University of South Bohemia in České Budějovice
Faculty of Science

Cloning and Expression of two selected Antigenic Proteins and their characterization as diagnostic markers against Relapsing Fever Disease

Bachelor Thesis

Laboratory of Molecular Ecology of Vectors and Pathogens
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Annotation: The aim of the thesis was to clone and express the two selected proteins Lactate dehydrogenase and *Human Complement Plasminogen A* and analyzing their diagnostic potential for *Borrelia duttonii* infection.

Affirmation: I declare that I am the author of this qualification thesis and that in writing it I have used the sources and literature displayed in the list of used sources only.

České Budějovice, 05.05.2024 Esma Özcan

Acknowledgement

I want to sincerely thank Dr. Ryan Rego, my supervisor, for all of his help and support during the writing of my thesis. His knowledge and perceptive criticism have greatly influenced my research, and his support has inspired me to pursue greatness.

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They have been a great source of strength and inspiration throughout this whole challenging yet very enriching process.

Thank you very much for your valuable contribution to my academic experience.

Abstract

This research study aimed at cloning, expressing and characterizing diagnostic markers for *Borrelia duttonii* infection, focusing on the Lactate dehydrogenase (LD) and Human Complement Plasminogen A (*hcpA*). The research was driven by the need to develop more effective diagnostic tools for relapsing fever Borrelia, which continues to be a significant public health challenge.

Through a series of PCR experiments, both LD and hcpA genes were successfully amplified, with genomic DNA from the 1120K3 strain proving most effective for this purpose. While cloning was successful, particularly for the LD gene, the expression phase encountered significant obstacles. Protein detection was problematic, indicating potential issues with protein expression, or stability.
Despite these challenges, the study highlights the potential of molecular techniques in advancing diagnostics for infectious diseases.

Future research should explore alternative expression systems and refine detection methodologies to enhance the solubility and visibility of these critical diagnostic markers.

1 Table of Content

2 Introduction

2.1 Relapsing Fever *Borrelia*

Relapsing Fever (RF) is a bacterial infection caused by spirochetes, a member of the *Borrelia* genus. These spirochetes are known for their ability to cause recurrent bouts of fever in infected individuals, giving rise to the name “Relapsing Fever”. This vector-borne illness is categorized into two groups: tick-borne relapsing fever (TBRF) and louse-borne relapsing fever (LBRF).

TBRF is transmitted primarily by argasid vectors, commonly referred to as 'soft ticks,' and infrequently by ixodid vectors or 'hard ticks.' Currently, the only identified *Borrelia* species causing RF is *Borrelia miyamotoi*, which is transmitted by hard ticks of the Ixodes genus. The LBRF group comprises a single species, *B. recurrentis*, which is transmitted by the clothing louse *Pediculus humanus*. While tick-borne RF is a prevalent disease found in various regions, including Europe, Africa, Central and Western Asia, as well as South and North America, visualized in Figure 1, it remains an area of limited research and is often underestimated. Each group includes several species with distinct characteristics. [1,2,3,4,5] *Borrelia* encompasses a diverse group of bacteria, comprising more than 30 identified species. Except for *B. duttonii* in Africa, which appears to be exclusively human and has no known animal reservoir, bacteria are all maintained in an enzootic cycle with humans serving as accidental hosts.[6]
RF Borrelia are located all over the world, including the United States, Mexico and Central America as well as throughout the Mediterranean area, Middle East, Central Asia, parts of Japan and Africa. The distinct types of RF Borrelia are closely regionally related to their vector species and, consequently, they can be found naturally in the geographical area where it is located.[19]

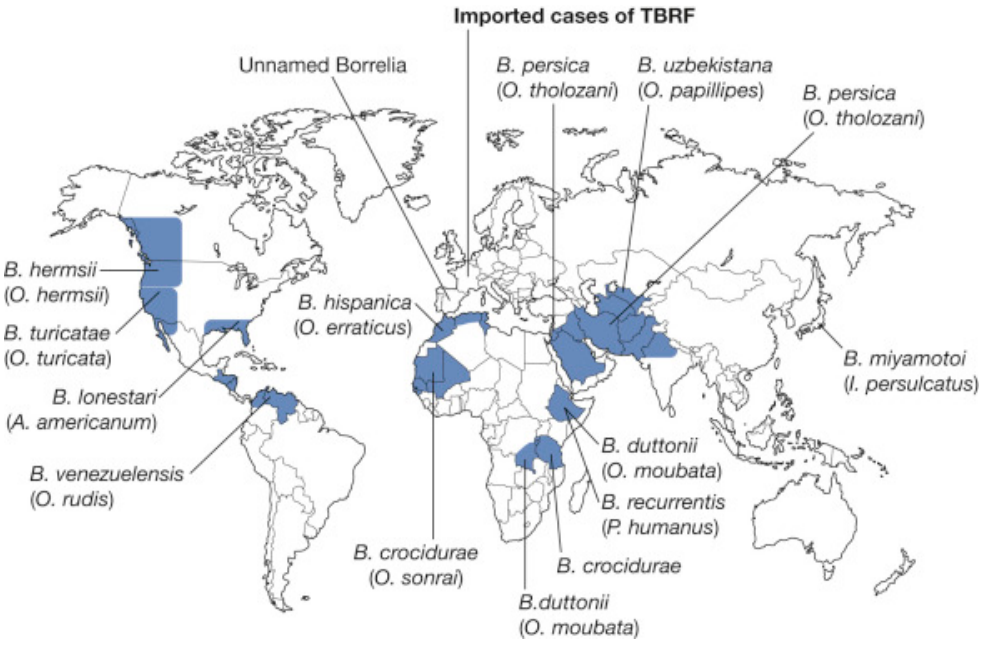

Figure 1: distribution of tick-borne relapsing fever agents. (from Fotso et al. 2015[4])

2.1.1 Disease Progression and Diagnosis

Some known symptoms are recurrent episodes of high fever, severe headache, myalgia, abdominal complaints, tachycardia, vomiting, neurological symptoms, and shaking chills. All symptoms have the potential to return, resulting in the recognizable pattern of a three-day fever, followed by an asymptomatic episode of seven days, and then another three days of a febrile period.[5,6, 20, 21]

Although this illness with malaria-like symptoms is responsible for moderate to dangerous spirochetemia, it is classified as a neglected tropical disease and has not been researched as well as other tick-borne diseases like Lyme disease. The diagnosis of this infection is challenging due to non-specific indications. Since the geographical range of RF spirochetes intersect with that of malaria and the malaria-like clinical manifestations of this disease, it has often been misdiagnosed. Most of the individuals suffering from RF were treated for malaria because of the lack of knowledge and diagnostics. [3,4]

While the symptoms of relapsing fever should not be overlooked, it is essential to acknowledge the shortage of diagnostic markers specific to RF *Borrelia*. Historically, only a single antigen, known as *Borrelia* immunogenic protein A (BipA), was identified in *B. hermsii* and *B. turicata*.[7,8] Additionally, researchers have explored the use of glycerophosphodiester phosphodiesterase (GlpQ) in PCR assays to detect the presence of *B. persica* in blood samples.[9] The discovery of the immunodominant enzyme GlpQ in *B. hermsii* has paved the way for the creation of diagnostic assays. When individuals are infected with spirochetes from the relapsing

fever group, their serum exhibits a positive reaction with GlpQ, making it a potential target for diagnostic purposes. Given the evident close relationship between *B. lonestari a*nd spirochetes from the relapsing fever group, researchers are actively investigating the presence of a GlpQ ortholog in *B. lonestari* DNA. This exploration is undertaken to develop specialized diagnostic assays capable of distinguishing between different *Borrelia* species, including B. *lonestari*.[10] These limited diagnostic markers highlight the critical need for further research in this area.

2.2 Borrelia genus

Bacteria of the *Borrelia* genus, classified within the spirochete's phylum, display common morphological features, including a spiral-shaped cell structure and three distinct modes of movement. Their cellular structure consists of a protoplasmic cylinder complex, encompassing the cytoplasm, an inner cell membrane, and peptidoglycan, all enclosed by an outer cell membrane. Spirochetes also harbor flagella in the periplasmic space, positioned between the outer cell membrane and the protoplasmic cylinder. Notably, despite their classification as Gram-negative bacteria, *Borrelia* is distinguished by the absence of lipopolysaccharides (LPS) in their outer membrane.[9,12] The bacteria species exhibit variations in characteristics such as length, diameter, coil tightness, coil regularity, and the number of periplasmic flagella. The spirochete enters the bloodstream after a tick bite and leads to an osmotic infection that is characterized by symptoms of remitting fever. The helical shape of spirochetes like *Borrelia duttonii* allows them to move through viscous environments, such as the bloodstream and tissues, using their corkscrew motion.[11]

2.3 Soft ticks (*Argasidae* family)

Soft ticks of the *Argasidae* family serve as the primary vectors for most *Borrelia* species. These ectoparasitic, bloodsucking arachnids, belonging to the subclass *Acari*, exhibit nocturnal behavior and typically inhabit animal burrows or human dwellings. Their feeding activity occurs during the night, after which they retreat to conceal themselves in tiny cracks and fissures. [22,23]

Within the *Argasidae* family, comprising an estimated 193 species across five genera, *Ornithodoros* and the *Argas* genus stand out with a higher number of species. Notably, soft ticks lack a dorsal scutum at both nymph and adult stages, distinguishing them morphologically from ticks in the Ixodidae family. They possess a leathery cuticle and a centrally located dorsal plate covered in small mammillae. The capitulum, housing teeth and mouthparts, is

subterminally positioned and concealed from a dorsal view. In species with eyes, they are typically situated on the ventral face of the body. [23]

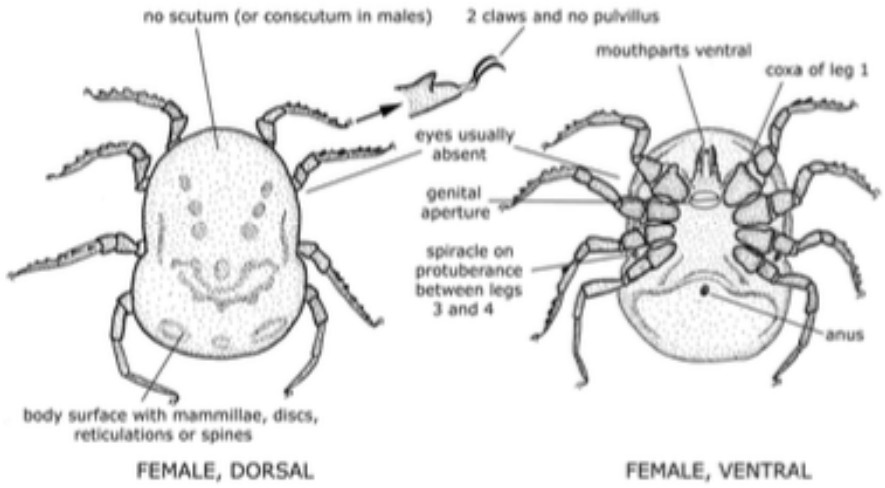

Figure 2:Morphological characteristics of soft ticks (Family Argasidae) are exemplified by the adult female of the Ornithodoros genus. (from Barker et al. 2014 [24])

One distinctive feature of soft ticks is their notably short blood meal duration, ranging from 15 to 60 minutes during the nymph and adult stages. This characteristic allows them to minimize exposure to adverse external conditions that may arise when the host departs from the tick's microhabitat. The mating process occurs several weeks after the blood meal, making research on small ticks advantageous due to the shorter analysis time compared to their hard tick counterparts. [25]

In contrast to ixodid ticks, which only go through one nymphal stage, argasid ticks usually go through two to eight nymphal stages before molting into adults. The temperature and the size of the blood meal control the development. Except for certain larvae of the *Ornithodoros moubata* complex, six-legged larvae undergo a genuine metamorphosis after hatching from the eggs following embryogenesis. An eight-legged nymph is produced via larval shedding, and it goes through multiple nymphal instars before molting into an adult. In a matter of weeks, each nymphal developmental stage is finished.

The process of molting into the subsequent nymphal stage is contingent upon receiving a bloodmeal. Soft ticks mate off the host, unlike hard ticks, and after each bloodmeal, the mated adult females lay numerous egg batches (up to 500 eggs). Unlike adult ixodids, which perish after laying eggs, they are able to feed and reproduce several times during their lifetime. Additionally, because they can retain their sperm, females can oviposit without having previously copulated. [26,27]

Argasid ticks are commonly found in arid regions with high temperatures and tropical or temperate climates. The leathery cuticle, a distinctive feature of soft ticks, serves as an adaptation to survive adverse conditions by minimizing water loss. Despite their resilience to challenging environments, these ticks inhabit sheltered locations such as caves, woodland crevices, walls, burrows, and soil surfaces beneath rocks covered with sand or dust.

Most Argasid species are nidicolous, preferring hosts that intermittently visit nesting areas for brief periods. This behavior allows them to endure extended periods of starvation, thereby extending their life cycle over several years. Additionally, soft ticks exhibit a multi-host life cycle. Infection is acquired when feeding on a host already carrying *Borrelia*. Within a few hours of tick attachment, spirochetes migrate and establish infection in the midgut and salivary glands. The subsequent transmission of pathogens occurs during a blood meal on another host. [28,29]

2.3.1 Ornithodoros moubata

The presence of the bacterium *B. duttonii* closely aligns with the geographical distribution of its vector, the soft tick known as *O. moubata*. This motility is crucial to its ability to spread inside the host while evading its immune system. The tick predominantly inhabits Sub-Saharan Africa, specifically regions such as Tanzania, Uganda, Kenya, and Sudan.[11]

In addition to being the primary vector for the spirochete *B. duttonii,* the central focus of this thesis, this tick species is also implicated in the transmission of the African swine fever virus to pigs and the West Nile virus. Pigs, humans, rodents, and birds serve as the primary hosts during the transmission of these diseases. [26,30]

2.4 Importance of Antigenic Proteins in Diagnosis

Antigenic proteins serve as valuable diagnostic markers in the field of medicine. Their specificity, ability to trigger antibody production, and application in various diagnostic assays make them indispensable tools for detecting and monitoring infectious diseases. Despite the clinical significance of Relapsing Fever caused by *Borrelia* species, there is a notable absence of well-defined diagnostic markers. In contrast, Lyme disease caused by *B. burgdorferi* benefits from a more comprehensive set of diagnostic markers. DbpA, known as Decorin binding protein A, plays a crucial role in binding to host tissue, recognized by the immune system during infection. Several laboratories have demonstrated that DbpA serves as a protective antigen, showing efficacy in preventing experimental *B. burgdorferi* infection in mouse models of Lyme borreliosis.[13] Also, during transmission to humans, *B. burgdorferi* expresses Outer surface

protein C (OspC), a highly immunogenic major antigen on its surface during early infection, commonly targeted in diagnostic assays.[14]

These and numerous other well-established markers contribute to the accurate diagnosis of Lyme disease, enabling clinicians to effectively identify and manage cases.

2.4.1 Human Complement Plasminogen A

Human Complement Plasminogen A (*hcpA*) is a versatile surface lipoprotein with the capability to bind to human complement regulators and host protease plasminogen. It has previously been explored in the context of *B. recurrentis* but has not been extensively studied in other tickborne relapsing fever *Borrelia* species. This is the primary motivation behind our investigation. Immunofluorescence analysis of *B. recurrentis* was conducted to precisely determine the localization of *hcpA* on the cell surface, complemented by corresponding differential interference contrast images. To elaborate, also the impact of Proteinase K and trypsin treatment on the surface expression of native *hcpA* was explored.[15]

2.4.2 Lactate Dehydrogenase

Lactate dehydrogenase (LD) is characterized as an authentic intracellular enzyme of the anaerobic metabolic pathway, primarily owing to its remarkable tissue specificity. In typical conditions, tissue concentrations of LD surpass serum levels by approximately 500-fold. It is typically localized on the surface of various cells.[16, 17]

Intriguingly, LD has been identified as an antigenic marker associated with *Plasmodium falciparum malaria* in prior research. This unique characteristic prompts exploration into LD's potential as a diagnostic marker, offering an innovative avenue for further investigation, even though its origins lie in a parasite rather than bacteria. [16]

3 Aims

This bachelor’s project focuses on cloning and expressing two selected proteins and analyzing their diagnostic potential.

4 Materials and Methods

The primary focus of this project centers on the identification, cloning, and expression of two selected proteins with a specific emphasis on evaluating their diagnostic potential. The following methodology outlines the key steps undertaken:

4.1 Identification

4.1.1 Polymerase Chain Reaction (PCR)

First, PCR was employed to amplify the target gene using gene-specific primers, including LD, *hcpA*, and *Vlp.* These primers were designed by Dr. Rego

Three different DNA samples (Table 1) were tested to determine the most suitable for amplification.

For all PCR reactions, the master mix was prepared using the OneTaq® Hot Start Quick-Load® 2x Master Mix with Standard Buffer from New England Biolabs®. The specific composition is provided in Table 2. The cycle involving denaturation, annealing, and elongation was repeated 30 times for all PCR programs, the temperature program details are listed in Table 3.

Table 1: used DNAs

A	1120K3
B	Ly
C	1120K3 gDNA

Table 2: amounts of Master Mix for each reaction

Master Mix	Amount [µl]
One Taq HS Quick-Load 2x MM	10
Milli-Q H2O	7
Primer F+R	1
DNA	2
Total	20

Table 3: PCR settings

PCR Setting			
#	PCR Step	T [°C]	t [sec]
1	Initial Denaturation	95	30
2c	Denaturation in cycle	94	30
3c	Annealing in cycle	55	45
4c	Elongation in cycle	72	60
5	Final Elongation	68	600
6	Hold	16	∞

Table 4: used primers and their sequences. The highlighted sequences represent the restriction enzyme sites used for cloning into the expression vector.

hcpA (forward)	CCT**CTCGAG**ATGGTAATGAGGTTA
hcpA (reverse)	ACA**AAGCTT**CTATGAAAGATAATTCATTACTC
Vlp (forward)	CCT**CTCGAG**ATGAATAAAGAGAAAAAAGGAG
Vlp (reverse)	ACA**AAGCTT**TTAATTAGCTGCTTTTGG
LD (forward)	CCT**CTCGAG**ATGGAGATGGTTATGCT
LD (reverse)	ACA**AAGCTT**TTAAAATTCTATTTTATCAAG

4.2 Cloning

The initial step involved a cloning reaction using the TOPO_TA vector using the TOPO TA Cloning® Kit, pCR®2.1-TOPO® Vector (Thermo Fischer Scientific), followed by the transfer of the construct into DH5α *E. coli* cells. Subsequently, a DNA isolation using NucleoSpin® Plasmid (Macherey-Nagel) and a restriction digestion reaction was performed using the EcoRI enzyme and the NE 2.1 buffer (New England BioLabs®).

4.2.1 Clone Reaction

The DNA topoisomerase enzyme possesses the ability to cleave and rejoin DNA at specific sequences. In the TOPO TA cloning process, the vector ends with T overhangs are coupled with the TOPO enzyme. Through a stable connection between the vector and insert ends, facilitated by AT base-pairing, the topoisomerase enzyme effectively seals the junctions.[18]

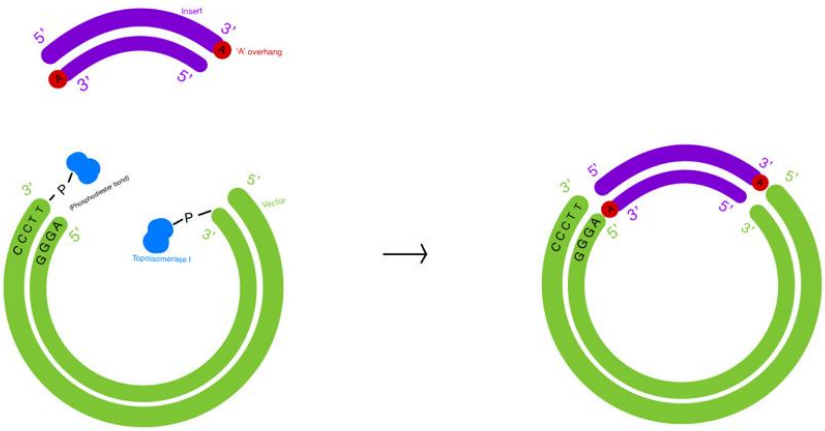

Figure 3: attachment of vector and insert with TOPO enzyme

The cloning reaction was initiated by combining the TOPO TA vector, the salt solution included in the kit, and the PCR products LD_C and $hcpA_C$. The precise quantities of these components can be found in Table 5. Subsequently, the mixture was incubated at room temperature for a duration of five minutes.

Table 1: composition's volumes of the clone reaction

Cloning reaction	Amount [µl]
TOPO TA vector	0.5
Salt solution	0.5
PCR product LD_C / $hcpA_C$	2

4.2.2 Transformation

Following the cloning step, the sample was chilled on ice alongside the *E. coli* tube. For the transformation process, 1.5 µl of the cloning reaction was combined with 25 µl of *E. coli* cells, and this mixture was left on ice for 30 minutes. Afterward, the sample underwent a heat shock by submerging the tube in 42°C water for 30 seconds. Once the heat shock was completed, the sample was returned to ice immediately for two minutes. To this *E. coli* mixture, 250 µl of SOC Outgrowth Medium (New England BioLabs®) was added, and it was placed in the horizontal shaker for 1 hour and 30 minutes. During this time, two carbenicillin plates for each PCR product were warmed in the incubator (37°C) for 1 hour. Subsequently, 40 µl of X-Gal (Thermo Scientific ™) was evenly spread on each plate and allowed to sit for 15 minutes. Following this, 100 µl and 150 µl of *E. coli* cells were applied to the plates and left to incubate overnight in the incubator. The following day, colonies displaying blue and white colors were observed, with the white ones indicating the presence of recombinant DNA.

4.2.2.1 Inoculation

Five pure white colonies were delicately collected using the tip of a pipette and transferred into 15ml falcon tubes containing 5ml of Lysogeny Broth (LB) medium. The specific composition of the LB medium can be found in Table 6. Subsequently, these tubes were placed in a horizontal shaker at 37°C and left to grow overnight.

Table 2: composition of 100ml LB medium. To adjust pH to 7.0 NaOH was used

100ml LB medium	Amount
Tryptone	1.0g
Yeast extract	0.5g
NaCl	1.0g
Distilled water	100ml

4.2.3 DNA Isolation

The following day, the tubes were centrifuged to separate the contents. The supernatant was removed, and the pellet was fully resuspended by adding 250 µl of A1 buffer from the Nucleospin® plasmid QuickPure Kit (Macherey-Nagel). The resulting mixture was then transferred into a 1.5ml Eppendorf tube. To this mixture, 250 µl of A2 buffer were added, and the tube was gently inverted a few times for thorough mixing. Subsequently, it was left to incubate for five minutes at room temperature.

Following the incubation, 300 µl of buffer A3 was introduced, and the solution was mixed until it became colorless. This mixture was then transferred into a NucleoSpin® tube and centrifuged for one minute at 11000 RPM. The flowthrough was discarded.

To wash the silica membrane, 500 µl of the buffer AW was added and centrifuged for one minute. This step was repeated using 600 µl of A4 buffer, and once again, the supernatant was disposed of.

To dry the silica membrane completely, the tubes were centrifuged for 1 minute at 11000 RPM, and any remaining flowthrough was discarded. The NucleoSpin® column was carefully placed into a 1.5ml Eppendorf tube, and 50 µl of Buffer AE were added for DNA elution. After a one-minute incubation at room temperature, it was centrifuged for one more minute at 13000 RPM.

4.2.4 Restriction Digestion Reaction and Sequencing

To create samples for the restriction reaction, 4 µl of the isolated DNA were mixed with 16 µl of a specialized reaction mix, as outlined in Table 7. These prepared samples were then placed at 37°C for one hour.

Following the incubation, about 2 µl of 6x DNA Loading Dye (Thermo Scientific™) was introduced into the samples. Subsequently, the samples were loaded onto an agarose gel for analysis.

Table 3: composition of reaction mixture per sample

Reaction mix	Amount [µl]
NE buffer 2.1	2
EcoRI	0.5
Milli-Q H2O	13.5

Table 4: composition of agarose gel

Agarose gel	amount
Agarose	2g
TAE (Tris-acetate-EDTA) buffer	200ml
GelRed® Nucleic Acid Stain (Biotium)	5 µl

The procedure was conducted once more with *hcpA*, this time using a different annealing temperature Table 9. In inoculation step 2.5 µl of 100mg/ml carbenicillin was additionally added to the LB media.

Table 5: PCR settings for repeated process of hcpA

PCR Setting			
#	PCR Step	T [°C]	t [sec]
1	Initial Denaturation	95	30
2c	Denaturation in cycle	94	30
3c	Annealing in cycle	46.9	45
4c	Elongation in cycle	72	60
5	Final Elongation	68	600
6	Hold	16	∞

The resulting constructs (TOPO_LD1, TOPO_LD2 and TOPO_*hcpA1*) were then sent for sequencing and the sequences were scrutinized by aligning them with other sequences available in the NCBI database. The analysis revealed that the experiment should proceed with TOPO_LD1 and TOPO_*hcpA1*.

4.2.5 Digestion and Ligation of the insert with expressing vector pBAD

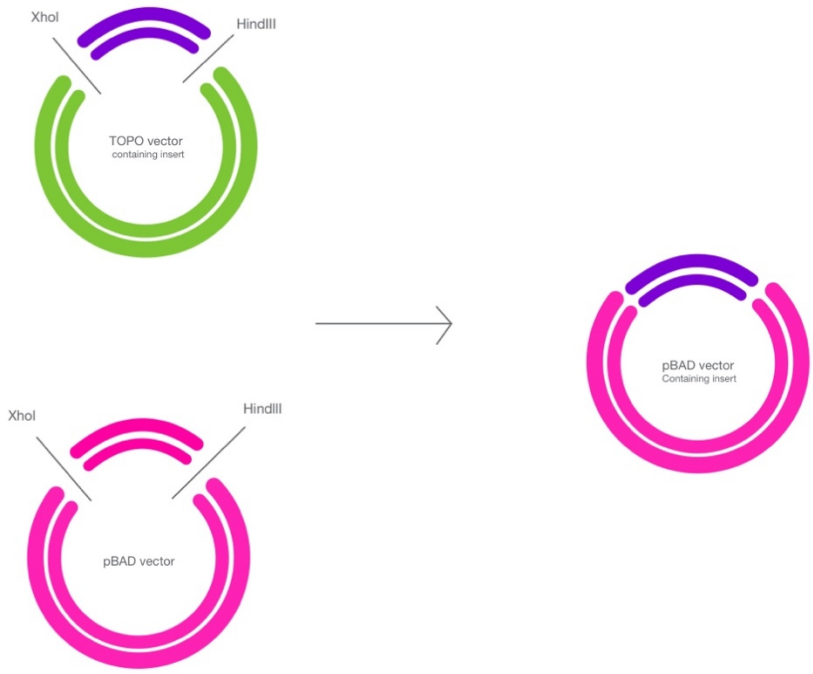

Figure 4: the DNA containing insert from the TOPO vector and the pBAD vector were cut using restriction enzymes XhoI and HindIII and subsequently were ligated together.

4.2.5.1 Restriction Digestion Reaction

This time XhoI and HindIII enzymes, along with the Cut Smart® buffer (New England BioLabs®) were employed (Table 10). Following the Master Mix preparation, 15 µl of the mix was combined with each insert and the vector. The specific quantities used are provided in Table 11.

Table 6: volumes of used reagents

1x Master Mix	Amount [µl]
Cut Smart	3.5
XhoI	1
HindIII	1
Milli-Q H2O	10

Table 7: specific amounts of inserts and vector

TOPO_LD1	222.9 ng/ µl
TOPO_*hcpA*1	307.1 ng/ µl
pBAD	260.7 ng/ µl

Once the samples were prepared, they underwent a three-hour incubation at 37°C. Following this incubation, they were loaded onto a 0.8% agarose gel, and subsequently, both the pBAD plasmid band and the insert's band of interest were excised.

4.2.5.2 Gel extraction

The gel extraction of LD1 insert was accomplished utilizing QIAquick Gel Extraction Kit (QiagenTM).

Despite numerous attempts, the isolation of the *hcpA* insert DNA using the same kit repeatedly failed. Therefore, a decision was made to switch methodologies. Consequently, the extraction of the *hcpA* insert was successfully performed using the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel), which proved more effective for this particular application.

4.2.5.2.1 DNA Extraction of LD

The gel extraction protocol was executed as follows: Initially, the gel fragments were accurately weighed. Subsequently, three volumes of Buffer QG were added to each volume of the gel. The mixture was incubated at 50°C until complete gel dissolution. Following this, one volume of isopropanol was introduced and thoroughly mixed. The resulting sample was then transferred into the provided spin column and subjected to centrifugation for one minute at 13 000 RPM. The flowthrough was carefully discarded.

Next, 500 µl of Buffer QG was applied, and another round of centrifugation was performed for one minute, after which the flowthrough was again discarded. Subsequently, 750 µl of Buffer PE was added and allowed to incubate for three minutes, followed by another one-minute centrifugation step. Once more, the flowthrough was discarded. The column was then placed into a 1.5ml Eppendorf tube. Finally, 15 µl of Milli-Q water was added, and the solution was incubated for three minutes before undergoing an additional one-minute centrifugation step.

4.2.5.2.2 DNA Extraction of *hcpA*

In this step, 200 µl of NTI buffer were introduced to the gel fragments and incubated for 5-10 minutes at 50°C, ensuring complete gel dissolution. Subsequently, 700 µl of the prepared sample was transferred to the column and centrifuged for 30 seconds at 13,000 RPM, with the flowthrough being discarded. Following this, 700 µl of NT3 buffer were added, and the column was centrifuged for 30 seconds, discarding the flowthrough once more. The column was then centrifuged for one additional minute and subsequently placed on a heating block for five minutes at 70°C.

Finally, the column was carefully positioned into a 1.5ml Eppendorf tube, and 15 µl of Milli-Q water were added. The solution was allowed to incubate for three minutes at room temperature and then underwent another one-minute centrifugation.

4.2.5.3 Ligation

The ligation process involved incorporating the insert into the pBAD vector, employing TA DNA ligase along with the 10x Buffer for TA DNA ligase with 10mM ATP (New England BioLabs®). The exact amounts used can be referenced in Table 12. Subsequently, the mixture was incubated for a duration of 16 hours at 16°C.

Table 8: specific quantities of the used reagents

	Vector: insert (1:1) [µl]	Vector: insert (1:2) [µl]
Insert LD/*hcpA*	2	4
Vector pBAD	2	2
10x TA DNA ligase buffer	1	1
TA DNA ligase	1	1

The next step in this phase involved transferring the construct into Subcloning Efficiency™ DH5α™ competent cells (Invitrogen™) utilizing the identical transformation procedure as described in section 4.2.2.

4.2.5.4 Colony PCR, Digestion and PCR

Six pure white colonies were gently picked from the carbenicillin plates using the tip of a pipette and were streaked on the bottom of a 0.2ml Eppendorf tube. Colony PCR was then carried out following the same procedure as a standard PCR, as described in section 4.1.1.

The used tips were placed in a 15ml Falcon tube containing 2ml LB media with 2 µl of 100mg/ml carbenicillin and were incubated in a horizontal shaker overnight.

The next day, 1ml of the mixture from the Falcon tube was transferred into another 15ml Falcon tube containing 5ml LB medium mixed with 5 µl 100mg/ml carbenicillin and incubated for 5 hours with continuous shaking. From the remaining solution of the 2ml LB medium, 500 µl were transferred into 2ml Eppendorf tubes as a backup. In a separate 2ml Eppendorf tube, 1ml of the 5ml LB medium solution was mixed with 300 µl of glycerol and stored in the freezer for future experiments. The remaining solution was isolated, and subsequently used for a restriction digestion reaction and a PCR to verify the presence of the insert.

4.3 Expression and Purification

4.3.1 Expression

Continuing with the cloning process, the pBAD plasmid containing the gene of interest was introduced into BL21 cells for expression by transformation (section 4.2.2). Colonies were selected and subjected to a colony PCR for amplification, followed by a digestion with the same restriction enzymes and buffer as in section 4.2.5.1 to confirm the presence of the insert.

4.3.1.1 Regulation of Conditions

The subsequent steps involve optimizing conditions for inducible promoters, including adjustments to temperature and pH to optimize expression parameters. The objective is to cultivate cell cultures under suitable conditions that allow for the growth and multiplication of the cells, ultimately increasing protein production. Hence, the glycerol stock of BL21 cells harboring the LD1cl5_pBAD plasmid was used to inoculate 5 mL of LB medium supplemented with 5 µL of 100 mg/mL carbenicillin. This culture was incubated at 37°C overnight with continuous shaking. The following day, 3 mL of the overnight culture was transferred to 10 mL of LB medium along with 10 µL of 100 mg/mL carbenicillin. This subculture was allowed to grow for 2-16 hours until the OD600 measurement reached 0.6-0.8.

Subsequently, 1 ml of the culture was harvested and subjected to centrifugation for cell collection. Following this, 20 µL of 4 M urea and 20 µL of SDS-PAGE sample buffer were introduced. This solution was labeled as "BI" (Before Induction).

Next, four 1 mL aliquots of the culture were drawn, and each milliliter was combined with a specified quantity of L-arabinose (20%, 1.3 M stock), as detailed in Table 13.

Table 9:specific quantity of L-arabinose for each concentration

concentration L-arabinose [%]	Amount of L-arabinose [µl]
0.02	10
0.25	125

Following this, the tubes were incubated for three hours at 37°C, with the same OD600 adjustment as in the case of sample BI. After centrifugation, the cells were resuspended in 20 µL of 4 M urea and 20 µL of SDS-PAGE sample buffer. These prepared mixtures were subsequently labeled as "AI" (After Induction).

The remainder of the culture was centrifuged, and the collected cells were transferred into a 2ml Eppendorf tube. These cells were suspended in 300 µl of a solution containing 20mM Tris, 0.3M NaCl, and 1mM BME, with particular attention given to adjusting the pH to 7.9. The solution was then subjected to sonication for 1-2 minutes at 45 amplitude using 0.5 seconds pulse on/off cycles, with intermittent waiting periods of five minutes.

After sonication, 100 µl of the sonicated mixture was centrifuged for ten minutes at 16,000 RPM. The resulting supernatant was collected and labeled as "SF" (soluble fraction). The pellet obtained after centrifugation was washed with a solution of 20mM Tris, 0.3M NaCl, and 1mM BME, and then centrifuged again.

The cells in the pellet were suspended with 50 µl of 4M urea and 50 µl of 0.1% SDS, resulting in the "IF" (insoluble fraction). Finally, 20 µl of SF and IF were mixed with 20 µl of SDS-loading buffer.

SDS-PAGE (Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis) gels for the Western blot and Coomassie Brilliant Blue Stain were prepared using the TGX™ and TGX Stain-Free™ FastCast™ Acrylamide Kit (Bio-Rad). These gels were made using on 0.75 mm glass plates, containing specific quantities of reagents, listed in Table 14. All the prepared samples, along with a positive control, were loaded onto two gels.

Table 10: reagents and volumes for preparation of each gel

	12% separation gel [µl]	5% stacking gel [µl]
30% AA (acrylamide)	2000	330
4x separation buffer	1250	-
4x stacking buffer	-	500
TCE (trichloroethylene)	25	10
water	1670	1120
TEMED (tetramethylethylenediamine)	2.5	3
10% APS	50	30

Subsequently, SDS-PAGE gel electrophoresis was carried out at 120V for approximately 1 hour. Following that, the blotting arrangement was assembled as followed: blotting paper, nitrocellulose membrane, gel, and another layer of blotting paper. The nitrocellulose membrane was pre-soaked in a 1:10 dilution of 25 mM Tris-base and 192 mM glycine in distilled water

(1x blotting buffer) for 5 minutes before being utilized. The blotting paper was also briefly soaked with the same buffer. The transfer was conducted at 25 V for a duration of 30 minutes. To block the membrane, a 5% blocking solution was formulated by dissolving 10 g of powdered milk in 200 ml of 1x TBS Tween 20. Following the transfer process, the membrane was extracted from the setup and immersed in the blocking solution for 2 hours while placed on a shaking platform. Simultaneously, primary antibody solutions were prepared by combining 5 ml blocking solution with 1.2 µl THE™ His Tag Antibody [HRP], mAb, Mouse (R&D Systems®). After blocking, the solution was discarded, and the prepared primary antibody mixture was poured over the membrane and incubated in the fridge overnight. The next day, the membrane underwent a triple wash with 1x TBS Tween 20, with each wash lasting 15 minutes.

In the final washing step, the preparation of Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific™) involved the mixing 1 ml of Detection Reagent 1 Peroxide Solution and 1 ml of Detection Reagent 2 Luminol Enhancer Solution (Thermo Scientific™). Subsequently, the membrane was immersed in this substrate solution for a 5-minute incubation. Finally, the membrane was arranged on a transparency sheet, and the outcomes were observed using the Chemi-Doc system (Bio-Rad).

Following the blotting step, a Coomassie Brilliant Blue (CCB) staining procedure was conducted as an additional process.

Therefore, the gel was rinsed once with distilled water, and then the water was removed. Subsequently, CBB stain (Coomassie Blue, Bio-Rad) was applied to cover the gel, and it was gently agitated for 2 hours.

After discarding the stain, distilled water was added to the gel, and it was gently agitated using a small piece of clean white paper towel. This process was continued until the background of the gel reached an acceptable level of destaining.

5 Results

5.1 Identification

5.1.1 Polymerase Chain Reaction

The PCR phase of my project aimed to amplify specific target genes, initially encompassing LD, *hcpA*, and Vlp. Although *Vlp* reached the sequencing stage, the results guided us to concentrate exclusively on the LD and *hcpA* genes. I conducted numerous PCR reactions using gene-specific primers and identified optimal DNA samples for amplification, thus facilitating subsequent cloning and expression experiments.

5.1.1.1 Target Gene Amplification

Following multiple PCR experiments, the LD and *hcpA* target genes were successfully amplified. The amplified products were of the expected size, further confirming their presence in our DNA samples. Among the various DNA samples tested, the genomic DNA sample C extracted from the 1120K3 strain proved to be the most suitable for the amplification of both LD and *hcpA*, owing to its high purity and concentration.

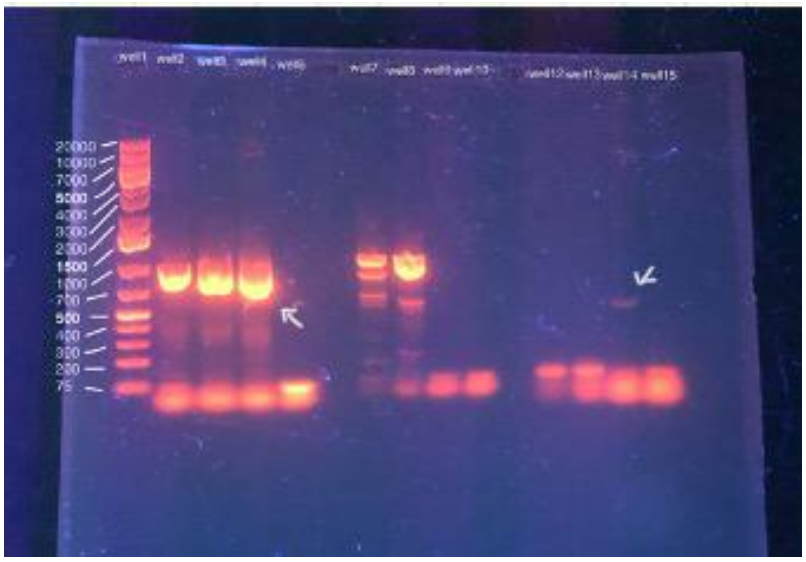

Figure 5: band visualization of loaded samples.
well1- protein ladder, well2- LD(A), well3- LD(B), well4- LD(C), well5-negative control;
well7- Vlp(A), well8- Vlp(B), well9- Vlp(C), well10- negative control;
well12- hcpA(A), well13- hcpA(B), well14- hcpA(C), well15- negative control;

5.2 Cloning

The cloning phase was pivotal in our research, focusing on transferring the amplified LD and *hcpA* genes into the TOPO_TA vector for subsequent analyses.

5.2.1 DNA Isolation

The DNA was successfully isolated from the pure white colonies that resulted from the cloning and transformation processes for further analyses.

5.2.2 Restriction Digestion Reaction and Sequencing

To verify the presence and fidelity of our target genes within the TOPO_TA vector, we subjected the purified DNA samples to a restriction digestion reaction using appropriate enzymes and buffers. Since only colony 1 and colony 2 for LD were discernible on the following electrophoresis (Figure 6), the whole process was repeated with *hcpA* again using a different annealing temperature, and during inoculation, an additional 2.5 µl of 100mg/ml carbenicillin was added to the LB media (Figure 7).

Following the digestion, we conducted DNA sequencing to analyze the sequences. This phase identified the most promising constructs, notably TOPO_LD1 and TOPO_*hcpA1*, for further investigations.

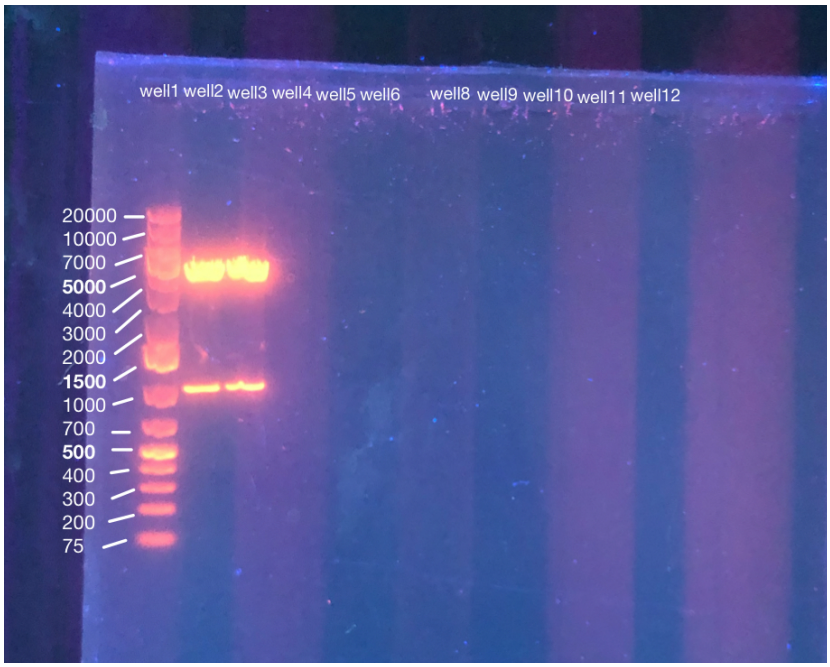

Figure 6: visualization of samples after restriction digestion reaction. well1- protein ladder, well2- LD1, well3- LD2, well4- LD3, well5-LD4, well6- LD5; well8- hcpA1, well9- hcpA2, well10- hcpA3, well11- hcpA4, well12- hcpA5

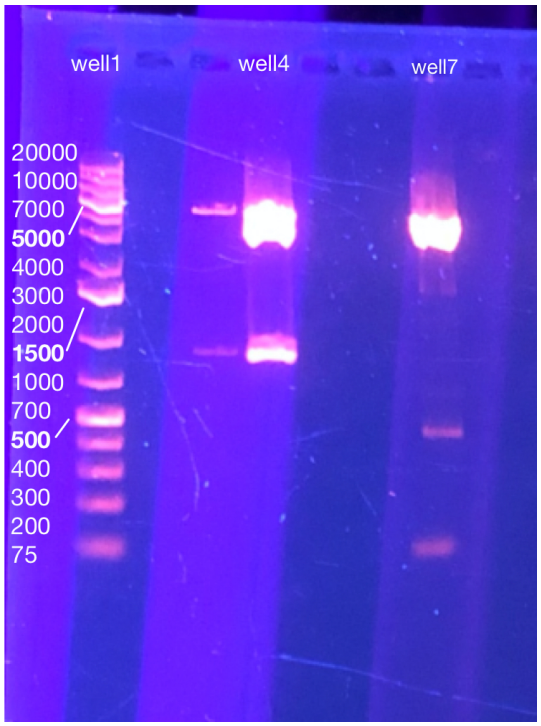

Figure 7: visualization of hcpA after changing annealing temperature.
well1- protein ladder, well4- hcpA1, well7- not relevant for this project

5.2.3 Digestion and Ligation of the insert with expression vector pBAD

After the ligation process, no colonies for *hcpA* were visible on the selection medium. Consequently, the experiment proceeded exclusively with the LD gene from this point onward.

5.2.4 Cloning into BL21 cells

Following the introduction of the pBAD plasmid with the LD insert into competent BL21 cells, it was observed that among the selected colonies, only LD1cl5_pBAD_DH5α harbored the desired insert, which is visualized in Figure 8, 9 and 10.

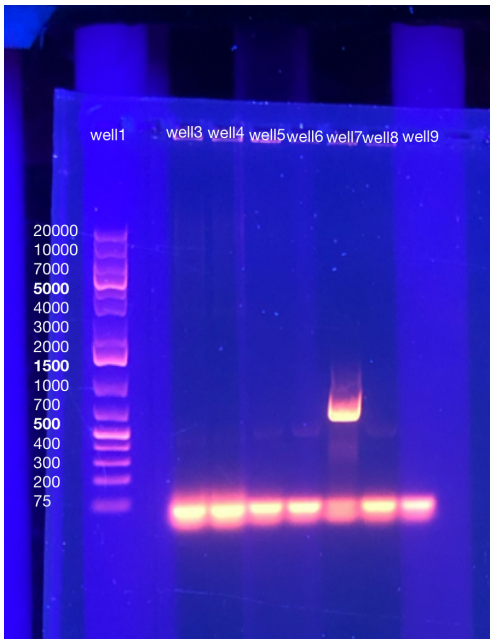

Figure 8: colony PCR. well1- protein ladder, well3- LD1cl1, well4- LD1cl2, well5- LD1cl3, well6- LD1cl4, well7- LD1cl5, well8- LD1cl6, well9- negative control;

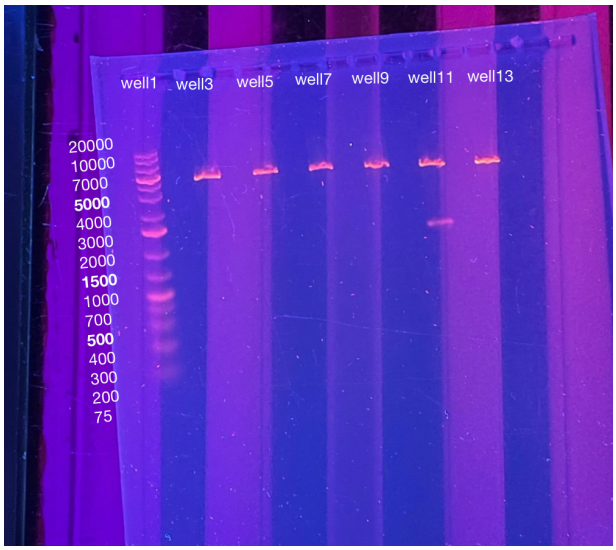

Figure 9: visualization after digestion. well1- protein ladder, well3- LD1cl1, well5- LD1cl2, well7- LD1cl3, well9- LD1cl4, well11- LD1cl5, well13- LD1cl6;

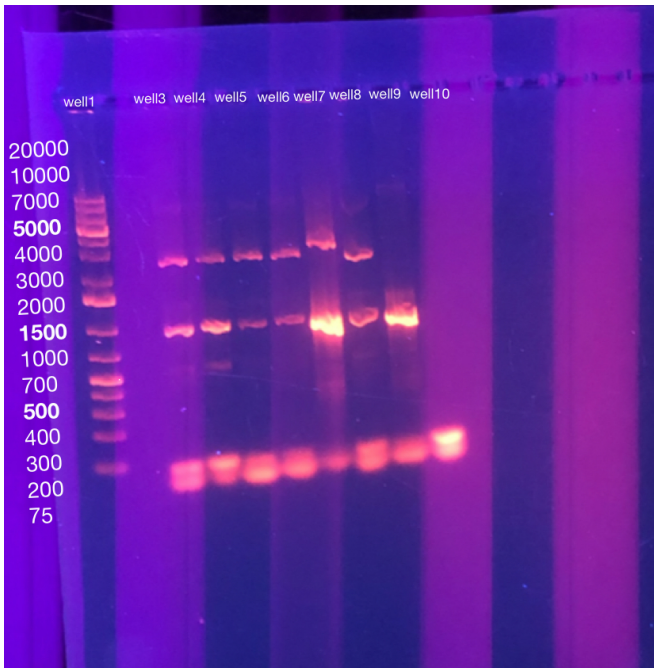

Figure 10: visualization PCR. well1- protein ladder, well3- LD1cl1, well4- LD1cl2, well5- LD1cl3, well6- LD1cl4, well7- LD1cl5, well8- LD1cl6, well9- positive control, well10- negative control

5.3 Expression and Purification

The expression and purification phase focused on producing the target protein, LD, for diagnostic analysis.

5.3.1 Expression of LD

During the expression trials, we observed clear bands on the CBB-stained gel in both the BI and AI samples.

In the samples representing the IF and SF, no distinct bands were detectable. The absence of bands in the IF suggests that the target protein did not form significant amounts of inclusion bodies, which are typically indicative of misfolded or aggregated proteins. Similarly, the absence of bands in the SF points to a lack of detectable levels of the soluble target protein in the lysate.

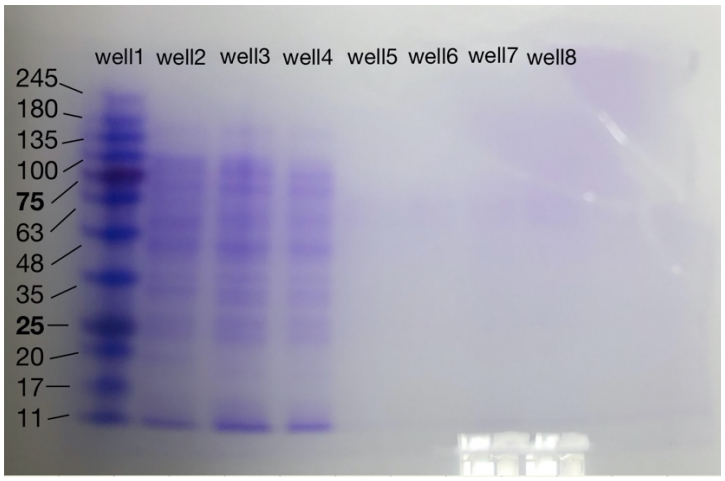

Figure 11: CBB stain visualization- expression of proteins in E. coli at different concentrations of L-arabinose. Two different concentrations: A-0,02%- 10µl L-arabinose, B- 0,25%- 125µl L-arabinose. well1- protein ladder, well2- BI, well3- AI (A), well4- AI (B), well5- IF (A), well6- IF (B), well7- SF (A), well8- SF (B)

Despite careful observation of the outcomes on the membrane, no visible bands were detected, indicating potential issues in the protein expression that may require further investigation.

Given the absence of successful *hcpA* cloning and the lack of bands for LD expression, no further steps in the cloning, expression, and purification of these proteins were undertaken.

6 Discussion

The preceding sections have detailed the methodological processes and outcomes of this study, which was aimed at identifying and characterizing potential diagnostic markers for *B. duttonii* infection. The results of our investigation underscore the complexity and challenges inherent in developing diagnostic tools for infectious diseases such as relapsing fever. Below, we explore the implications of these results, the challenges encountered, and propose future directions based on our findings.

6.1 Implications of Results

The core objectives of this study were to identify and characterize diagnostic markers, specifically the *hcpA* and LD proteins, for *B. duttonii* infection. Significant steps have been made towards these goals:

6.1.1 Challenges in *hcpA*

Our attempts to characterize *hcpA* encountered significant challenges, which underscore the complexities typically faced in the early stages of cloning.

6.1.2 Successful cloning of LD and *hcpA* gene

The successful cloning of the LD gene underscores the potential of molecular cloning techniques in developing diagnostic tools.

Notably, the cloning of the *hcpA* gene represents a significant milestone, as this is the first time an *hcpA* sequence from a relapsing fever species of the Old World has been cloned. Previously, available sequences were only of *B. recurrentis*, associated with louse-borne relapsing fever (LBRF). This advancement expands our genetic understanding of relapsing fever *Borrelia* and enhances the potential for developing region-specific diagnostic assays.

6.2 Comparison with BipA and GlpQ Studies

In prior research, the cloning and expression of BipA and GlpQ have been instrumental in developing specific diagnostic tools and therapeutic targets. For instance, studies on BipA in *B. hermsii* have demonstrated its role in immune evasion and interaction with host tissues, leading to the development of specific inhibitors that could potentially reduce bacterial virulence.[7,8] Similarly, GlpQ has been used as a biomarker in diagnostic assays due to its unique presence in *Borrelia* species that cause relapsing fever, differentiating them from Lyme disease-causing *Borrelia.*[10]

6.3 Application to *B. duttonii*

Adapting these approaches for *B. duttonii* could involve investigating the specific functions and interactions of the LD and *hcpA* proteins within the host-pathogen dynamics. Given the genetic distinctions and ecological niches of *B. duttonii*, understanding these proteins' roles could lead to novel insights into the pathogenesis of Old-World relapsing fever and enhance diagnostic accuracy. For example, the expression profiles of *hcpA* under different environmental stresses or host conditions could reveal how *B. duttonii* adapts and persists in endemic regions. Additionally, comparing the protein structures of *hcpA* from different *Borrelia* species might identify conserved regions that are potential targets for broad-spectrum diagnostics or therapies.

6.4 Future Directions

The results of this research set the stage for future investigations into diagnostic markers for *B. duttonii* infection. Several promising avenues for further research can be considered:

6.4.1 Exploring Alternative Expression Systems

Considering alternative expression systems that might provide better environments for protein folding and stability, such as yeast or mammalian cell systems, could potentially increase the solubility and stability of LD. Exploring various concentrations of L-arabinose may also prove beneficial.

7 Conclusion

This study embarked on the exploration and development of diagnostic markers for *Borrelia duttonii* infection, focusing specifically on the cloning and expression of the LD and *hcpA* genes. Our research highlights the inherent complexities and challenges encountered in the molecular diagnostics of infectious diseases like relapsing fever.

The PCR and cloning phases demonstrated that while we could successfully amplify and clone the LD gene, the *hcpA* gene presented significant challenges. These included repeated failures in extraction and cloning, which were not resolved even after methodological adjustments. This highlights the unpredictable nature of working with molecular techniques and the need for adaptable experimental strategies.

In particular, the successful cloning of the LD gene represents a significant achievement and illustrates the potential of molecular cloning techniques to advance the development of diagnostic tools. However, the subsequent expression trials revealed difficulties in protein detection, which were evident from the lack of visible bands in both the insoluble and soluble fractions. This suggests issues with protein solubility or stability, or possibly with the detection methods themselves.

The results of this study set a foundation for further research, specifically recommending the exploration of alternative expression systems. Systems such as yeast or mammalian cell cultures could potentially offer more favorable conditions for protein folding and stability, thereby increasing the likelihood of successful protein expression. Additionally, refining detection methods and exploring different concentrations of L-arabinose could improve the visibility and quantifiability of target proteins.

In conclusion, while this research faced several significant challenges, it also laid important groundwork for future investigations. These efforts are crucial for advancing our understanding of *Borrelia* diagnostics and for the eventual development of effective tools to combat this persistent and problematic disease.

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