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EPITOPE MAPPING OF MONOCLONAL ANTIBODIES AGAINST THE STRUCTURAL PROTEINS VP1, VP2 AND VP3 OF ACUTE BEE PARALYSIS VIRUS

Diploma thesis

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submitted by

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1 LIST OF ABBREVIATIONS

aa – Amino Acid(-s)

ABPV – Acute Bee Paralysis Virus

APS - Ammonium persulphate solution

(k)bp – (kilo)base pair

kDa – kilo Dalton

ddH_2O – Double distilled water

DNA – Desoxyribonucleic acid

Dpn1 – Restriction enzyme cleaving Gm6A^TC-sites

DWV – Deformed wing virus

EcoRI-HF – EcoRI-High fidelity restriction enzyme cleaving palindromic G^AATTC-sites

EtOH - Ethanol

ELISA – Enzyme Linked Immunosorbent Assay

fw. – Forward (primer)

HRP – Horseradish peroxydase

i. – Induced

IAPV – Israeli acute paralysis virus

IPTG - Isopropyl-β-D-thiogalactopyranosid

KBV – Kashmir bee virus

LB-Ampicillin medium – lysogeny broth ampicillin medium

MeOH – Methanol

MluI-HF – MluI-High fidelity restriction enzyme cleaving A^CGCGT-sites

n.i. – Not induced

nt(-s) – Nucleotide(-s)

PBS-T – Phosphate buffered saline with tween

PCR – Polymerase chain reaction

PvuI-HF – PvuI-High fidelity restriction enzyme cleaving CGAT^CG-sites

TEMED - Tetramethylethylendiamin

SDS-PAGE – Sodium Dodecylsulfate Polyacrylamide Gel

rev. – Reverse (primer)

2 INTRODUCTION

The western honey bee *(Apis mellifera L.)* is essential to our ecologic system. Its dutiful work of pollinating plants and making of high quality products like royal jelly, wax and honey contributes a lot to agriculture and life quality of human life. Not only play these little insects a big role for our environment, but they also have a big economic impact, which adds up to billions of dollars worldwide, as of their role in food production (Ullah et al., 2020).

As there have been observed and reported more and more instances of whole bee colony losses all over the world, bee health has become an ever more important aspect in scientific research. Over the last decades, the usage of harmful pesticides as well as climatic changes have caused more and more troubles for bee health. It makes them potentially more susceptible to pathogens, therefore resulting in increased losses of colonies. Climate change also enables dangerous parasites to spread to new regions causing novel challenges to the immune system of the bees by potentially conveying new diseases. Another factor has been the increasing global trade of honey bee products and live honey bees as potential danger to transport diseases to new areas. Bees can be affected by many different pathogens like viruses, bacteria, fungi or parasites. All pathogens named can have major influence on colony health (Tantillo et al., 2015; Schittny et al 2020). This investigation will focus on a virus that causes disease in bees, namely the *Acute bee paralysis virus (ABPV)*. It is of importance to better understand the pathogenic mechanisms in both the individual animal and the effects on colony levels, as well as possible interactions with other pathogens to try prevent the loss of honey bee colonies (Van Engelsdorp et al., 2009; Chejanovsky 2020).

The *acute bee paralysis virus (ABPV)* was first described by Bailey et al., 1963 in Great Britain. The icosahedral virus is a non-enveloped single-stranded RNA virus and a member of the family *Dicistroviridae*, within the superfamily *Picornavirales*. Its genome has a length of 9470 nucleotides and displays high variability including genetic recombinants. The ABPV genome encodes two open reading frames, namely ORF1 and ORF2. The ORF1 sequence spans from nucleotides 605 to 6325 encoding a replicase polyprotein, consisting of a helicase, a protease and an RNA-dependent RNA polymerase. Sequence similarities to other virus families like the *Picorna-, Como-, Calici-* and *Sequiviruses* are observed. ORF2 (nt 6509 and 9253) contains three major structural proteins (VP1, VP3, VP2) and a minor protein (VP4) according to Govan, who ordered the proteins according to their molecular mass. (Govan et al 2000). The figure

below graphically shows the organization of *ABPV* by de Miranda et al., 2010 that uses a slightly different taxonomy, as the denomination of VP2 and VP3 is exchanged. While the structural proteins are named differently to facilitate a better comparison between the different bee viruses, the structural protein VP2 (Govan et al., 2000) is equivalent to VP3 (de Miranda et al., 2010). The nomenclature used in this thesis is based on the newer work by de Miranda et al., 2010.

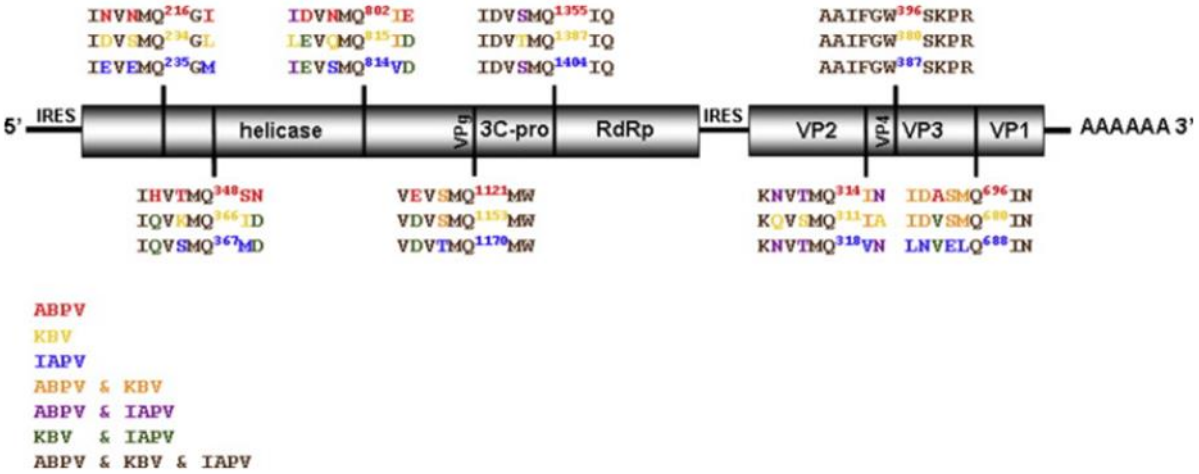


Fig. 1: Genome organization of *ABPV* with ORF1 and ORF2 marked in red (de Miranda et al., 2010). ORF2 encodes the structural proteins VP1-VP4. VP3 in this genome organization is analogous to VP2 by Govan et al., 2000.

The main clinical symptoms in the colony are paralysis of adult bees resulting in high mortality. Affected classes are worker bees and drones, but the queen is also susceptible (Amiri 2020). Horizontal transmission can occur via trophallaxis, glandular secretions and direct body contact. ABPV has been found in honey bee eggs and in early larval stages of bee queen development (Chen et al., 2006; Žvokelj et al., 2020) so that venereal or transovarial infections are possible. In addition, the *Varroa destructor* mite is suspected to transmit ABPV to the bee brood. The beekeeper is also a risk factor for transmitting ABPV between colonies or apiaries if hygiene is not respected (Schittny et al., 2020).

The disease has been associated with the Colony collapse disorder, same as the *Kashmir paralysis virus* and the *Israeli acute paralysis virus* (Dittes et al., 2020). The Colony collapse disorder is a phenomenon that describes large scale losses in bee colonies of different cause. The term has been used in the US since 1869, when distinctive bee losses have been first described (Van Engelsdorp et al., 2009). *Acute bee paralysis virus* is the second most detected

bee virus in Austria, the most prevalent being the *Deformed Wing virus*. Berényi described that in hives showing symptoms of bee losses, sudden deaths, paralysis, dark colour together with *Varroa destructor* infestation, ABPV is frequently occurring (Berény, et al., 2006). A subsequent study by Morawetz et al., 2019, discovered clinical Varroosis in 21% of inspected apiaries, the numbers of mite- and ABPV- coinfected hives being threefold higher in summer and autumn compared to spring. The Varroa mite feeds on bee tissues, mainly the fat body, promoting a direct ABPV transmission overcoming the natural defence mechanisms (Traynor et al., 2020).

Diagnostic methods to detect *ABPV* include a clinical examination of the hive including the individual castes in their different stages of development. Bees showing any kinds of ABPV typical abnormalities are taken as samples. Standard method for detecting honey bee viruses is reverse-transcriptase PCR (RT-PCR) or quantitative RT-PCR (RT-qPCR (Ciglenečki & Toplak, 2012; Dittes et al., 2020). The RT-qPCR protocol is faster than conventional PCR and provides quantitative results indicating the viral burden of the infected bees (Chantawannakul et al., 2006).

PCR is an extremely sensitive method, detecting down to 10 – 100 molecules in the reaction. Unfortunately, the costs of the analysis, including sampling a shipment, are often too high for the application in apiaries. As a new alternative, a serological test system was developed that detects ABPV antigens with the help of monoclonal antibodies. To this end mice were immunized with purified ABPV or recombinantly expressed VP1, 2 or 3 proteins. The aim is the induction of specific B cells that are immortalized to form hybridomas that are selected in order to produce large amounts of a single IgG species. Technically these antibodies are then applied in ELISA or a Lateral Flow Assay format. Both methods are inexpensive and offer the advantage of applying the tests directly at the apiary. As an effort in the joint project “Zukunft Biene 2”, Murine monoclonal antibodies were raised against the structural proteins VP1, VP2 and VP3 of ABPV (Brodschneider, Morawetz and Seitz., 2021).

Several candidate monoclonal antibodies have been prepared in extensive preparatory work that detect and bind to ABPV virus particles with high specificity and sensitivity. The aim of this diploma thesis was to identify the structural protein that is detected. As next step, the precise location of the epitopes detected by candidate antibodies αABPV-VUPLA7, αABPV-RF9, αABPV-RC11 was to be determined.

3 MATERIALS AND METHODS

3.1. MATERIALS

3.1.1. ANTIBODIES

3.1.1.1. PRIMARY ANTIBODIES

The anti-APBV antibodies investigated in this study have been generated at the Institute of Virology, Vetmeduni Vienna, within the framework of the project Zukunft Biene 2. These murine monoclonal antibodies αABPV-VUPLA7, αABPV-RF9, αABPV-RC11 were selected in serial screenings for high affinity binding to native antigen as it is exposed in the virus particle (ZUBI-2 Endbericht). The murine anti-HisTag monoclonal antibody (αHis-10B6) was used as a control for successful protein expression

3.1.1.2. SECONDARY ANTIBODIES

As a secondary antibody, a horse raddish peroxidase (HRP) conjugated goat α mouse IgG (Jackson ImmunoResearch) was used at a concentration of 1:10.000 diluted in Phosphate buffered saline including 0.05% Tween20 (PBS-T).

3.1.2. ENZYMES

All enzymes were purchased from *New England Biolabs, USA*. (NEB).

1) Dpn1

2) EcoRI(-HF)

3) MluI(-HF)

4) PvuI(-HF)

3.1.3. LABORATORY EQUIPMENT AND CONSUMABLES

96-well plates – Sarstedt AG & Co. KG, Germany

Caps (15 ml, 50 ml) – Sarstedt AG & Co. KG, Germany

Chemidoc – Bio-Rad Laboratories, USA

Eppendorf tubes (0,2, 1,5, 2 ml) – Eppendorf AG, Germany

Eprouvettes

Fitting glas pipette tips (5ml, 10ml)

Petri dishes (10cm) with LB-Ampicillin-Agar

Petridishes, 10cm

Pipetboy

Pipettes of various gauge (10, 20, 100, 200, 1000µl)

Pipette tips of various gauge

Scalpel

MiniSpin® Centrifuge - Eppendorf AG, Germany

Mini Trans-Blot® Cell – Bio-Rad Laboratories, USA

NanoVue Plus Spectrophotometer - Biochrom Ltd, England

Rocky® platform shaker

Safety cabinet LaminAir HB 2448 – Heraeus Group, Germany

Vacuum pump - Carl Roth, Germany

Vortex-Genie 2 – Scientific Industries, Inc., USA

3.1.4. PLASMIDS

All plasmids were generated using a pet11a vector backbone (Fa. Novagen) for protein expression and a N-terminal polyhistidine tag. The figure below shows the first two constructs

that were designed for the capsid protein VP3 (pKS98). To keep the figure clearer, only the first two plasmids that were generated are shown. For a detailed overview of pKS157 (N-terminal end of capsid protein VP3) and pKS158 (C-terminal end of capsid protein VP3) see Figure 2.

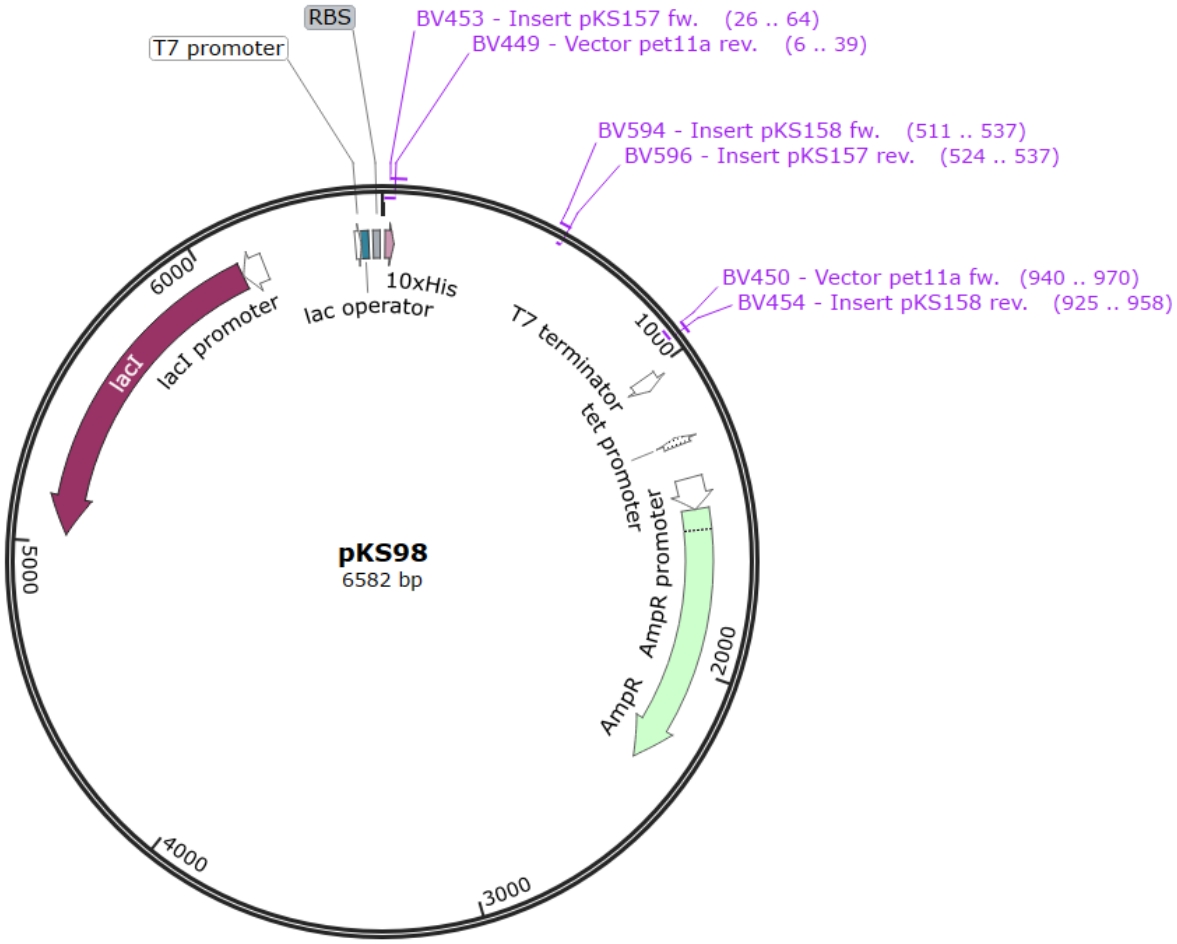


Fig. 2: Plasmid map of the original plasmid pKS98 of the capsid protein VP3 generated by SnapGene Viewer (version 7.0.2). The numbers in brackets represent the number of base pairs. The pet11a vector makes up the majority of the plasmid and contains an Ampicillin resistance gene (green arrow) as well as a lac repressor (purple arrow), which inhibits the transcription of E. coli by binding to the lac operator. The addition of IPTG suppresses this inhibition, which is essential for induction of the protein expression. The lavender marks show the regions of the inserts pKS157 (N-terminal part of capsid protein VP3) and pKS158 (C-terminal part of capsid protein VP3). The primers were designed to overlap between vector and inserts to make the following Gibson Assembly possible.

pKS97 – Plasmid for expression of the VP1 structural protein of ABPV

pkS98 - Plasmid for expression of the VP2 structural protein of ABPV

pKS99 – Plasmid for expression of the VP3 structural protein of ABPV

pKS157 – Plasmid for expression of the N-terminal of VP2

pKS158 – Plasmid for expression of the C-terminal part of VP2

pKS209 – Plasmid for the expression of VP2, minus 264 nts (88 aa) from the C-terminal part

pKS212 – Plasmid for the expression of VP2, minus 171 nts (57 aa) from the C-terminal part

pKS211 - Plasmid for the expression of VP2, minus 84 nts (29 aa) from the C-terminal part

pKS217 – Plasmid for the expression of VP2, minus 36 nts (12 aa) from the C-terminal part

pKS215 – Plasmid for the expression of VP2, minus 12 nts (4 aa) from the C-terminal part

pKS218 – Plasmid for the expression of VP2, minus 3 nts (1 aa) from the C-terminal part

pKS219 – Plasmid for the expression of VP2, minus 6 nts (2 aa) from the C-terminal part

3.1.5. PRIMERS

Primers were designed with the ambition to shorten the plasmid via PCR in means of finding the epitope. All primers were ordered from *Eurofins Genomics Germany GmbH*.

primer	Strand orientation	PCR product	sequence (5‘-NNN-3‘)
BV449	Rev.	Vector pET11a for all constructs; Template:	ATGATGATGATGGTGATGGTGATGGTG ATGGCTA

		pKS98 (capsid protein VP3)	
BV450	Fw.	vector pET11a for all constructs; Template: pKS98 (capsid protein VP3)	TAGGATCCGGCTGCTAACAAAGCCCGAAAGG
BV453	Fw.	used for generation of pKS157 (C-terminal part of capsid protein VP3), pKS209 (capsid protein VP3 -88 aa), pKS212 (capsid protein VP3 -57 aa), pKS211 (capsid protein VP3 -29 aa), pKS215 (capsid protein VP3 -4 aa), pKS217 (capsid protein VP3 -12 aa), pKS219 (capsid protein VP3 -2 aa), pKS218 (capsid protein VP3 -1 aa); Template:	ACCATCATCATCATTCAAAACCTAGAAATTTAGAACAAG

		pKS98 (capsid protein VP3)	
BV454	Rev.	insert pKS158 (N-terminal part of capsid protein VP3); Template: pKS98	TGTTAGCAGCCGGATCCTATTGCATTGAAGCATC
BV596	Rev.	insert pKS157 (C-terminal part of capsid protein VP3); Template: pKS98 (capsid protein VP3)	AGCCGGATCCTACGTCGTCGTTATTG
BV676	Rev.	Insert pKS211 (capsid protein VP3 -29 aa); Template: pKS98 (capsid protein VP3)	AGCCGGATCCTAAGGTTCTACTACTACTACATCTT
BV690	Rev.	insert pKS209 (capsid protein VP3 -88 aa); Template: pKS98 (capsid protein VP3)	AGCAGCCGGATCCTAAAACATTTTATTTGAAAC
BV692	Rev.	insert pKS212 (capsid protein VP3 -57 aa); Template: pKS98 (capsid protein VP3)	AGCAGCCGGATCCTATGTGACAGGTCTTATACAC

BV695	Rev.	insert pKS215 (capsid protein VP3 -4 aa); Template: pKS98 (capsid protein VP3)	AGCCGGATCCTAATCTATAGTATT GATAGAATCTGCGGA
BV697	Rev.	insert pKS217 (capsid protein VP3 -12 aa); Template: pKS98 (capsid protein VP3)	AGCCGGATCCTAGGAAGTTACAGG TGGTTGAAAC
BV699	Rev.	Insert pKS219 (capsid protein VP3 -2 aa) ; Template: pKS98 (capsid protein VP3)	GTTAGCAGCCGGATCCTATGAAGC ATCTATAGTATTG
A10	Fw.	generic vector pET11a	GGAATTGTGAGCGGATAAC
A11	Rev.	generic vector pET11a	GGTTATGCTAGTTATTGCTCGA

Tab. 1 List of primers

3.1.6. REAGENTS

Acrylamide

APS 10% - 1g Ammonium persulphate/10 ml dH_2O

Glycerol – Sigma-Aldrich, USA

NEB 3.1 buffer – New England BioLabs, USA

NEBCutSmart® buffer – New England BioLabs, USA

NEBuilder HIFI DNA Assembly Master Mix – New England BioLabs, USA

2x Phanta Max Master Mix – Vazyme Biotech Ltd.

One Taq Quick Load 2x Master Mix – New England Biolabs, USA

peqGreen – VWR International, USA

TEMED - Roth Chemie GmbH, Germany

3.1.6. KITS

Amersham ECL Prime Western Blotting - Detection Reagent - Cytiva

FavorPrep™ Plasmid DNA Extraction Mini Kit – Favorgen Biotech Corp., Taiwan

Monarch® DNA Gel Extraction Kit (5µg) – New England Biolabs, USA

Monarch® PCR & DNA Cleanup Kit (5 µg) – New England Biolabs, USA

Trans-Blot Turbo Transfer System RTA Transfer Kit, Bio-Rad Laboratories, Inc

3.1.7. SOLUTIONS, MEDIA AND BUFFER

6X Protein Loading Dye for SDS-PAGE: 6M urea, 62,5 mM Tris, 2% SDS, 10% Glycerin, 0,025% bromophenol blue

Agarose gel: 1x TAE buffer, agarose (0,8%), MidoriGreen Advance DNA stain (NipponGenetics, Europe; 16 µl/400ml buffer)

Anode buffer for SDS-PAGE: 0,2 M Tris-HCl, pH 8,9

Cathode buffer for SDS-PAGE: 0,1 M Tris-HCl,pH 8,9, 0,1 M Tricin, 0,1% SDS, pH 8,25

Coomassie Dye: 0,5 % (w/v) Coomassie Brilliant Blue R250, 50 % (v/v) Methanol, 7 % (v/v) acetic acid

Destaining solution for Coomassie: 20 % (v/v) methanol, 7 % (v/v) acetic acid

LB-Ampicillin medium: 500ml of lysogeny broth, 500 µl Ampicillin (%?)

P1: 50mM Tris + 0,1mg/ml RNase A, pH=8,0

P2: 0,2mM NaOH + 1% SDS (Natriumdodecylsulfat)

P3: 2,55M KaAc, pH=5,5

PBS-T: 137 mM NaCl + 2,7 mM KCl + 10 mM Na2HPO4 + 1.8 mM KH2PO4 + Tween® 20 detergent, 0,1% (w/v) washing buffer for various applications

0,5% TAE (Tris-acetate-EDTA)-buffer for gel electrophoresis: 20 mM Tris, 10 mM trifluoroacetic acid, 0,5 mM EDTA, pH of 50X = 8,4

Tris-Tricin-buffer for SDS-PAGE: 25 mM TRIS, 192 mM glycine, 0,1% m/V SDS (pH 8,3)

Western blot buffer: 39mM Glycin, 48mM Tris, 0,037% SDS, 20% MeOH

rCutSmart Buffer (or NEBuffer 4 + rAlbumin)

3.2. METHODS

3.2.1. WORKING WITH DNA

3.2.1.1. POLYMERASE CHAIN REACTION (PCR)

The goal of PCR is to amplify a specific part of a DNA-sequence in large quantity and in a short time. Other uses for PCR are modification of DNA or RNA samples, gene cloning, sequencing and expression studies. The PCR consists of three steps, which are performed in a thermocycler. First, the DNA template is denatured at 92-95°C, then the annealing of the primers is performed at 50-70°C, the temperature depending on the chosen primers and finally the sample(-s) of DNA are extended. The annealing temperatures were calculated using the NEB T_M Calculator. These three steps are performed generally for 30-40 cycles. The primers are single-stranded DNA molecules, consisting of 18-25 nucleotides, which are complementary to the template sequence (Bevan et al 1992; Gupta 2019).

The PCRs were performed in 25/50 µl reactions. All reagents were combined in 0,2 ml Eppendorf tubes. Those products showing specific bands under UV light after being run on the agarose gel electrophoresis were further processed. Sizes of the samples were checked through

specific bands using the *1 kb DNA Ladder* (New England Biolabs® Inc.). The PCR was programmed for 40 cycles, annealing for 30 minutes, and elongation of the insert for 15 seconds, for the vector 1 minute 30 seconds.

Reagent	**Volume (µl)**
2x Phanta Max Mastermix	25
10 µM Forward Primer	2
10 µM Reverse Primer	2
Plasmid pKS98	0,5
ddH_2O	20,5

Tab. 2 Standard PCR protocol for the 2x Phanta Max Mastermix

PCR Products	**Forward**	**Reverse**	**Annealingtemp.**	**Elongation**
Vector (for all constructs)	BV449	BV450	69°C for 30 sec	1 min 30 sec
pKS157 (Insert) = N-terminal part of VP3	BV453	BV596	71°C for 30 sec	15 sec
pKS158 (Insert) = C-terminal part of VP3	BV594	BV454	69°C for 30 sec	15 sec
pKS209 (Insert) = C-terminal part of VP3	BV453	BV690	69°C for 30 sec	15 sec
pKS212 (Insert) = C-terminal part of VP3	BV453	BV692	69°C for 30 sec	15 sec
pKS211 (Insert) = C-terminal part of VP3	BV453	BV676	69°C for 30 sec	15 sec
pKS215 (Insert) = C-terminal part of VP3	BV453	BV695	69°C for 30 sec	15 sec

pKS217 (Insert) = C-terminal part of VP3	BV453	BV697	69°C for 30 sec	15 sec
pKS219 (Insert) = C-terminal part of VP3	BV453	BV699	69°C for 30 sec	15 sec
pKS218 (Insert) = C-terminal part of VP3	BV453	BV698	69°C for 30 sec	15 sec

Tab. 3 PCR protocol for the vector and inserts.

3.2.1.2. AGAROSE GEL ELECTROPHORESIS OF PCR PRODUCTS

The goal of an agarose gel electrophoresis is to separate DNA fragments of a sample by their molecular size using an electric current for the separation. The concentration of the gel depends on the size of the DNA fragment to be separated. Lesser concentrated gels allow the fragments to travel faster, therefore the bigger the sample the lower a concentration is to be used. After staining the gel with a DNA-binding dye, the results can be visualized by illuminating the gels with UV light using the BioSpectrum® Imaging System (Voytas 2001).

In this work, 0,8 % agarose gels were used to visualize the DNA fragments. Usually, 2 µl of the PCR reaction was mixed with 1µl Gel loading dye and the mixture was pipetted into these purses. As a size marker, 3 µl of a DNA ladder (Carl Roth GmbH + Co. KG) was used. The chamber was set for 30 minutes at 100V. Afterwards the gel was visualized under UV light using *the BioSpectrum® Imaging System*. For purification of DNA fragments, a 0,8% agarose gel was used and at least 25 µl of the PCR reaction was used as sample, mixed with 5 µl of *Gel loading dye purple* (in a ratio of 1:6) and pipetted into the gel wells. The chamber was again set for 30-45 minutes at 100V. After that, the gels were set on a UV light station and the bands were cut from the gel with a scalpel.

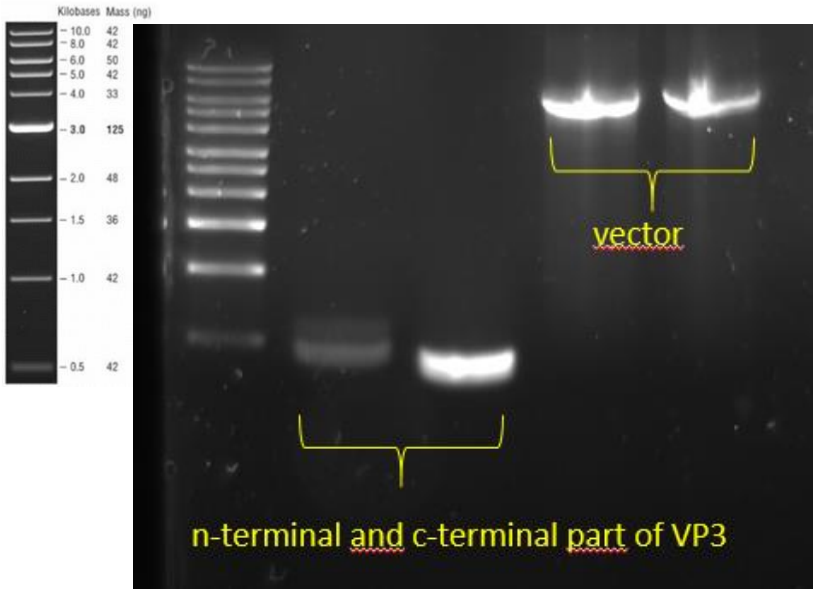


Fig. 3: Exemplary depiction of agarose gel electrophoresis results made visible under UV light. The inserts are approximately at the 500 bps mark, the pET11a vector is 5.677 bps long (1 kb ladder by New England Biolabs GmbH; https://international.neb.com/products/n3232-1-kb-dna-ladder#Product%20Information, access: 22.08.23).

3.2.1.3. PURIFICATION OF PCR PRODUCTS

The PCR products were purified using the *Monarch® DNA Gel Extraction Kit (5µg)/ Monarch® PCR & DNA Cleanup Kit (5 µg)* according to manufacturer's instructions.

Briefly, the bands were excised from the gel using a scalpel, transferred into 2ml Eppendorf tubes and weighed. After that, they were filled up with 4x volume of *Monarch® Gel Dissolving Buffer*. The tubes were put into the thermoshaker at 37°C and occasionally vortexed to speed up the solution of the gels. Then, 700 µl of the solution were transferred onto the column, spun for 1 min at 13.000 rpm and the flow-through discarded. This was repeated until all solution was used. Then in each tube 200 µl of *Monarch® DNA Wash Buffer* was added and again spun down for 1 minute at 13.000 rpm and the flow-through discarded. This step was done twice and then the tubes were dry centrifuged for 2 min at 13.000 rpm. Now the collection tubes were put into a new 1,5 ml Eppendorf tube and 14 µl DNAse free H_2O was added for elution of the bound DNA. The tubes were incubated for 1 min and spun down for 1 minute at 13.000 rpm. The concentration of the eluate was measured using a Spectrophotometer, the *NanoVue Plus Spectrophotometer*. The following table is showing selected purified products and is included exemplary.

Sample	Concentration
V1 (vector used for pKS157 and 158)	183,0 ng/µl
pKS157 (insert; N-terminal part of capsid protein VP3)	158,0 ng/µl
pKS158 (insert; C-terminal part of capsid protein VP3)	146,0 ng/µl

Tab. 4: Concentrations of purified PCR products

3.2.1.4. GIBSON ASSEMBLY

The Gibson Assembly is a DNA cloning method, where gene constructs get assembled and many fragments can be cloned into a vector at once (Chen 2018). The Gibson assembly is a method for DNA cloning into any vector within one reaction. For the Gibson Assembly to work the primers need to be designed to create overlapping ends (containing 20-40 bp) between vector and the fragment(-s), that is (are) meant to be amplified (Share Biology®. https://sharebiology.com/gibson-assembly/, (access 15.08.23)).

The products of vector and inserts were ligated following instructions of this protocol used for all samples:

5µl Master Mix (Hifi NEB …) + vector:insert 1:2 + x µl H_2O

adding up to 10 µl.

product	**Fragment 1 (insert)**	**Fragment 2 (vector)**	**H_2O**
pKS 157	1 µl (89,5 ng/µl)	0,5 µl (163,5 ng/µl)	3,5 µl
pKS 158	0,8 µl (151 ng/µl)	2 µl (123,5 ng/µl)	3,2 µl
pKS 209	4 µl (24 ng/µl)	0,5 µl (163,5 ng/µl)	0,5 µl
pKS 212	3 µl (27 ng/µl)	1 µl (163,5 ng/µl)	1 µl
pKS 211	3 µl (23 ng/µl)	1 µl (163,5 ng/µl)	1 µl

Tab. 5: Amounts of PCR products used for the Gibson Assembly exemplary for some of the constructs

The samples were incubated at 50°C for 15 minutes and then put on ice.

3.2.1.5. TRANSFORMATION IN HB101

HB101 are E. coli cells with high competence for transformation (Inoue et al. 1990). E. coli are gram negative and rod-shaped bacteria, laboratory strains in general can be considered non-pathogenic. They are a standard choice as host for the construction of plasmids. Positive aspects of working with E. coli are it´s fast growth rate especially under aerobic but also under anaerobic conditions, they are easy to manipulate at comparatively low cost (Rosano et al., 2019; Tuttle et al., 2021).

The transformation was done following given protocol:

1.) 10 µl ligation (on ice) + 50 µl of competent cells (thawed on ice for 30 minutes)

2.) reaction mix set on ice for 20 minutes,

3.) heat shock at 37°C for 1 minute 30 seconds,

4.) 2-3 minutes incubation on ice,

5.) addition of 450 µl of LB-medium (without ampicillin),

6.) regeneration for 20 minutes at 37°C shaking

7.) 200 µl of the reaction mix is placed on two 10 cm petri dishes with LB-ampicillin agar and incubated at 37°C overnight

3.2.1.6. RETRANSFORMATION IN EXRESSION STRAIN BL21_ROSETTA

It was performed using the following protocol:

1.) competent cells are thawed on ice for at least 30 minutes

2.) 30µl of competent cells are added to 50ng of plasmid DNA

3.) reaction mix is incubated on ice for 15 minutes

4.) heat shock the sample for 1 minute 30 seconds at 37°C

5.) incubation on ice for 1 min

6.) addition of 200 µl of LB-medium to the mix and incubated for 30-60 min at 37°C to allow the expression of the resistance gene

7.) 50 µl of the transformation are immediately plated on LB – agar with 50µg/ml ampicillin

8.) incubation overnight at 37°C

3.2.1.7. PLASMID PREPARATION

12 colonies growing on the LB-ampicillin agar were picked and transferred to 2,5 ml of LB-ampicillin medium and cultured at 37°C for 6-12 h. The plasmids were prepared using the following alkaline lysis protocol:

2 ml of the culture were transferred into 2 ml Eppendorf tubes and spun in the centrifuge at 13.000 rpm for 1 minute, the supernatant was discarded using the vacuum pump

- 200 µl of resuspension buffer (P1) were added to each tube and then vortexed until the bacterial pellet was resuspended
- 200 µl of P2 were added and carefully inverted 5-6x, followed by an incubation for 5 minutes at room temperature
- 200 µl of P3 were added and mixed thoroughly

Next, the tubes were centrifuged for 5 minutes at 13.000 rpm, meanwhile 1,5 ml Eppendorf tubes were prepared with 500 µl isopropanol pipetted into each tube. After the centrifugation the clear supernatant was pipetted into the prepared tubes and mixed well. The tubes were centrifuged for 1 minute at 13.000 rpm and the supernatant carefully discarded using the vacuum pump. To wash the pellet, 100 µl of 70% Ethanol were added and centrifuged for 1 minute at 13.000 rpm. The supernatant was removed with the vacuum pump. Next the tubes were transferred into the *Thermomixer compact* for 3-5 minutes at 37°C with the lid open, so that the remaining EtOH could vaporize.

- For resuspension, 25 µl H_2Odd were added to the pellet and the reaction incubated in a thermomixer at 37°C and 700 rpm for 10 minutes.

The tubes were then vortexed and stored at -20°C.

3.2.1.8. ANALYTICAL RESTRICTION DIGEST

For the analytical restriction digest enzymes are needed. To be able to do DNA analysis and cloning, type II restriction enzymes are used. These are produced by prokaryotes for the purpose of battling bacteriophages. Restriction enzymes have the ability to detect specified sequences

in the DNA and cleave at the restriction sites or close to them (Pingoud et al. 2014). This technique was used to verify the generated plasmids.

The master mix was prepared for 20 samples and consisted of following components:

134 µl H_2Odd + 20 µl 10x buffer NEB + 3 µl restriction enzyme 1 + 3 µl restriction enzyme 2 = 160 µl in total + 40 µl plasmid DNA (20x2) = 360 µl in total

On a 96 well plate 8 µl Master Mix were pipetted into each well and 2 µl of the plasmid added. The filled wells were closed with tape so that the samples could not evaporate and incubated at 37° for 45 minutes. The reaction was loaded onto an 0,8% agarose gel and visualized under UV light.

The chosen restriction enzymes were MluI-HF and EcoRI-HF using the *CutSmart Buffer* respectively MluI and EcoRI using the *3.1 Buffer*.

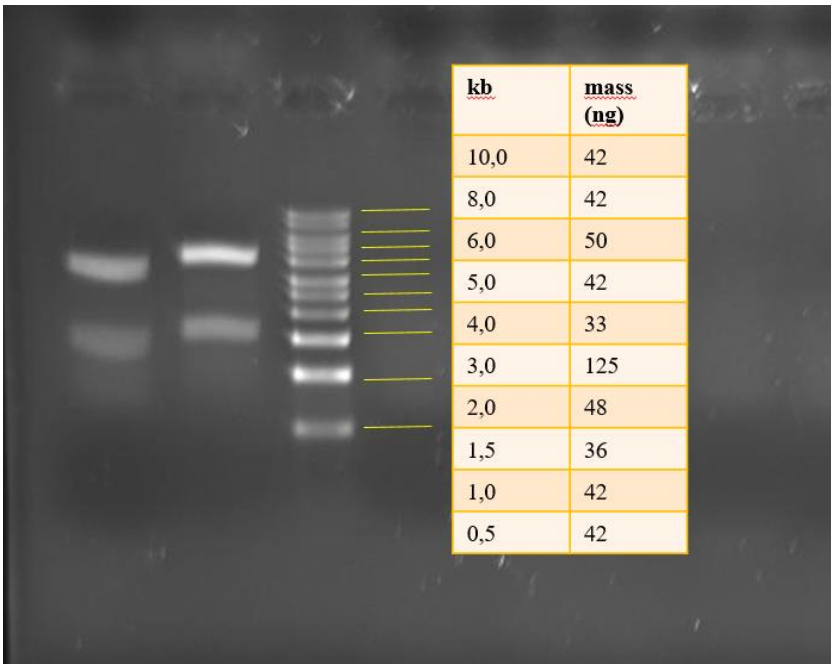


Fig. 4: Exemplary depiction of the analytical restriction digest of a preparative plasmid preparation of the n-terminal part of capsid protein VP3 (pKS157) with the enzymes MluI-HF (lane 1) and EcoRI-HF (lane 2) using the *CutSmart Buffer*.

3.2.1.9. COLONY-PCR

A colony PCR was used to screen many colonies in a short amount of time. This is a faster method than the plasmid preparation, followed by analytical restriction digest.

For the test PCR following protocol was used:

For 24 samples: 135 µl One Taq Quick Load 2x Master Mix
125 µl H_2O
5 µl Forward Primer (10 µM)
5 µl Reverse Primer (10 µM)
Adding up to 270 µl in total (10 µl used for each sample)

The PCR was programmed for 40 cycles at an initial denaturation temperature of 94°C, annealing temperature of 48°C for 30 seconds and the elongation at 68°C for 30 seconds. The PCR products were separated on an 0,8% agarose gel for 30 min at 100 V and visualized under UV-light.

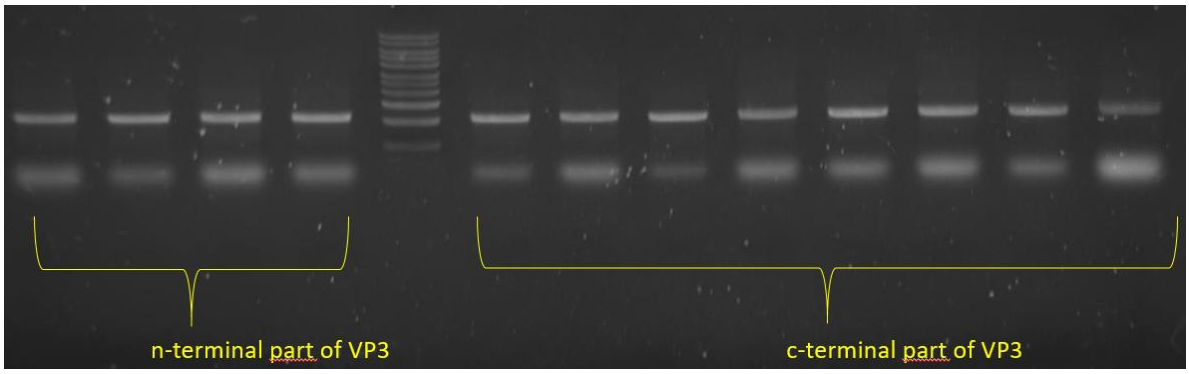


Fig. 5: Exemplary depiction of verification of the constructed N- and C-terminal part of capsid protein VP3 through colony PCR. Results have been made visible under UV light. The 4 samples on the left of the protein ladder show the N-terminal part, the ones on the right C-terminal part. They are all at the same height indicating the right size of ~500 bps (1 kb ladder shown in the pictures above for measurement).

3.2.3. WORKING WITH PROTEINS

3.2.2.1. TEST-EXPRESSION OF PROTEINS

The test expression of proteins was done in BL21 Rosetta cells in LB-medium, which has ampicillin and chloramphenicol added to prohibit unwanted bacterial overgrowth. To start the test expression, colonies are picked from the petri dishes with pipette tips and then transferred into 2,5 ml of LB-medium. The sample preparations were incubated at 37°C in a thermoshaker until they reached an optical density of 1. The samples were then divided and filled up with fresh LB-medium to reach an optical density of approximately 0,6. For induction of the test expression two different concentrations of IPTG were used to see if either one would improve

the protein yield (addition of 10/100 µl). IPTG was chosen over lactose, as the bacteria would consume the lactose over the time of induction. The samples were incubated at 37°C for 3 h. An aliquot of 2 ml was removed and transferred into a 2 ml Eppendorf tube every 30 minutes to determine the peak of expression. The tubes were centrifuged at 13.000 rpm for 1 minute, the supernatant discarded, and the process repeated a second time, to limit the amount of liquid to a minimum. The remaining pellets were stored in between sample collections at -20°C. After collecting six samples over a 3 h time lapse, the pellets were then washed twice. First 200 µl 1% Tritonx-100 solution was added, the tubes were vortexed and then centrifuged for 3 minutes at 13.000 rpm. The supernatant was discarded again. Secondly 300 µl urea were added and the tubes were vortexed to dissolve the insoluble components. The next step in the workflow was the Western blot and the following steps are further described in the following paragraphs. For the protein expression of the structural proteins VP1, VP3 and VP2 eprouvettes were prepared and filled with 2,5 ml LB-amp. Then the samples in form of glycerol stocks were picked with pipette tips, which were then dropped into the medium and further processed as described above. The plasmid preparation for the N- and C-terminal part of the capsid protein VP3 was performed identically and the induction was performed with 1 mM/0,1 mM IPTG to evaluate if the different molarity would result in a better outcome. For the following constructs of the C-terminally shortened plasmids the induction was started with a concentration of 1 mM IPTG. The Western blot method was used to continue the work flow.

3.2.2.2. GLYCEROLSTOCKS

Glycerol stocks were manufactured of the final plasmid containing bacteria with the desired sequences for long term storage.

The following protocol was used:

1000 µl bacterial culture in LB – medium + Ampicillin

+ 400 µl 86% glycerol

The mixture has to be vortexed well, afterwards the tubes can be stored at -80°C.

3.2.2.3. SDS-PAGE

SDS-PAGE is used to separate and fractionate proteins by their molecular size (Magdeldin et al 2014).

To prepare the polyacrylamide-gels the following protocol was performed:

Four separating gels 7,5%: 7,6 ml H_2O

5,4 ml Tris-tricin buffer

3,0 ml acrylamide (40%)

4 µl TEMED

80 µl 10% APS

Four stacking gels 4%: 3,9 ml H_2O

1,5 ml Tris-tricin buffer

0,6 ml acrylamide

6 µl TEMED

60 µl 10% APS

As soon as the gels have polymerized, they can be transferred into the chamber and filled up with cathode buffer. Now the combs can be removed and the samples loaded into the small cavities of the gels.

After the completed protein expression, Eppendorf tubes were prepared and filled with 50 µl *Protein Loading Dye 6x* + 100 µl of the samples and incubated at 95°C in the Thermomixer for 5 minutes before being pipetted into the gel cavities. The same amounts were used for all samples: 3 µl *Color Protein Standard* and 8/10/15 µl of the samples.

The next step is to insert cassettes into the electric field and run it for about 1 h at 100 V. The gels can now either be dyed in Coomassie blue for visualization of the samples or further processed with the Western Blot method, to show specific proteins. If the gel is only to be dried, it is to be taken off the glass and just coated with the dye on the shaker for about 1 h. After that the gel is to be transferred into the de-staining solution for at least 2 h. Each expressed protein was stained with Coomassie blue to show a timeline of the protein being expressed.

3.2.2.4. WESTERN BLOT

For this work, a semidry Western blot system was used. Briefly, following SDS-PAGE the acrylamide gel was removed from the glass support, the stacking gel was removed carefully and discarded. The "Western blot sandwich" was built using the *Trans-Blot Turbo Transfer System RTA Transfer Kit* following the subsequent instructions. The buffer of the kit was already prepared and consisted of 200 ml of 5x transfer buffer + 600 ml of distilled water + 200 ml ethanol which sums up to 1 l of 1x transfer buffer. First, the nitrocellulose membranes and transfer stacks need to be wetted with the transfer buffer for 2-3 minutes. One of the wetted stacks can be placed on the cassette, on top of that comes the wetted nitrocellulose membrane. Next, the gel can be placed on top and then another wetted transfer stack on top. The last step is to close the lid of the cassette and insert it in the instrument to start the transfer. For the transfer, the program for mixed molecular weight was used (7 min 25 V). After this, the nitrocellulose membrane was removed from the Western blot sandwich and directly transferred into the blocking solution consisting of 5% skimmed milk powder in PBS-T for 1 h.

The blocked nitrocellulose membrane is washed in PBS-T three times for 10 minutes. The Hybridoma supernatant of the antibodies has been diluted 1:5 with PBS-T for incubation. Then, the first antibody was put onto the nitrocellulose for 1 h. The membrane was washed three times for 10 minutes. Next the secondary antibody, was diluted at a ratio of 1:10.000 respectively 1:20.000 with PBS-T and the same procedure is done as following the first antibody. The washing process is usually performed 3 times for 10 min but can be repeated as often as needed, it only enhances the quality of the blot as it removes background that masks specific signals. To reveal the proteins the western blots were incubated with the *Cytiva Amersham™ ECL™ Prime Western-Blot-Detection reagent.* Following the protocol of the kit 500 µl of Solution A and 500 µl of solution B are combined in an Eppendorf tube. The nitrocellulose is taken out of the PBS-T and coated with 1ml of the combined mixture. The scanning process was performed with *Mini Trans-Blot® Cell* (BIO-RAD, USA) respectively with *ChemiDoc MP* (BIO-RAD, USA).

4 RESULTS

4.1. TEST-EXPRESSION AND WESTERN BLOTS OF THE PLASMIDS VP1, VP3 AND VP2

The first step in the workflow was the test expression of the pre-existing capsid proteins VP1, VP3 and VP2 as described earlier. The samples were then run via SDS-PAGE and Western blot method. Figure 6 shows the Coomasie blue stained gel of structural proteins VP1-3, Figure 7 shows the three capsid proteins marked with the murine anti-HisTag monoclonal antibody (αHis-10B6). All constructs generated in this work encode a N-terminal homopolymeric His tag consisting of 10 histidine residues.

The SDS-PAGE following the protein expression of the structural proteins was performed in following manner: Six Eppendorf tubes were prepared and filled with 50µl *Protein Loading Dye 6x* + 100µl of the samples and incubated at 95°C in the Thermomixer for 5 minutes before being pipetted into the gel purses in the following order*:* 3 µl *Color Protein Standard*, 15 µl plasmid VP1 n.i., i., 15 µl plasmid VP3 n.i.,i. and 15 µl plasmid VP2 n.i.,i.

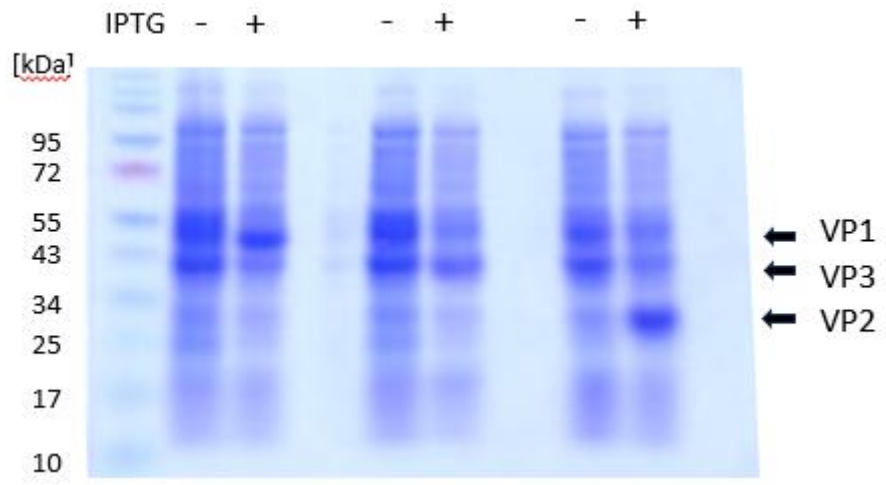


Fig. 6: Coomassie stained gel of the structural proteins VP1, VP3 and VP2. Samples were taken from glycerol stocks for protein expression. They were induced with IPTG to reach a molarity of 1mM each. The right columns are the induced samples, whereas the left ones were not induced. To assess the apparent molecular mass, a prestained *Protein Standard* was used. The arrows mark the expressed structural proteins.

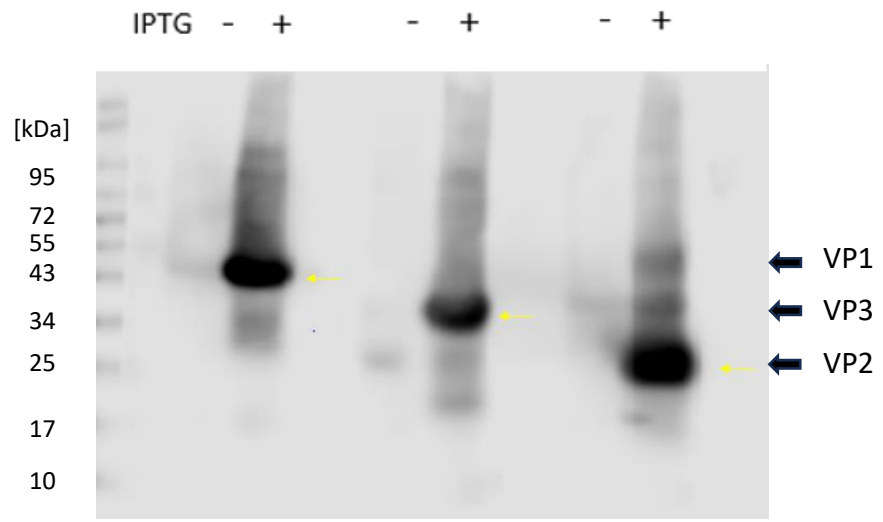
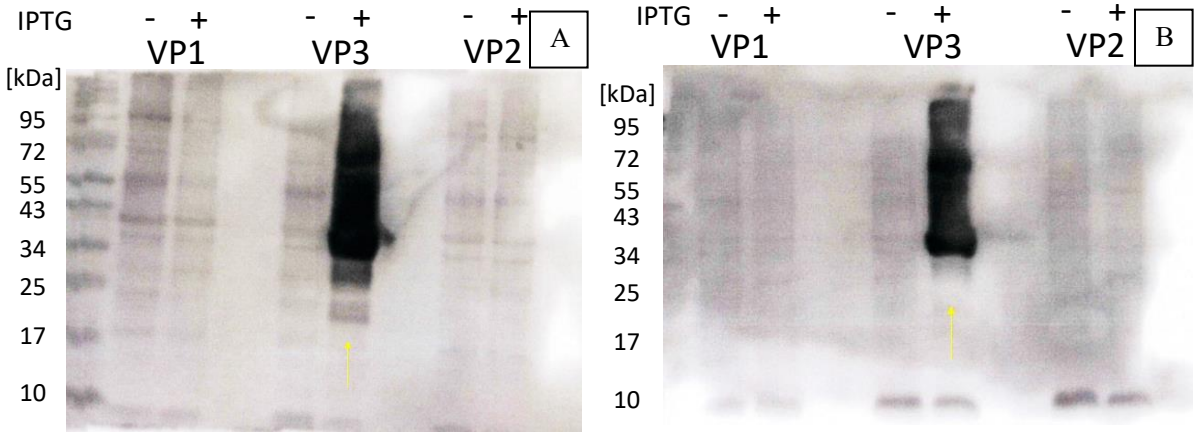


Fig. 7: Western blot after the protein expression of the structural proteins VP1 (35 kd), VP3 (33 kd) and VP2 (24 kd) marked with anti-HisTag. First antibody is the anti HisTag, second antibody goat α mouse IgG. The arrows mark the capsid proteins.

After verifying the already existing plasmids contained the capsid proteins VP1, VP3 and VP2, the Western blots were coated with the three murine monoclonal antibodies αABPV-VUPLA7, αABPV-RF9, αABPV-RC11 to check, which capsid protein is responsible for the binding of the antibodies. All three blots show a distinct signal corresponding to the proteins expressed by the capsid protein VP3 plasmid. The bands correspond to the expected size of VP3. Although one gel for was damaged during the electrophoretic transfer, the membrane was incubated with the primary antibody αABPV-RF9 and resulted in a clear reactivity with VP3.

The following figures show that all three antibodies bind to capsid protein VP3.



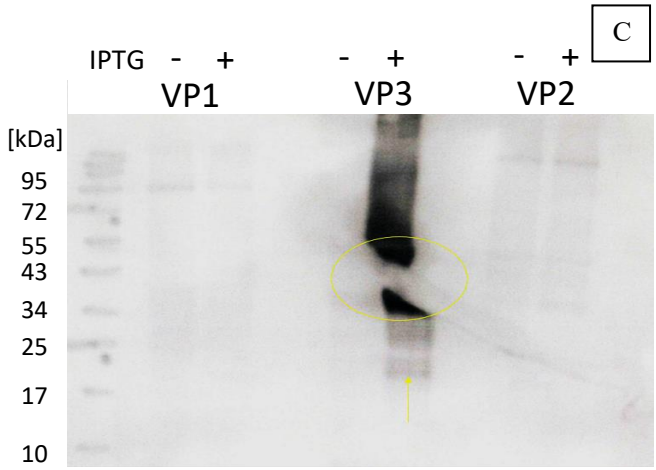


Fig. 8: Western blots of the capsid proteins VP1-3 detected with A) murine monoclonal antibody αABPV-VUPLA7, B) murine monoclonal antibody αABPV-RC11 and C) murine monoclonal antibody αABPV-RF9. Secondary antibody used for all blots was horseradish peroxidase (HRP) conjugated goat α mouse IgG. All three blots show that the antibodies recognize the capsid protein VP3.

Following these Western blots it can be concluded that VP3 is the protein of interest and therefore is chosen for further analyses.

4.2. MAPPING OF THE EPITOPES WITHIN VP3

The vector PCR for all constructed plasmids was performed using the primers BV449 fw. and BV450 rev. The N-terminal part of VP3 (insert pKS157) was created via the primers BV453 fw. and BV596 rev., for the C-terminal part of VP3 (insert pKS158) the designed primers were BV594 fw. and BV454 rev. The plasmid template named pKS98 encodes the structural protein VP3. The primers used for the next steps are shown in Tab. 1. They were designed to divide the capsid protein VP3 gene in two parts of approximately the same size. The two halves were about 500 bps each, reaching from 20-520 and 520-940 bps. The new products were named pKS157 (N-terminal part of VP3) and pKS158 (C-terminal part of VP3) as shown in Fig. 2.

Following the test expression of the N- and C-terminal part of VP3, the samples were further processed for SDS-PAGE. The SDS-PAGE following protein expression of the N- and C-terminal part of VP3 was performed as follows: The samples were incubated at 95°C for 5 minutes. 3 µl *Color Protein Standard* were pipetted into the gel purses and 15 µl of each sample

(each timepoint) to make the gradient protein expression visible. Approximately at 2h post induction with IPTG the expression of the structural protein is well discernible and becomes more apparent. This is shown in the figure below.

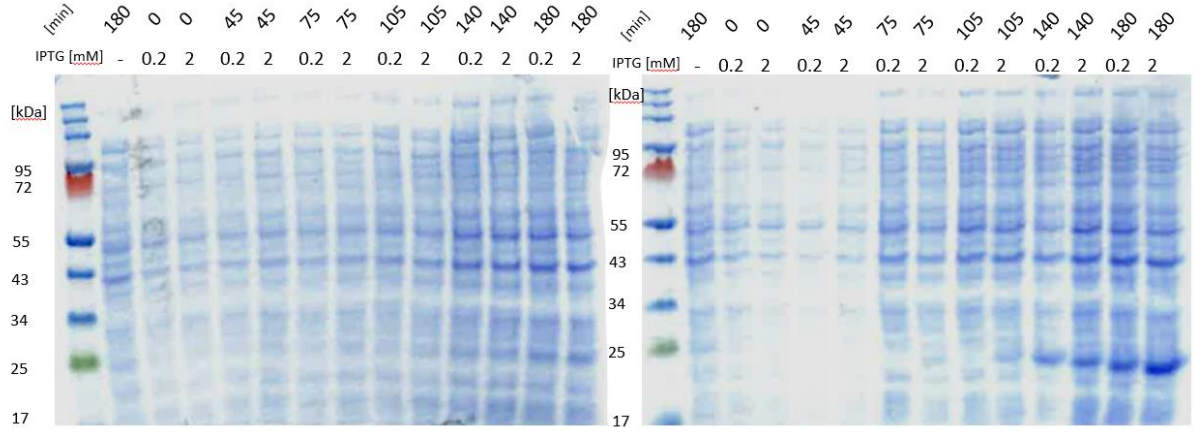


Fig. 9: Coomassie-blue dyed gel showing the induction of the capsid protein VP3 over a 3h timeframe (12:00-15:00). All samples were induced twice with 10 µl respectively 100 µl 1M IPTG in 50 ml medium resulting in a molarity of 200 µM respectively 2 mM. The gel shows no differences between the varied molarity. It is clearly visible that with more time, more intense staining of the gel is discernible, therefore more proteins get expressed over a longer time period. The structural protein is much more perceivable in the C-terminal expression construct of the VP3.

Next the Western blots were again probed with the murine monoclonal antibodies αABPV-VUPLA7, αABPV-RF9, αABPV-RC11. The following images show that all three antibodies bind to the C-terminal part of the capsid protein VP3.

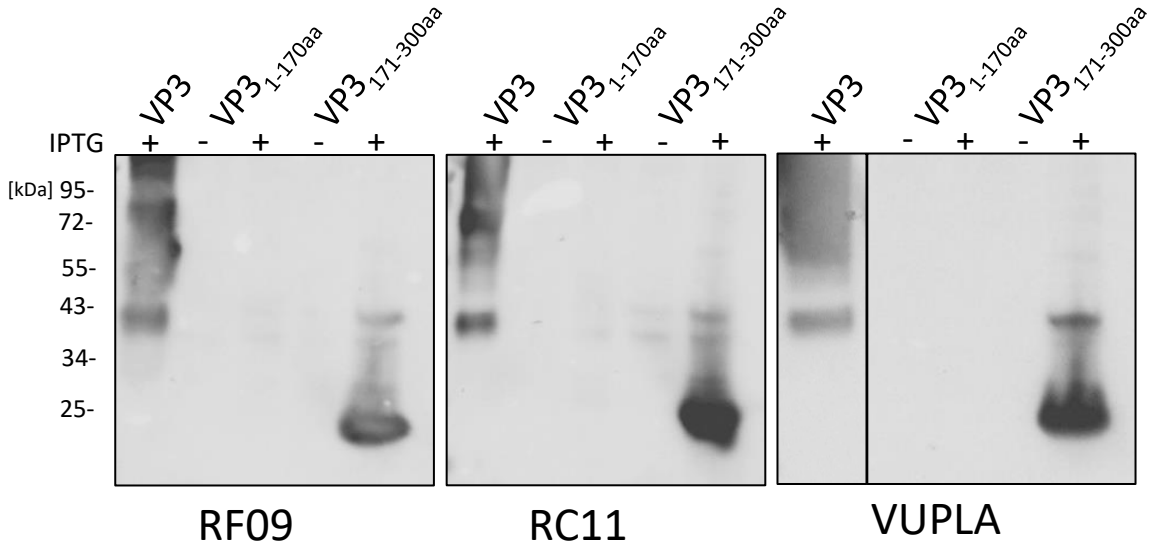


Fig. 10: Western blots of the capsid protein VP3 (N- and C-terminal part) probed with A) murine monoclonal antibody αABPV-VUPLA7, B) murine monoclonal antibody αABPV-RC11, and C) murine monoclonal antibody αABPV-RF9. Secondary antibody used for all blots was horseradish peroxidase (HRP) conjugated goat α mouse IgG. All three blots show that the antibodies are recognizing the C-terminal part of the capsid protein VP3.

In accordance to these results the next steps were to shorten the C-terminal part of the structural protein VP3 from the C-terminal end. To do so the same fw. primer BV453 was used for all samples and rev. primers BV690, BV692 , BV676, BV695, BV697, BV699 and BV698 designed to shorten the plasmid by -88 aa, -57 aa, -29 aa, -12 aa, -4 aa, -2 aa and -1 aa. To narrow down the search for the epitope but to avoid the production of relatively small inserts, the original plasmid pKS98 of encoding the VP3 gene (942 nucleotides) was chosen for further processing.

Following the PCR tests the samples were further processed for SDS-PAGE and the Western blot. For the SDS-PAGE following the protein expression of the shortened capsid protein VP3 (-88 aa, -57 aa and -29 aa) the samples were incubated at 95°C for 5 minutes. 3 µl *Color Protein Standard* were pipetted into the gel slots and 15 µl of each sample. The figure below shows the proteins of the original template (structural protein VP3) and the new constructs.

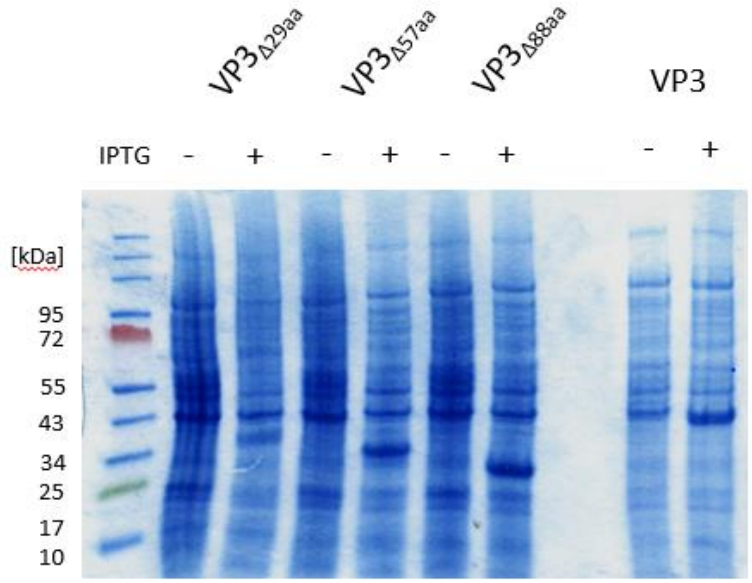


Fig. 11: Coomassie gel of the original template for reference (capsid protein VP3; positive control) and capsid protein VP3 shortened by -88 aa, -57 aa and -29 aa to visualize the proteins.

To verify that the right samples have been expressed, the blots were again checked with the murine anti-HisTag monoclonal antibody (αHis-10B6). Figure 12 shows the three shortened plasmids of the capsid proteins marked with it.

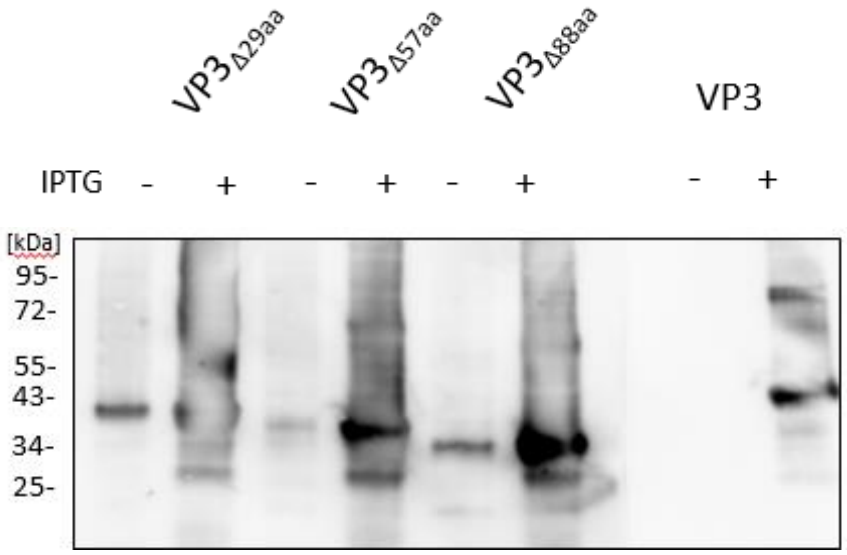


Fig. 12: Shortened plasmids encoding shortened VP3 capsid proteins (-88 aa, -57 aa and -29 aa) were transformed in E. coli and induced with IPTG. Non-induced samples were processed side by side as a reference. The samples were blotted and incubated with the murine anti-HisTag and secondly with goat α mouse IgG. The original template of capsid protein VP3 was added for reference.

Next the blots were incubated with the three monoclonal antibodies RF09, RC11 and VUPLA7. While the positive control (full length VP3) was clearly recognized, none of the truncated VP3 variants was detected by any of the antibodies, which is depicted in the following images.

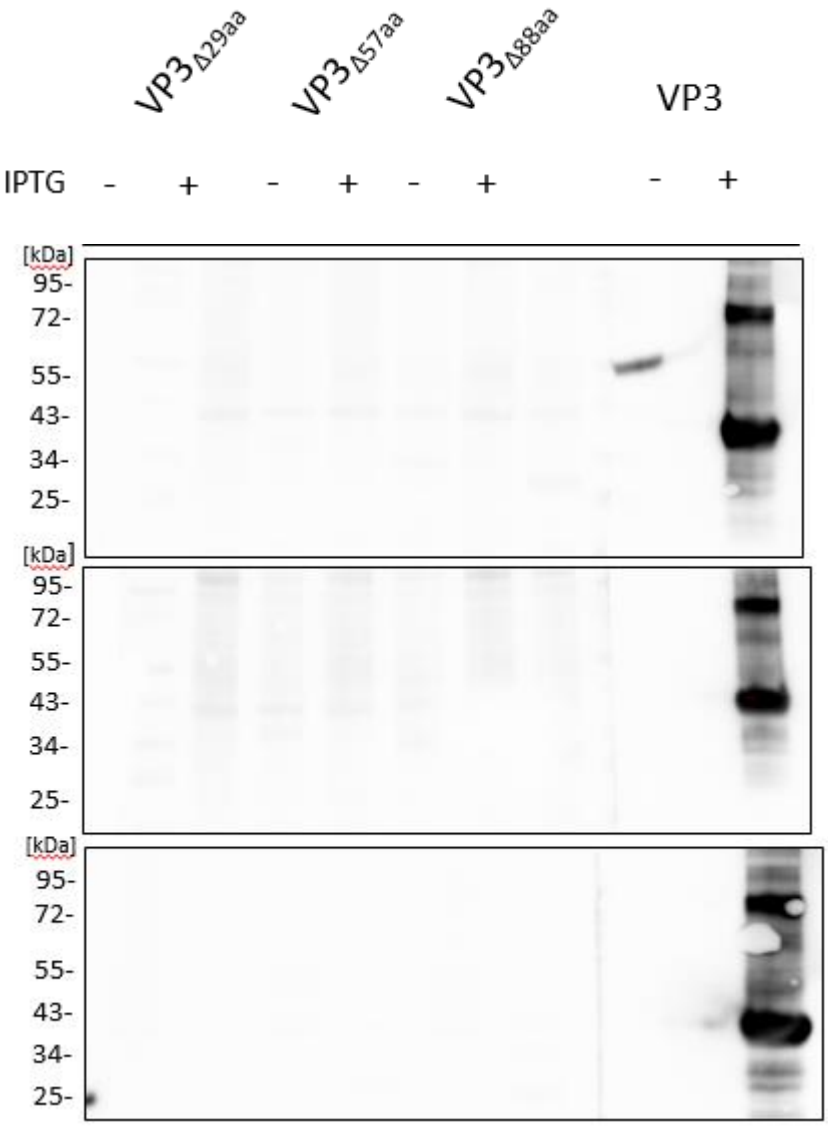


Fig. 13: Western blots of the shortened VP3 capsid proteins (-88 aa, -57 aa and -29 aa). The original template is shown as a positive control (column on the far right). Neither one of the antibodies was bound by any of the proteins; from top to bottom: αABPV-RC11, αABPV-RF9 and αABPV-VUPLA7 did not detect any binding sites in the shortened proteins.

Following the assumption that the epitope might be closer to the C-terminal end of the VP3 capsid protein and might have been "cut off" by the deletion, new constructs were designed to shorten the capsid protein VP3 using smaller deletions. The SDS-PAGE following the protein expression of the plasmids of VP3 capsid proteins shortened by -12 aa, -4 aa, -2 aa and -1 aa. Again samples were diluted in sample buffer and incubated at 95°C for 5 minutes. 3 µl *Color Protein Standard* were pipetted into the gel slots and 15 µl of each sample. The figure below shows the proteins of the new constructs.

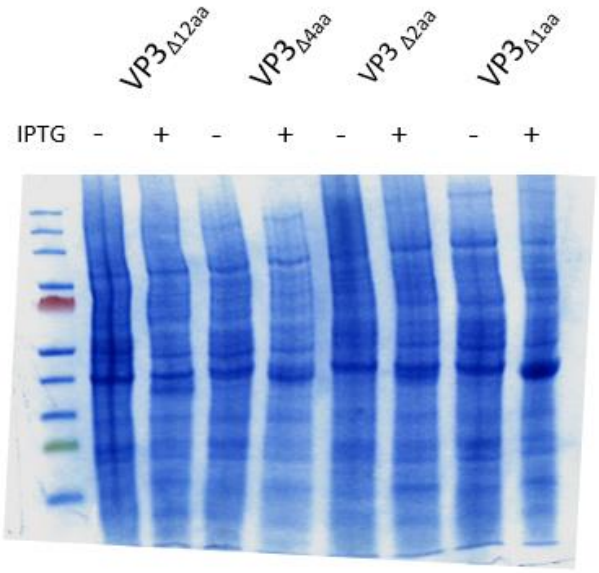


Fig. 14: Coomassie gel of the shortened VP3 constructs (-12 aa, -4 aa, -2 aa and -1 aa).

Lastly the constructs of VP3 capsid proteins shortened by -12 aa, -4 aa, -2 aa and -1 aa at the C- terminus were separated on 4 gels in parallel and transferred to membranes. One blot was incubated with the murine anti-HisTag (αHis-10B6) (Fig.15) and the others with the monoclonal antibodies αABPV-VUPLA7, αABPV-RC11 and αABPV-RF9, respectively (Fig 16). None of the VP3 specific antibodies recognized the C-terminally truncated VP3, only the full length VP3 (positive control) was detected. The most likely explanation is the essential presence of the C-terminal amino acid for the integrity of the epitope. Surprisingly all three monoclonal antibodies share the same epitope.

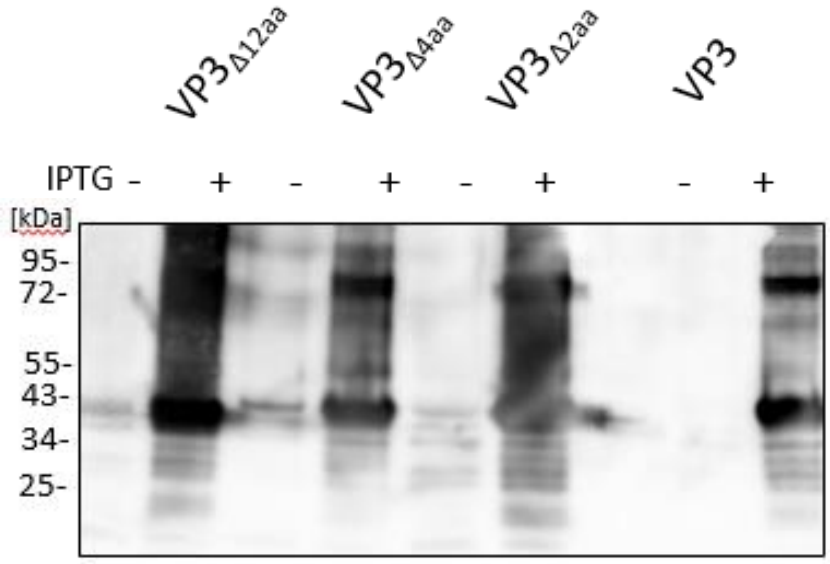


Fig. 15: Western blot of the constructed plasmids of VP3 -12 aa, -4 aa and -2 aa. The structural protein VP3 was tagged with the murine anti-HisTag (10B6). Full length VP3 served as a positive control.

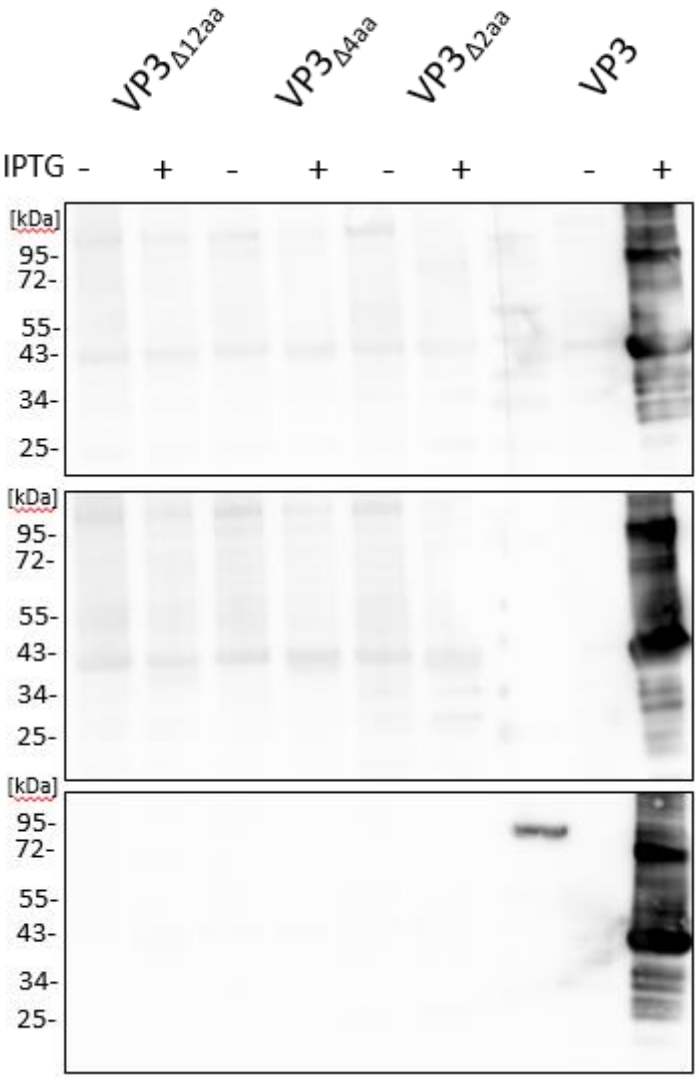


Fig. 16: Western blots of the capsid proteins VP3 Δ12 aa, Δ4 aa and Δ2 aa. Full length VP3 served as positive control. The monoclonal antibodies αABPV-VUPLA7, αABPV-RC11 and αABPV-RF9 did not recognize the C-terminally truncated VP3 proteins.

5 DISCUSSION

The detection of ABPV has previously been performed via serological testing. In the means of improving the testing methods the project called “Zukunft Biene 2” was aiming at developing monoclonal antibodies that are able to detect native antigens. The question to be answered by this thesis was if the generated monoclonal antibodies of the “ZuBi 2” are able to detect the same epitope, which capsid protein is responsible for the formation of the epitope and the complete sequence of the epitope. The main methods used in this project to identify the epitope for the three monoclonal antibodies (αABPV-VUPLA7, αABPV-RC11 and αABPV-RF9) investigated in this work were PCR for the generation of the shortened genes and Western blot for the actual identification of the antibody binding site(s). Both methods are very sensitive, fairly easy to conduct and have semi-quantitative outcomes. In total, three antibodies against ABPV were investigated in regard to their epitope. These antibodies were generated to make the detection of ABPV easier and cheaper for the beekeeper, in order to mitigate honey bee colony losses. ABPV might occur in apiaries without affecting the bees at all and losses are more correlated to an extreme infestation with the mite *Varroa destructor,* so quantitative tests are required. Another point to take into account is, that different strains of ABPV exist with different levels of virulence (Šimenc, et al., 2021). A study by Schurr et al., 2019 assessed viral loads and showed symptoms of naturally affected honeybees to be able to differentiate between infections that were of relevance to the apiaries. They set the threshold at ≥ 5 $\log_{10}$ genome copies/bee or larva. This was confirmed by Šimenc, et al., 2021 although they appointed their threshold higher at 7.21 $\log_{10}$ viral copies/bee for affected hives that showed symptoms like depopulation, death and again infestation with *Varroa destructor*.

The identification of this specific epitope for all three antibodies proved to be a little more difficult as it seems to be positioned at the very end of the structural protein VP3. Unfortunately only the construct of VP3 shortened by -2aa is shown in this thesis as the -1aa has not been conducted in the Western blot. It would be of utter interest to recheck the -1 aa shortened construct via Western blot method to have proof of the importancy of the last amino acid to the epitope. After the positive identification of the epitope of all three antibodies being located at the C-terminal part of VP3 (using the construct pKS158), none of the constructs built following pKS158 yielded a positive signal. The positive control pKS98 (capsid protein VP3) was still recognized in all Western blots and the expression of the constructs was verified via Coomassie staining and incubation with an anti-His antibody, as all constructs generated have a polyhistidine tag at the 5’ end. Furthermore, there were even more verification steps included

in the beginning of the workflow to check that the right plasmid has been constructed and multiplied, like using an analytical restriction digest, Colony-PCR, and sequencing. The identity and integrity of each construct was verified via sanger sequencing including the deletions at the C-terminal end.

However, to confirm the epitope detected in this study, further investigations are necessary. However, that would have gone beyond the scope of this diploma thesis. One possible while not very likely explanation why the binding site could not be defined is that the epitope might not be linear but discontinuous. Conformational or discontinuous epitopes are the most frequently detected targets of antibodies but often difficult to confirm by denaturing Western blot assays (Forsström et al., 2015). The structure of conformational epitopes is described by "regions", these encase at least three antibody-contacting residues which have to be interrupted by 3 or less residues. These regions can be classified as straight, curved or folded. Better methods to identify non-linear epitope sequences is the usage of protein 3D-structures and similar computational assisted techniques (Haste-Andersen et al., 2006; Moreau et al., 2008). Important information of structural composition of epitopes is gathered through their size, amino acid composition or shape (Ferdous et al., 2019). Against the hypothesis of a discontinuous epitope speaks the fact that the full length VP3 is well detectable in Western blot, hence folding is not an issue, unless the terminal amino acids (M_{299}-Q_{300}) are decisive for the secondary structure.

As second possibility, the C-terminal amino acids (M_{299}-Q_{300}) are an essential part of the epitope because all the C-terminally shortened constructs yielded no positive signal in Western blots. This means, that the antibodies failed to bind even after removing the two amino acids from the C-terminal part of VP3. Because a B cell epitope usually comprises in average 15 amino acids the residues M_{299}-Q_{300} likely are an essential part of epitope itself (Potocnakova et al., 2016). The figure below shows the composition of the genome of ABPV, the region that contains the epitope is marked by a red circle.

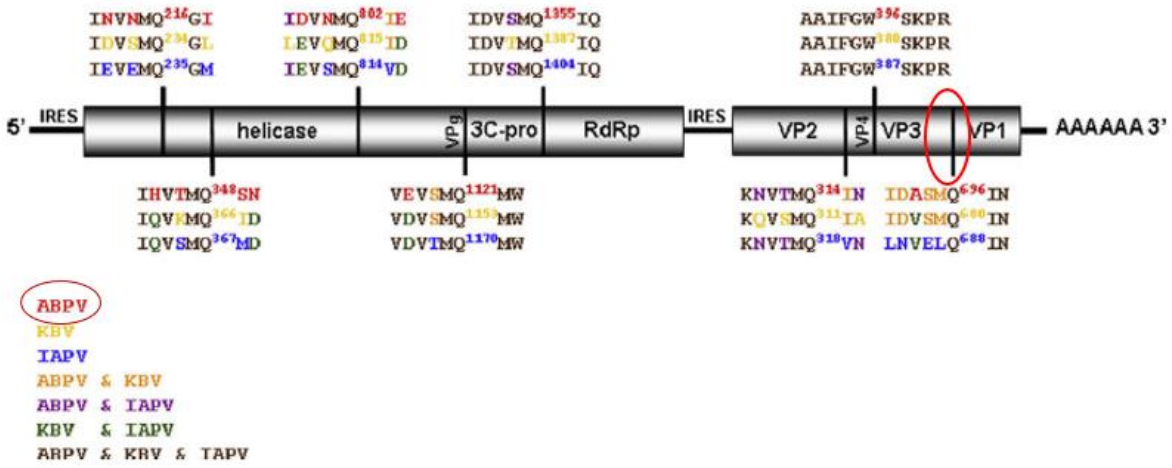


Fig. 17: Composition of the genome of ABPV (as well as IAPV and KBV to show their close relation). The region of interest (IDASMQ) at the C-terminal end of VP3 is marked by a red circle (de Miranda et al., 2010).

The figure down below (Fig. 18) shows a 3D-description of the protein structure of a very closely related virus, namely the Israeli acute paralysis virus (Mullapudi et al., 2017). The red circle demonstrates the glutamic acid at the end of the capsid protein VP3, which is the main part of the epitope. In the figure one can see, that the structure proteins are overlapping and that the last amino acid is indeed protruding out of the viral capsid. This would make this epitope a good target for antibodies an could be another explanation why the antibodies were not able to bind to the epitope in the later constructs which contain less amino acids and potentially miss part(-s) of the epitope.

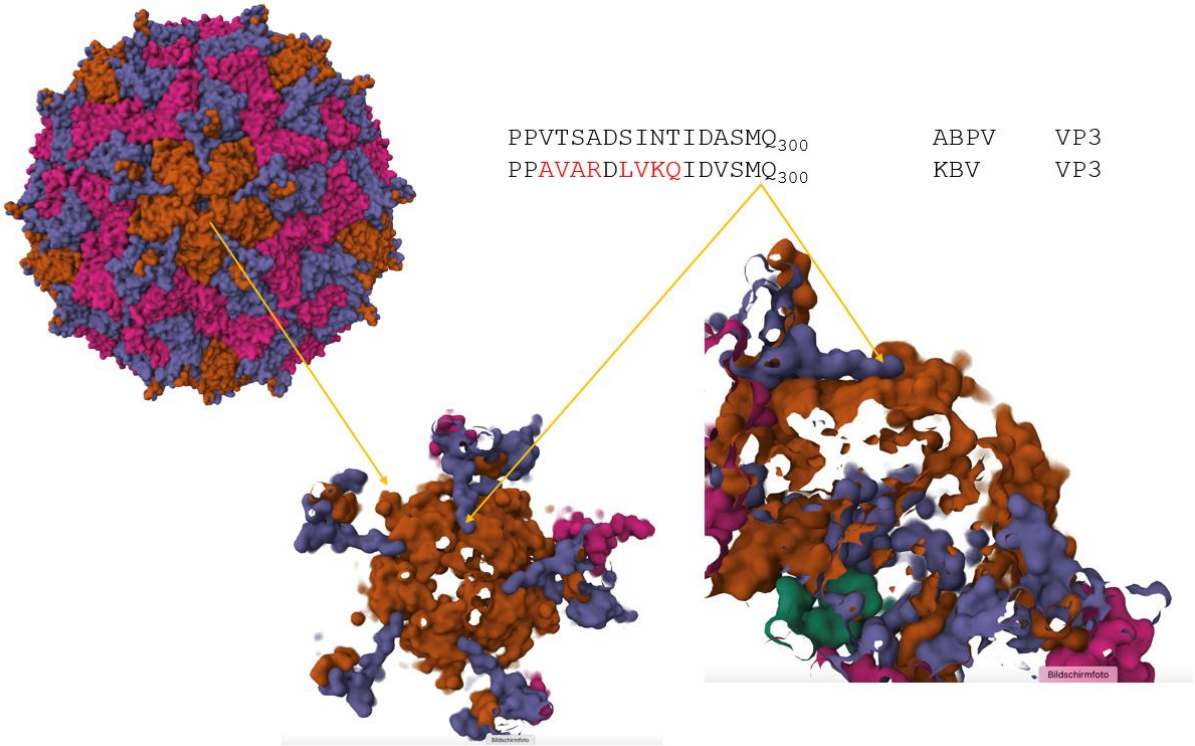


Fig. 18: Description of the protein structures of the Kashmir bee virus which is closely related to *ABPV* (AKI-complex). The amino acid sequence of interest is situated at a quite exposed position and is coloured purple in the 3D-model. The findings suggest that it is essential to the epitope and its position is marked by yellow arrows (https://www.rcsb.org/3d-view/7BGK/1).

Furthermore, a BLAST protein search was performed to check if the C-terminal part of the sequence of VP3 was conserved among various isolates of ABPV. The amino acid sequence IDASMQ was part of the majority of the results shown. Below the whole amino acid sequence of the capsid protein VP3 is shown, the final part that seems to be conserved is marked in red.

SKPRNLEQVNLYQNVPGWGYSLYKGIDNSVPLAFDPNNELGDLRDVFPSGVDE
MAIGYVCGNPAVKHVLSWNTTDKVQVPISNGDDWGGVIPVGMPCYSKIIRTTE
NETTQTKTEVMDPAPCEYVCNMFSYWRATMCYRIAIVKTAFHTGRLEIFFEPGR
IPITTTKDNISPDLTQLDGIKAPSDNNYKYILDLTNDTEITIRVPFVSNKMFMKST
GIYGGNAENNWDFSESFTGFLCIRPVTKLMCPETVSNNVSIVVWKWAEDVVVV
EPKPLLSGPTQVFQPPVTSADSINTIDASMQ

Fig. 19 Amino acid sequence of the structure protein VP3 of *ABPV*

	VUPLA7	RC11	RF9
$PPVTSADSINTIDASMQ_{300}$	+	+	+
$PPVTSADSINTIDASM_{299}$	-	-	-
$PPVTSADSINTIDA_{297}$	-	-	-
$PPVTSADSINT_{293}$	-	-	-
$PPVTS_{287}$	-	-	-

Fig. 20: Depiction of the C-terminal amino acid sequences of *ABPV*. As soon as the C-terminal part is removed the three murine monoclonal antibodies (αABPV-VUPLA7, αABPV-RC11 and αABPV-RF9) tested in this thesis are not able to detect a binding site anymore, which supports the theory that the epitope is at the C-terminal end of the capsid protein VP3.

Another option to find the epitope is to repeat the workflow but shortening the VP3 coding sequence from the N-terminal end additionally to the C-terminal end. This would prohibit the problem of losing the essential part at the C-terminal end of VP3 and might result in the finding of the epitope or prove the theory that the epitope is complete only in the presence of the C-terminal amino acid.

6 SUMMARY

The Acute bee paralysis virus has various negative impacts on bee colony health. Investigating this disease and other factors contributing to the “colony collapse disorder” in times of immense bee losses is of utter interest. The knowledge gathered by epitope mapping, is not only serving as basic research but can enable other scientists to discover new ways to diagnose this disease via rapid tests or help develop new treatment plans. Within the framework of this thesis, it was possible to show via the Western blot method, that all three monoclonal antibodies investigated in this study bind to the structural protein VP3. By shortening the template VP3 it was possible to show with certainty, that the very last part, especially the last amino acid of VP3 is essential for the antibody-binding capability. Further investigations need to be conducted to confirm the complete epitope or if the epitope consists only of the last amino acid of structure protein VP3. Possible further steps are n-terminal shortening of template VP3 or cloning the c-terminal end into another sequence and testing if the antibodies are still able to detect a binding site.

7 ZUSAMMENFASSUNG

Das Akute Bienenparalyse Virus hat vielfältige negative Einflüsse auf die Gesundheit von Bienenvölkern. Forschung an dieser Krankheit und weiteren Faktoren, die im Zusammenhang mit dem sogenannten „*colony collaps disorder*“ stehen, ist in Zeiten, in denen die Bienensterblichkeit ein geradezu dramatisches Ausmaß angenommen hat, von immenser Wichtigkeit. Das Wissen, welches durch Epitopkartierung erhoben wird, dient nicht nur der Grundlagenforschung, sondern kann auch in der Entwicklung von Schnelltests zur leichteren Feststellung von einem Befall mit dem besagten Virus oder neuen Therapieansätzen eingesetzt werden. In dieser Arbeit konnte über die Westernblot Methode gezeigt werden, dass das Strukturprotein VP3 von allen drei in dieser Arbeit untersuchten monoklonalen Antikörpern gebunden wird. Jedoch muss das vollständige Epitop noch erhoben werden. Durch Verkürzungs-PCRs konnte gezeigt werden, dass die letzte Aminosäure des Strukturproteins VP3 mit Sicherheit essenziell für die Antikörperbindung ist. Mögliche weiterführende Schritte wären beispielsweise die Verkürzungs-PCRs vom N-terminalen Ende der Sequenz durchzuführen oder den hinteren Teil des Strukturproteins VP3 in eine andere Sequenz hinein zu klonieren, um zu sehen ob die Antikörper die Sequenz immer noch erkennen können.

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