IUT de LAVAL DEPREAUX Juliette

Département Génie Biologique.

Place of the internship : Institute for Experimental Pathology, University of Iceland, Keldur.

Production and purification of *Culicoides* hyaluronidase, an allergen in Insect Bite Hypersensitivity in horses (IBH)

Internship supervisor : Sigurbjorg Torsteinsdottir **Confidential** : No

Internship from : 28th of March **to** 24th of June **Tutor** : L. Poisson

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Abbreviations

AcMNPV : *Autographa californica* NucleoPolyhedroVirus

B-Cells : B Lymphocytes

bp : base pair

DNA : Desoxyribonucleic Acid

APC : Antigen-Presenting Cell

Fw : Forward

h : hour

HBM : Honey Bee Melittin

High-Five : Cell ligne from *Trichopulsia ni*

IBH : Insect Bite Hypersensitivity

Ig : Immunoglobulin

IL : Interleukin

kb : kilo base

kDa : kilo Dalton

mA : milliamp

min : minute(s)

o.n : over night

PEST : Penicillin Streptomycin

PCR : Polymerase Chain Reaction

PVDF : PolyVinylide Fluoride

QS : Quantity sufficient

Re : Reverse

RT : Room temperature

sec : second(s)

Sf-9 : Cell ligne from *Sporoptera frugiperda*

T-Cells : T Lymphocytes

TBS-T : Tris-Buffered Saline + Tween 20

TEMED : Tetramethylethylenediamine

Th : T Helper Cells

Treg : T regulatory cells

UM : User Manual

V : volt

WB : Western Blot

Institute presentation

Founded in 1948, the Institute for Experimental Pathology (Keldur) is a research institute linked to the faculty of medicine, University of Iceland. Keldur is located in Reykjavik, capital city of Iceland, the employees are around 50 plus a dozen students.
This area is composed of several different buildings. Three of them are offices and laboratories, and there is a biosafety level 3 laboratory. There is also a house for small animals, stables for horses and sheep and pastures for them.
Keldur is an academic establishment with a special governing board and an independant budget. The studies are mainly carried out in the veterinary field.
The specialities are immunology, prionology, pathology, parasitology, biochemistry, bacteriology and molecular biology. Many of the studies are carried out in association with international groups.

The building in which this internship took place is divided in two levels : the ground floor and the first floor. On the ground floor there are rooms for sterilisation, measurments (ELISA, PCR, FPLC), sterilised manipulations (hoods) and storage (fridge, freezer and dry room). Up the first floor, there are working places, labs and a staff rest area.
On both levels, there are changing rooms where the « outside equipments » are stored. Outside shoes mustn't be worn in the labs, and lab coat mustn't be hanged out of the lab.

Keldur has many main objectives, consisting mostly on basic researches on animal and human, and development of drugs and vaccines on veterinary purpose. Even if the service research is based on studying veterinary and medicine diseases, the supervision of animal health is most prominent.

On the first days of an internship, the trainees are taken around the place to be informed of the security procedures and the safety equipments.

Introduction

1 Insect Bite Hypersensitivity

a) Epidemiology

IBH is also known as the summer eczema in horses. This is a recurrent allergic dermatitis in response to the bite of midges of the genus *Culicoides*. It affects all breeds of horses (Schaffartzik, 2012). The Icelandic horse is a pure breed and has never been crossed with other breeds. As a result of their isolation from other horses, very few infectious diseases are known in the breed within Iceland. Therefore, the Icelandic horse has little acquired immunity against continental diseases and the importation of horses is forbidden in Iceland (Bjornsdottir et al., 2006). Because there are no *Culicoides* in Iceland, they are very sensitive to IBH when they are exported to the continent (Schaffartzik et al, 2009).

b) Clinical signs

The bite of the *Culicoides* causes skin sensitization that involves severe itches at the base of the mane and the tail. It leads to alopecia (hair loss), hyperkeratosis (thickening of the skin) and excoriation (skin lesion). The itches start in the summer when the *Culicoides* are active and cease in late autumn (Baker and Quinn, 1978).

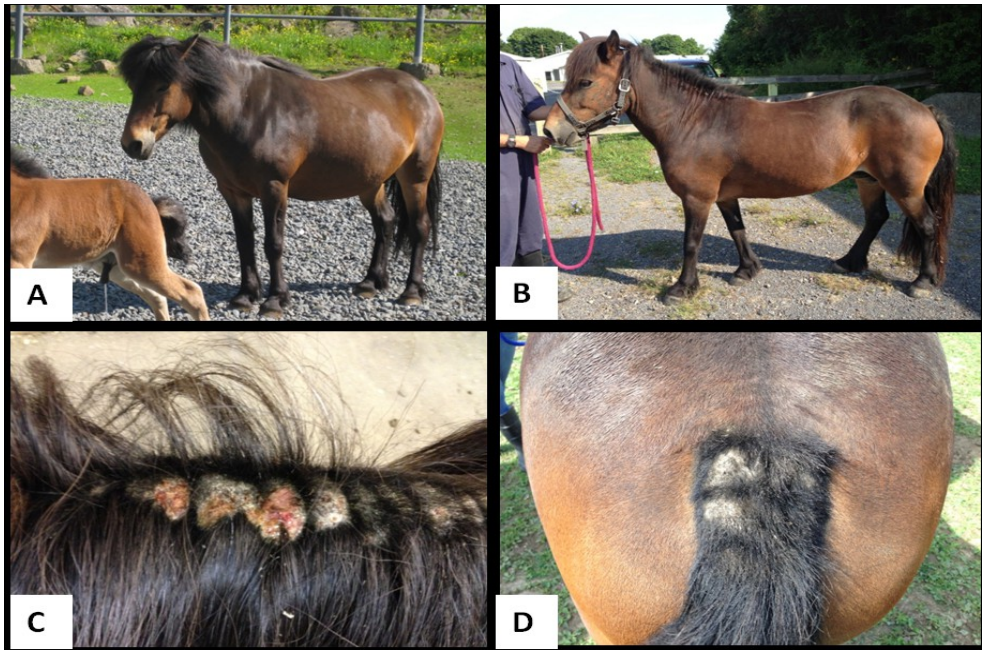

Figure 1: Clinical signs of IBH. A. An Icelandic horse before leaving Iceland **B.C.D.** After three years in USA, the horse present IBH clinical signs. Photos : Sigurbjorg Torsteinsdottir and Bettina Wegner

There is no vaccine for this disease and only symptomatic treatments. To avoid the midges, blanckets are used to cover the horses. In severe cases, IBH makes the horse periodically unavailable for riding.

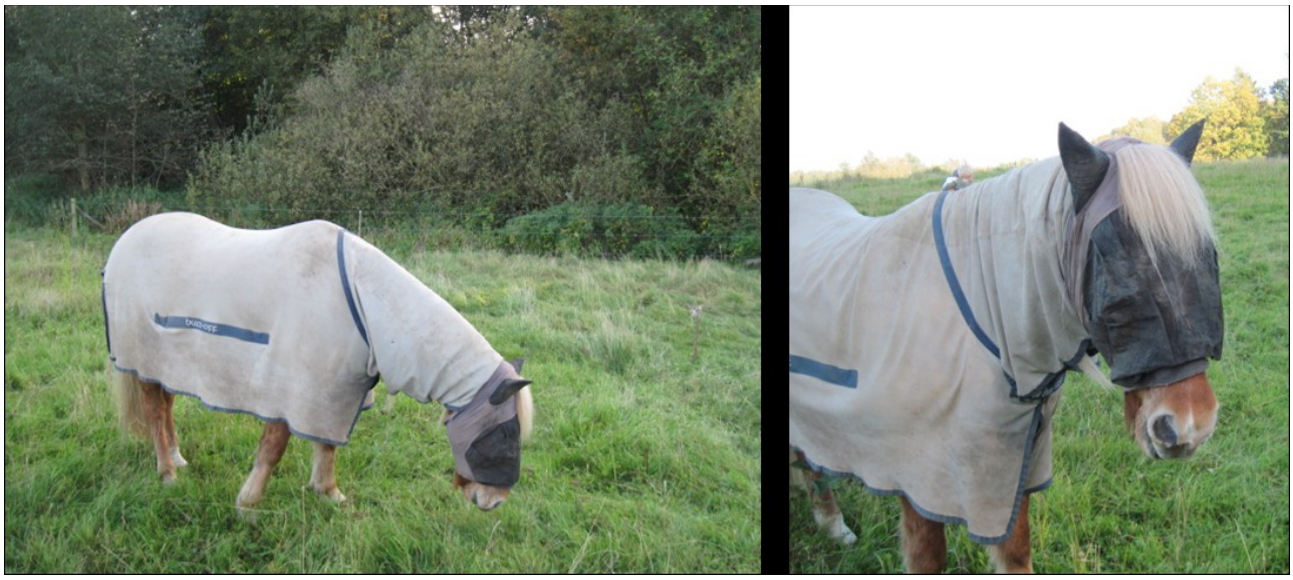

Figure 2: A horse wearing a blancket as a protection against the *Culicoides* bites.
Photos : Thorunn Gudmundsdottir.

The only way to prevent this disease would be to vaccinate the horses before exporting them to other parts of the world. Keldur aims in developing an immunotherapy against IBH (Summer eczema in horses, Keldur website home page).

c) Pathogenesis

When a midge bites the horse it injects saliva containing various proteins and in some horses these proteins cause allergy. An allergy, or hypersensitivity type 1, is an IgE mediated overreaction of the immune system. This reaction is against substances which are usually harmless and the proteins or antigens involved are called allergens (Janeway's Immunobiology, Kenneth Murphy 2012).

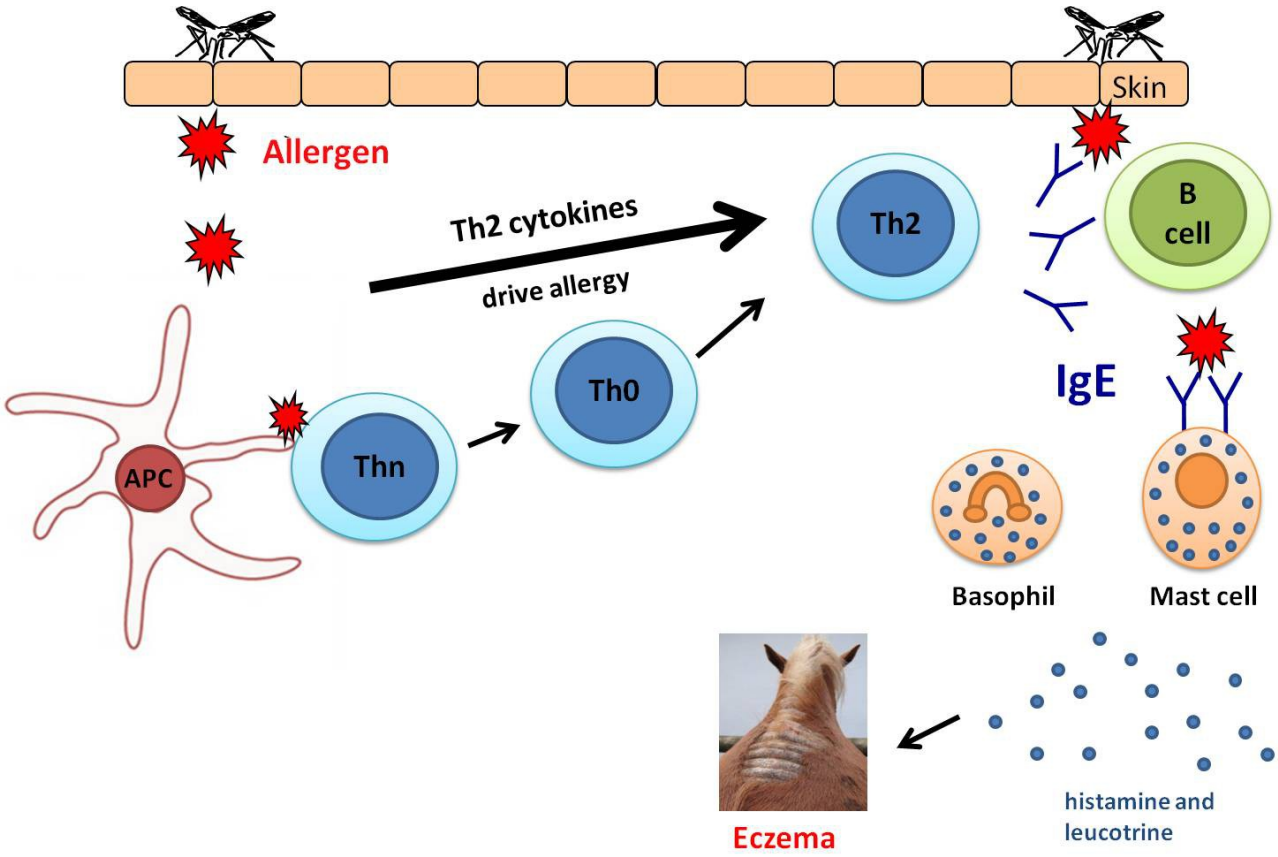

Figure 3: IgE mediated response in IBH. Immune response on a first exposure to the allergen, then allergic reaction due to a re-exposure to the allergen. Schematic picture : Sigurbjorg Torsteinsdottir

A midge bites the horse, it secretes the allergens with the saliva into the skin. The Antigen presenting cells (APC) present this allergen to naive $CD4^+$ T helper cells (Thn). They differentiate into Th2 cells which induce B-cells to produce IgE antibodies specific to the allergen presented. The IgE binds to high affinity receptors FcεRI found on mast cells and basophils. This is the first exposure and the horse is now sensitized to the allergen.

When the horse encounters the midges again, mast cells and basophils already have the specific antibodies ready to cross-link the antigen. They release inflammatory mediators, and this process causes the allergic response.

It is suggested that there is an imbalance between Th1, Th2 and T regulatory cells (Treg) in exported Icelandic horses (Hamza et al., 2007). The immunotherapy could be used to rebalance these T cells responses.

d) *Culicoides*

The *Culicoides* is a genus of haematophagous biting midges present all over the world except in Iceland, Antartica, New Zealand and far South of America. Over 1400 species were recorded. These insects feed on many animals included horses. The saliva of *Culicoides* contains several compounds that break the host defenses to permit the feeding. These compounds can sometimes become allergens (Ribeiro and Francischetti, 2003).
C. nubeclosus can easily be bred in laboratory. *C.nubeculosus* is principally found in the United States of America and *C.obsoletus* is mainly present in Europe. They are both studied in this report. IBH affected horses can react to *Culicoides* species that they have never been exposed to (Anderson et al, 1993).

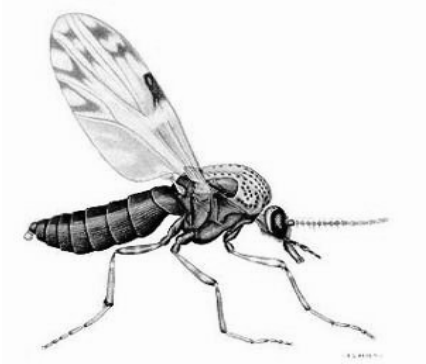

Figure 4: *Culicoides spp.*

2 *Culicoides* allergens

Allergens originating in the midges salivary glands have been isolated and expressed from three *Culicoides* species : *C. sonorensis, C. nubeculosus and C. obsoletus.* They are named according to the systematic allergen nomenclature (see table 1).
Hyaluronidases are hydrolytic enzymes found in the salivary glands of some bloodsucking and stinging insects such as wasps and honey bees (Hoffman, 2006). The hyaluronidase can degrade hyaluronic acid (HA) which is one of the principle component of the extracellular matrix in all vertebrates. Hyaluronidase is present in almost all venoms and facilitates the penetration of other harmful venom components.
The hyaluronidases from *C. nubeculosus* (Cul n 2, 46.7 kDa) and *C. obsoletus* (Cul o 2, 42.3 kDa) are studied here. Cul n 2 is expressed as a partial protein (e16) to try to ease the purification.

An allergen is considered as major allergen when it binds IgE antibodies from more than 50% of allergic individuals (Deifl and Bohle, 2011). Cul n 2 and Cul o 2 are major allergens since they bind 56,5% and 62% of the IgE antibodies from IBH horses respectively.

Allergen	Homology to	% positive sera
Cul s 1	Maltase	87,5% / 22%
Cul n 1	Antigen 5 like protein	35%
Cul n 2	Hyaluronidase	56,50%
Cul n 3	Putative cysteine endopeptidase	47,80%
Cul n 4	Secreted salivary protein	45,70%
Cul n 5	Secreted salivary protein	43,50%
Cul n 6	Secreted salivary protein	34,80%
Cul n 7	Unknown salivary protein	30,40%
Cul n 8	Maltase	21,70%
Cul n 9	D7-related salivary protein	26,10%
Cul n 10	Secreted salivary protein	15,20%
Cul n 11	Trypsin	13,00%
Cul o 1	Kunitz protease Inhibitor	45%
Cul o 2	D7 protein	40%
Cul o 3	Antigen 5	
Cul o 1	Maltase (Cul s 1)	43%
Cul o 2	Hyaluronidase (Cul n 2)	62%
Cul o 3	Antigen 5 like protein (Cul n 1)	60%
Cul o 4	Trypsin (Cul n 11)	39%
Cul o 5	Unknown saliv protein (Cul n 7)	67%
Cul o 6	D7-related saliv protein (Cul n 9)	38%
Cul o 7	Secreted saliv protein (Cul n 4)	43%

Table 1: List of isolated and expressed *Culicoides* allergens. The allergens studied in this report are in grey.

3 Protein expression system

E.coli is the most commonly used method for the production of recombinant proteins. The bacteries are fast growing, easy to use and less expensive than other systems. Nevertheless, *Culicoides* proteins produced in *E. coli* do not have post translational modifications and *E.coli* proteins give too much background in some immunological tests. Baculoviruses are used as vector system to produce proteins in insect cells such as moths cells. This system allows eukariotic post translational modifications, good secretion and it is suitable for antigen production and therapeutic proteins (Schmidt and Hoffman, 2002).

First the gene of interest is cloned into pFastBac vector and transformed in *E.coli* cells. The *E.coli* are cultured and the colonies containing the gene of interest are selected with antibiotics. The bacmids from the positive cultures (containing the gene) are isolated.

The recombinant viruses are produced in Sf-9 cells and the recombinant proteins are produced in High 5 cells .

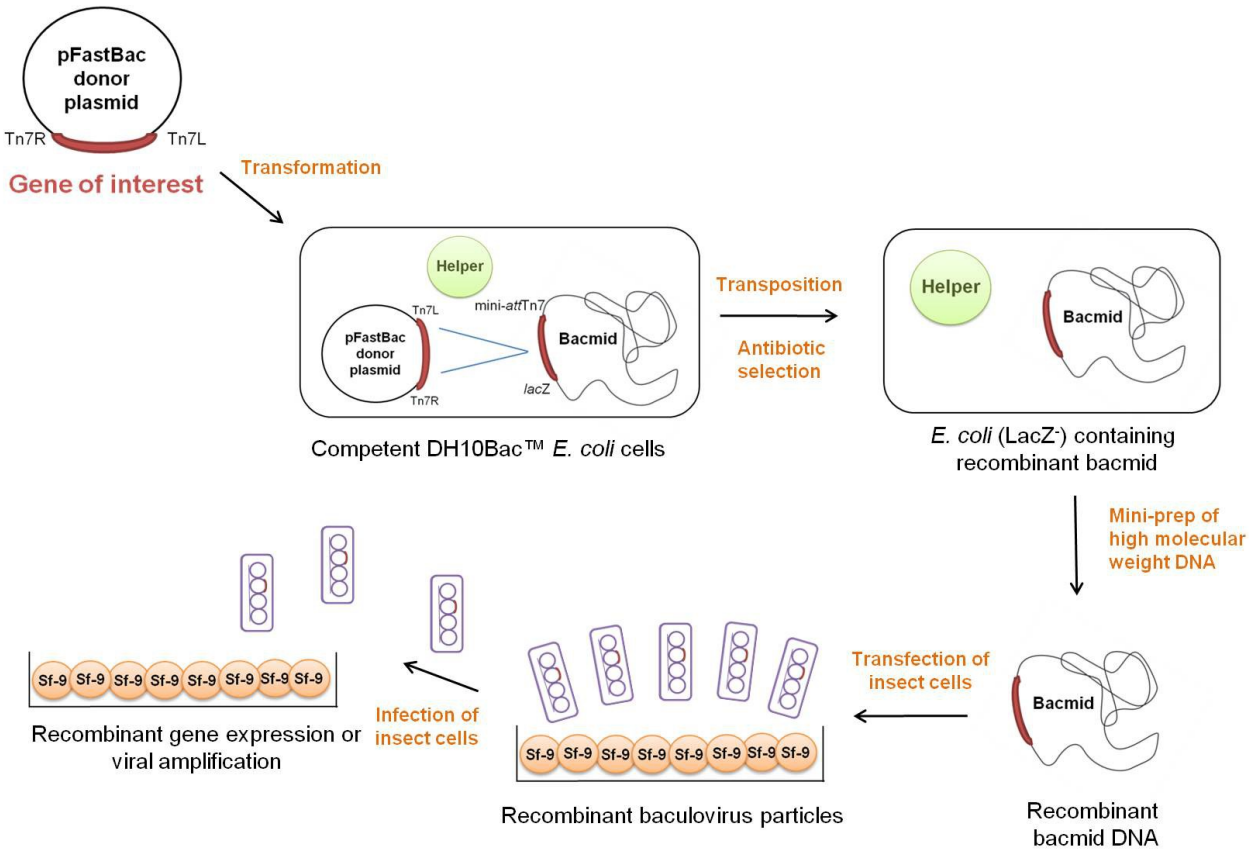

Figure 5: Bac-to-Bac Baculovirus Expression System (Invitrogen).

4 The project

The *Culicoides* Hyaluronidase, which is an important allergen in IBH, has proven to be very difficult to purify on a native form from the baculovirus system. The *C. nubeculosus* in full length had been expressed using different constructs but purification on native form and resuspension in non-toxic buffer was not successful (Sara's thesis).

Therefore, the hyaluronidase from *C. obsoletus* was codon optimised and expressed to investigate if that would ease the purification. The *C. nubeculosus* was expressed as a partial protein Cul n 2-e16.

Materials and methods

Culicoides n 2 and *Culicoides* o 2 gene accession numbers are HM145950 and KC339672 respectively in the GenBank.

1 Cloning with Phusion® Hot Start Flax DNA polymerase PCR

The amplification of Cul n 2 (e16) was made with a PCR from a FastBac vector. The Phusion® Hot Start Flax DNA polymerase was used as Taq DNA polymerase.
These reactions were performed in DNA Engine® Peltier Thermal Cycler (PTCC-200) from MJ Research.

PCR Reaction solution		**PCR Reaction**
Variable volume of DNA		1- Denaturing : 98°C → 30 sec
25 µL Phusion		2- Denaturing : 98°C → 10 sec
1,5 µL Forward Primer (20mM)	x30	3- Annealing : 56°C → 30 sec
1,5 µL Reverse Primer		4- Elongation : 72°C → 1 min/kb
21 µL ddH_2O		5- Elongation : 72°C → 10 min
Total : 50 µL		4°C forever

A previous PCR showed that the best temperature is 56°C for the annealing part.
For the reaction, a volume of 1µL of DNA was used, for a concentration of 254 ng/µL.

2 Electrophoresis

The PCR products were run on a middle size agarose gel. For the gel, 50 mg of Agarose were weighted and diluted in 50 mL of TBE (Tris Borate-EDTA) buffer. Before solidification, two drops of Ethidium were added to reveal the nucleic acids of the DNA under the UV light after migration.
Restriction buffer in 5µL was added to each sample. The electrophoresis was carried out at 70 volts for 55 minutes (depending of the size of the fragments). 2-log ladder was loaded to compare the results to a theorical size. After the migration, the results were analyzed under UV light in InGenius using the software GeneSnap.

This DNA was extracted from the gel with the GeneJet Gel Extraction Kit (Thermo scientific) under UV light. A binding buffer was added, the tube was centrifuged 1 min and the flow-through discarded. The washing buffer was added and the tube was centrifuged again. An elution buffer was added. After 1 min centrifugation, the DNA was stored at -20°C.

The concentration of DNA in the final samples was measured by the spectrophotometer NanoDrop®ND-1000 (NanoDrop Technologies Inc).

3 Vectors and TOPO® Cloning

The Bac-to-Bac® TOPO® Cloning kit (Invitrogen) was used to clone the gene Cul n 2-e16 in the vector HBM TOPO.

The size of the gene was 840 bp. The concentration of the vector was10 ng/µL and the gene was 24,4 ng/µL. The ratio was 2:1.

The volumes for the TOPO® Cloning reaction mix :

Gene : depend of the concentration and dilution

Salt Solution : 1 µL

ddH_2O : QS 6 µL

Vector : 0,5 µL (or 1 µL) **Final volume :** 6 µL according to the UM

The cells used to grow and multiply the vector were One Shot® Mach1™ $T1^R$ Chemically Competent *E. coli*.

The competent cells were slowly thawed on ice for 30 min with 2 µL of the TOPO® Cloning reaction mix. After a 30 second-heat-shock at 42°C without shaking, they were put again 2 min on ice. The S.O.C (Super Optimal Broth) medium was added in 250 µL and the TOPO® Cloning reaction mix was incubated 37°C for 1h with horizontal shaking. Finally it was put on pre-heated LB (lysogeny broth) agar plates medium with 100 µg/mL ampicillin, and incubated for 18 hours at 37°C.

4 Taq DNA polymerase PCR

After culture, the colonies were put into a liquid LB medium (6 col into 2 ml). Positive colonies were confirmed by PCR and incubated under horizontal shaking.

PCR Reaction solution

Variable volume of culture
16 µL buffer
16 µL dNTP (2mM)
8 µL Fast bac TOPO Fw Primer (20mM)
8 µL Reverse Primer specific to insert
1,6 µL Taq Polymerase
102,4 µL ddH_2O
Total : 152 µL and 20 µL for each tube

PCR Reaction

1- Denaturing : 95°C → 30 sec
x30 (
2- Denaturing : 98°C → 30 sec
3- Annealing : 56°C → 30 sec
4- Elongation : 72°C → 1 min/kb
)
5- Elongation : 72°C → 10 min
4°C forever

The electrophoresis was run at 75 V, 400 mA and 55 min.

5 Plasmid purification and sequencing

The plasmid isolation was made with the Nucleospin®Plasmid Kit (Macherey-Nagel). Bacterial cells were centrifuged 30s at 11 000g and lysed by three different buffers according to the manufacturer. The lysate was clarified by centrifugation. The NucleoSpin® column was put on a collecting tube for the binding of the DNA. The silicia membrane was washed with a AQ buffer given by the manufacturer and centrifuged until it was dried. The DNA was eluted when the column was placed in a 1,5mL tube and filled with a AE buffer given by the manufacturer then centrifuged.

The DNA concentration was measured with the NanoDrop®ND-1000.

For the sequencing the samples were mixed to a sequencing reaction made with different primers. Several primers were used to make sure that all the bases of the DNA were covered. The sequencing was performed using a BigDye Terminator v3,1 Cycle Sequencing Kit that optimized the signal balance.

Cycling Sequencing Reaction solution

Variable volume of DNA
1,5 µL buffer
1 µL BigDye
1,6 µL Primer (2mM)
4,9 µL ddH_2O
Total : 10 µL for each tube

Cycling Sequencing Reaction

1- Denaturing : 95°C → 5 min
x30 (
2- Denaturing : 95°C → 20 sec
3- Annealing : 50°C → 15 sec
4- Elongation : 60°C → 4 min
)
4°C forever

A precipitation was performed by adding 75% isopropanol to each tube and centrifuged. The pellet was dissolved in HiDi Formamide and each 200 to 500 ng/µL sample was put on a 96 wells sequencing plate. The sequencing was analyzed with the Sequencher™ program from Gene Codes Corporation.

6 Expression of recombinant proteins in Sf-9 cells

a) Transformation of DH10Bac™ *E.coli*

The expression was made with the MAX Efficiency® DH10Bac™ Competent Cells Transformation kit (*E.coli*). This kit contained a bacmid and an helper plasmid. The bacmid had a 136 kb baculovirus genome with mini-attTn7 target site, a Kanamycin resistance gene, a mini-F replicon allowing stable replication. It also contained a LacZα gene and that confirmed that the transposition is at the correct site by giving a $LacZ^-$ phenotype.
The helper plasmid encodes the transposase and gives resistance to tetracycline.

The competent cells were put in 100 µL in chilled tubes. The pFastbac-gus control DNA was put in 5 µL with the cells and incubated on ice for 30 min. The transformation reaction mix was heat-shocked for 45 sec at 42°C and incubated on ice for 2 min. S.O.C medium was added in 0,9 mL and the solution was put under shaking at 37°C for 4h. The Miniprep was spread on LB agar plates as well as the experimental reaction. The plates were incubates for 48h at 37°C. The LB agars contained 50µg/mL Kanamycine, 7 µg/mL gentamicin, 10 µg/mL tetracyclin, 100 µg/mL X-gal and 40 µg/mL IPTG.
Colonies containing the $LacZ^-$ recombinant bacmid (white) were picked and placed in LB medium jars with the same antibiotics at the same concentrations than previously. The medium was incubated 48h at 37°C under shaking.

b) Isolation of r-bacmid

The colonies from the liquid medium were tested with PCR using M13 primers and gene specific primers (e16).
The positive colonies containing the recombinant bacmid were purified via the PurLink™ HiPure Plasmid DNA Purification kit (Invitrogen).
Resuspension Buffer, Lysis Buffer and Precipitation buffer were added in 0,4 mL each. A centrifugation was made at 15000g for 10 min and the supernatant was loaded on a

column. After the draining the supernatant was washed two times. Elution Buffer in 0,9 mL of and isopropanol in 0,63 mL were added. A centrifugation was made at 15000g for 30 min at 4°C. 70% ethanol was added in 1 mL and the same centrifugation was done again for 5 min. The pellet was dried and resuspend in 50 µL of TE Buffer and store at -20°C.
Another PCR was made to test the isolated recombinant bacmid.
The Sf-9 cells were cultured in a 12 well plate the day before the transfection. The culture medium was SF-900™II medium (Life Technologies™).
The day of the transfection, the medium was aspirated from the wells and 500 µL of the Grace's medium were added and aspired to wash the cells. The Grace's medium was then added in 800 µL. The concentration of the recombinant bacmid was measured with the NanoDrop. The r-bacmid solution was heated at 72°C for 5 min to avoid contamination.

c) Transfection of Sf-9 insect cells

Two mix were prepared.

	Mix 1	Mix 2
r-bacmid (DNA wells)	a) 1 µg rBacmid + Grace's media up to 100 µL b) 0,5 µg rBacmid + Grace's media up to 100 µL	a) 8 µL cellfectin + 100 µL Grace's media b) 8 µL cellfectin + 100 µL Grace's media
Cellfection control	100 µL Grace's media	8 µL cellfectin + 100 µL Grace's media
Cell control	100 µL Grace's media	100 µL Grace's media

The mix 2 was kept at RT for 5 min and then added to the mix 1. The final mixes were kept at RT for 20 min. They were put in labeled wells of a 12 well plate and incubated 5 hours at 27°C. After 5 hours the Grace's medium was aspired and 900 µL of SF-900II medium with PEST and 1% FCS were added. The plate was incubated for 3 to 7 days. The wells containing the cells were read in microscope every day to follow the cytopathic changes. When the cytopathy is very clear all the wells were mixed and pipetted to sterile eppendorf tubes. The tubes were spun down at 14 000 rpm for 3 min and the supernatant was collected. The pellet was dissolved in 50 µL of lysis buffer. For the first passage, 200 µL of the supernatant was transfered into fresh Sf-9 cells in 12 well plate. These cells were

grown for 5 days. When the cytopathic effects were clearly visible, the supernatant was transferred to fresh Sf-9 cells in T75 flask (Nunc) in complete Sf-9 medium and incubated for four days at 27°C. Harvesting was done by spining down the cells at 18 800g for 3 min. The virus stock was kept at 4°C.

d) Cloning and production of r-baculovirus

The cloning was done from the supernatant harvested from the first passage in a 96 well plate. Sf-9 cells were added in 100 µL per well. The r-baculovirus was added in seven dilutions (from $1x10^{-5}$ to $5x10^{-9}$) in complete Sf-9 medium. The cytopathy of the cells was followed and scored after 14 days incubation. The clones with cytopathy from the highest dilution were transferred (180 µL) to fresh Sf-9 cells in a 24 well plate. When cytopathy was observed, the supernatant was used for a second passage in the same condition in T75 flasks. The second passage virus was used to make a large viral stock of third passage virus. The cell pellets from each passage were tested in WB. The r-baculoviruses were kept in 4°C and frozen at -80°C.

e) Production of r-allergens in High 5 insect cells.

The time did not allow to perform this part but it will be done according to Sara's thesis references.

7 Protein purification and analysis

a) Protein purification with nickel affinity gel under native conditions

The protein purification was done with former produced *C. obsoletus* hyaluronidase (Bac-1 Cul o 2 hya and HBM Cul o 2 hya), $100x10^6$ High 5 cells. The HIS-select® HF Nickel Affinity Gel (Sigma-aldrich) was washed in dH_2O with 10x the volume of the gel and equilibrated in lysis buffer with 10x the volume of the gel. It was centrifuged at 800g for 8 min.

The cells were lysed by a 8mL lysis buffer with 160 µL Protease Inhibitor Cocktail (PIC) (Sigma-Aldrich). They were then placed on ice and sonicated five times 10 sec with 20 sec interval. The pellet was centrifuged two times five minutes at 10 600g at 4°C. The HIS-select® HF Nickel Affinity Gel was added to the supernatant and mixed under orbital

shaking for two hours at 4°C. It was then spun down at 800g for 5 min and the supernatant was collected.
The pellet was resuspended in wash buffer (10 times the volume of the gel), mixed 5 min and spun down again at 800g for 5 min. Four series of washing with two times 10 mM Imidazol buffer and two times 20 mM Imidazol buffer were made with 5 min orbital shaking and 5 min centrifugation. Four elutions with two times pH 8 elution buffer and two times pH 6,5 elution buffer were undertaken in the same conditions. Samples were collected on a plastic column. The elution fractions were stored at 4°C and tested with Coomassie Blue staining and Western Blot.

b) SDS-PAGE (Sodium Dodecyl Sulfate PolyAcrylamid Gel Electrophoresis)

A gel was made to separate proteins in a Mini-protean II (Bio Rad) system.
The separating gel was produced with dH_2O, 1,5 M Tris buffer pH 8,8, 40% acrylamid/bis, 10% SDS, 10%APS and TEMED. The stacking gel was composed of dH_2O, 0,5 M Tris buffer pH 6,8, 40% acrylamid/bis, 10% SDS, 1% Bromophenol blue, 10% APS and TEMED. The combs were added. When it had solidified, the samples and the ladder were placed in the wells.The migration was run from 60 to 80 minutes, at 200V, 65mA.

c) Coomassie Blue Staining

After a SDS-PAGE, the gel was stained with four different solutions (see recipies in appendix III). For each bath in the stain solution, the gel was heated in the microwave and then washed with water. After staining the gel was dried between two cellophane sheets o.n with storage solution.

d) Western Blot

After a SDS-PAGE, the samples on the gel were transferred to a PVDF membrane by a wet transfer. A membrane was washed in methanol for 15 sec, water for 2 min and transfer buffer for 5 min.
A sandwich was made according to figure 6.

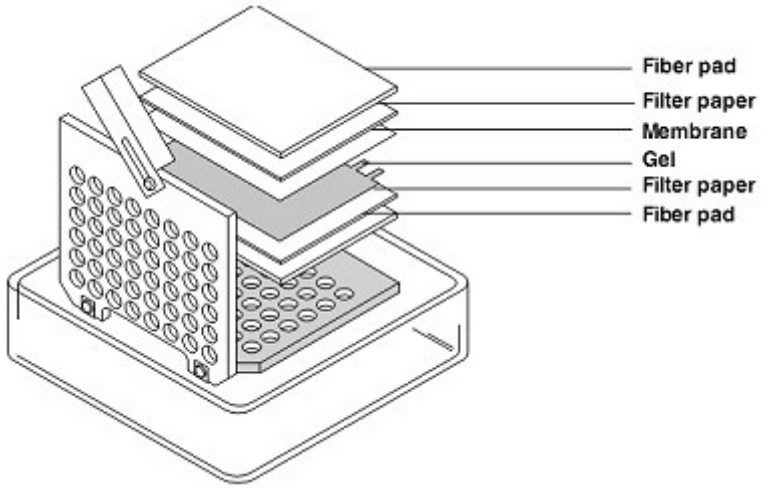

Figure 6: Sandwich organisation of the gel-to-membrane transfer of proteins, in Western Blot

The transfer was made in the Miniprotean II system (Bio-Rad). Transfer buffer, a bloc of ice and a magnetic stirrer were put into a transfer container and run at 100 V, 250 mA for 1h.

After the transfer, the membrane was blocked with TBS-T and Tween for 30 min at RT. The membrane was washed three times for 5 min with TBS-T. The primary antibodies were added and stayed o.n under horizontal shaking at 4°C. The membrane was washed five times for 5 min and the secondary antibodies were added for 1h at RT. These secondary antibodies were Alkaline Phosphatase-conjugated Affinity Pure Goat Anti-Mouse IgG. The membrane was washed five times 5 min with TBS-T. The development was made with BCIP/NBT, washed with dH_2O and the membrane was dried. The time of the revelation was recorded.

e) Bradford Protein assay

The Bradford protein assay is a colorimetric assay used to determine the concentration of a protein, using Coomassie Plus (Bradford) Assay Kit (Thermo Scientific). Six standards of different concentrations were put on the plate as well as the samples, and water was used as a blank. It was read in a micro-plate spectrometer (VICTOR^3 Perkin Elmer). The absorbance was read at 600 nm.

f) Dialysis

The dialysis was done on the purified protein under native conditions in a Slide-A-Lyzer® 40 kDa Dialysis Cassette G2 (Thermo Scientific). The samples were loaded in the cassette and put in a beaker filled with elution buffer (at least 200x of the volume of the sample). It stayed o.n at 4°C under stirring. The buffer was changed and left for 2 hours under stirring. The samples were removed from the cassette and spun down at 18 800g for 3 min. The supernatant was collected and the protein samples were filtered and stored at 4°C.

Results – Discussion

1 Cul n 2 (e16)

a) Amplification and cloning of Cul n 2

The gene was amplified by PCR and the concentration of DNA was measured by NanoDrop®. The cloning was made in an HBM TOPO vector. To clone the gene, the plasmid was introduced in *E.coli* cells. The colonies were tested with PCR.

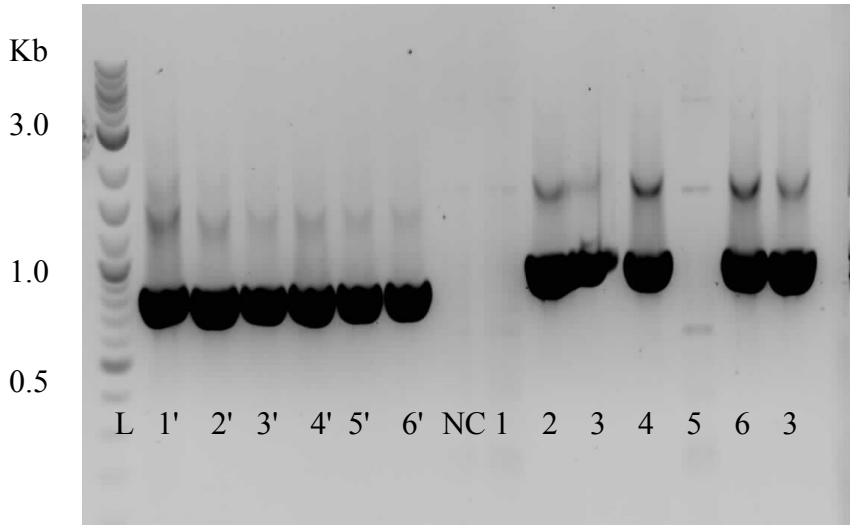

Figure 7: PCR. L : Ladder. **1'-6' :** Gene specific primers **NC :** Negative Control. **1 to 6 :** gene and vector specific primers.

In Miniprep 2, 3, 4, 6 the gene was inserted successfully in the plasmid, miniprep 1 and 5 were negative. The positive cultures were tested with NanoDrop and the Miniprep 2 and 4 showed strongest concentrations of recombinant plasmid, from 200 to 300 ng/µL. They were chosen for further work.

b) Sequencing

Miniprep 2 and 4 were sequenced. The first one wasn't complete, the primers didn't cover all the sequence of the gene of interest, especially the His taq. This part is important to know if the insert is in the right correctly oriented way in the plasmid. Four new primers were designed. The second sequencing didn't cover the very beginnig of the sequence but

at least it covered the His taq. This part of the sequence was at the end of the insert. It confirmed that the gene of interest was in the plasmid and that it had turned the right way.

b) Isolation of the recombinant bacmid

After transformation of DH10Bac with Miniprep 2, enough colonies grew to test five samples with PCR.

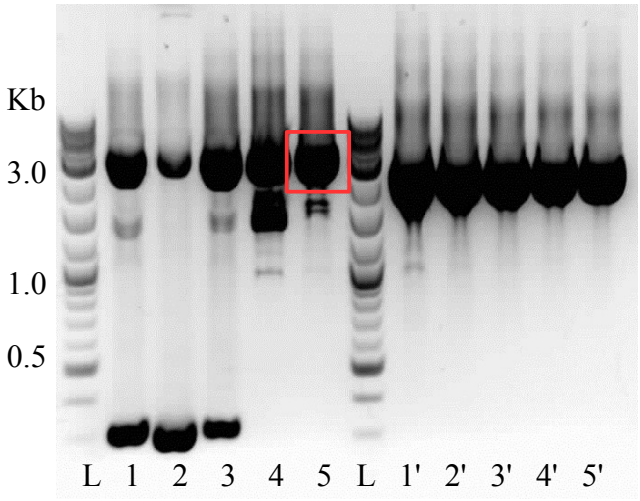

Figure 8: Cul n 2 bacmid PCR
L : Ladder ; **1** → **5 :** gene and vector specific primers ; **1'** → **5' :** gene specific primers

Samples 1' to 5' contained gene specific primers to see if the gene was present. Samples 1 to 5 contained gene and vector specific primers to see if they were clean. The samples 1, 2 and 3 had extra bands. They were bad quality because they were taken too close from negative colonies.The sample 5 was the cleanest, it was the one used for the expression in insect cells.

c) Expression of r-allergen in Sf-9 cells

The Sf-9 cells were transfected with the recombinant bacmid to generate recombinant virus. The transfection was done with Cellfectin. Pictures were taken after five days to see the cytopathy, with the inverted light microscope, Leica CM IL LED, magnified 200x.

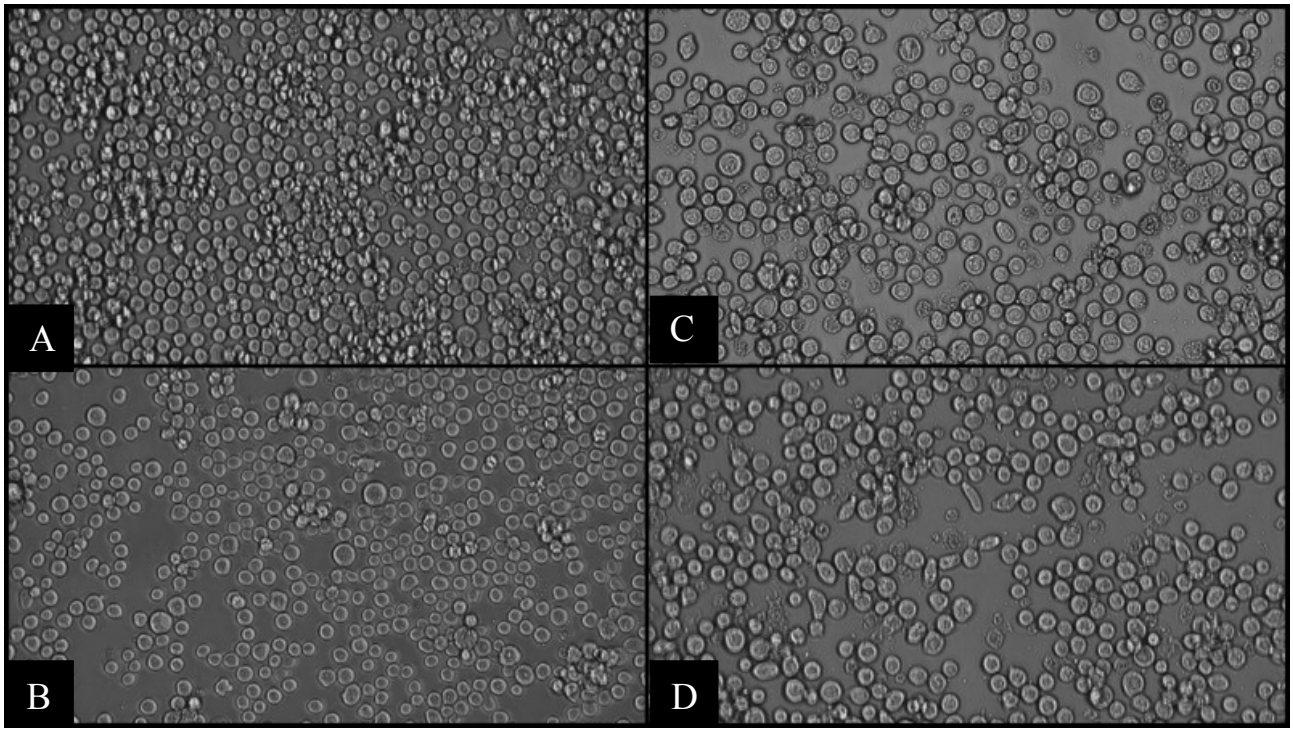

Figure 9: Sf-9 cells transfection
A : Infected cells with 0,5 µg bacmid **B :** Infected cells with 1 µg bacmid **C :** uninfected cells **D :** uninfected cells with Cellfectin

As seen on the pictures A and B, the uninfected Sf-9 cells are round and small. The growth is more pronounced in healthy cells. In picture C and D there are some irregular, big and elongated cells. They are infected Sf-9 cells. More infected cells can be seen on the picture D because of the higher concentration of the bacmid.

A western blot was done after the transfection to make sure that the virus was expressing the e16 partial protein. Two different antibodies were used to confirm the expression of anti Cul n 2 protein specific and anti His specific.

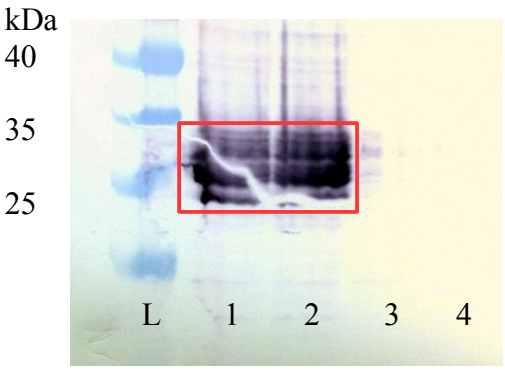

Figure 11: e16 western blot with anti His

L : Ladder **1 :** 0,5 µg bacmid **2 :** 1 µg bacmid **3 :** Cell control (uninfected) **4 :** Cellfectin control (uninfected)

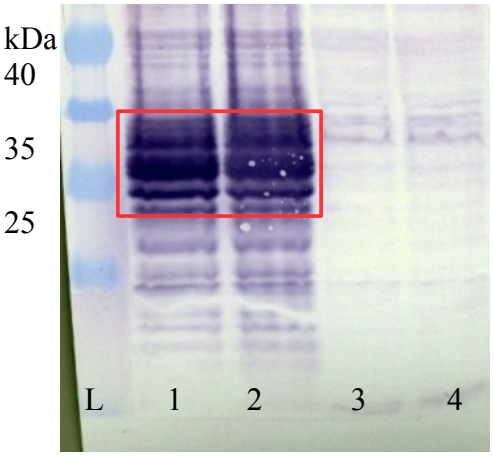

Figure 10: e16 western blot with anti Cul n 2

L : Ladder **1 :** 0,5 µg bacmid **2 :**1 µg bacmid **3 :** Cell control (uninfected) **4 :** Cellfectin control (uninfected)

Using both anti Cul n 2 and anti His antibodies, the partial protein e16 appeared as a band of 36 kDa.

The previous try to produce Cul n 2 was not successful, but it was successfully expressed this study. All the methodological process was recorded carefully to compare with the unsuccessful production process.

d) Cloning of r-Bac-Cul-n-2-e16 virus in limiting dilution

The cloning was set up according to the methods in Sara's thesis.

2 Cul o 2

a) Purification of Bac-1-Cul-o-2 and Bac-HBM-Cul-o-2

Bac-Cul-o-2 with and without pFastBac™HBM TOPO® vector were purified ($50x10^6$ cells) with the HIS-select® HF Nickel Affinity Gel under native conditions and compared with Coomassie and Western Blot to test if either one can be purified on native form.

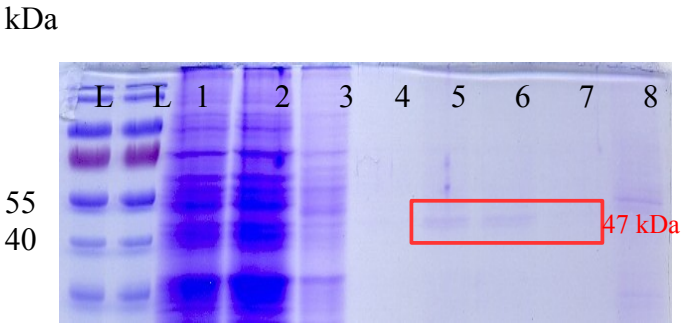

Figure 12: Bac-1-Cul-o-2 Coomassie
L : Ladder(s) **1 :** after sonication **2 :** proteins that didn't bind to the beads **3 :** washing 1 **4 :** washing 3 **5 :** elution 1 **6 :** elution 2 **7 :** elution 3 **8 : b**eads

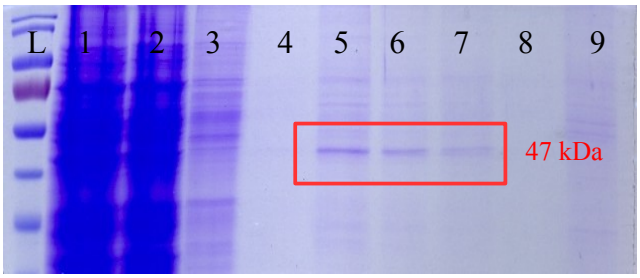

Figure 13: Bac-HBM-Cul-o-2 Coomassie
L : Ladder **1 :** after sonication **2 :** proteins that didn't bind to the beads **3 :** washing 1 **4 :** washing 3 **5 :** elution 1 **6 :** elution 2 **7 :** elution 3 **8 :** elution 4 **9 :** beads

The 47 kDa bands matched for the protein of interest.

The Coomassie showed that the bands from the purification of the Bac-1-Cul-o-2 were rather weak and nearly invisible. They appeared stronger on the purification with the Bac-HBM-Cul-o-2.

In the meantime, the same samples were tested in Western Blot and showed the same results. Therefore, the Bac-HBM-Cul-o-2 was used for the further tests and purifications.

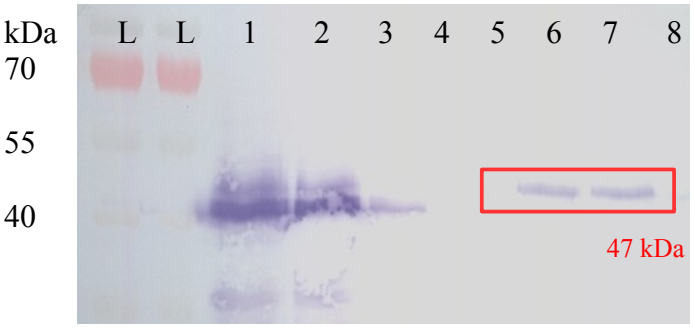

Figure 15: Bac-1-Cul-o-2 Western Blot

L : Ladder **1 :** after sonication **2 :** proteins that didn't bind to the beads **3 :** washing 1 **4 :** washing 2 **5 :** elution 1 **6 :** elution 2 **7 :** elution 3 **8 :** beads

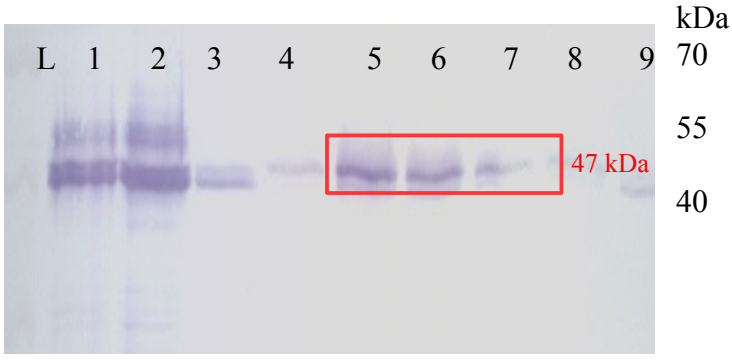

Figure 14: Bac-HBM-Cul-o-2 Western Blot

L : Ladder **1 :** after sonication **2 :** proteins that didn't bind to the beads **3 :** washing 1 **4 :** washing 3 **5 :** elution 1 **6 :** elution 2 **7 :** elution 3 **8 :** elution 4 **9 :** beads

b) Dialysis on Bac-HBM-Cul-o-2

The Bac-HBM-Cul-o-2 was purified a second time ($100x10^6$ cells). A dialysis was made on this solution. Samples were taken before and after the dialysis to be tested with Coomassie blue staining, Bradford and Western Blot.

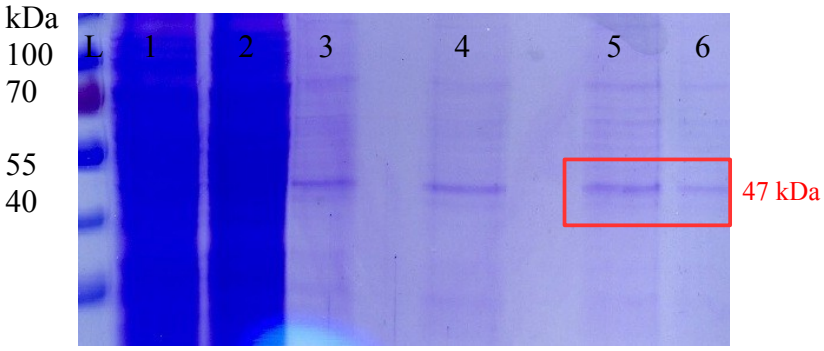

Figure 16: Bac-HBM-Cul-o-2 dialysis coomassie
L : Ladder **1 :** after sonication **2 :** proteins that didn't bind to the beads **3 :** elution 1 **4 :** elution 2 **5 :** before dialysis **6 :** after dialysis

The concentration of proteins was 180 ng/µL before the dialysis and 80 ng/µL after. The sample was successfully dialysed. The coomassie confirmed that the protein of interest was still there.

The purification of Bac-HBM-Cul-o-2 was successful. More hyaluronidase from this *C.obsoletus* will have to be purified. An important amount of this allergen is needed for further tests and for the development of the immunotherapy.

Conclusion

The Institute for Experimental Pathology has a long term project which is the development of an immunotherapy against IBH in horses. Purified proteins are essential to succeed in this process but they can be hard to obtain in a suitable form.

The previous purifications of Cul n 2 in native form were not successful. During this internship, the partial protein e16 was produced with promising results. It will be purified in native form in future works.

The purification of Cul o 2 was successful, and following that, more purifiction will be done the same way to obtain more hyaluronidase.

Personal review of the internship

During this internship I have improved what I learnt during my DUT, even if I still have a lot to learn in specific fields and specific methods. Working in a research lab allowed me to experiment the everyday tasks of searchers. Having good results is a long term process, when something didn't work out I had to find a solution and start again. Time is needed to achieve a goal so I learnt patience and willingness.

Eventhough I know that I'm not going to work in a lab in my future life, it was a great experience and I am grateful I learnt so many things. Working in the research field was gratifying since I contributed to horse healthcare at my scale. Being part of a team project was more motivating for me than working on my own.

Doing this internship abroad was rewarding in term of personal experience as well. I liked discovering new cultures and new languages, it gave me more self-confidence and autonomy.

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Appendix I – Cul n 2 sequence

ATGTGGTTGAACGTGGTAAATGTCTCACAATTTATGACAGCATGGGCGACCTTTAATTTGATTAATGCACA
ACAACTGATACAAGTGGGACCAGAAAATGTACCATATGAAATTGTAGATGCAAAGGACGATGCATCTGAAA
GCAGAGGAATATTTTTTAATAACTTCATCACATCAAAAAATAACGATGATAAAAGACATGATTTTACCTTTTA
CTGGAACATCCCATCATTTATGTGTTCAAAATACAATGTAACATTCACTGACATGCCTTCATCATATAATATC
GTACAAAATAAAGATGATAAATGGCGTGGTGACCAGATCATAATTTTATATGATCCTGGTAAATTTCCGGCT
TTATTAGAGCATCAAGGAAAATTATATAGACGAAATGGTGGTGTACCACAAGAAGGGAATTTACAAGAACA
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TCTTGAGTCATGGCGACCGATTTATCGTCAAAATTCAGGTGTGTTACAGCCATATAAGGATTTATCATATAA
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GGACCAATTGTACTATCAGCAAATGACAATACACCAAAAATTCTTACCCCAAATTTGTCATAA

Appendix II – Primers designed and used for e16 sequencing

1 - DNA Oligo Name : e16_130_rev

Oligo ID : 160421J1C03
5'-CAC TCC AAT GCC TGA GAA GT-3'

2- DNA Oligo Name : e16_188_rev

Oligo ID : 160421J1B01
5'-ATG GCT GTA ACA CAC CTG AA-3'

3- DNA Oligo Name : e16_675_fw

Oligo ID : 160421J1G02
5'-CAA AGA GCA AAA GGG TAG TGG-3'

4- DNA Oligo Name : e16_707_fw

Oligo ID : 160421J1A01
5'-GGG GCA GTT CAT ATG ATG TCA-3'

Appendix III – Transfer buffer

700 mL dH_2O

200 Ml Methanol

100 mL 10x electrophoresis buffer

Appendix IV – Coomassie Blue Staining (Wong et. al, 2000)

1 Stock solution

0,2% PhastDel Blue R : 1 tablet in 80 mL dH_2O, stir 5-10 min. 120 mL methanol, stir till it is disolved.

2 Buffer A – D

Fairbanks A : Staining solution
25 mL 0,2% coomassie stock
10 mL isopropanol
25 mL acetic acid
40 mL dH_2O

Fairbanks B : Staining solution
2,5 mL 0,2% coomassie stock
10 mL isopropanol
10 mL acetic acid
77,5 mL dH_2O

Fairbanks C : Staining solution
1 mL 0,2% coomassie stock
10 mL acetic acid
89 mL dH_2O

Fairbanks D : Destaining solution
10 mL acetic acid
90 mL dH_2O

Abstract – Résumé

The IBH disease is the insect bite hypersensitivity on horses. This allergy affects exported icelandic horses and is due to the bite of *Culicoides* midges. It causes itches at the base of the mane and the tail, and can involve infected wounds. The Institute for Experimental Pathology, Keldur, aims to develop a vaccine to prevent this disease.

One of the major allergen in this disease is the hyaluronidase, produced in the salivary glands of the midges such as *C. nubeculosus* and *C. obsoletus*. The hyaluronidases worked with are named Cul o 2 and Cul n 2 respectively.

Cul o 2 has already been produced and was purified and tested with Western Blot, Coomassie Blue staining and Bradford assay. Hyaluronidase was successfully purified but this process will have to be repeated to get more of the protein for further tests. Cul n 2, in the form of the partial protein e16, was cloned, expressed in baculovirus and produced in Sf-9 Cells. These processes were successful and the next step will be to purify it in native way.

La dermatite saisonnière du cheval, ou IBH, est une allergie proéminante chez les chevaux islandais exportés sur le continent. Cette réaction est due aux piqûres d'insectes, et particulièrement celles des moucherons hématophages du genre *Culicoides.* Elle provoque des démangeaisons à la base de la queue et de la crinière, qui peuvent s'infecter. Le centre de recherche Keldur s'est donc posé comme objectif de développer un vaccin pour palier à cette maladie.

L'une des protéines majoritairement responsable de l'IBH est la hyaluronidase. Elle est produite dans les glandes salivaires des *Culicoides obsoletus* et *Culicoides nubeculosus.* Les deux allergènes sont nommés respectivement Cul o 2 et Cul n 2-e16.

Cul o 2, ayant déjà été produit, a été purifié pour extraire la hyaluronidase et testé sous Western Blot et Coomassie. Cette purification s'est avérée fructueuse, néanmoins elle devra être recommencée. Il faut obtenir une quantité suffisante de hyaluronidase afin d'enchainer d'autres tests et procéder à la mise en place d'une immunothérapie. Cul n 2-e16 a été cloné, exprimé dans un baculovirus et produit dans des cellules Sf-9. La prochaine étape sera sa purification sous forme native.