250/200.

```
mt144/177/200    GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                           M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
mtVP7/VP2-250    GTTTAAATTCGGTTAGGATGGACGCGATAGCAGCAAGAGCCTTGTCCGTTGTACGGGCAT  43
                           M  D  A  I  A  A  R  A  L  S  V  V  R  A  C  15
                 ************************************************************

mt144/177/200    GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                   V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
mtVP7/VP2-250    GTGTCACAGTGACAGATGCGAGAGTTAGTTTGGATCCAGGAGTGATGGAGACGTTAGGGA  103
                   V  T  V  T  D  A  R  V  S  L  D  P  G  V  M  E  T  L  G  I  35
                 ************************************************************

mt144/177/200    TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                   A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
mtVP7/VP2-250    TTGCAATCAATAGGTATAATGGTTTAACAAATCATTCGGTATCGATGAGGCCACAAACCC  163
                   A  I  N  R  Y  N  G  L  T  N  H  S  V  S  M  R  P  Q  T  Q  55
                 ************************************************************

mt144/177/200    AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                   A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
mtVP7/VP2-250    AAGCAGAACGAAATGAAATGTTTTTTATGTGTACTGATATGGTTTTAGCGGCGCTGAACG  223
                   A  E  R  N  E  M  F  F  M  C  T  D  M  V  L  A  A  L  N  V  75
                 ************************************************************

mt144/177/200    TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                   Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
mtVP7/VP2-250    TCCAAATTGGGAATATTTCACCAGATTATGATCAAGCGTTGGCAACTGTGGGAGCTCTCG  283
                   Q  I  G  N  I  S  P  D  Y  D  Q  A  L  A  T  V  G  A  L  A  95
                 ************************************************************

mt144/177/200    CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                   T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
mtVP7/VP2-250    CAACGACTGAAATTCCATATAATGTTCAGGCCATGAATGACATCGTTAGAATAACGGGTC  343
                   T  T  E  I  P  Y  N  V  Q  A  M  N  D  I  V  R  I  T  G  Q  115
                 ************************************************************

mt144/177/200    AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                   M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
```

```
mtVP7/VP2-250    AGATGCAAACATTCGGACCAAGCAAAGTGCAAACGGGGCCTTATGCAGGAGCGGTTGAGG  403
                   M  Q  T  F  G  P  S  K  V  Q  T  G  P  Y  A  G  A  V  E  V  135
                 ************************************************************

mt144/177/200    TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
mtVP7/VP2-250    TGCAACAATCTGGCAGATATTACGTACCGCCCGGGGAATTCCTCGAGCAAGGTCGAACGC  463
                   Q  Q  S  G  R  Y  Y  V  P  P  G  E  F  L  E  Q  G  R  T  R  155
                 ************************************************************

mt144/177/200    GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                   G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
mtVP7/VP2-250    GTGGTGGGTACATCAATTCAAATATTGCAGAAGTGTGTATGGATGCAGGTGCTGCGGGAC  523
                   G  G  Y  I  N  S  N  I  A  E  V  C  M  D  A  G  A  A  G  Q  175
                 ************************************************************

mt144/177/200    AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGAGGCCT----------------------  561
                   V  N  A  L  L  A  P  R  L  Q  R  P                         187
mtVP7/VP2-250    AGGTCAATGCGCTGCTAGCCCCAAGGCTGCAGGAGAGTAAGAGAAAAGCAATCCTTGATC  583
                   V  N  A  L  L  A  P  R  L  Q  E  S  K  R  K  A  I  L  D  Q  195
                 *********************************

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-250    AGAATAAGATGTCTAAGGTTGAACAATGGAGAGATGCGGTTAATGAAAGGATTGTGAGTA  643
                   N  K  M  S  K  V  E  Q  W  R  D  A  V  N  E  R  I  V  S  I  215

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-250    TCGAACCAAAGCGAGGTGAGTGCTATGATCACGGAACCGACATTATCTACCAATTCATAA  703
                   E  P  K  R  G  E  C  Y  D  H  G  T  D  I  I  Y  Q  F  I  K  235

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-250    AAAAGCTGAGATTTGGAATGATGTACCCACACTATTATGTTTTGCATAGTGATTACTGTA  763
                   K  L  R  F  G  M  M  Y  P  H  Y  Y  V  L  H  S  D  Y  C  I  255

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
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mtVP7/VP2-250   TTGTACCAAATAAGGGGGGAACTAGTATTGGATCATGGCATATAAGAAAACGTACTGAGG  823
                  V  P  N  K  G  G  T  S  I  G  S  W  H  I  R  K  R  T  E  G  275

mt144/177/200   ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-250   GTGATGCGAAAGCTTCTGCTATGTATTCTGGAAAAGGTCCACTGAATGACTTACGAGTTA  883
                  D  A  K  A  S  A  M  Y  S  G  K  G  P  L  N  D  L  R  V  K  295

mt144/177/200   ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-250   AAATTGAGCGGGATGATTTATCTCGAGAGACAATTATTCAGATCATTGAGTACGGTAAGA  943
                  I  E  R  D  D  L  S  R  E  T  I  I  Q  I  I  E  Y  G  K  K  315

mt144/177/200   ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-250   AATTTAATTCATCAGCAGGTGATAAGCAGGGGAACATTTCAATTGAAAAATTGGTAGAGT  1003
                  F  N  S  S  A  G  D  K  Q  G  N  I  S  I  E  K  L  V  E  Y  335

mt144/177/200   ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-250   ATTGTGATTTTTTGACAACATTCGTTCATGCGAAGAAGAAAGAAGAGGGTGAGGATGATA  1063
                  C  D  F  L  T  T  F  V  H  A  K  K  K  E  E  G  E  D  D  T  355

mt144/177/200   ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-250   CTGCTCGACAGGAGATAAGAAAAGCATGGGTTAAGGGGATGCCTTATATGGATTTCTCAA  1123
                  A  R  Q  E  I  R  K  A  W  V  K  G  M  P  Y  M  D  F  S  K  375

mt144/177/200   ------------------------------------------------------------  561
                                                                              187
mtVP7/VP2-250   AACCGATGAAAATCACGCGTGGATTCAACAGAAATATGCTTTTCCTTGCGGCGCTCGATT  1183
                  P  M  K  I  T  R  G  F  N  R  N  M  L  F  L  A  A  L  D  S  395

mt144/177/200   ------------------------------------------------------------  561
                                                                              187
```

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mtVP7/VP2-250    CATTCAGAAAGAGGAACGGTGTAGATGTTGATCCGAATAAGGGTAAGTGGAAAGAACATA  1243
                   F  R  K  R  N  G  V  D  V  D  P  N  K  G  K  W  K  E  H  I  415

mt144/177/200    ------------------------------------------------------------  561
                                                                               187
mtVP7/VP2-250    TAAAGGAGGTAACCGAAAAATTGAAGAAAGCGCAAACCGAAAATGGAGGACAACCATGCC  1303
                   K  E  V  T  E  K  L  K  K  A  Q  T  E  N  G  G  Q  P  C  Q  435

mt144/177/200    --GCGCGCAGGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTT  619
                   A  R  R  G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  207
mtVP7/VP2-250    AAGCGCGCAGGGGGGACGCAGTCATGATCTATTTCGTTTGGAGACCGTTGCGTATATTTT  1363
                   A  R  R  G  D  A  V  M  I  Y  F  V  W  R  P  L  R  I  F  C  455
                   ************************************************************

mt144/177/200    GTGATCCTCAAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTT  679
                   D  P  Q  G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  227
mtVP7/VP2-250    GTGATCCTCAAGGTGCGAAGCTTTCTAGAGTCGACTCACTTGAGAGCGCTCCAGGAACTT  1423
                   D  P  Q  G  A  K  L  S  R  V  D  S  L  E  S  A  P  G  T  F  475
                   ************************************************************

mt144/177/200    TTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTG  739
                   V  T  V  D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  247
mtVP7/VP2-250    TTGTCACCGTTGATGGAGTAAATGTTGCAGCTGGAGATGTCGTCGCATGGAATACTATTG  1483
                   V  T  V  D  G  V  N  V  A  A  G  D  V  V  A  W  N  T  I  A  495
                   ************************************************************

mt144/177/200    CACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTAT  799
                   P  V  N  V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  267
mtVP7/VP2-250    CACCAGTGAATGTTGGAAATCCTGGGGCACGCAGATCAATTTTACAGTTTGAAGTGTTAT  1543
                   P  V  N  V  G  N  P  G  A  R  R  S  I  L  Q  F  E  V  L  W  515
                   ************************************************************

mt144/177/200    GGTATACGTCCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAA  859
                   Y  T  S  L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  287
mtVP7/VP2-250    GGTATACGTCCTTGGATAGATCGCTAGACACGGTTCCGGAATTGGCTCCAACGCTCACAA  1603
                   Y  T  S  L  D  R  S  L  D  T  V  P  E  L  A  P  T  L  T  R  535
                   ************************************************************

mt144/177/200    GATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGA  919
                   C  Y  A  Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  307
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mtVP7/VP2-250    GATGTTATGCGTATGTCTCTCCCACTTGGCACGCATTACGCGCTGTCATTTTTCAGCAGA  1663
                   C  Y  A  Y  V  S  P  T  W  H  A  L  R  A  V  I  F  Q  Q  M  555
                 ************************************************************

mt144/177/200    TGAATATGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTG  979
                   N  M  Q  P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  327
mtVP7/VP2-250    TGAATATGCAGCCTATTAATCCGCCGATTTTTCCACCGACTGAAAGGAATGAAATTGTTG  1723
                   N  M  Q  P  I  N  P  P  I  F  P  P  T  E  R  N  E  I  V  A  575
                 ************************************************************

mt144/177/200    CGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAA  1039
                   Y  L  L  V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  347
mtVP7/VP2-250    CGTATCTATTAGTAGCTTCTTTAGCTGATGTGTATGCGGCTTTGAGACCAGATTTCAGAA  1783
                   Y  L  L  V  A  S  L  A  D  V  Y  A  A  L  R  P  D  F  R  M  595
                 ************************************************************

mt144/177/200    TGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACC  1099
                   N  G  V  V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  367
mtVP7/VP2-250    TGAATGGTGTTGTCGCGCCAGTAGGCCAGATTAACAGAGCTCTTGTGCTAGCAGCCTACC  1843
                   N  G  V  V  A  P  V  G  Q  I  N  R  A  L  V  L  A  A  Y  H  615
                 ************************************************************

mt144/177/200    ACTAGTGGCTGCGGTGTTGCACGGTCACCGCTTTCATTAGTGTCGCGTCGGTTCTTATGC  1104
                   Stop
mtVP7/VP2-250    ACTAGTGGCTGCGGTGTTGCACGGTCACCGCTTTCATTAGTGTCGCGTCGGTTCTTATGA  1845
                   Stop
                 ************************************************************
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