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A proteomic study for serum biomarkers for variations of two genotypes of Anaplasma phagocytophilum in infected lambs

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Abstract

The work in this thesis was collaboration between the Norwegian School of Veterinary Science (NSVS), IRIS (International Research Institute of Stavanger) and the University of Stavanger. NSVS inoculated to genotypes of the bacteria *Anaplasma phagocytophilum* in four different lambs, two with variant 1 and two with variant 2. The request from NSVS was to find possible biomarkers for the disease "Sjodogg" caused by *A. phagocytophilum* transmitted by the tick *Ixodes ricinus*. The samples received from NSVS were selected from peaks in the infection cycle based on rickettsemia of *A. phagocytophilum* in the blood of lambs.

In the process of finding biomarkers several methods were attempted to find protein peaks in the SELDI mass spectra. It turned out to be a challenge working with serum samples. The only protein peaks present in the mass spectra was Albumin. To remove Albumin various methods were attempted including; Precipitation with Acetone, HPLC with HiTrap Blue column, LC with SAX and SCX columns and ProteinChip® serum fractionation kit. The HiTrap Blue column separated the sample into one "protein fraction" and one "albumin fraction" (Chapter 4.1). The "protein fraction" from the experiment on HPLC was run on LC with SAX and SCX in an attempt to fractionate the residual proteins. The results were satisfying, but they yielded poor spectra on SELDI analysis. Finally a kit from BioRad; ProteinChip® serum fractionation kit was attempted. The results from the following SELDI analysis was good, hence this was our method of choice.

The spectra, more than 5000 in all, were treated and analysed in Ciphergen Software to find biomarkers for infected lambs. Variant differences was unveiled using PRIMER 6. Four up-regulated and one unique (not present in controls or pre inoculated lambs) peak were found as possible biomarkers for the infected lambs. This might indicate both immuneresponse from the lamb and presence of extracellular protein from the bacteria. PRIMER 6 indicated that variant 1 and 2 were able to separate from each other by peak intentions in the spectra from Ciphergen ProteinChip® Software.

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Abbreviations

Aa	Amioacids
DNA	Deoxi
EAM	Energy Absorbing Molecules
ExPASy	Expert Protein Analysis System
FTIR	Fourier Transform Ion Resonance
HM	High mass
HPLC	High Performance Liquid Chromatography
kDa	kilo Dalton
LC	Liquid Chromatography
LM	Low Mass
NAD+	Nicotinmide Adenine Dinucleotide (oxidized form)
NADP+	Nicotinamide Adenine Dinucleotide phosphate (oxidized form)
NCBI	National Center for Biotechnology Information
MALDI	Matrix-Assisted Laser Desorption/Ionization
MDS	MulitiDimensional Scaling
MMDB	Molecular Modeling DataBase
MS	Mass Spectra
MW	Moleculat Weight
m/z	mass-to-charge ratio
PCR	Polymerase Chain Reaction
PCA	Principal components analysis
PDB	Protein DataBank
pH	*pondus Hydrogenii* or *potentia Hydrogenii*
PRIMER	Plymouth Routines In Multivariate Ecological Research
PSGL-1	P-selectine glycoprotein ligand-1
RP	Reverse Phase
rRNA	ribosomal Ribo Nucleic Acid
SAX	Strong Anion eXchange
SCX	Strong Cation eXchange
SELDI	Surface-Enhanced Laser Desorption/Ionization
TBE	Tick-Borne Encephalitis
TBF	Tick-Borne Fever
TOF	Time Of Flight

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1 Introduction

The aim of this study was to investigate if it was possible to find biomarkers for the disease Sjodogg, and variations between to genotypes of the disease using SELDI-TOF-MS. Sjodogg is an Anaplasmosis in farm animals such as sheep, goats, cattle and in ruminants like; deer, elk and roe deer, and even in human, transmitted by the tick *Ixodes ricinus* (Taxonomy id: 34613) (Stuen, Engvall et al. 1998; Stuen, Bråten et al. 2008). Serum samples were received from The Norwegian School of Veterinary Science (NSVS) (Table 1.1). After sample collection at NSVS the samples were left in room temperature to coagulate. Thereafter they were spun to precipitate blood cells and fibrinogen and the supernatant were frozen at -75 °C(Stuen, Bråten et al. 2008).

Several variants of *Anaplasma phagocytophilum* (Taxonomy id; *Anaplasma*: 768, *A. phagocytophilum*: 948) have been found in Norwegian mammals by sequencing of the 5' part of the 16S rRNA gene of the organism. Five of them have been identified in sheep. It has been established that these genetic variants may interact in infected hosts. The lambs used in this thesis were infected with two different 16S rRNA variants of *A. phagocytophilum*. They were followed up for three months for monitoring of the occurrence of variants(Stuen, Bråten et al. 2008).

Table 1.1: Overview of samples received from The Norwegian School of Veterinary Science. The serum volume for all samples were 0.5 ml

Lamb number	Sampling date	Variant
4203	14.09.04 (pre-inoculation) 13.10.04 09.11.04	Variant 1
4210	14.09.04 (pre-inoculation) 29.09.04 13.10.04 09.11.04	Variant 1
4202	14.09.04	Variant 1
4249	14.09.04	Variant 1
5019	14.09.05 (pre-inoculation) 28.09.05 12.10.05 09.11.05	Variant 2
5029	14.09.05 (pre-inoculation) 28.09.05 12.10.05	Variant 2
5041	14.09.05	Variant 2
5076	14.09.05	Variant 2

Eight lambs from the bread; Norwegian White sheep, six months old were used for the experiments(Stuen, Bråten et al. 2008). One experiment took place in 2004 with variant 1, and one in 2005 with variant 2. The lambs were tested negative for *Mycoplasma ovis* (*Taxonomy ID:* 171632) in advance(Granquist, Stuen et al. 2007), they had not previously been on tick pasture, and were kept indoor without any chance of contact with ticks during the 13 week experiment period(Granquist, Stuen et al. 2007; Stuen, Bråten et al. 2008). Two 16S rRNA variants of *A. phagocytophilum* were used as inoculum, variant 1 (GenBank accession number M73220) and variant 2 (AF336220). Two lambs were infected with approximately 2.0 x 10^6 neutrophils containing variant 1 and two lambs with variant 2 in the same way. Temperature and clinical variables were recorded. Sera were collected once a week from the lambs and analysed for antibodies to *A. phagocytophilum*. Presence of *A. phagocytophilum* was also established using PCR analysis(Stuen, Engvall et al. 1998; Stuen, Bråten et al. 2008).

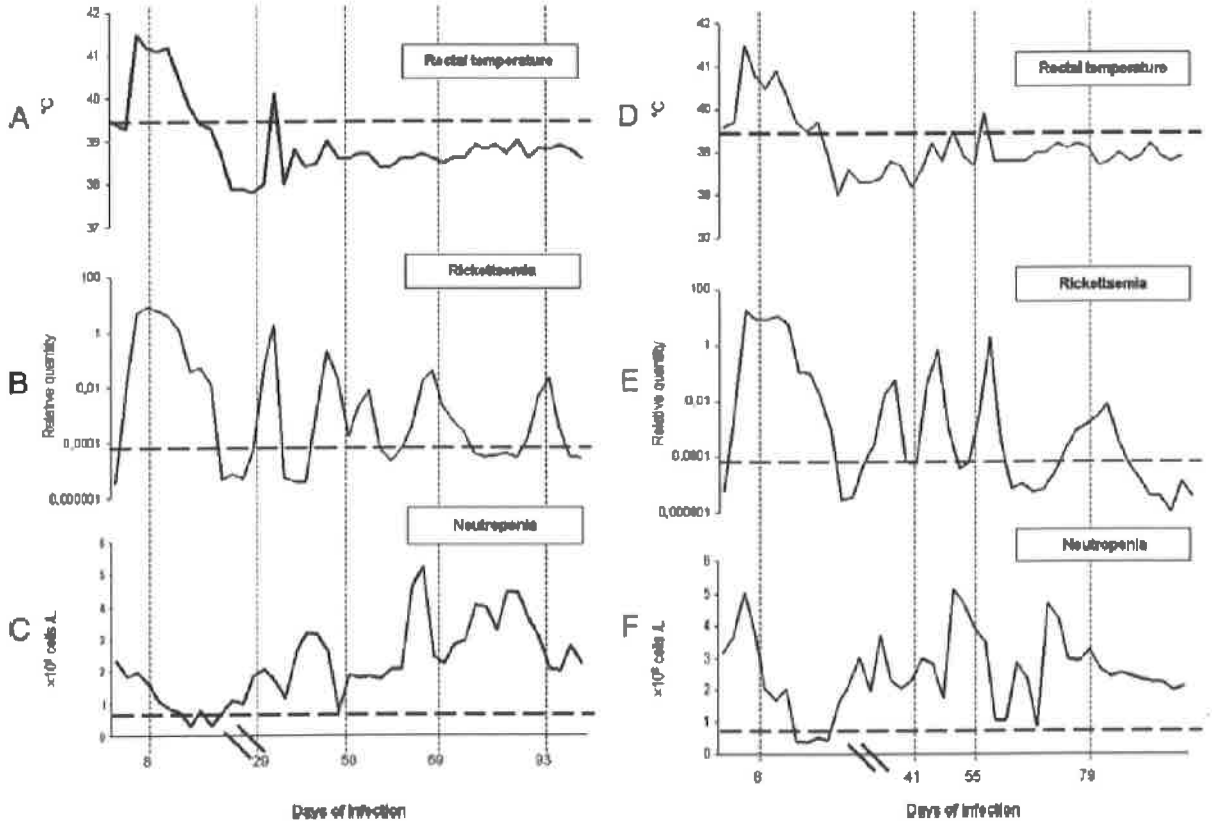


Figure 1.1: Clinical parameters of *Anaplasma phagocytophilum* infected lambs, 4203 and 4210 from A to F. A, B and C represent lamb 4203 and D, E and F represent lamb 4210. B and E represent rickettsemia in lambs, presented as relative quantitative data on a logarithmic graph. Horizontal dotted lines represent body temperature (°C), positive threshold level (C_1) for rickettsemia and value (< 0.7 x 10^9 cells/liter) below significant neutropenia recorded, respectively for A and D, B and E, and C and F. Vertical dotted lines represent sampling times in days after infection. Hash marks on the x-axis indicate a scale change.

The graph in Figure 1.1 is from the experiment in 2004, variant 1. For variant 2; see figure 4.17 in 4.4 Discussion.

Blood samples were collected into EDTA every second day for a period of 3 months. These samples were analyzed on PCR (amplification of DNA), and established rickettsia. The samples in this project were chosen from the peaks in spectra for Rickettsia (Figure 1B and E) made from PCR analysis results.

The lambs showed signs of inappetence and depression the first two days after infection. The fever started at day 3 and lasted for about 6 days and the highest temperature was measured to 41.8 °C (Figure 1A and D). Neutropenia was observed from day 13 after infection at lasted for about 10 days (Figure 1C and F)(Granquist, Stuen et al. 2007; Stuen, Bråten et al. 2008). Neutropenia is an abnormally low number of neutrophils in the blood. The neutrophils are the largest part of the defense of the body against acute bacterial and some fungal infections. 45 to 75 % of the leucocytes in the blood stream are neutrophils(Beers, Porter et al. 2003).

The EDTA-blood samples were analyzed by real-time PCR and showed a cyclic pattern. The rickettsemia lasted for several days. In between the positive peaks there were negative samples ($C_{p,}$ >35). Both of the lambs, 4203 and 4210 showed five rickettsemic peaks each (Fig. 1B and E). Samples were collected from time points corresponding to these peaks(Granquist, Stuen et al. 2007).

During the first 90 days after inoculation the two lambs infected with variant 1 had four and five relapses respectively. Variant 2 had one or two relapses of *bacteriaemia*. The reasons of variations are unknown; it might be difference in growth rate of the pathogenic bacteria, immunogenicity and antigenic variation could interact between host and microbe. Variant 1 seems to be the most widespread and patogenetic variant. The results from this experiment determined that different variants of *A. phagocytophilum* cycles differently in individual lambs(Granquist, Stuen et al. 2007).

To work with serum samples is a challenge because of the high abundance of Albumin and IgGs (Immunoglobulins); more than 60 % of the total amount of proteins in serum (Chen, Lin et al. 2004). Therefore it was necessary to deplete them from the serum prior to analysis on SELDI-TOF-MS. SELDI-TOF-MS is a proteomic method for analyzing peptides, proteins and other molecules in very small sample volumes. The technique relies on time-of-flight mass spectrometry (TOF-MS) for accurate measurement of mass-to-charge ratio (m/z) of peptides and proteins. The samples can, according to BioRad, be added directly to spots on the protein chips without any need for removal of salt or detergents. The SELDI reader is capable of running up to 192 samples at the time. This is making this method a time-saving and efficient way to treat the samples(Bio-Rad Laboratories 2007).

2 Theory

2.1 Ticks

The phylum *Arthropoda* (*Taxonomy ID:* 6656) includes the classes: insects, arachnids and crustaceans. Ticks belong to the class *Aracnida* (*Taxonomy ID:* 6656) and the subclass *Acari* (*Taxonomy ID:* 6933). Approximately 850 species of ticks are known, subdivided into two major families; *Argasidae* (Taxonomy id: 6936) and *Ixodidae* (Taxonomy id: 6939), respectively "softbacked" and "hardbacked" ticks. The "softbacked" ticks have a flexible leathery cuticle, while the "hardbacked" ticks have a sclerotized dorsal scutal plate. Morphology, habitat and lifecycle differ between the two families. The soft ticks (argasids) take several meals within short duration, while the hard ticks (ixodids) take one large meal during each instar(Sonenshine 1991). The ixodids are considered to be one of the most important endoparasites (organisms which are living on the surface of a host) in addition to their ability to transfer zoonotic infections (infections that cause disease in both human and animals).

2.1.1 Classification

The ixodids is classified as prostriate or metastriate based on morphology and variations in life cycle; like the ability of some prostriate males to undergo spermatogenesis and to mate without having a prior blood meal(Gray 2003). The prostriate ticks have inherited arrangement of the mt (Metallothionein) genes of the arthropods (Arthropods are animals belonging to the Phylum Arthropoda), while the metastriate ticks has an arrangement of eight genes and copies of controllregions(Shao, Aoki et al. 2004). Most of the important ixodids are metastriate, but the single pro-striate genus *Ixodes* includes species that in the latter part of the 20th century are notorious as the vector for several zoonotic diseases in the northern hemisphere. For instance Lyme borreliosis (LB), ehrlichiosis, babesiosis and tick-borne encephalitis (TBE)(Gray 2003).

2.1.2 *Ixodes ricinus*

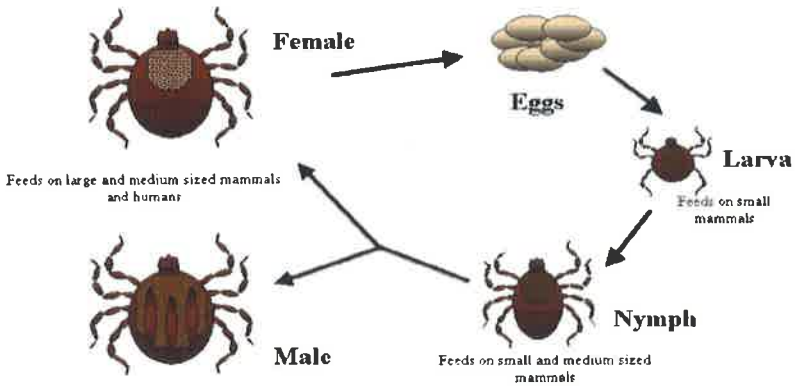


Figure 2.1: Lifecycle of a three host tick(Hunt 2008).

Figure 2.2: *Ixodes ricinus* waiting for passing host(Krásenský 2007).

Twelve species of ticks are known in Norway according to reports from animals(Mehl, Sandven et al. 1987), but only *I. ricinus* (figure 2.2) is important as a vector for tick-borne diseases in this country and in Europe. The ticks have four stages of development: egg, larval, nymph and adult (Figure 2.1). The total life cycle takes around three years, approximately one year for each stage (larvae, nymphs and adults). All ticks feed on blood from vertebrates. After a blood meal on a host the female tick drops to the ground and lays her eggs, between 2000 and 3000. Later the eggs hatch to larvae. Larvae feed on small mammals or birds but are also capable of feeding on bigger animals. After blood feeding for two to four days, the larval drops to the ground and develop into a nymph. Next year the nymph tries to find a small or medium sized vertebrate. After this blood meal it drops to ground to develop into adult stage, female or male. Adult females feed for seven to ten days. Usually it is only the female tick who feeds on hosts, while the male tick feeds infrequently. The main task for the male tick is to mate feeding females(Sonenshine 1991).

I. ricinus has been known for hundreds of years in Norway. The tick has several local geographical names; flått, hantikk, lyngbobb, påte, skaumann, skogflått and sugar(Stuen 2003). *I. ricinus* is found along the coastal areas of southern Norway and north to the contry of Nordland(Mehl, Sandven et al. 1987). However, during the last 10 years *I. ricinus* has been found further north in both Norway and Sweden(Lindgren, Talleklint et al. 2000)

Huge number of ticks may cause serve blood loss and anaemia, especially in young animals. However, the main challenge with ticks, for instance *I. ricinus*, is the pathogens it may transmit to animals and humans, such as arboviruses, *Babesia* (*Taxonomy ID:* 5864), *Borrelia*(*Taxonomy ID:* 138), *Ehrlichia* (*Taxonomy ID:* 943) and *Rickettsia* (*Taxonomy ID:* 780)(Stuen 2003). Totally, 11 tick-borne bacterial pathogens have been described in Europe during the last 20 years(Parola and Raoult 2001).

2.1.3 Effects on the host

On the host the tick slits the skin with the scalper-like mouth parts and inserts a barbed proboscis. Cement, seceded by salivary glands, collaborated with the proboscis anchors the tick to the host. The tick remains on the host for several days. The duration depends on the stage of development. During this time the gut and cuticle grows to adjust to the enormous amount of blood from the meal. The tick does not pierce blood vessels but secrets vasoactive mediators and immunomodulators. The vasoactive mediators effect the size of the vessels and immunomodulators has an effect on the immune system that keeps the blood flowing and reduces capability of resistance against the process. During the meal the blood is concentrated by extraction of water and secreted back into the host by special salivary gland cells. This is an important tool in transfusion of tick-borne pathogens. When the tick is fully engorged it drops to the ground where it digests the blood meal and develops to the next instar. The digestive process includes pinocytosis (microphagocytosis) and endocytosis of blood components by cells lining the gut. The process is followed by intracellular enzymatic digestion. Lack of digestion enzymes in the tick gut favours the microorganisms and may explain why ticks transfers a wider spectre of microorganisms than any other arthropods(Gray 2003).

2.1.4 Tick-borne infections in Norway

Tick-borne diseases in animals have been registered along the south-western coast of Norway for more than a century. Briefly, the first tick-borne disease diagnosed in Norway was babesiosis in cattle in 1901(Stuen 2003). In 1939, Tick-borne fever (TBF) or "sjodogg" as it is called in Norway, was diagnosed the first time in sheep(Thorshaug 1940). The name "sjodogg" was mentioned in the literature as a disease in ruminants in Hardanger in already in 1780(Stuen 2003). One explanation for the name "sjodogg" (sjo = sea, dogg = moisture) is the observation that sheep became ill on pastures near the sea, and that they may have had a lot of moisture in their wool(Stuen 1997).

The most common and widespread tick-born disease in domestic ruminants in Norway is TBF. In 1995, more than 11,000 sheep flocks were treated prophylactically against the disease (Norwegian Animal Disease Report 1995). It has been estimated that more than 300,000 lambs are infected each year with *Anaplasma phagocytophilum* (the microorganism causing the disease transmitted by the tick)(Stuen and Bergstrom 2001). For a historical review of tick-borne diseases see table 2.1.

Table 2.1: A historical review of tick-borne infections in Norway(Stuen 2003).

Disease	Year of first description/variation
Sjodogg (TBF)	1780
Babesiosis in cattle	1901
TBF in sheep	1939
Borreliosis in humans	1955
TBF In cattle	1962
Louping-ill in goats	1978
TBF in goats	1979
Louping-ill in sheep	1982
Borrelia infection in sheep*	1988
Borrelis / *A. phagocytophilum* infection in dogs*	1996
Tick-borne encephalitis (TBE) in humans	1997
A. phagocytophilum infection in humans	1998
A. phagocytophilum infection in horses	1999
A. phagocytophilum infection in moose / roe deer	1999
A. phagocytophilum infection in red deer	2000

* Only seropositive samples

2.2 Anaplasma phagocytophilum

2.2.1 Classification

Microorganisms in the genus *Ehlichia* (established in 1945 to honour the German bacteriologist Paul Ehrlich (1845-1915)) of the family *Rickettsiaceae*(Taxonomy id: 775) are Gram-negative, small (0.2-1.5 μm), obligate intracellular organisms that infect principally monocytes and granulocytes(Rikihisa 1991). *A. phagocytophilum* gets inserted by the host cell in separate phagosomes. Phagosomes are membrane combined vacuoles inside the cytoplasm(Stuen 2003). The bacterium has a genome consisting of 1.47 million base pairs with 1411 annotated features(Nelson, Herron et al. 2008).

Table 2.2 New classification of *genus Anaplasma, Ehrlichia* and *Neorickettsia* in the Family *Anaplasmataceae* (*Taxonomy ID:* 942)(Dumler, Barbet et al. 2001).

	Genus		
	Anaplasma	***Ehrlichia***	***Neorickettsia***
Species	*A. marginale*	*E. canis*	*N. risticii*
	A. bovis	*E. chaffeensis*	*N. sennetsu*
	A. phagocytophilum	*E. ewingii*	
	A. platys	*E. muris*	
		E. ruminantium	

Genus *Ehrlichia* was divided into three genogroups as determined by 16S rRNA sequencing similarities, such as *E. phagocytophila*, *E. equi* and the agent causing human granulocytic ehrlichiosis (HGE). These three agents are very closely related based on the 16S rRNA gene sequences(Rikihisa 1996). Recently, a reclassification of the genus *Ehrlichia* was proposed, and based on phylogenetic studies, the granulocytic *Ehrlichia* has now been renamed *Anaplasma phagocytophilum*(Dumler, Barbet et al. 2001).(Table 2.2).

2.2.2 Characteristics

A. phagocytophilum principally invades leucocytes, which are in charge of protecting the immune system(Bjålie, Haug et al. 1998; Granick, Reneer et al. 2008). An *A.phagocytophilum* infection causes moderate to considerably trombocytophenia, which means reduction in number of trombocytes in the blood plasma(Bjålie, Haug et al. 1998; Granick, Reneer et al. 2008). With lack of trombocytes the blood has difficulties coagulate. In leucocytes the bacterium uses glycolysed ligands, usually Sialic acid and primary P-selectine glycoprotein ligand-1, PSGL-1, to enter the host cell. PLGS-1 gets expressed in a great number of haematopoietic cells, including megacaryocytes(Granick, Reneer et al. 2008).

A. phagocytophilum is a fragile bacterium that causes distinctively challenge for culture, isolation, enumeration and labelling. *A. phagocytophilum* has a cytotoxic effect on HL60 cells (Human promyelocytic leukemia cells) and induces cell lysis and apoptosis if they become too infected. Routine methods of bacterial enumeration are not possible because of their small size and intracellular location. Use of top vented cell-culture flasks is recommended for the growth of these bacteria, and this causes a risk for secondary bacterial contamination. Membrane active agents cannot be used because they are deleterious to *Ehrlichial* viability. The fact that the organism is fragile requires gentle sonication. Foaming of media induces cell lysis. Extracellular organisms rapidly become inactivated. Completely removal of the host cell by DNAase or trypsin has not yet succeeded(Borjesson 2007; Borjesson 2008).

Intensive research has demonstrated interactions with signal transduction of host cells and possible eukaryotic transcription. This interaction causes permutation of leucocytes functions and can permit immunopathological changes, serious illness and opportunistic (infections that causes disease in mammals with diminished immune system). Further studies is necessary to define the immunology and pathogen mechanism, and to understand why some people develop serious illness and why some animals become long-term permissive reservoir hosts for the organism(Dumler, Choi et al. 2005).

2.2.3 Transmission

Transmission of *A. phagocytophilum* from vector to host occurs generally 24 hours after the tick attaches. The bacteria survive through the moulting process of *I. ricinus*, i.e. transstadial transmission occurs. However, transovarial transmission of *A. phagocytophilum* has not yet been recorded. The possibility of co-feeding transmission between different stages of *I. ricinus* has to be elucidated(Stuen 2003).

2.2.4 Prevalence

The prevalence of *A. phagocytophilum* infection in *I. ricinus* in Europe varies from area to area and between stages of development of the tick. The prevalence in nymphs is usually higher than in the adult stage, and has been found to vary from 0.25 – 25%. The high infection prevalence in nymphs combined with higher density and wider feeding preferences in nymphs than adults, makes nymphs the predominant source of exposure for humans, livestock and wild deer(Walker, Alberdi et al. 2001).

In Norway, TBF is diagnosed mainly along the south, southwest and west coast of southern Norway. The northernmost case of TBF found so far is Brønnøysund in Nordland(Stuen, Oppegaard et al. 2005).

2.2.5 Variants of *A. phagocytophilum*

Different 16S rRNA and msp4 genotypes of *A. phagocytophilum* have been reported in sheep and red deer in Norway(Ladbury, Stuen et al. 2008).

Biological and ecological differences clearly exist between variants of *A. phagocytophilum*, including varying vectors, DNA sequence, geographical distribution and host pathogenicity(Massung, Mauel et al. 2002).

2.2.6 Clinical symptoms

Sjodogg is normally a benign disease in domestic and wild ruminants. The most characteristic symptoms of TBF in domestic ruminants are high fever, inclusions in neutrophils and severe neutropenia(Foggie 1951; Stuen 2003). Neutropenia has been observed from day 13 after infection and this lasts for about 10 days. Neutrophile cells protect the body against infection, and this number will decrease as the fever descends and will continue descending for a couple of weeks. This impairs the lambs immune system, and the lamb is more susceptible for other infections, ewes may miscarry and rams may get temporary sterile. Infected animals will typically have an enlarged spleen(Stuen, Engvall et al. 1998).

Sheep exposed to infected ticks develop clinical signs 4-14 days. The clinical disease is characterized by sudden rise in rectal temperature (often over 41°C). The fever may last for one to two weeks(Foggie 1951; Stuen 2003). The fever reaction may vary according to the age of the animals, the variant of *A. phagocytophilum* used, the host species and immunological status of the host(Stuen 2003). In sheep, other clinical signs are often absent or mild(Øverås, Lund et al. 1993; Stuen 1996). The injury from the tick bite itself is almost slight, even if some lambs may lose blood and get anaemic if they are highly infected. It is the transfer of microorganisms that causes infection with sheep on pasture(Stuen, Engvall et al. 1998). TBF in sheep is often followed by complications such as tick pyaemia and *Pasteurella* (*Taxonomy ID:* 745)(*Mannheimia* (*Taxonomy ID:* 75984)) septicaemia. The most important aspect of the infection is its implication as a predisposing factor for other infections(Stuen 2003), like septicaemia (blood poisoning) and arthritis. Bacteria that cause this kind of diseases are *Pasteurella* and *Staphylococcus*(*Taxonomy ID:* 1279)(Stuen, Engvall et al. 1998).

It should be mentioned that clinical manifestations in humans range from a mild self-limited febrile illness, to a life-threatening infection. On average, patients develop a nonspecific influenza-like illness(Bakken, Dumler et al. 1994). However, most human infections probably result in minimal or no clinical manifestations(Bakken, Haller et al. 2002).

2.2.7 Practical problems

Sjodogg may cause great losses for farmers. In certain areas a third of the livestock has been lost because of the disease and the complications it brings out. Diminished production and smaller lambs leads to losses for the farmer as well. It has been proven several strains of *A. phagocytophilum*. One livestock or even one lamb may be infected with several strains. The different strains seem to cause different symptoms. One infected cell is enough to cause infection, and the symptoms do not depend on dose. The infection is able to remain in the flock through one season to another, and from one pasture to another(Stuen, Engvall et al. 1998).

2.2.8 Diagnosis

A blood smear is easily made because about 90 per cent of the neutrophile cells will be infected. In the acute phase, organisms are visible in the leucocytes in a blood smear(Stuen, Engvall et al. 1998).

Microscopy of blood smears taken in the fever period is normally sufficient to confirm the diagnosis. Stained with May-Grünwald Giemsa, the organisms appear as blue cytoplasmatic inclusions in monocytes and granular leucocytes, especially neutrophils.

PCR techniques for the identification of *A. phagocytophilum* infection in blood samples has been established(Massung, Owens et al. 2000).

2.2.9 Prophylaxis and treatment

No vaccines for protection against either *I. ricinus* or *A. phagocytophilum* are available for human or veterinary use. In endemic areas, regular dipping or pour-on treatment with synthetic pyrethroids against ticks may be necessary(Brodie, Holmes et al. 1986). In the treatment of *A. phagocytophilum* infected man or animals, the drug of choice is tetracycline (Woldehiwet and Scott 1993; Dumler 1996).

2.3 Proteins

2.3.1 Peptides and proteins

The word protein came from the Greek *proteios*, meaning first. They are a class of organic compounds present in and vital to every living cell. Proteins come in forms of enzymes, hormones, antibodies and globulins among others, all with different functions like catalysis and regulation to protect the biological organism. Hemoglobin, myoglobin and various lipoproteins effect oxygen transportation within an organism. Generally proteins are regarded as beneficial and necessary for all animals. But some proteins possess harmful properties, they are called toxins. Proteins are classified as a single chemical family because of their molecular structure which is sufficiently similar. Compared to carbohydrates and lipids, proteins are different in fundamental composition. Lipids are mostly hydrocarbon in nature, consisting of 75-80% carbon. Carbohydrates consists of 50% oxygen, and like lipids, less than 5% nitrogen, or none nitrogen at all. Proteins and peptides are composed of 15-25% nitrogen and about an equal amount of oxygen. Peptides are small proteins with a molecular weight less than 10 000.

Proteins and peptides are chains of amino acid units formed when amine and carboxylic functional groups shape amide bonds together(Reusch 2004). The same 20 amino acids are used as building blocks for the assembly of all proteins in every living organism. Variation in amino acid order and the number of amino acids per protein allow for an almost limitless variety of protein molecules. Proteins are translated from mRNA which is transcribed from genes embedded in the DNA of an organism. Hence the amino acid sequence of a protein is dictated from the nucleotide sequence of a gene(Horton, Moran et al. 1996).

2.3.2 Structures

Proteins and peptides are biopolymers that in general are amino acid residues interlinked by amide bonds. There are four levels of protein structures; primary, secondary, tertiary and quaternary structure.

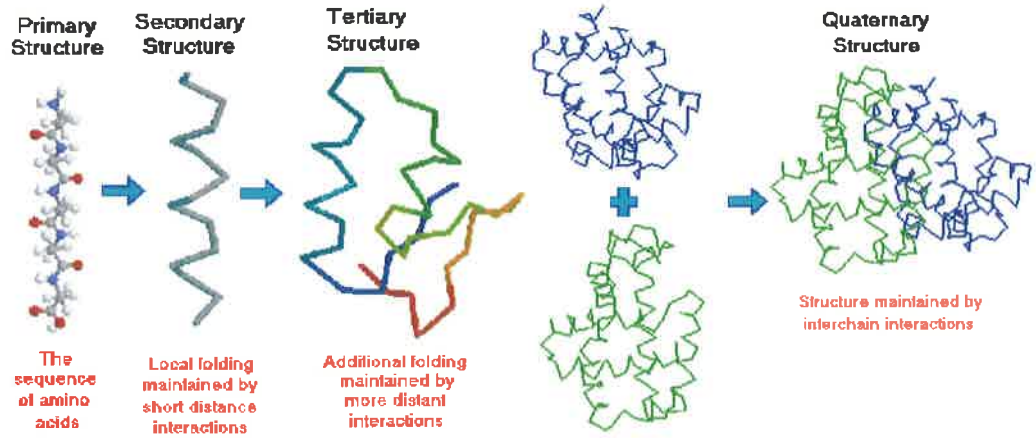


Figure 2.3: Proteinstructures; primary, secondary, tertiary and quaternary structure. Showing the sequence, local folding, additional folding and interchain interactions respectively.

Primary structure: The amino acid sequence of the protein.

Secondary structure: Local conformations within the protein, examples are alpha-helix and beta sheet.

Tertiary structure: Global conformations within the protein, examples are helix-loop-helix and beta-barrels.

Quaternary structure: Describes the interactions between different domains

Proteins exhibit several physical and biological properties. Two general categories of simple proteins are commonly recognized; Fibrous proteins and globular proteins(Wampler 1996).

2.3.3 Fibrous proteins

Fibrous proteins has fibre like structures, hence the name, and serve as head structural material in various tissues. These structures are relatively insoluble in water and are not affected by moderate changes in temperature and pH. Subgroups of Fibrous proteins are; Collagens (figure 2.4) and elastins (connective tissues, tendons and ligaments), keratins (major component of skin, hair, feathers and horn) and fibrin (formed when blood coagulates)(Reusch 2004).

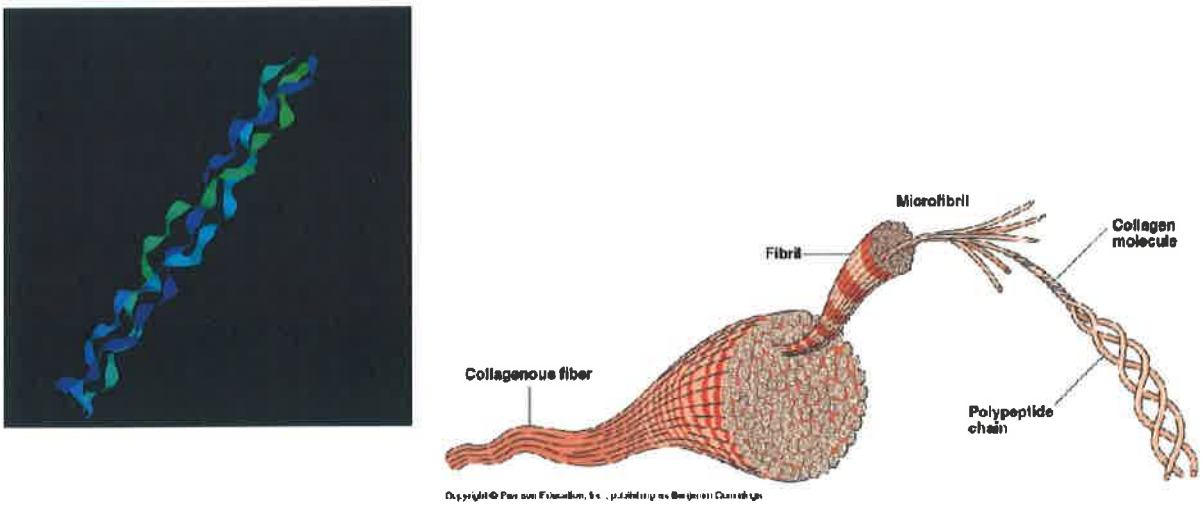


Figure 2.4: Left; Three helixes in the fibrous protein collagen. Right; Collagenous fiber, fibril, microfibril, collagen molecule and polypeptide chain (Bella, Eaton et al. 2003; Dr. Mallery 2008)

2.3.4 Globular proteins

Globular proteins serve regulatory, maintenance and catalytic roles in living organisms and include hormones, antibodies and enzymes. They dissolve or form colloidal suspensions in water. Proteins in this class are generally more sensitive to temperature and pH change than fibrous proteins. Example: hemoglobin (figure 2.5)(Reusch 2004).

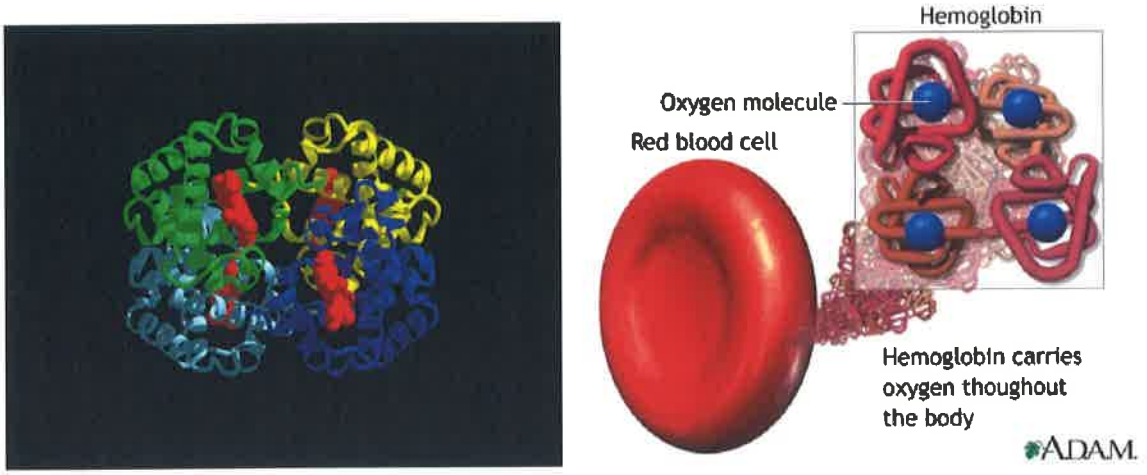


Figure 2.5: Left; Hemoglobin protein with the four domains colored green, blue, light blue and yellow. The heme groups are colored red. Right; Red blood cell consisting of hemoglobin inter alia(Palmer 1994; Commission 2007).

2.3.5 Proteinfolding

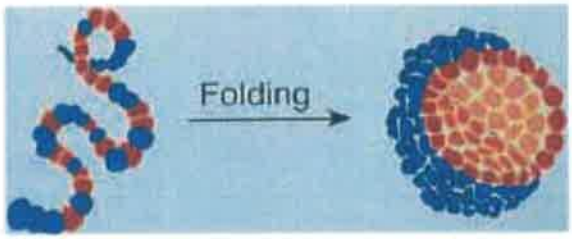


Figure 2.6: The folding of a protein. The pearls illustrate the amino acids(Chan and Dill 1993).

Some proteins only functions when folded as shown in Figure 2.6, e.g. hemoglobin which transports oxygen in the blood. Not only does the protein need to fold, it has to fold in a certain way. The function of a protein often depends on amino acids located far from each other making contact when folded. This folding process might takes seconds or minutes.

The process of folding is controlled by an interaction between electrical energy among the amino acids, the ambient water and random collisions between the water molecules and the protein. The temperature, which determines the velocity distribution of the water molecules, is also an important factor.

The electrical energy and dynamics in the system is well understood. Nevertheless it is practically impossible for computers today to manage all the amino acids and collision with water molecules. Therefore physicists are working on simplified models for protein folding, which have given new understanding. Still there is a long way to go before we can estimate which folded structure a protein will turn in to, or to specify which amino acid sequence that is needed to end in a certain, desired folded structure(Einevoll).

2.3.6 What we know about protein folding

The native conformation of a protein is the most stable shape possible under normal conditions(Horton, Moran et al. 1996).

The forces contributing to protein folding are non-covalent interactions; van der Waals interactions, charge to charge interactions, the hydrophobic effect, disulfide bridges and hydrogen bonding(Horton, Moran et al. 1996).

There are more than 50,000 known structures from X-ray crystallography and 2D NMR studies today. Structure data base is available for analysis in protein data bank (PDB – 54,559 structures) and in Molecular Modelling Database (MMDB – NCBI > 40,000 structures)(NCBI 2008; PDB 2008).

The structure of folded proteins is stable and the folding possibilities are quite limited. Motifs of folding within a protein are used repetitively and similar function typically has similar structure. Structure similarity tends to be more conserved than the exact sequence of amino acids with similar proteins(Wampler 1996).

2.4 Proteomics

Proteomics is the study of the proteome using large-scale protein separation and identification techniques. The proteome is the whole set of proteins produced by a species. The term proteomics was formed in 1994 by Marc Wilkins defining it as "the study of proteins, how they're modified, when and where they're expressed, how they're involved in metabolic pathways and how they interact with one another"(Wiley and Sons 2008).

The complete sequence of many genomes, including human, which was identified in 2003, is now known(Patterson and Aebersold 2003; ExPASy 2008). Still, the understanding of probably half a million human proteins encoded by less than 30 000 genes is not complete. The complexity of biological systems is still a mystery. The concept called proteome (PROTEin complement to a genOME) has emerged and should help to understand biochemical and physiological mechanisms of complex multivariate diseases at the functional molecular level. Proteomics has been initiated to complement physical genomic research. Definition of proteomics could be *the qualitative and quantitative comparison of proteomes under different conditions to further unravel biological processes*(ExPASy 2008).

As the human genome was identified in 2003 everyone expected a revolution in molecular biology. This revolution was postponed, even with all the human genes sequenced. This can be attributed to the fact that the genes are coding for proteins that often are multifunctional and that they are parts of metabolic pathways. A gene is also able to code for more than one protein. Depending on physical and biochemical parameters it can occur various modifications from gene to transcript, and from transcript to protein (i.e. mRNA editing, splicing etc.). A mature protein may also modify in many other ways (post-translational modifications) that affects its functionality. This will naturally interfere with its pathway in different ways.

The word proteomics was born in the early 90'ies and was supposed to be complementary to genomics. Traditional methods for separation and identification like 2D-gels or Edmann degradation has lately been exchanged with more advanced instruments. Especially mass spectrometers in different forms, most often in combination with HPLC (High-Performance Liquid Chromatography). The development of more and more sensitive and accurate instruments in combination with more advanced algorithms has formed a new era in proteomics(Patterson and Aebersold 2003).

Different techniques are used for identification and characterisation of proteins. The most popular methods are MALDI (Matrix Assited Laser Desorption Ionisation), ESI-Q-TOF (ElectroSpray Ionisation – Quadrupole - Time of Flight), Ion Trap and FTIR (Fourier Transform Ion Resonance). These instruments are capable of fragment peptides digested by enzymes at specific amino acids (Aa). The result is a fragmentation spectrum that is subsequently analysed to an Aa sequence. The masses obtained from this spectrum are compared to masses from "*In Silico*" (computer analysis).

Databases are available from the Internet and extend continually. From all over the world new sequence data is daily reported, and the growth has in recent years been

exponential. The protein database at NCBI (*National Center for Biotechnology Information)* possesses more than 6.7 million protein sequences(NCBI 2008).

Everything living contains proteins; mammals, plants, bacteria or virus. The aim of proteomics is to get an overview over the function, biochemical characteristics and the relevant pathways of the proteins. The diversity is enormous as well as the challenges for the researchers all over the world working on proteomics while they are pursuing the understandings of life(Patterson and Aebersold 2003).

2.5 Biomarkers

A biomarker is widely defined as any response detected within a living organism or biotic system liked to the presence of an adverse stressor. A frequently used definition was suggested by Peakall (1994) "A change in biological response, ranging from molecular through cellular and physiological responses to behavioural changes, which can be related to exposure to or toxic effects of environmental chemicals". Another more limiting definition: "A biomolecular response signal of an adverse effect condition in a toxicant exposed organism". It has become common to categorise the biomarker in functional classes based on user area and topic (Jonny Beyer, Personal Communication).

2.5.1 Biomarkers in proteomics

In proteomics a biomarker could be a protein peak in a mass spectrum conspicuous in contrast to other spectra, e.g. a peak present in an infected individual absent in the healthy individual, or contrary; the absent of a biomarker in an infected individual, but present in the controls. Traditionally it is common to look for one biomarker in a biologic sample or system. More recently it has been accepted that not only one biomarker is needed to recognize an altered biological state, but several proteins as biomarkers for a biological alteration. From a proteomic point of view, not only presence or absence of a protein but also up- and down regulation of different proteins might indicate an altered biological response due to an external stress factor. This is measured quantitatively by calculating the area under a peak in a mass spectrum. For the proteins of interest quantitative analysis might tell us about how different proteins acts in concert according to up- and down regulation.

2.6 Chromatography

2.6.1 Chromatography

The word 'Chromatography' is Greek for 'colored drawing' (*chroma* and *graphein*), and the term was used the first time in 1906 by the Russian botanist Mikhail Tswett. He used it to describe the separation that occurred when solutions of plant pigments were passed through columns of calcium carbonate or alumina, using petroleum ether. Each pigment showed a different degree of readiness to attach itself to the adsorbent. The pigments got separated in different-layers in the tube. The result of the analysis was latterly written in color, hence the name. Like Tswett's discovery, modern Liquid Chromatography (LC), uses a liquid mobile phase to transport the sample components through a column packed with a solid material; the stationary phase(Meyer 2005).

Chromatography is a collective term for techniques based on the fact that compounds separates due to different division between two phases; one moving (Mobile phase) and the other stagnant (Stationary phase)(Greibrokk, Lundanes et al. 1994).

2.6.2 HPLC High Performance Liquid Chromatography

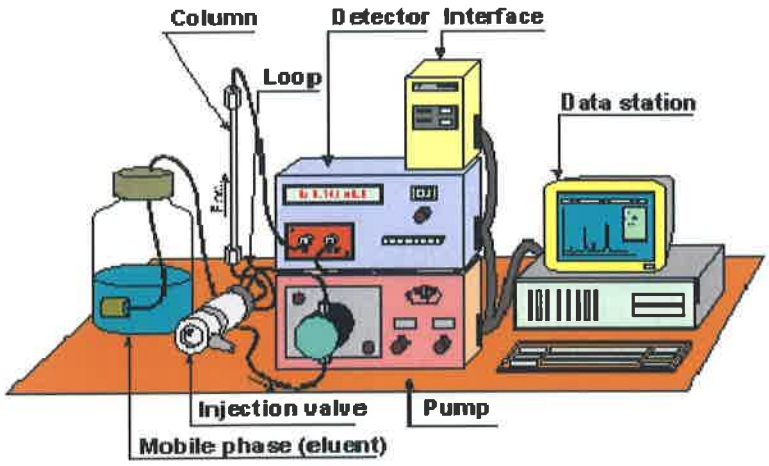


Figure 2.7: HPLC system illustrating the devices of the workflow.

The principal for LC and HPLC are the same. LC is preparative, components comprise larger dimension; larger columns and higher flow rates e.g. while HPLC is analytical.

The HPLC instrumentation includes a pump, injector, detector and recorder or data system. This is connected as shown in figure 2.7. The most important arrangement of the system is the column where the separation occurs. Because of the micrometer sized porous particles in the stationary phase, the system needs a high pressure pump to move the mobile phase and sample through the column. The separation occurs due to differences in interactions between analytes in the sample and the stationary phase. As the analytes and mobile phase pass through the column, the separation begins. The eluted components appear as peaks in a chromatogram. Depending on the detector used, the detection of the eluted components can be either selective or universal. Each

component gives a response to the detector and gets displayed on a screen as a part of a chromatogram. Computers, software and various data processing equipments are used to collect, store and analyze the chromatographic data(Kazakevich and LoBrutto 2007).

HPLC is very efficient and yields excellent separation in a short time. HPLC are sometimes referred to as *high-pressure liquid chromatography.* This is because the stationary phase requires very small particles and high pressure to force the mobile phase through the column. *Martin* and Synge were aware of this already in 1941, and are considered to be the inventors of modern chromatography.

The chromatogram is responses given to the detector from the amplified analytes plotted against time. The chromatographic peak height or peak area is related to the quantity of the analyte. Determination of actual amount of the compound, peak area or height is compared against standards of known concentration(Meyer 2005).

There are several liquid chromatographic separation modes;

Adsorption chromatography; Reverse-Phase (RP) chromatography and normal phase chromatography, where RP is the most common for proteins. Separation based on hydrophobic and hydrophilic interactions, the interaction is reversible.

Ion-Exchange chromatography; Anion and cation exchange. Ion exchange matrial as stationary phase. Mobile phase is usually water soluble solutions of salts, acids or bases.

Size-exclusion chromatography: gel filtration, separation on size. The smallest compounds resides longer in the column (stationary phase)

Affinity chromatography: Separation on biologic interaction such as between antigen and antibody, enzyme and substrate or receptor and ligand. Combines size fractionation with a stationary phase that reversibly binds to a known subset of molecules. One of many types of affinity chromatography is Ion-pair chromatography (Greibrokk, Lundanes et al. 1994; Meyer 2005)

2.7 SELDI-TOF-MS

Figure 2.8: SELDI-TOF-MS PBSII

The ProteinChip® SELDI system detects and calculates the mass of proteins and peptides using suface-enhanced laser desorption/ionization (SELDI) technology. The system is constituting of ProteinChip® arrays (figure 2.#), a ProteinChip® SELDI reader (figure 2.9), and a ProteinChip® software to analyze samples by measuring time-of-flight (TOF). The ProteinChip® SELDI system is able to precise mass analysis of peptides and proteins from complex biological samples(Bio-Rad Laboratories 2007)

2.7.1 ProteinChip® Arrays

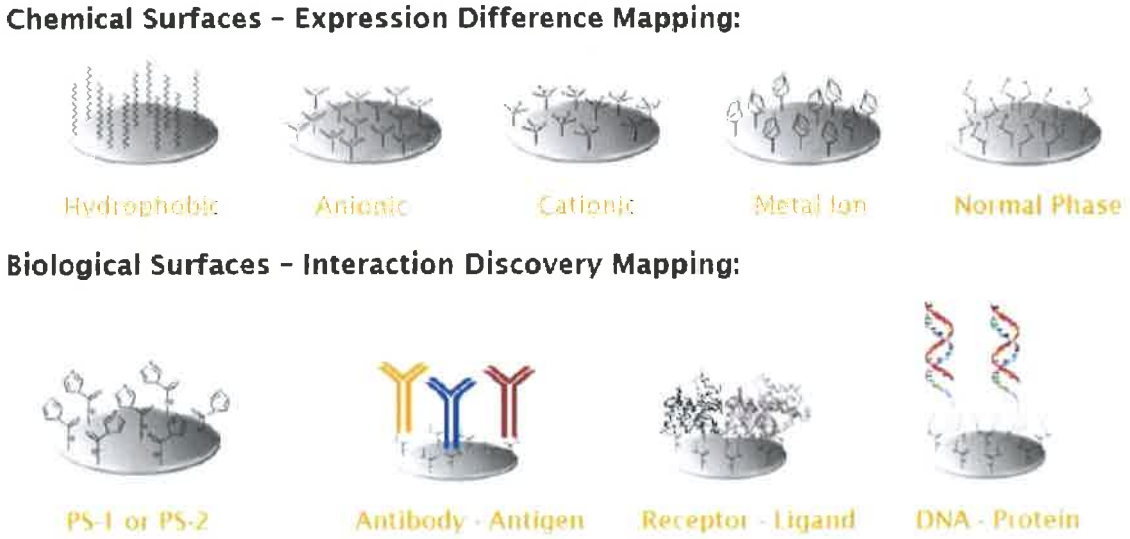


Figure 2.9: Different ProteinChip® Arrays(Bio-Rad Laboratories 2007).

SELDI-TOF-MS (Surface-Enhanced Laser Desorption Time-Of-Flight Mass Spectrometry) is a ProteinChip® Array technology where a protein containing sample is added to a chip with a pre activated surface. The surfaces on the different chip types have different affinity and will therefore bind to different classes of proteins. The chips have chromatographic properties; chemical sufaces: cation exchange, anion exchange, hydrophobic, normal phase, metal affinity etc. Biological surfaces: PS-1 or PS-2, Antibody – Antigen, Receptor – Ligand, DNA – Protein (figure 2.9). Biological surfaced will not be discussed here as they have no relevance for the experiment(Bio-Rad Laboratories 2007).

CM10 (Cationic Exchange Array)

The CM10 chip has an anionic surface derivatized with Carboxylate groups. The interaction is electrostatic and the proteins bind through positively charged residues such as Lysine, Arginine and Histidine. The optimal binding conditions are low pH (4.5) and low salt(Bio-Rad Laboratories 2007).

Q10 (Anionic Exchange Array)

The surface on the Q10 chip is cationic and derivatized with Ammonium groups. The interaction is electrostatic and the proteins bind through negatively charged residues like Aspartic acid and Glutamic acid residues. Optimal binding conditions for this surface are high pH (8.5) and low salt(Bio-Rad Laboratories 2007)

NP20 (Normal Phase Array)

The NP20 chip is pre-activated with SiO_2 on the surface and the interactions are electrostatic and dipole-dipole. The proteins bind through hydrophilic and charged residues on protein surface. Binding condition for this surface is a buffer, generally PBS (phosphate buffered saline)(Bio-Rad Laboratories 2007).

Processing

The cassettes containing the protein chips are placed in the autosampler device in the instrument and mechanically moved to its appropriate location. The first proteinchip is lowered into the compartment for laser desorption and ionization. Starting with spot A, the laser shoots according to the parameters set in the spot-protocol (Chapter 3. Material and Methods)(Bio-Rad Laboratories 2007).

The laser beam "shoots", with user-directed intensity, towards the spots on the chip and makes the protein loosen (desorption) (figure 2.10). Crystallized matrix molecules (usually SinaPinic Acid, SPA) enhance the ionization of the desorbed proteins. The laser beam hitting the matrix molecules makes them excite and convert into thermal energy. The crystal heats to about 1000 K in less than one nanosecond and the overheated part of the crystal explodes together with the proteins in an ionic cloud(Bio-Rad Laboratories 2007).

The ionized proteins are directed into the Time-Of-Flight (TOF) tube. This is a 1 meter long flying tube located in an approximated vacuum. The ionic cloud, with charged proteins and matrix molecules reaches the TOF-part of the instrument. All ions have the same energy as the flying starts, and the small molecules will fly faster than the bigger

ones. A molecule with two charges will fly twice as fast as the same single-charged molecule. Therefore a two-charged molecule will be detected on exact half the mass according to the single-charged mother ion. The mass spectrum confirms this(Bio-Rad Laboratories 2007).

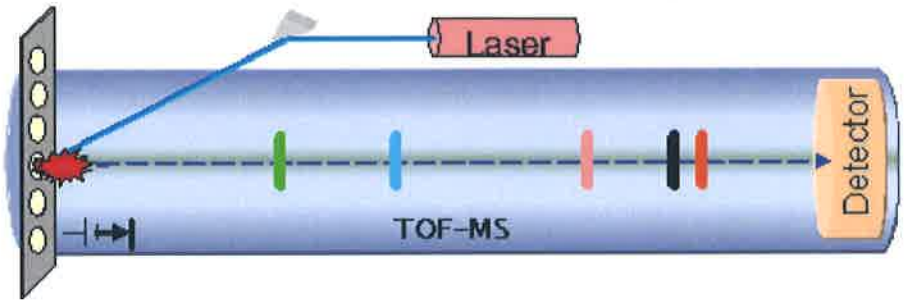


Figure 2.10 TOF-MS The figure illustrates how the laser hits the chip and makes the ions fly through the tube and hit the detector(BioRad 2003)

The result from a SELDI analysis is a protein profile, in the shape of a mass spectrum. The detector measures the period from the electrical field is turned on to the particle hits the detector(Dijkstra, Vonk et al. 2006). The time of flight for each molecule is easily converted to m/z – values (equation 1 and 2) and visualized as a mass spectrum.

The relations between TOF and m/z may be calculated by using the Law of energy conservation:

1. $zV = \frac{1}{2}mv^2$
2. $TOF = x/v = x\sqrt{(m/2zV)}$, where x is the length of the flying tube

A typical mass spectrum (figure 2.11) shows protein peaks with mass over charge (m/z – value) along the x-axis and relative peak intensity along the y-axis.

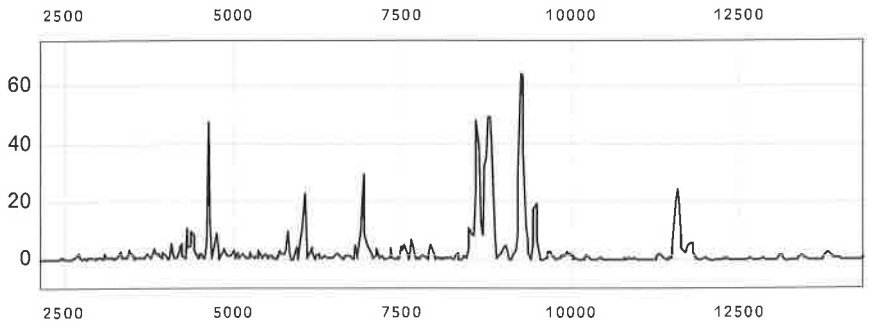


Figure 2.11: Mass spectrum from SELDI analysis on samples obtained from NSVS. The spectra shows protein peaks eight weeks post inoculation in lamb 5019 (Chapter 1. Introduction).

2.8 SOFTWARE

2.8.1 Ciphergen ProteinChip® Software 3.0

The ProteinChip® Software controls all aspects of the ProteinChip® Reader and facilitates data collection and analysis. The software uses a Microsoft Windows NT interface. The features of the software includes automatic reading of ProteinChip® Arrays, multiple spectrum comparison for different protein display and discovery of biomarkers, several alternative visualization options for data and a user-friendly interface.

ProteinChip® Software stores the spectra and experiment files into databases, rather than as files. The data can be easily rearranged into different experiments for analysis. Location and sorting data is done by key data properties such as date to data acquisition, Array type, laser intensity, and additional fields. This simplifies the construction of experiments containing a consistent group of conditions for expression analysis.

A new database needs to be created to manage the spectra formed by the software. After labeling, the spectra are calibrated against LM and HM spectra with known MWs. The detector will detect background noise and the baseline has a tendency to vary, to avoid this baseline may be adjusted.

Biomarker Wizard

Biomarker wizard clusters peaks and recognizes patterns in protein profiles. Plots intensity vs. cluster and allows rapid visualization of potential patterns to distinguish e.g. variants.

Spectra with similar condition are divided into sample groups and assigned to sample groups. Biomarker Wizard groups peaks of similar molecular weight across sample groups of spectra. The Biomarker Wizard data plot is a splitter-window above the experiment. Colors used in the data plot correspond to a different sample group. The clusters are used for analysis in PRIMER 6 (Chapter 2.8.2)

2.8.2 PRIMER 6

An infectious disease, like Sjodogg, is not necessarily recognized by the presence of one unique protein in a serum sample. The proteome will often respond in concert, increasing the samples due to up and down regulation of different proteins. This might happen at the transcription- or translation level. The disease might also affect the PTM

(post translational modifications: the enzymatic processing of a polypeptide chain after translation from mRNA after peptide bond formation has occurred. Examples include glycosylation, acylation, limited proteolysis, phosphorylation, isoprenylation(Dark 2007).) of a protein, thus changing or inhibit its biological function. To uncover changes in the proteome at this level the need for multivariate analysis indispensable.

PRIMER (Plymouth Routines In Multivariate Ecological Reasearch) is a software for multivariate analysis with a broad range of univariate, graphical and multivariate routines for analyzing species/sample abundance matrices arising in biological monitoring of environmental impact and more fundamental studies in community ecology, together with associated chemical data. The methods make with few, if any, assumptions about the form of the data and concentrate on approaches that are straightforward to understand and explain. 'Non-metric' ordination and permutation test are fundamental to the approach. A workspace explorer makes it easy to keep track of the workflow(Clarke and Ray 2008).

2.8.2.1 MDS (MultiDimensional Scaling)

MDS is used for data of non-metric nature, not time or distance related. The purpose of the ordination is to represent the samples as points in a low-dimensional space (2D or 3D) such that the relative distances apart of all points are in the same rank order as the relative dissimilarities of the samples. The relative distances and dissimilarities are measured by an appropriate resemblance matrix calculated from (often transformed) input data.

The algorithm is iterative and not guaranteed to converge to the optimal solution, hence the need to run it for a number of restarts. This is implemented in PRIMER v6.

In an MDS plot; points that are close together are very similar in composition (community composition, environmental variables, biomarker responses, particle size distribution etc), and points that are far apart correspond to very different values of the variable set.

Peak intensities were defined as non-metric values. Although there is a rationale for claiming that relative intensities are distances. For resemblance matrixes of non-metric nature the Bray-Curtis similarity (equation below) is used ~~(Appendix)~~ and the MDS is chosen for representing the data.

If the samples were defined as distances, the resemblance matrix would be calculated based on Euclidean distance. PCA (Principal Component Analysis) would be used to represent the data. PCA is an ordination in which samples, regarded as points in the high dimensional variable space are projected onto a "best-fitting" plane, or to other low-dimensional solution.

$$BC_{ij} = \sum \frac{|n_{ik} - n_{jk}|}{(n_{ik} + n_{jk})}$$

3 Experiments

3.1 List of Instruments and Equipments

Name	Manufacturer	Serial number
AT200	METTLER	
Centrifuge 5415R	Eppendorf	
Eppendorf tubes	Eppendorf	
IKA®	SCHÜTTLER MTS 4	
Labofuge 400R	Heraeus Instruments	
Microsentrifuge 157.MP	OLE DICH Instrumentmakers AC	
Microsep 1K Omega	PALL Life Science	P/N OD001C45
Model 250A	Orion	
MK100	Heildolph	
ProteinChip Q-spin column	BioRad	07B5434
Purelab Ultra	ELGA	
Purelab Prima	ELGA	
Rotator SB3	Stuart	
SPA EAM 5 mg	BioRad	
Spectrophotometer	NanoDrop ®	ND-1000
SELDI-TOF-MS	Ciphergen	PBS II
Vivaspin 2	Satorious Stedim Biotech	VS02H92
Vortex	VWR International	

3.2 List of Chemicals

Name	Manufacturer	Lot number
2-propanol	Merck	1.00998.2500
ACN (Acetonitrile)	VWR international	152856K
Chaps	Merck	1.11662.0010
DTT (Dithiothreitol)	SIGMA	086K13071
Hepes 2[4-(Hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid	Merck	1.10110.0250
IAA (Iodoacetamide)	BioSciences	786-228
Acetone	Merck	1.00021.2500
Lysosyme from chicken egg white lyophilized powder, ~50,000 units/mg protein (E1%/282)	SIGMA	L700
Protease inhibitor cocktail for use with mammalian cell and tissue extracts, DMSO solution	SIGMA	P8340
Trifluoroacetic acid	SIGMA	302031
Trizma-base	SIGMA	S-1503
Tri-natriumcitrat-dihydrat	Merck	1.06448.1000
Sodium Acetate	Merck	1.0628.1000
Sodium chloride	Merck	1.06404.1000
Sodium Phosphate	SIGMA	S-0751
Urea	SIGMA	U-6504

3. 3 SELDI - TOF – MS

The samples were analysed on SELDI-TOF-MS to check for proteins in the samples. Peaks in spectra indicated presence of proteins in spectra. The samples were added to protein chips with different pre-activated surfaces. In this project CM10, Q10 and NP20 was used(Chapter 2.7 SELDI-TOF-MS for theory). The protein chips were placed in the SELDI instrument for analysis. The results of analysis were displayed in mass spectra in Ciphergen ProteinChip Software (Chapter 2.8.1 Ciphergen ProteinChip® Software 3.0 for software details). Figure 3.1 illustrates the workflow.

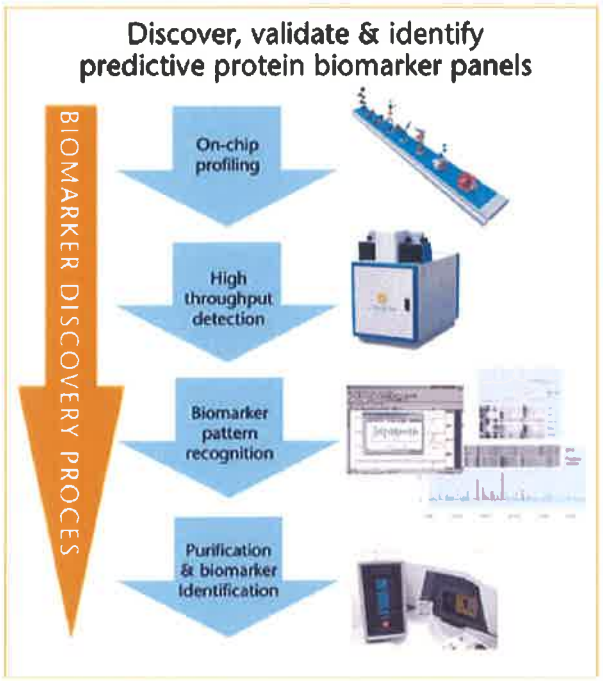


Figure 3.1: The biomarker discovery process. From top: The samples added to protein chip with different affinity, protein chip analysed in the SELDI instrument, biomarker pattern recognition using software, purification and identification of biomarkers. The last step was not performed in this experiment.

3.3.4 LM and HM protocol

LM

1: Set high mass to 30000 Daltons, optimized from 1000 Daltons to 15000 Daltons.
2: Set starting laser intensity to 170.
3: Set starting detector sensitivity to 7.
4: Focus by optimization center.
5: Set Mass Deflector to Auto
6: Set data acquistion method to Seldi Quantitation
7: Set Seldi acquisition parameters 20. delta to 5. transients per to 5 ending position to 80.
8: Set warming positions with 2 shots at intensity 175 and Don't include warming shots.
9: Process sample.

HM

1: Set high mass to 200000 Daltons, optimized from 25000 Daltons to 150000 Daltons.
2: Set starting laser intensity to 180.
3: Set starting detector sensitivity to 8.
4: Focus by optimization center.
5: Set Mass Deflector to Auto
6: Set data acquistion method to Seldi Quantitation
7: Set Seldi acquisition parameters 20. delta to 5. transients per to 5 ending position to 80.
8: Set warming positions with 2 shots at intensity 185 and Don't include warming shots.
9: Process sample.

3.3.4 Buffers

One buffer for each chip type was prepared in advance (table 3.1).

Table 3.1: Concentration and pH adjustments for buffers for different ProteinChip® types. pH adjusted with 1M HCl and 1M NaOH.

ProteinChip types	CM10	Q10
Buffer	NaAc	Tris-HCl
Concentration	50 µM	50 µM
pH adjusted to	4.0 - 4.5	8.0 – 8.5

3.3.5 Sample handling with different chip types

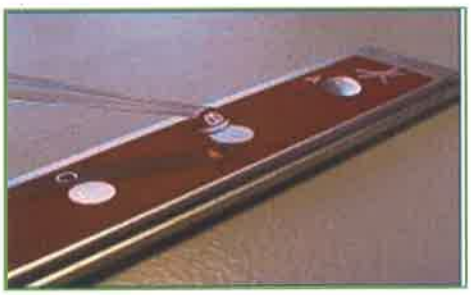


Figure 3.2: Adding sample on a spot on a protein chip

NP20

2 µl sample was added by pipette directly to the spots on the chip and dried in room temperature (illustration in Figure 3.2). 1 µl Matrix was added to the spots twice and evaporated between the steps.

CM10

Prior to the sample addition the chips were washed with NaAc (Natrium Acetate) buffer. 2 x 200 µl buffer was applied to each well in the Bioprocessor. The Bioprocessor was placed on a shaker for 5 minutes. The buffer was removed. Another 200 µl buffer was applied, shaken and removed. 150 µl sample was then added on wet spot. The Bioprocessor containing the samples was incubated overnight at 4 °C. Sample solution was throen out. The spots were then washed 3 x 5 minutes with 200 µl NaAc buffer on a shaker, and subsequently 2 x 200 µl distilled water was added and removed immediately.

The Bioprocessor was disassembled and the spots were allowed to dry. 2 x 1 µl Matrix was added and evaporated twice in room temperature.

Q10

This procedure was similar to the one for CM10, but with Tris-HCl buffer instead of NaAc.

3.3.6 Standards and controls

Low mass standard (LM) and High Mass standard (HM) (LM and HM are mixtures of peptides/proteins with clearly defined masses) were used for calibrating the spectra. With each run there should be spots with LM, HM, positive control and negative control. Positive control ('in house' trypsinated BSA) was used to check if the experiment was successful or not. If the positive control could be trusted and the rest of the spectra were flat, there would probably be something wrong with the samples. Negative control (the respected buffer) was used to check for contaminations either in the buffer itself or in some of the other solutions used in these experiments.

3.3.1 Preparing for SELDI-TOF-MS analysis

After standard washing and sample procedure (3.1.5) the chips were loaded into a cassette, and placed in the ProteinChip® reader on the SELDI instrument. Spot-, Chip- and cassette protocol were programmed in the ProteinChip® software. Spot- and Chip protocols had to be prepared only once because they were constant for every run. The cassette protocol had to be prepared for each run, because the number of chips would vary between experiments. Look up Chapter 2.8 SELDI-TOF-MS for details.

3.3.2 Matrix (EAM - Energy Absorbing Molecules)

1% TFA: 198 µl distilled water was added to 2 µl 100% TFA in an eppendorf tube. 200 µl ACN was added to a tube with SPA powder and added the 1 % TFA. The Matrix mixture was vortexed for 10 minutes.

3.3.3 Bioprocessor

A bioprocessor (figure 3.3) is an appliance to hold the chips during the washing procedures and sample loading. NP20 do not require washing prior to analysis in the SELDI reader, hence the bioprocessor is not used for this chip type.

Figure 3.3: Bioprocessor holding protein chips during the washing process

3.4 Albumin removal

3.4.1 Precipitation

The first method tried was found in an article called *A modified protein precipitation procedure for efficient removal of albumin from serum* (Chen, Lin et al. 2004).

200 µl sample was precipitated with quick additive of 800 µl ice cold Acetone with 10 % w/v TCA and vortexed. The mixture was incubated at - 20 °C for 90 minutes. Subsequently it was centrifuged at 1500 x g, 4 °C for 20 minutes. The supernatant was collected. The precipitate was washed with 1 ml ice cold Acetone. The sample was incubated on ice for 15 minutes and centrifuged with the same conditions as above. The Acetone with the supernatant was removed and the precipitate was lyophilized. 1 ml ice cold Acetone was added to the 10 % TCA/Acetone supernatant to precipitate the proteins completely. The precipitated pellet was dried in an incubator at 37 °C for 20 minutes. The remaining pellet was attempted solved in 10 x diluted PBS, but would no dissolve. The pellet was then attempted solved in 1 ml 1:1 DMSO(dimethyl sulfoxide)/H_2O, but the pellet was only partly dissolved.

Methanol was also attempted as dissolving agent, but the pellet would not decompose.

The partly solved sample was run on SELDI. Results were poor; no peaks in the spectra.

3.3 Albumin removal with HiTrap Blue on HPLC

HiTrap Blue (GE Healthcare) is a column pre-packed with blue sepharose and intended to purify albumin, enzymes like NAD^+ and $NADP^+$, interferones and related proteins. The non-binding proteins will elute as flow through, while the albumin fraction will elute with a steep salt gradient. The column was assembles to a HPLC (Waters™ Corp.) and a syringe was connected to the system.

The HiTrap Blue column was mounted on a Waters™ HPLC system (Waters™ Autosampler 717 plus, Waters™ Controller 600 and Waters™ detector 486). A syringe (? ml) was mounted onto an injector with a 500 ul loop. Fractions from this experiment was reanalyzed with ion-exchange on LC (Chapter 3.5 LC)

For mobile phase A and B; 20 mM Na_2HPO_4 was prepared and adjusted to pH 7 with HCl. The solution was divided in two and mobile phase B was added 2.0 M NaCl.

Three solutions were made for injection:

1. 1 ml Na_2PO_4 20 mM, 100 μL 5 % BSA
2. 1 ml Na_2PO_4 20 mM, 100 μL 5 % BSA + 0.6 mg Lysozyme from chickens egg white
3. 1 ml Na_2PO_4 20 mM, 0.9 mg Lysozyme

The best dilution seemed to be 1:20 after testing the column. It was assumed that a total protein concentration was 50 mg/ml in the serum samples(Jacobs, Adkins et al. 2005).

Serum from lamb 4202 and 5041 was diluted 1:20 in mobile phase A. For both samples, 0.5 ml was injected into the system, respectively. For details on the HPLC run (table 3.2).

Table 3.2: Desalination program for HiTrap Blue on HPLC

Desalination program	
Time	25 minutes
Injection volume	0.5 ml
Wavelength	280 nm
Flow	1ml/ min

See figure 4.2 for chromatogram.

Both the non-binding proteins (the protein fraction) and the albumin fraction were collected in Eppendorf tubes for further analysis.

3.5 Denaturation

3.5.1 Albumin denaturation with DTT and IAA

A lot of proteins have high affinity for albumin. Also the less abundant proteins that might be of interest in biomarker discovery can bind to albumin. Therefore, in an attempt to unbind these proteins, albumin was denaturated with DTT and IAA.

The albumin fraction from the HiTrap Blue experiment was thawed. Spin columns, Vivaspin 2 (Satorius Stedim biotech) were used for salt removal and buffer exchange.

2 ml sample was added to the vivaspin columns. The solution centrifuged for 10 minutes, 4000 x g at 4 °C. For washing the samples 1 ml Ambic (Ammoniumbicarbonate) was centriguged as described above to approximately 200µl. 1M DTT and 1M IAA were prepared. 1 µl DTT was added to 99 µl sample. This solution incubated for 45 minutes at 56 °C. The sample was cooled to room temperature and 5.5 µl IAA was added. The solution incubated for 30 minutes. The samples were analyzed on SELDI-TOF-MS.

3.5.2 Denaturation using urea

It is known that a strong Urea solution will efficiently disrupt the noncovalent bonds in proteins.

8M Urea was made by adding 24 g Urea to a flask and diluted in 50 ml 20 mM $NaPO_4$ (Sodium phosphate) buffer, pH 7.

0.5 ml sample of the albumin fraction from the HiTrap Blue experiment was mixed with 1.5 ml 8M Urea and incubated at 4 °C for 24 hours.

After incubation the solution was spun twice with spin columns; cutoff 10k and 30k respectively. They were spun with 7500 x g at 4 °C for 10 minutes. The phases over and under the filter was tested on NP20 chip along with crude sample from the denaturation. The experiment was unsuccessful because the drops on the protein chips crystallized. Probably because of urea.

The samples were put on NP20 as well after using 1k spin columns without success.

The denatured Albumin was then tested on Q10 and CM10 chips. The samples were spun with 1k spin columns for 3 hours prior to the analysis with SELDI-TOF-MS. The spectra showed that Albumin still overshadowed other proteins.

3.6 LC (Liquid chromatography)

For the SAX and SCX experiments the Akta explorer 900 (Amersham Biosciences) LC connected to a fraction collector (Frac. 950) was used.

For the anion and cation exchange experiments an Ãkta explorer LC (GE life sciences) was used, connected to a fraction collector.

Table 3.3: Program for run on SAX with HiTrap QXl column and SCX with HiTrap SPXl column on LC.

Variables	SAX	SCX
Column	HiTrap QXL 1 ml	HiTrap SPXL 1 ml
Flow rate	1.00 ml/min	1.00 ml/min
Pump A inlet	Mobile phase A: Tris 50 mM pH 8.5	Mobile phase A: NaAc 50 mM pH 4.5
Pump B inlet	Mobile phase B: Tris 50 mM pH 8.5 + 1M NaCl	Mobile phase B: NaAc 50 mM pH 4.5 + 1M NaCl
Injection Flowrate	1.0 ml/min	1.0 ml/min
Volume of Sample	40.0 ml (10.0 ml sample to 40.0 ml mobile phase A)	43.0 ml (9.0 ml sample to 43.0 ml mobile phase A)
End Fraction at	100 %B	100 %B
Eluate Fraction size	2 ml	2 ml
Length of Gradient	16.70 ml	20.00 ml
Wavelength 1	280 nm	280 nm
Wavelength 2	215 nm	215 nm
Wavelength 3	254 nm	254 nm

See chromatogram in figure 4.4

3.6.1 SAX (Strong anion exchange) on LC with HiTrap QXL column

The column (HiTrap Q XL 1 ml, GE life science) was equilibrated with mobile phase B; 50 mM Tris, pH 8.5 + 1M NaCl, and the system was thereafter purified with mobile phase A; 50 mM Tris, pH 8.5 (table 3). All the samples from the protein fraction 4202 I, from the HiTrap Blue experiment, was collected in a tube and constituted 10 ml. This sample was diluted 1:3 with mobile phase A to 30 ml total volume and then loaded onto the column (See table 3.3 for LC run details). The sample volume was programmed to 40.0 ml because the injecting device was somewhat inaccurate. The back pressure from the column renders the injection pump unable to supply the programmed flow.

The flow through fraction was collected in a large tube, while the elution within the gradient was collected in 2 ml fractions (table 3.3, figure 4.4).

3.6.2 SCX (Strong cation exchange) on LC with HiTrap SPXL column

The procedure for SCX was similar to the one for SAX only with another column, HiTrap SPXL (HiTrap SP XL 1 ml, GE life science), 9 ml sample; 5041 from the HiTrap Blue experiment was diluted to 45 ml in mobile phase A, 50 mM NaAc pH 4.5 (table 3.3, figure 4.5).

3.7 ProteinChip® serum fractionation kit

The kit contained elution columns with Q HyperD F anion exchange beads delivered with storage buffer and one buffer called U9. The fractionation kit is done based on pH. The kit is also called "Q-kit".

3.7.1 Preparing buffers

- U1 buffer was made from U9 buffer diluted 8:1 with rehydrating buffer, Tris, pH 9.

Table 3.4: Buffers made for fractionation steps, QF1, QF2, QF3, QF4 and QF5, with ProteinChip® serum fractionation kit. Buffers were added 0.1 % OGP subsequently.

Buffer	Concentration	pH	Fraction
Tris	50 mM	9	QF1
Hepes	50 mM	7	QF2
Sodium Acetate	100 mM	5	QF3
Sodium Acetate	100 mM	3	QF4
tri-Sodium Citrate	50 mM	2	QF5

Table 3.5: Contents of the organic wash buffer.

Chemical	Amount
Iso-propanol	33.3 %
ACN (acetonitrile)	16.7 %
TFA (trifluoracetic acid)	0.1 %

3.7.2 Fractionating by pH with BioRad kit

Table 3.6: Samples used with fractionation kit

Lamb number	Sampling date
4203	14.09.04 (pre-inoculation) 13.10.04 (Four weeks post inoculation) 10.11.04 (Eight weeks post inoculation)
4210	14.09.04 (pre-inoculation) 29.09.04 (two weeks post inoculation) 13.10.04 (Four weeks post inoculation) 10.11.04 (Eight weeks post inoculation)
4249	14.09.04 (control)
5019	14.09.05 (pre-inoculation) 28.09.05 (two weeks post inoculation) 12.10.05 (Four weeks post inoculation) 09.11.05 (Eight weeks post inoculation)
5029	14.09.05 (pre-inoculation) 28.09.05 (two weeks post inoculation) 12.10.05 (Four weeks post inoculation)
5076	14.09.05 (control)

3.7.2.1 Preparing sample

The samples (table 3.6), U9 buffer and U1 buffer were thawed prior to the experiment. 100 µl protease inhibitor 1 (diluted 1:1000, SIGMA mammalian protease inhibitor cocktail) was added to the samples to obtain the native conditions of the proteome. Subsequently, 150 µl U9 buffer was added to 100 µl serum and incubated for 20 minutes at room temperature on a rotating wheel. While incubating the samples, the spin columns were prepared and equilibrated as described below.

3.7.2.2 Q Ceramic Hyper D Anion Exchange column equilibration

The eluting column was consisted of the following parts; one filter (filter: Q Ceramic Hyper D Anion Exchange beads) column with top lid for sample additive and bottom lid for removal of eluted sample (figure 3.4).

Figure 3.4: Q Ceramic Hyper D Anion Exchange column

The bottom lid was removed and the column was placed in a 2 ml manifold for elution collection. Pressure was applied to the column to elute the storage buffer through the beads, when air bubbles appeared the elution was complete.

Washing the column: The beads were allowed to re-pack before the bottom lid was replaced. 500 μl *U1 buffer was added. The column was equilibrated by replacing the top lid and hand mixing (turn the column upside down several times) for 1 minute. Bottom lid was removed and the column was placed in a collecting tube. The column was eluted by gravity. The eluted solution, waste, was thrown away. The washing was repeated twice.

3.7.2.3 Sample fractionation

After 20 minutes of incubation the serum/U9 buffer mixture (3.6.2.1 Preparing samples) was diluted with 250 μl *U1 buffer. The mixture was added to the washed spin column and incubated on the rotating wheel for 30 minutes. Seven 2.0 ml micro centrifuge tubes were labelled for each sample with: QFT, QF1, QF2, QF3, QF4, QF5 and QF6, one for each fractionation buffer(table 3.4 and 3.5).

After 30 minutes of incubation, the serum sample was first eluted in the tube labelled QFT, this was the Flowthrough fraction. It was eluted until there was no fluid left in the column. The collecting tube was kept on ice and the column was recapped and left for the next fraction. 500 μl Tris buffer, pH 9 was added. The column was placed on the rotating wheel for incubation for 5 minutes at room temperature, thereafter the column was placed in the collecting tube labelled QF1. The fraction was eluted as described above. All the eluted fractions was kept on ice through the experiment. The column was prepared for the next fraction.

These steps were repeated for QF2 with buffer pH7, QF3 with buffer pH 5, QF4 with buffer pH 4, QF5 with buffer pH3 and QF6 organic fraction (table 3.7).

All the fraction samples were stored at – 80 °C for later analysis on SELDI-TOF-MS.

Table 3.7: Overview over fractionated samples analysed on SELDI-TOF-MS.

4203 QFT	4210 QFT	4249 QFT	5019 QFT	5029 QFT	5076 QFT
4203 QF1	4210 QF1	4249 QF1	5019 QF1	5029 QF1	5076 QF1
4203 QF2	4210 QF2	4249 QF2	5019 QF2	5029 QF2	5076 QF2
4203 QF3	4210 QF3	4249 QF3	5019 QF3	5029 QF3	5076 QF3
4203 QF4	4210 QF4	4249 QF4	5019 QF4	5029 QF4	5076 QF4
4203 QF5	4210 QF5	4249 QF5	5019 QF5	5029 QF5	5076 QF5
4203 QF6	4210 QF6	4249 QF6	5019 QF6	5029 QF6	5076 QF6

Table 3.7 shows the fractionated samples analysed on SELDI-TOF-MS. Lamb 4249 and 5076 were control samples, thus one sampling date. Lamb 4203 and 5029, each had three sampling dates each (table 3.7). Lamb 4210 and 5019 had four sampling dates each. Assembled there were 16 samples in fractionated in seven fractions each; 112 fractionated samples. The cassette for the SELDI-TOF-MS could not hold two parallels of the samples (max 2 x 96), and an analysis in addition to sample preparation holds too much time to run both parallels in one day. All the 112 fractionated samples were put on three chip types; two parallels of CM10 and Q10, and one of NP20.

Successful experiment; finally the mass spectra from SELDI-TOF-MS showed protein peaks.

4 Results and discussion

4.1 Results

Samples from healthy and infected lambs, inoculated with *A.phagocytophilum* was collected from The Norwegian School of Veterinary Science. These samples were serum samples from different infected sheep and control samples. The infected blood was sampled on determined point in time during the cycle of the disease.

To find the protein profiles in serum from sheep the samples were diluted and put on chip for analyse with SELDI-TOF-MS. The spectra indicated almost exclusively peaks of Albumin (figure 4.1). The Albumin had to be removed. Removal of Albumin is not always required, even if the concentration overshadows everything else in a mass spectrum. Albumin has affinity for a lot of different proteins and among this there may be important proteins, and possibly biomarkers.

Different methods for albumin removal were attempted, e.g. precipitation with Acetone and 10% TCA. In theory albumin and some other proteins remain whole in solution when combined to TCA, while the residual precipitates. This experiment failed.

Subsequently the samples were injected on column (HiTrap Blue, GE-Healthcare) for

Albumin removal on HPLC. This was successful. The Albumin disappeared from the spectra (figure 4.1).

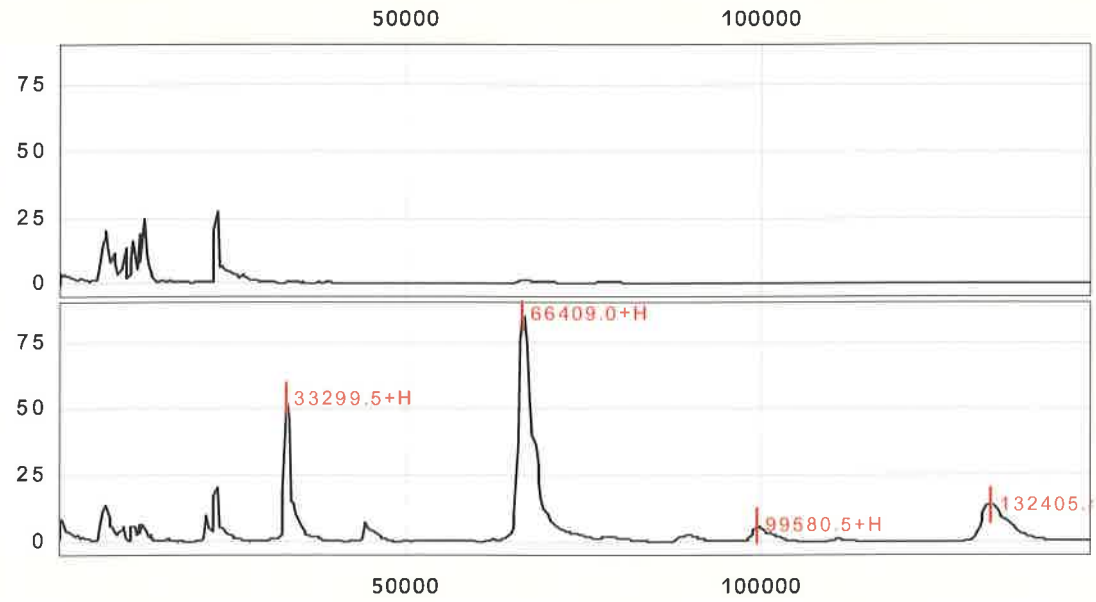


Figure 4.1: The upper spectrum shows that the Albumine is removed from the sample. The lower spectrum shows the Alumin peaks that were present before the column was used.

The run on HPLC divided the sample in two fractions; one with Albumin "Albumin fraction" and one with the other proteins "Protein fraction" (Figure 4.2). The fraction with proteins shows the absorption at 280 nm, but still, the spectra from the SELDI analysis were poor. This could be due to too much dilution in the HPLC system, or that the buffer which the sample elutes in has qualities that impair the binding on spot surface on the chips.

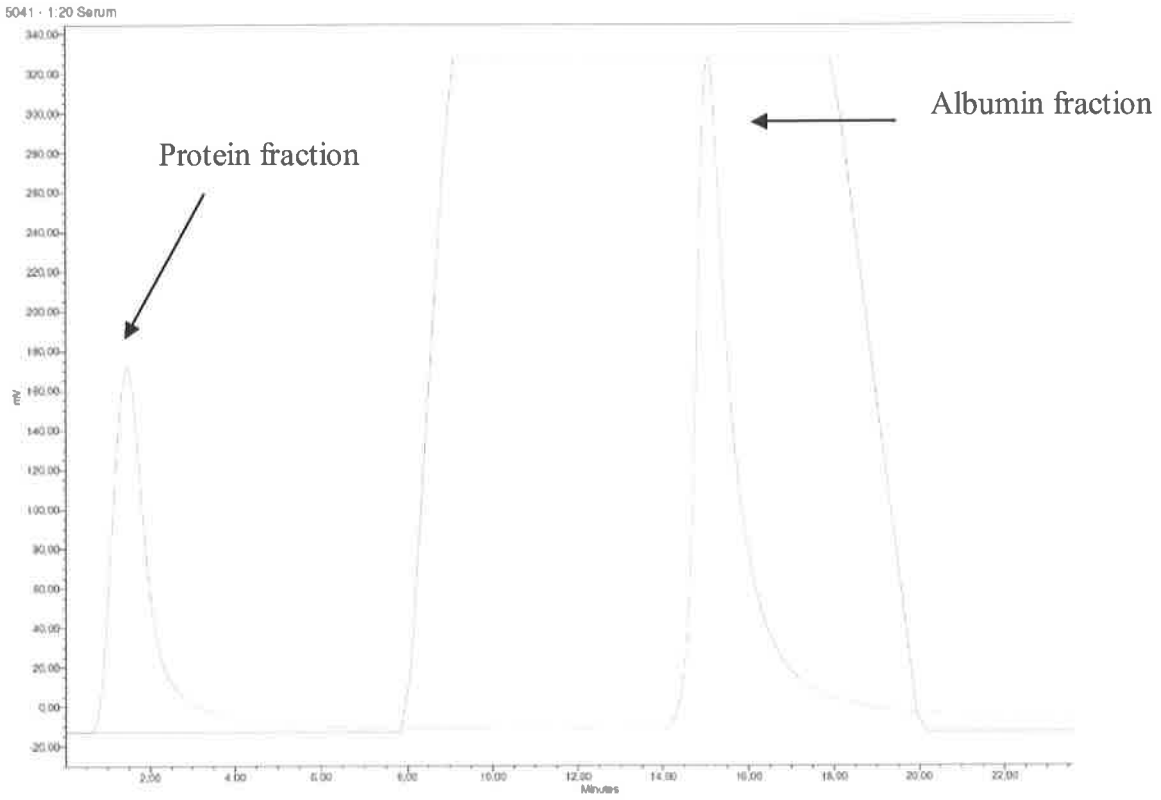


Figure 4.2: Chromatogram of serum samples from sheep. Separated on HiTrap Blue. X-axis; m/z –

Thereafter a fractionation kit, also called Q-kit (Proteinchip serum fractionation, Anion exchange, Q-kit – BioRad) was tested. This kit contains tubes with anion exchange materiale, the sample was added and eluated in fractions using buffers with different pH, in addition to an organic fraction. These fractions were accordingly analysed with SELDI. The fractions showed good spectra with several peaks (figure 4.3).

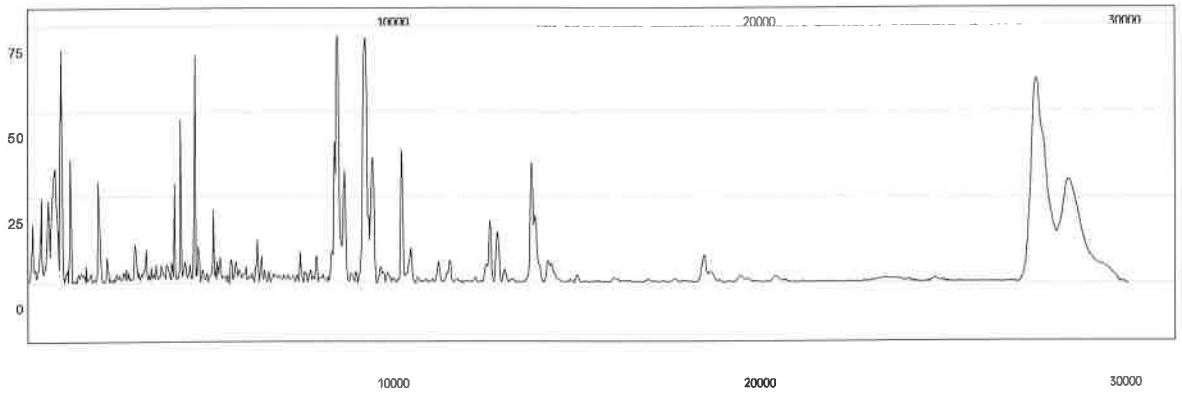


Figure 4.3: Typical mass spectrum that show the protein profile for the flowthrough fraction from the Q-kit. X-axis; m/z – ratio, Y-axis; relative peak intensity

4.2 SAX and SCX on LC

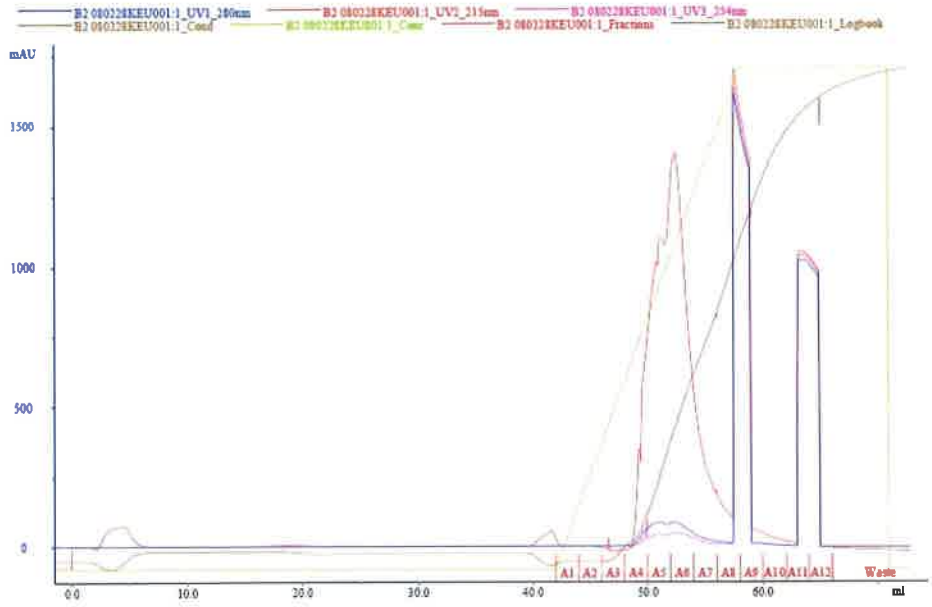


Figure 4.4: Chromatogram from SAX on LC with HiTrap QXL column. Pink graph illustrates absorbance at 215 nm, the green line is the programmed gradient and the brown is the actual gradient measured as conductivity. X-axis: ml (milliliter) injected, y-axis: mA (milliAmper)

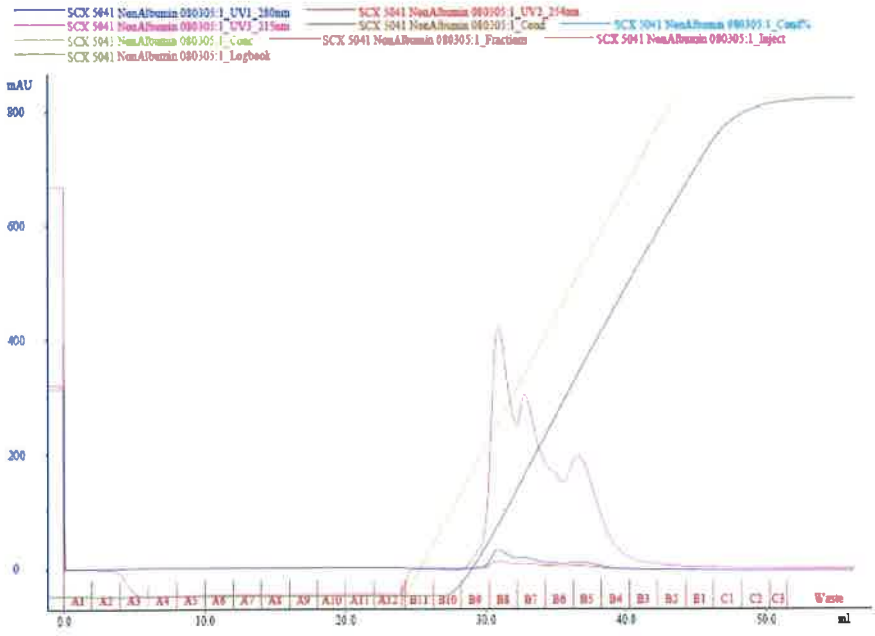


Figure 4.5: Chromatogram from SCX on LC with HiTrap SPXL. Pink graph illustrates absorbance at 215 nm, the green line is the programmed gradient and the brown is the actual gradient measured as conductivity. X-axis: ml (milliliter) injected, y-axis: mA (milliAmper)

Lamb 4202 and 5041 in the "protein fraction" from the HiTrap Blue experiment (Chapter 3.3) were used for the analysis. See "Table 1.1" for sample details.

The chromatogram for the both the SAX (figure 4.4) and SCX (figure 4.5) shows that proteins elutes within the salt gradient. The eluted fractions were collected in 2 ml tubes for further analysis on SELDI. Absorbance at 215 nm (pink) is evident in these chromatograms, while absorbance at 280 nm (blue) is modest. Protein containing fractions for the SAX A3-A11 and SCX B9-B3 were desalted prior to SELDI analysis. The square peaks arising to the right in the chromatogram are probably air bubbles that have entered the system with the sample injection.

4.3 Biomarkers for the disease, Sjodogg

The following results originate from the ProteinChip® serum fractionation kit.

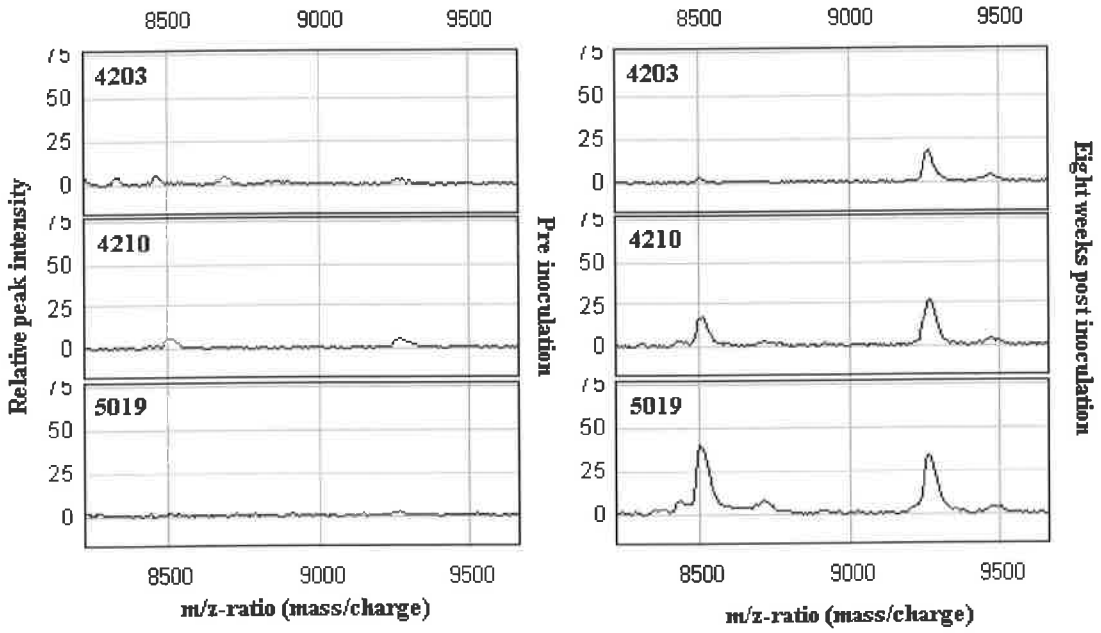


Figure 4.6: Mass spectra (Low mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF1 on Q10 chip (3 Experiments). The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 8.50 to 10.0 kDa. The spectra originate from serum samples from lamb 4210, 4203 and 5019 in parallel I, to the left; pre inoculation, sampling date 14.09.2008 and 14.09.2008, respectively (table #.1.1). To the right are spectra from the same lambs pre inoculation, sampling date 10.11.04 and 09.11.05, respectively (table #.1.1). The figure illustrates up-regulation of the protein peaks at 8.50 and 9.27 kDa. Note that the intensity increases from healthy to infected lambs.

The protein peaks in the spectra in figure 4.6 seem to be up regulated. The spectra which represent the infected lambs are from the sampling after eight weeks, 09. and 10. November, respectively. Lamb 5029 did not show a positive rickettsemia peak at that time (figure 1.1 in Introduction), thus no sample was received.

Lamb 4203 had an intensity difference of 2 and 3 for the 9.27 kDa peak (figure 4.6) parallel I and II respectively (table 4.1 and 4.2). Lamb 4210 had an intensity difference of 9 and 57 (I and II) for the 8.50 kDa peak, and 20.5 and 21.5 (I and II) for the 9.27 kDa peak.

For lamb 5019 the peak intensity difference were 40 and 70 (I and II) for the 8.50 kDa peak and 31.5 and 60 for the 9.27 kDa peak.

The other sampling dates are represented in table 4.1 and 4.2, but the difference at 8.50 kDa and 9.27 kDa is not significant due to the low intensities. It is difficult to partition them from the background noise.

These proteins did not, according to these results, upregulate until eight weeks of infection. The different parallels showed great variance according to each other. Generally the samples showed higher intensities and more peaks in parallel II (figure 4.6, table 4.1 and .2).

The parallels were not analyzed on the same day on the SELDI instrument because the cassette did not accommodate all the samples (max 2x12 chips). Thus the variance could occur as a result from drift in the SELDI instrument, calibration of the spectra or with sample handling related to the fractionation. Also the freeze and thaw processes, storage time in the freeze, delay in the laboratory process and environmental conditions might contribute to variance.

Lamb 4203 had a slight up-regulation at 9.27 kDa, but not significant, though the spectrum in parallel I was flat pre inoculation, 14.09.04 (table 4.1). In the respected spectrum in parallel II, the peaks 8.50 and 9.27 kDa had increased intensity of 3.5 and 13.5, respectively.

Lamb 4210 had a significantly higher increase of intensity in 8.50 and 9.27 kDa, this is apparent in figure 1. These proteins were also present in the sample collected before infection.

In samples collected pre-inoculation lamb 5019 showed intensities of 2.5 and 4.0 at 9.27 kDa for parallel I and II respectively. This lamb seemed to have the greatest up-regulation for both the 8.50 and 9.27 kDa peaks.

Table 4.1: Overview of peak intensities at 8.50 and 9.27 kDa for all the lambs, including controls; 4249 and 5076, pre and post inoculation. Parallel I

Sample name	Sampling date	Peak Intensity at 8.50 kDa	Peak Intensity at 9.27 kDa
4249 I	14.09.2004	2.5	9.0
5076 I	14.09.2005	5.0	8.5
4203 I	14.09.2004	5.0	2.5
4210 I	14.09.2004	5.0	5.0
5019 I	14.09.2005	-	2.5
5029 I	14.09.2005	-	-
4210 I	29.09.2004	-	2.5
5019 I	28.09.2005	-	2.0
5029 I	28.09.2005	-	-
4203 I	13.10.2004	-	3.0
4210 I	13.10.2004	-	-
5019 I	12.10.2005	-	-
5029 I	12.10.2005	-	2.0
4203 I	10.11.2004	3.0	17.5
4210 I	10.11.2004	16.0	25.5
5019 I	09.11.2005	40.0	34.0

Table 4.2: Overview of peak intensities at 8.50 and 9.27 kDa for all the lambs, including controls; 4249 and 5076, pre and post inoculation. Parallel II

Sample name	Sampling date	Peak Intensity at 8.50 kDa	Peak Intensity at 9.27 kDa
4249 II	14.09.2004	10.0	22.0
5076 II	14.09.2005	10.0	11.0
4203 II	14.09.2004	-	-
4210 II	14.09.2004	15.0	9.5
5019 II	14.09.2005	-	4.0
5029 II	14.09.2005	-	-
4210 II	29.09.2004	-	5.5
5019 II	28.09.2005	-	2.5
5029 II	28.09.2005	-	5.0
4203 II	13.10.2004	2.0	4.0
4210 II	13.10.2004	-	5.0
5019 II	12.10.2005	-	3.0
5029 II	12.10.2005	-	3.5
4203 II	10.11.2004	3.5	13.5
4210 II	10.11.2004	72.0	31.0
5019 II	09.11.2005	70.0	72.5

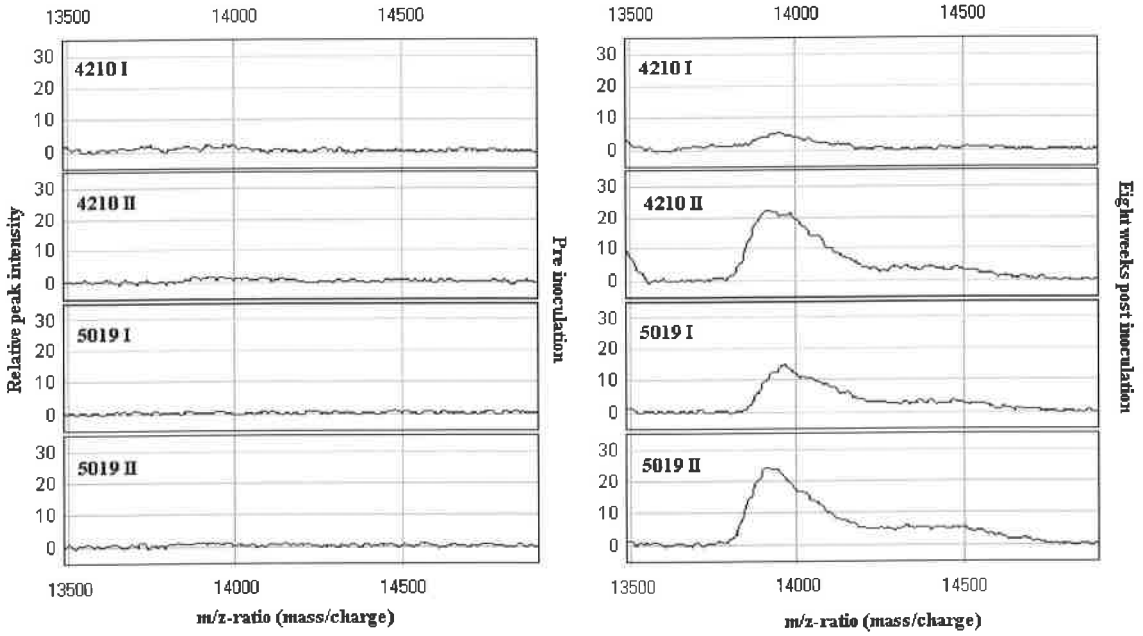


Figure 4.7: Mass spectra (High mass displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF1 on Q10 chip (3 Experiments) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 13.5 to 15.0 kDa. The spectra originate from serum samples from lamb 4210, 4203 and 5019 in parallel II, to the left; pre inoculation, sampling date 14.09.2008 and 14.09.2008, respectively (table 4.4). To the right are spectra from the same lambs pre-inoculation, sampling date 10.11.04 and 09.11.05, respectively (table 4.4). The figure illustrates up-regulation of the protein peak at 13.96 kDa.

The protein peak in the spectra to the right in figure 4.7 seemed to be up-regulated in lamb 4210 and 5019. The 13.96 kDa peak in lamb 4210 shows a great variation between the two parallels, intensities differs with 5.0 and 22.5, respectively. Lamb 5019 had a difference in intensity for the 13.96 kDa peak on 15.0 and 25.0 in parallel I and II, respectively. Lamb 4203 and 5029 had, according to table 4.3 and 4.4, no up-regulation at this mass. 4249 shows traces of peak 13.96 kDa in parallel I (table 4.3).

Table 4.3: Overview of peak intensities at 13.96 kDa for all the lambs, including controls, 4249 and 5076, pre- and post inoculation, parallell I

Sample name	Sampling date	Peak Intensity at 13.96 kDa
4249 I	14.09.2004	3.0
5076 I	14.09.2005	-
4203 I	14.09.2004	-
4210 I	14.09.2004	-
5019 I	14.09.2005	-
5029 I	14.09.2005	-
4210 I	29.09.2004	-
5019 I	28.09.2005	-
5029 I	28.09.2005	-
4203 I	13.10.2004	-
4210 I	13.10.2004	-
5019 I	12.10.2005	-
5029 I	12.10.2005	-
4203 I	10.11.2004	-
4210 I	10.11.2004	5.0
5019 I	09.11.2005	15.0

Table 4.4: Overview of peak intensities at 13.96 kDa for all the lambs, including controls, 4249 and 5076, pre- and post inoculation, parallell II

Sample name	Sampling date	Peak Intensity at 13.96 kDa
4249 II	14.09.2004	-
5076 II	14.09.2005	-
4203 II	14.09.2004	-
4210 II	14.09.2004	-
5019 II	14.09.2005	-
5029 II	14.09.2005	-
4210 II	29.09.2004	-
5019 II	28.09.2005	-
5029 II	28.09.2005	-
4203 II	13.10.2004	-
4210 II	13.10.2004	-
5019 II	12.10.2005	-
5029 II	12.10.2005	-
4203 II	10.11.2004	-
4210 II	10.11.2004	22.5
5019 II	09.11.2005	25.0

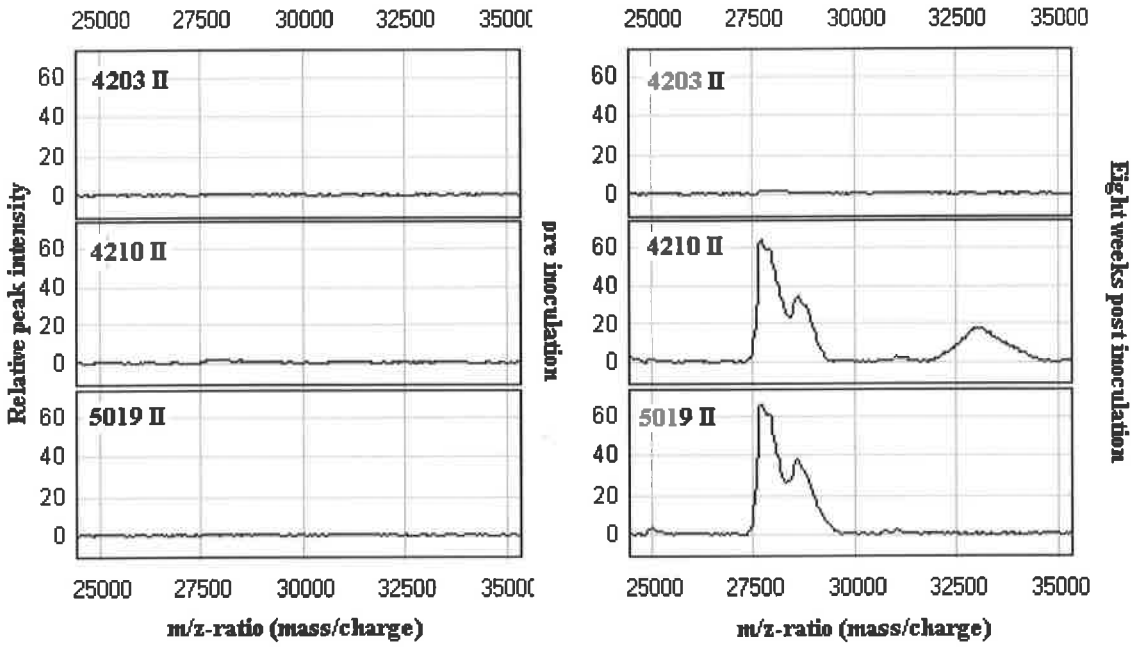


Figure 4.8: Mass spectra (High mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF1 on Q10 chip (3 Experiments) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 25.0 to 35.0 kDa. The spectra originate from serum samples from lamb 4210, 4203 and 5019 in parallel II, to the left; pre-inoculation, sampling date 14.09.2008 and 14.09.2008, respectively (table 4.6). To the right are spectra from the same lambs pro inoculation, sampling date 10.11.04 and 09.11.05, respectively (table 4.6). The figure illustrates up-regulation of the protein peaks at 27.6 kDa and slightly at 31.2 kDa. The spectra also show presence of the peak 32.8 kDa in lamb 4210.

The lambs 4203, 4210 and 5019 had an up-regulation of protein peak 27.6 kDa according to figure 4.8 and table 4.5 and 4.6.

Lamb 4203 had the lowest intensity for peak 27.6 kDa (table 4.5 and 4.6. Generally this lamb had the lowest intensities over the whole mass range.

The peak intensity for the 27.6 kDa peak in lamb 4210 was 29.0 and 64.0 in parallel I and II, respectively (table 4.5 and 4.6). Reasons for these differences between the parallels are discussed under figure 4.7. This lamb also had a peak at 32.8 kDa that only were present in this lamb eight weeks post inoculation in parallel I and II with peak intensity of 14.0 and 16.0 (table 4.5 and 4.6). This could be an individual response, an individual difference, or a contamination in the sample.

The highest up-regulation for the 27.6 k.Da peak was found in lamb 5019, where intensities was 55.0 and 65.5 in parallel I and II, respectively (table 4.5 and 4.6).

A shoulder appears at 870 Da upstream for the main peak. This might be two bounded proteins or a protein modificated several times.

According to the ProteinChip® software the 27.6 kDa peak is the same protein as in figure 4.7/table 4.3 and 4.4 at 13.96 kDa. The 13.96 kDa peak is thus double charged.

Lambs 4210 and 5019 had traces of peak 31.2 kDa in parallel II with an intensity of 2.5 (table 4.6).

Table 4.5: Overview of peak intensities at 27.6, 31.2 and 32.8 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell I

Sample name	Sampling date	Peak Intensity at 27.6 kDa	Peak Intensity at 31.2 kDa	Peak Intensity at 32.8 kDa
4249 I	14.09.2004	3.0	-	-
5076 I	14.09.2005	-	-	-
4203 I	14.09.2004	-	-	-
4210 I	14.09.2004	-	-	-
5019 I	14.09.2005	-	-	-
5029 I	14.09.2005	-	-	-
4210 I	29.09.2004	-	-	-
5019 I	28.09.2005	-	-	-
5029 I	28.09.2005	-	-	-
4203 I	13.10.2004	-	-	-
4210 I	13.10.2004	-	-	-
5019 I	12.10.2005	-	-	-
5029 I	12.10.2005	-	-	-
4203 I	10.11.2004	-	-	-
4210 I	10.11.2004	29.0	-	14.0
5019 I	09.11.2005	55.0	-	-

Table 4.6: Overview of peak intensities at 27.6, 31.2 and 32.8 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell II

Sample name	Sampling date	Peak Intensity at 27.6 kDa	Peak Intensity at 31.2 kDa	Peak Intensity at 32.8 kDa
4249 II	14.09.2004	-	-	-
5076 II	14.09.2005	-	-	-
4203 II	14.09.2004	-	-	-
4210 II	14.09.2004	-	-	-
5019 II	14.09.2005	-	-	-
5029 II	14.09.2005	-	-	-
4210 II	29.09.2004	-	-	-
5019 II	28.09.2005	-	-	-
5029 II	28.09.2005	-	-	-
4203 II	13.10.2004	-	-	-
4210 II	13.10.2004	-	-	-
5019 II	12.10.2005	-	-	-
5029 II	12.10.2005	-	-	-
4203 II	10.11.2004	2.0	-	
4210 II	10.11.2004	64.0	2.5	16.0
5019 II	09.11.2005	66.5	2.5	-

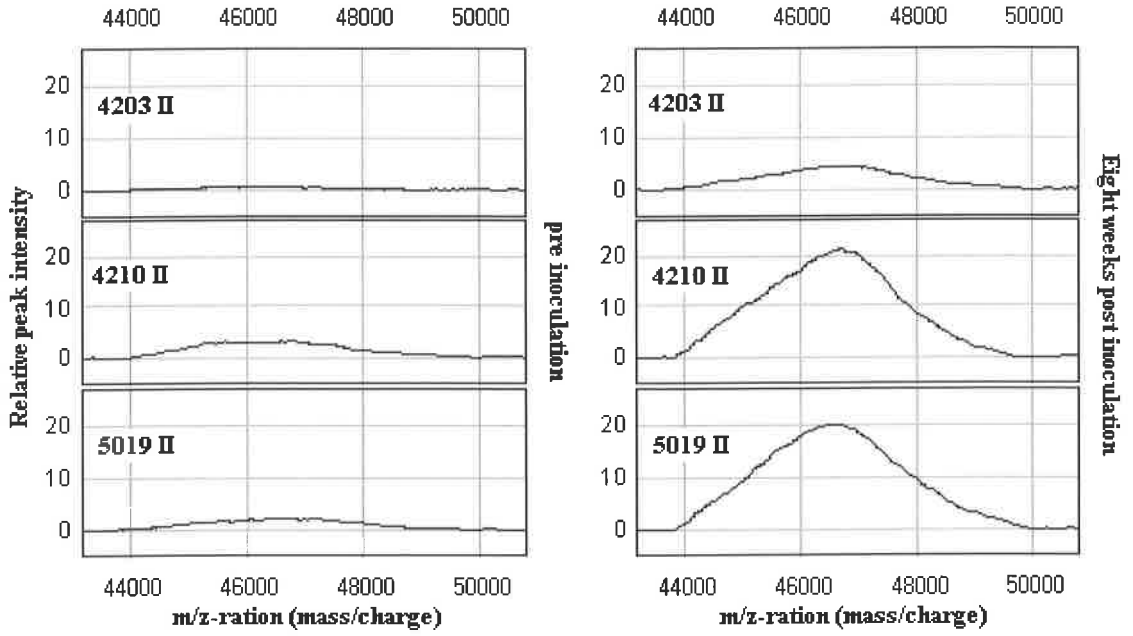


Figure 4.9: Mass spectra (High mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF1 on Q10 chip (see 3 Experiment) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 44.0 to 50.0 kDa. The spectra originate from serum samples from lamb 4210, 4203 and 5019 in parallel II, to the left; pre-inoculation, sampling date 14.09.2008 and 14.09.2008, respectively (table 4.8). To the right are spectra from the same lambs pro inoculation, sampling date 10.11.04 and 09.11.05, respectively (table #.4.2). The figure illustrates up-regulation of the protein peak at 46.6 kDa.

The peak at 46.6 Da illustrates up-regulation in lambs 4203, 4210 and 5019 in parallel II. Lamb 4203 had a poor up-regulation with a difference of 5.0 and 4.5 in intensity in parallel I and II, respectively(table 4.7 and 4.8)..

Lamb 4210 did have trace of the 46.6 kDa peak in both parallels with an intensity of 2.0 and 3.0 pre inoculation (table 4.7 and 4.8). The intensity differences were 8 and 19.5 in parallel I and II, respectively (table 4.7 and 4.8).

The intensity difference for lamb 5019 for the 46.6 kDa peak was 12 and 18 in parallel I and II, respectively (table 4.7 and 4.8).

Peak 46.6 kDa was also present in the control samples 4249 and 5076 with an intensity of 5.0 for 4249 in both parallels and 5.0 and 4.0 for 5076 in parallel I and II, respectively (table 4.7 and 4.8)

Table 4.7: Overview of peak intensity at 46.6 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell I

Sample name	Sampling date	Peak Intensity at 46.6 kDa
4249 I	14.09.2004	5.0
5076 I	14.09.2005	5.0
4203 I	14.09.2004	-
4210 I	14.09.2004	2.0
5019 I	14.09.2005	-
5029 I	14.09.2005	-
4210 I	29.09.2004	2.0
5019 I	28.09.2005	-
5029 I	28.09.2005	-
4203 I	13.10.2004	-
4210 I	13.10.2004	-
5019 I	12.10.2005	-
5029 I	12.10.2005	-
4203 I	10.11.2004	5.0
4210 I	10.11.2004	10.0
5019 I	09.11.2005	12.0

Table 4.8: Overview of peak intensity at 46.6 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell II

Sample name	Sampling date	Peak Intensity at 46.6 kDa
4249 II	14.09.2004	5.0
5076 II	14.09.2005	4.0
4203 II	14.09.2004	-
4210 II	14.09.2004	3.0
5019 II	14.09.2005	2.0
5029 II	14.09.2005	-
4210 II	29.09.2004	-
5019 II	28.09.2005	-
5029 II	28.09.2005	-
4203 II	13.10.2004	-
4210 II	13.10.2004	-
5019 II	12.10.2005	-
5029 II	12.10.2005	-
4203 II	10.11.2004	4.5
4210 II	10.11.2004	21.5
5019 II	09.11.2005	20.0

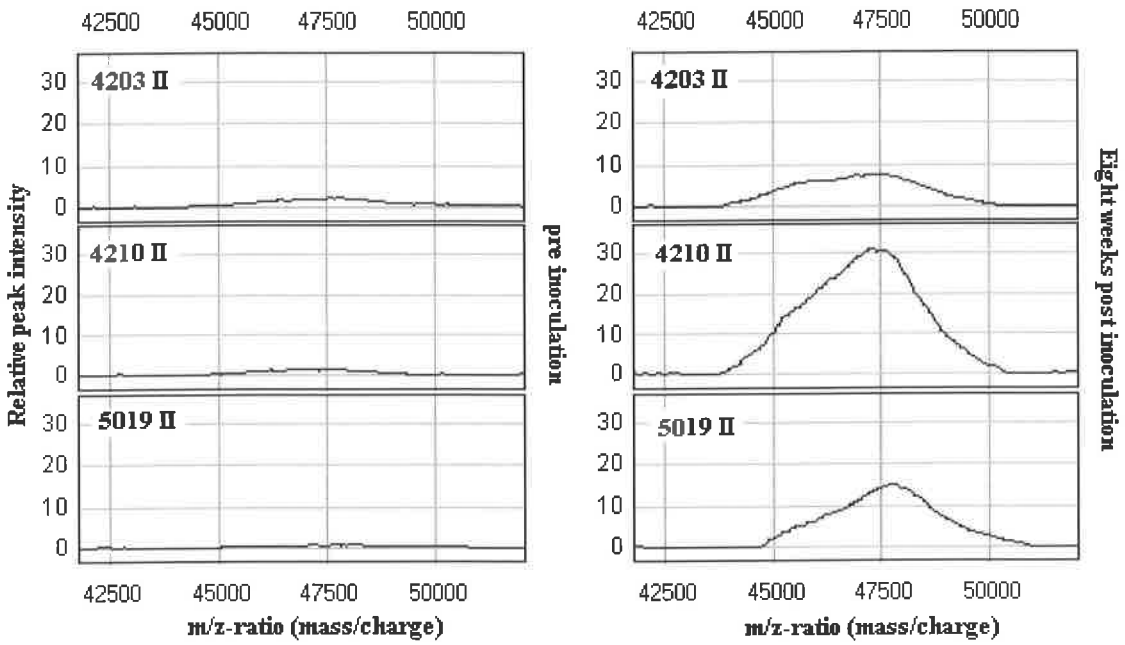


Figure 4.10: Mass spectra (High mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF1 on CM10 chip (see Chapter 3 Experiments) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 42.5 kDa to 5.25 kDa. The spectra originate from serum samples from lamb 4210, 4203 and 5019 in parallel II, to the left; pro inoculation, sampling date 14.09.2008 and 14.09.2008, respectively (table 4.10). To the right are spectra from the same lambs pre inoculation, sampling date 10.11.04 and 09.11.05, respectively (table 4.10). The figure illustrates up-regulation of the protein peak at 46.6 kDa.

The 46.6 kDa peak in figure 4.10 seem to be the same protein as in figure 4.9.

Lamb 4203 had an intensity difference of 6.5 and 5.5, in parallel I and II for the 46.6 kDa peak (table 4.9 and 4.10). For lamb 4210 the difference in peak intensity for the 46.6 kDa peak were 16.0 and 27.5 in parallel I and II, respectively (table table 4.9 and 4.10). Lamb 5019 had an intensity difference, for the 46.6 kDa peak, of 21.5 and 15.0 in parallel I and II, respectively (table table 4.9 and 4.10). Compared to the respected spectrum for 5019 in figure 4.9 the 46.6 kDa peak had poorer up-regulation with CM10 chip than with Q10 chip.

4210 had a slight up-regulation on 3.0 and 6.0 in parallel I and II, respectively, for the 46.6 kDa peak two weeks post inoculation (table 4.9 and 4.10). This is the acute phase of the disease.

The controls, 4249 and 5076, were in possession of this protein peak with intensities on 7.0 and 4.0 in parallel I (table 4.9), nothing in parallel II (table 4.10).

Table 4.9: Overview of peak intensity at 46.6 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell I

Sample name	Sampling date	Peak Intensity at 46.6 kDa
4249 I	14.09.2004	7.0
5076 I	14.09.2005	4.0
4203 I	14.09.2004	-
4210 I	14.09.2004	-
5019 I	14.09.2005	-
5029 I	14.09.2005	-
4210 I	29.09.2004	3.0
5019 I	28.09.2005	-
5029 I	28.09.2005	-
4203 I	13.10.2004	-
4210 I	13.10.2004	-
5019 I	12.10.2005	3.0
5029 I	12.10.2005	-
4203 I	10.11.2004	6.5
4210 I	10.11.2004	16.0
5019 I	09.11.2005	21.5

Table 4.10: Overview of peak intensity at 46.6 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell II

Sample name	Sampling date	Peak Intensity at 46.6 kDa
4249 II	14.09.2004	-
5076 II	14.09.2005	-
4203 II	14.09.2004	2.0
4210 II	14.09.2004	3.0
5019 II	14.09.2005	-
5029 II	14.09.2005	-
4210 II	29.09.2004	6.0
5019 II	28.09.2005	-
5029 II	28.09.2005	-
4203 II	13.10.2004	7.0
4210 II	13.10.2004	-
5019 II	12.10.2005	-
5029 II	12.10.2005	2.0
4203 II	10.11.2004	7.5
4210 II	10.11.2004	30.5
5019 II	09.11.2005	15.0

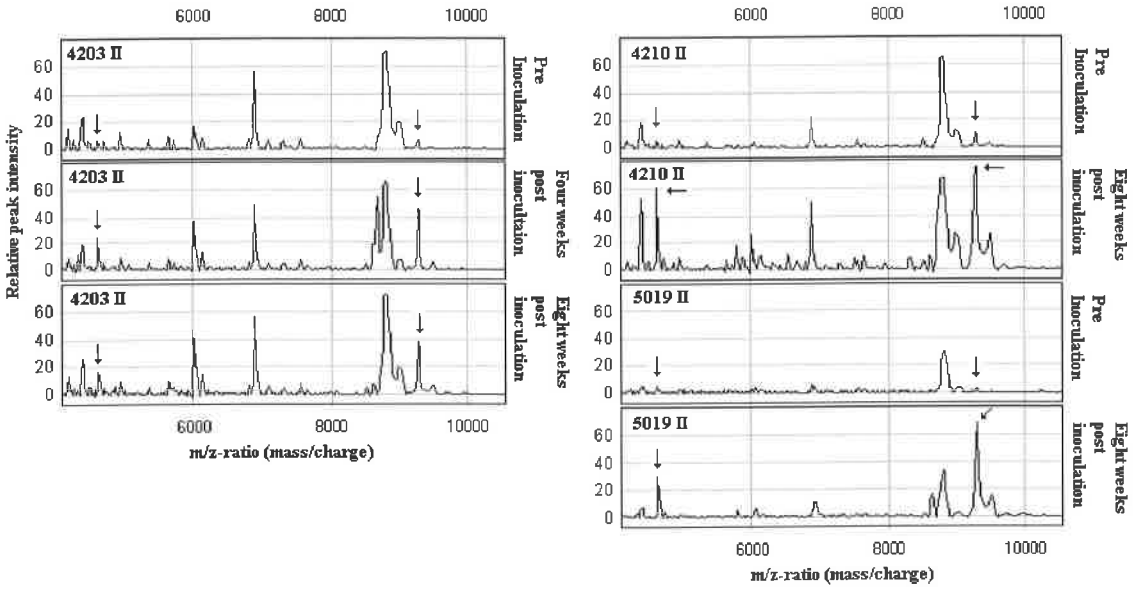


Figure 4.12: Mass spectra (Low mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF1 on CM10 chip (see Chapter 3 Experiments) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 4.0 kDa to 11.0 kDa. The spectra originate from serum samples from lamb 4203, 4210 and 5019 in parallel II, to the left; 4203 pre-inoculation, four and eight weeks post inoculation, sampling dates 14.09.2004, 13.10.2004 and 10.11.2004, respectively (table 4.12). To the right are spectra from lambs 4210 and 5019, pre-inoculation and eight weeks post inoculation, sampling dates 14.09.05 and 09.11.05, respectively (table 4.12). The figure illustrates up-regulation of the protein peaks at 4.6 and 9.3 kDa. Arrows point at the respected peaks.

The 4.6 kDa peak seemed slightly up-regulated four weeks post inoculation according to figure 4.12 and the tables: 4.11 and 4.12. The intensity increased with 4.5 and 16.5 in parallel I and II. In parallel II the intensity decreased eight weeks after inoculation in lamb 4203. While in parallel I the intensity increases from four to eight weeks post inoculation. This phenomenon repeats for the peak at 9.3 kDa (figure 4.12, 4.11 and 4.12).

For the 9.3 kDa peak the intensity increases with 6.0 in parallel I four weeks post inoculation and another 26.0 eight weeks post inoculation, whereas the intensity increases with 26.0 and decreases with 8 in parallel II (4.11 and 4.12). This could insinuate a mix-up in the samples four and eight weeks post inoculation, but the other peaks in the spectra for the respected samples show increasing with time.

Lamb 4210 had lower intensity (2.0 and 5.0, parallel I and II, respectively) for peak 9.3 kDa pre inoculation compared to lamb 4203 for the respected peak (table 4.11 and 4.12). Lamb 4210 had an increase in intensity on 38 and 55 in parallel I and II, respectively for the 4.6 kDa peak. In parallel I the 4.6 kDa peak decreased below 2 four weeks post inoculation. The intensity of the 9.3 kDa peak increased with 67.0 in both

parallel I and II in lamb 4210. Also at this peak the intensity decreased four weeks post inoculation.

Lamb 5019 had the lowest intensities pre inoculation for the 4.6 and the 9.3 kDa peaks (4.11 and 4.12). The difference in intensity was 41.0 and 66.0 for the 4.6 kDa peak in parallel I and II and 59.5 and 66.0 for the 9.3 kDa peak in parallel I and II, respectively. The spectrum illustrating protein peaks for lamb 5019, which represent variant 1 had fewer peaks than the lambs in variant 2. The peaks in variant 2 had greater intensity according to variant 1. There could be higher concentration of these proteins in the sample from 5019, thus these proteins would overshadow other potential proteins. In figure 4.12 peaks are visible, but with poor intensity, in lamb 5019 which has greater intensity in variant 1, 4203 and 4210.

Peak 9.3 kDa is the same as is figure 4.6 on Q10 chip.

Table 4.11: Overview of peak intensities at 4.6 and 9.3 kDa the all the lambs, including controls, 4249 and 5076, pre and post inoculation. Parallel I

Sample name	Sampling date	Peak Intensity at 4.6 kDa	Peak Intensity at 9.3 kDa
4249 I	14.09.2004	10.0	31.0
5076 I	14.09.2005	7.0	18.0
4203 I	14.09.2004	5.5	-
4210 I	14.09.2004	2.0	-
5019 I	14.09.2005	6.5	4.5
5029 I	14.09.2005	9.0	2.0
4210 I	28.09.2004	-	2.5
5019 I	28.09.2005	3.0	4.5
5029 I	28.09.2005	9.0	5.0
4203 I	13.10.2004	10.0	6.0
4210 I	13.10.2004	-	-
5019 I	12.10.2005	12.0	18.5
5029 I	12.10.2005	8.0	3.0
4203 I	10.11.2004	16.0	32.0
4210 I	10.11.2004	40.0	67.0
5019 I	09.11.2005	47.5	64.0

Table 4.12: Overview of peak intensities at 4.6 and 9.3 kDa the all the lambs, including controls, 4249 and 5076, pre and post inoculation. Parallel II

Sample name	Sampling date	Peak Intensity at 4.6 kDa	Peak Intensity at 9.3 kDa
4249 II	14.09.2004	5.5	4.5
5076 II	14.09.2005	7.5	24.0
4203 II	14.09.2004	6.0	7.0
4210 II	14.09.2004	5.0	10.5
5019 II	14.09.2005	5.0	2.0
5029 II	14.09.2005	6.0	5.5
4210 II	28.09.2004	7.5	10.5
5019 II	28.09.2005	14.0	12.0
5029 II	28.09.2005	4.0	8.0
4203 II	13.10.2004	22.5	45.0
4210 II	13.10.2004	8.0	7.0
5019 II	12.10.2005	3.0	3.0
5029 II	12.10.2005	8.0	10.5
4203 II	10.11.2004	16.0	37.0
4210 II	10.11.2004	60.0	74.0
5019 II	09.11.2005	28.0	68.0

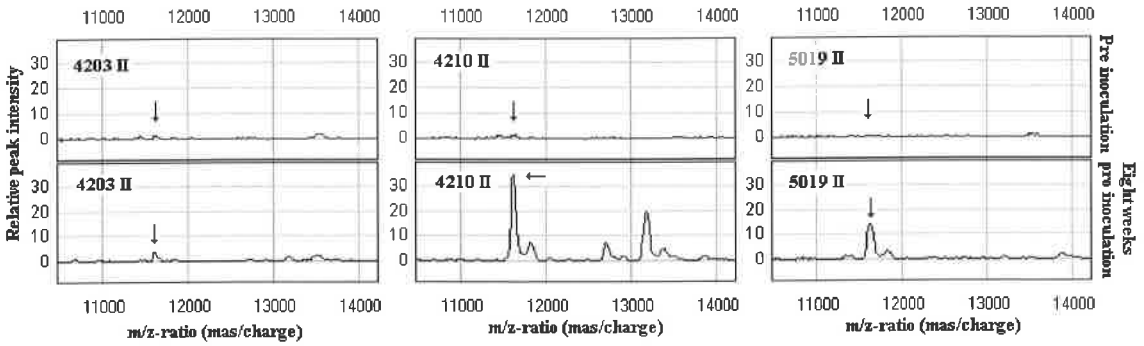


Figure 4.13: Mass spectra (High mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF1 on CM10 chip (see Chapter 3 Experiments) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 11.0 kDa to 14.0 kDa. The spectra originate from serum samples from lamb 4203, 4210 and 5019 in parallel II. All the upper spectra illustrate the samples pre-inoculation, sample dates 14.09.04 and 14.09.05(table 4.14). The lower spectra show samples eight weeks post inoculation, sampling dates 10.11.04 and 09.11.05 (table 4.14). From left to right; lambs 4203, 4210 and 5019. The figure illustrates up-regulation of the protein peak at 11.6 kDa. Arrows point at the respected peak.

In the figure:

For lamb 4203 the intensity difference for the 11.6 kDa peak were 4.0 and 3.5 in parallel I and II, respectively (table 4.13 and 4.14). The controls, 4249 and 5076, had an intensity at 3.5 and 2.5 in parallel I and >2.0 and 5.0 in parallel II, respectively(table 4.13 and 4.14).. The distinction between the infected lamb, 4203 and the controls 4249 and 5076 was rather poor and this may question the reliability on the respected peak as a biomarker.

The increase in intensity for the 11.6 kDa peak in lamb 4210 was 16.0 and 34.5 in parallel I and II, respectively(table 4.13 and 4.14).. For lamb 5019 the intensity difference were 24 and 14 in parallel I and II, respectively(table 4.13 and 4.14)..

Table 4.13: Overview of peak intensity at 11.6 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell I

Sample name	Sampling date	Peak Intensity at 11.6 kDa
4249 I	14.09.2004	3.5
5076 I	14.09.2005	2.5
4203 I	14.09.2004	-
4210 I	14.09.2004	-
5019 I	14.09.2005	-
5029 I	14.09.2005	-
4210 I	29.09.2004	3.0
5019 I	28.09.2005	-
5029 I	28.09.2005	-
4203 I	13.10.2004	-
4210 I	13.10.2004	-
5019 I	12.10.2005	2.0
5029 I	12.10.2005	-
4203 I	10.11.2004	4.0
4210 I	10.11.2004	16.0
5019 I	09.11.2005	24.0

Table 4.14: Overview of peak intensity at 11.6 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell II

Sample name	Sampling date	Peak Intensity at 11.6 kDa
4249 II	14.09.2004	-
5076 II	14.09.2005	5.5
4203 II	14.09.2004	-
4210 II	14.09.2004	-
5019 II	14.09.2005	-
5029 II	14.09.2005	-
4210 II	29.09.2004	3.5
5019 II	28.09.2005	-
5029 II	28.09.2005	-
4203 II	13.10.2004	5.0
4210 II	13.10.2004	-
5019 II	12.10.2005	-
5029 II	12.10.2005	-
4203 II	10.11.2004	3.5
4210 II	10.11.2004	34.5
5019 II	09.11.2005	14.0

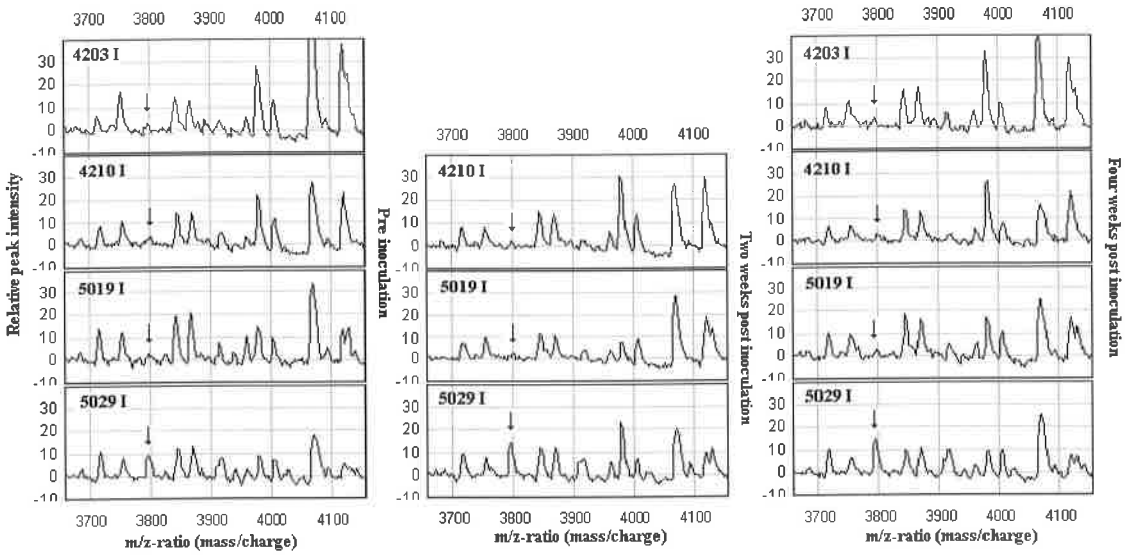


Figure 4.14: Mass spectra (Low mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF3 on Q10 chip (see Chapther 3 Experiments) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 36.5 kDa to 41.5 kDa. The spectra originat from serum samples from lamb 4203, 4210, 5019 and 5029 in parallel I, to the left; pre-inoculation, sampling dates 14.09.2008 and 14.09.2008, respectively (table 4.15). In the middle; two weeks post inoculation, lambs 4210, 5019 and 5029, sampling dates 29.09.04 and 28.09.05, respectively (table 4.15). To the right; four weeks post inoculation, lambs 4203, 4210, 5019 and 5029, sampling dates 13.10.04 and 12.10.05, respectively (table 4.15). The arrows point at the protein peak at 3.8 kDa present in all the 5029 lambs only.

Since there was no sample received for lamb 5029 eight weeks post inoculation, and most of the biomarkers were present at this stage of the disease, it was impossible to compare this lamb with the other ones. Figure #.8 shows a peak at 3.8 kDa only present in lamb 5029. The peak is present pre inoculation, sampling date 14.09.05, two weeks post inoculation, sampling date 28.09.05, and four weeks post inoculation, sampling date 12.10.05 (table 4.15 and 4.16).

Table 4.15: Overview of peak intensity at 38.0 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell I

Sample name	Sampling date	Peak Intensity at 3.8 kDa
4249 I	14.09.2004	-
5076 I	14.09.2005	5.0
4203 I	14.09.2004	3.0
4210 I	14.09.2004	3.0
5019 I	14.09.2005	3.0
5029 I	14.09.2005	9.0
4210 I	29.09.2004	2.5
5019 I	28.09.2005	2.5
5029 I	28.09.2005	14.0
4203 I	13.10.2004	3.0
4210 I	13.10.2004	3.0
5019 I	12.10.2005	2.5
5029 I	12.10.2005	15.0
4203 I	10.11.2004	-
4210 I	10.11.2004	2.0
5019 I	09.11.2005	3.0

Table 4.16: Overview of peak intensity at 38.0 kDa for all the lambs, including controls, 4249 and 5076, pre and post inoculation, parallell II

Sample name	Sampling date	Peak Intensity at 3.8 kDa
4249 II	14.09.2004	3.0
5076 II	14.09.2005	-
4203 II	14.09.2004	3.5
4210 II	14.09.2004	2.0
5019 II	14.09.2005	3.5
5029 II	14.09.2005	16.0
4210 II	29.09.2004	4.0
5019 II	28.09.2005	4.0
5029 II	28.09.2005	28.0
4203 II	13.10.2004	2.0
4210 II	13.10.2004	3.0
5019 II	12.10.2005	3.0
5029 II	12.10.2005	16.0
4203 II	10.11.2004	2.5
4210 II	10.11.2004	-
5019 II	09.11.2005	-

4.4 Variant differences shown in SELDI spectra

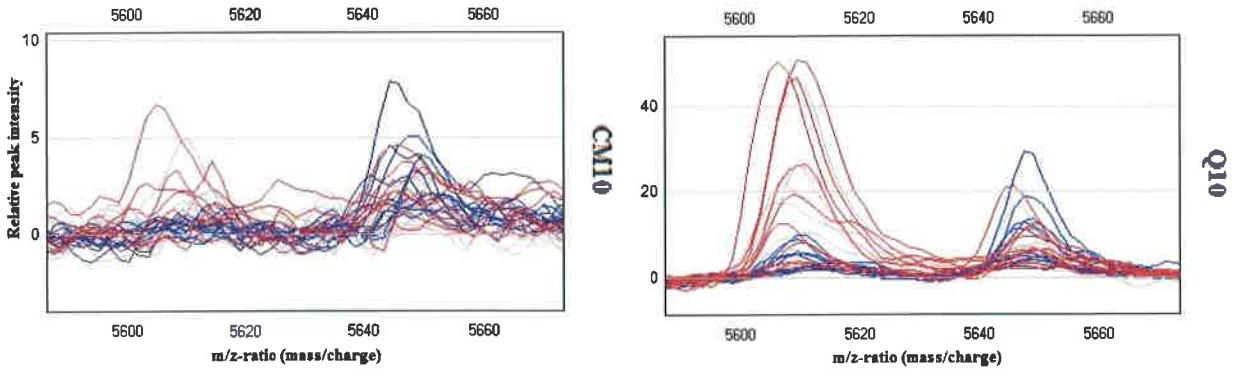


Figure 4.15: Overlaid mass spectra. Low mass, displaying protein peaks with Ciphergen ProteinChip® Software 3.1. The samples are from the fraction QF4 on CM10 chip (left) and Q10 chip (right) (seeChapther 3 Experiments) The x-axis shows m/z-ratio (m/z), the y-axis shows relative peak intensity. The spectra display the mass area from 5.58 kDa to 5.68 kDa. The spectra originate from serum samples from all the lambs; 4203, 4210, 5029, 5019, at all sample dates and controls; 4249 and 5076, in parallel I and II. The red spectra display the infected lambs from variant 1; 4203 and 4210, while the blue spectra display the infected lambs from variant 2, 5019 and 5029, the grey spectra illustrates the controls and both the variants pre inoculation.

CM10

The spectra in figure 4.15 shows overlaid mass spectra. The red colored spectra which represent variant 1, lamb 4203 and 4210, dominates the peak at 5.61 kDa and the blue spectra which represent variant 2, lamb 5019 and 5029, dominates the 5.65 kDa peak. Figure #.9 shows a variance difference.

Q10

As in figure 4.15 the 5.61 kDa peak and the 5.65 kDa peak illustrate a difference between variant 1, 4203 and 4210, and variant 2, 5019 and 5029. These spectra originate from samples on Q10 chip. The samples were treated with the ProteinChip® serum fractionation kit which renders the eluted proteins with negative charge. The Q10 chip has affinity for proteins with negative charge; hence the greater intensity on the protein peaks in the spectra from Q10 chip (right in figure 4.15) compared to the peaks in spectra from CM10 (left in figure 4.15)

4.3.1 Multivariate analysis with PRIMER 6

(PRIMER: Plymouth Routines In Multivariate Ecological Research)

All plots are generated from clustering spectra of protein profiles from SELDI analysis.

All datasets are treated in the same manner prior to the MDS analysis. Raw clustered data were square root transformed and a resemblance matrix, calculated with Bray-Curtis similarities, was obtained from the PRIMER v6 software. For information about MDS analysis see the Software chapter (Chapter 2.8.2 PRIMER)

Primer v6 allows for implementation of factors to the datasets. Two factors were applied to each of the datasets presented as MDS-plots below. The first factor is the fractions from the Q-Kit fractionation, visualized with different colors in the plots. The second factor is the Anaplasma phagocytophilum variant number, Variant 1 and 2, determined from PCR 16s mRNA analysis (see chapter 1 Introduction). These are visualized by numbers in the plots.

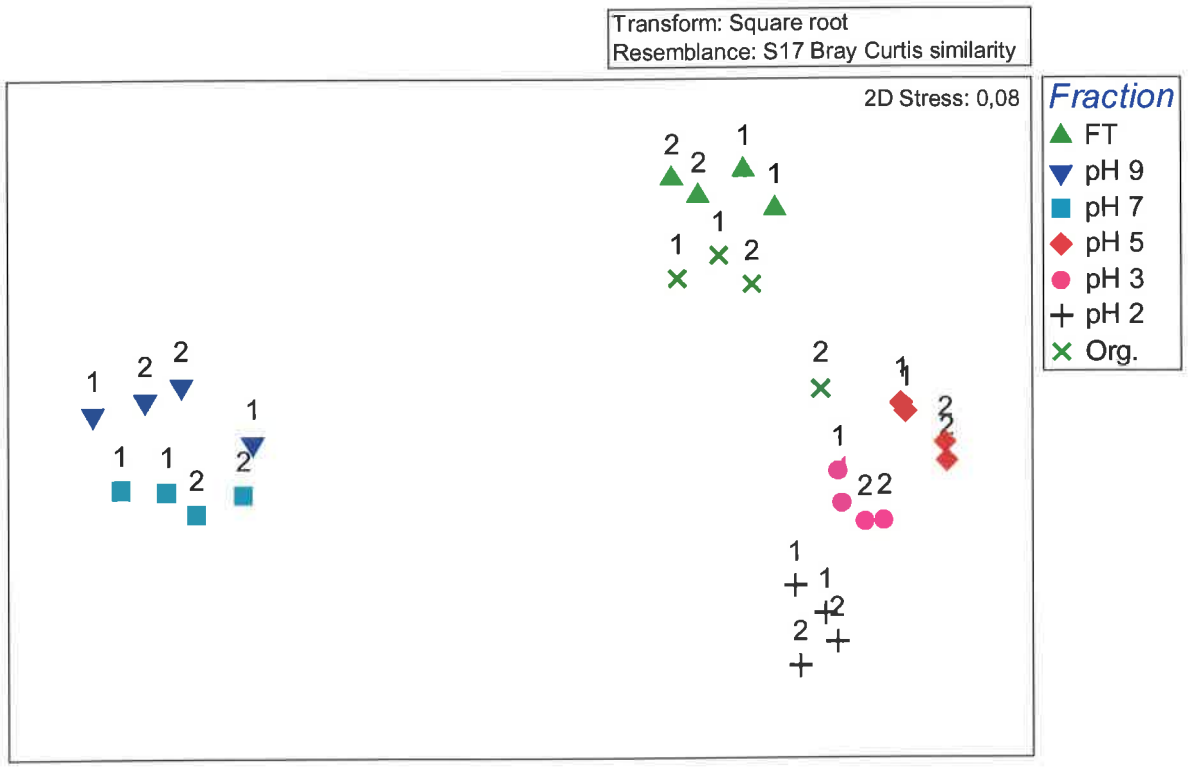


Figure 4.16: 2D MDS-plot of all fractions from lambs 4203 and 5029, Q10 chip, low mass area. 1 – variant 1, 2 – variant 2. QFT (FT), QF1 (pH 9), QF2 (pH7), QF3 (pH 5), QF4 (pH 3), QF5 (pH 2), QF6 (Org). Software used: Primer 6.

The dataset represented as MDS-plot in Figure 4.16 originates from the Q10 chip, low mass area (1000 Da – 30 kDa), hence small anionic proteins is the source of the distribution shown. As anticipated, the different fractions separate well. Altering pH in the ambient solution will change protein properties like charge state and might also change their conformation.

The overall tendency is that the pH 9 (QF1) and pH 7 (QF2) groups apart from the other fractions. Within the other fractions (pH 5, 3 and 2, FT and Org.) to the right in the plot, the flow through fraction (FT) and the organic fraction (Org.) also seem to group together. This tendency of grouping is not only seen on Q10, low mass area, but in the Q10 high mass area and in the CM10 datasets as well. Figure 4.16 also reveals that the two variants are separated from each other within each fraction. This is discussed in further detail below.

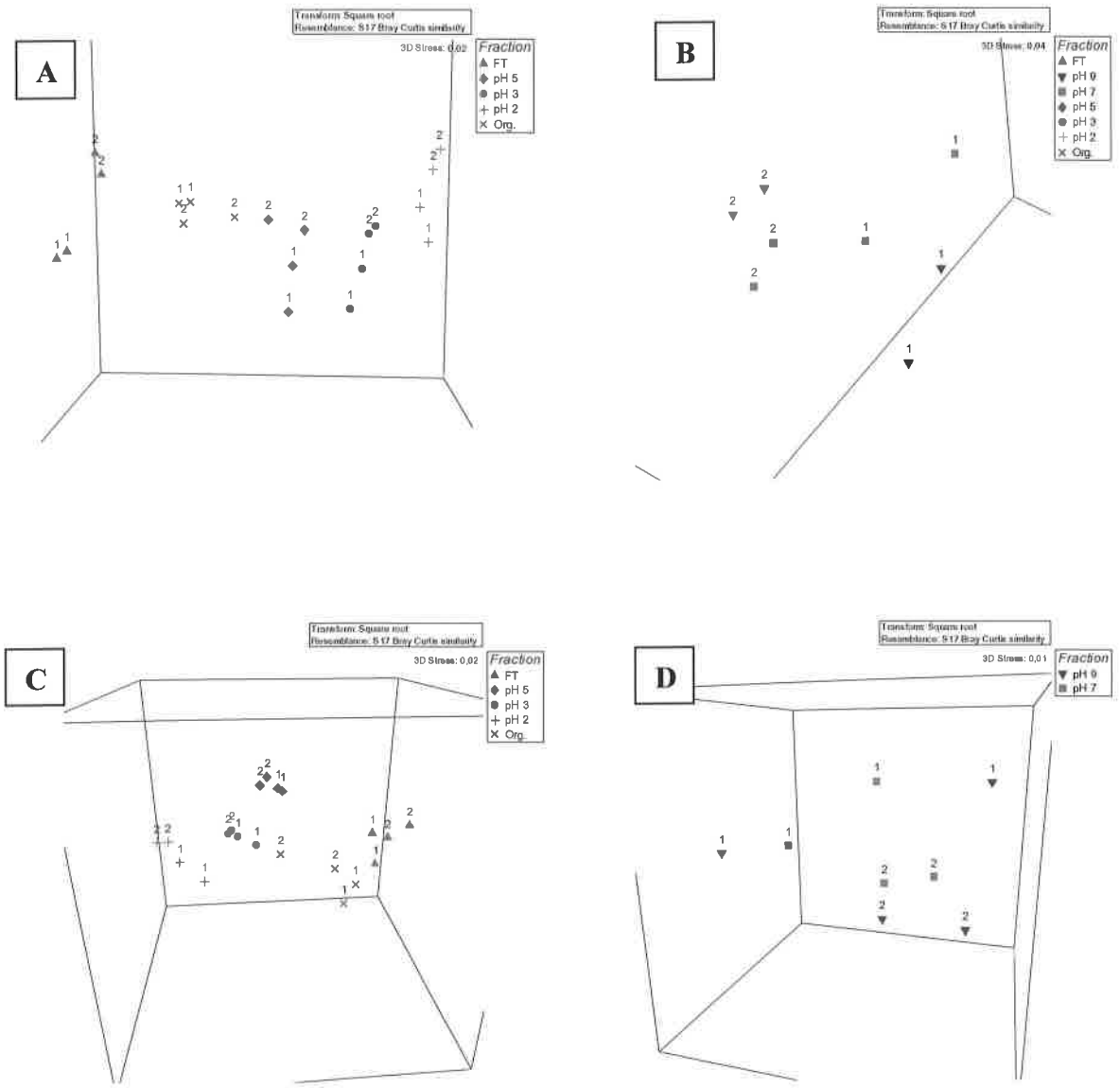


Figure 4.16: 3D MDS plot for lambs 4203 and 5029, Q10 chip. A and B Low mass area. C and D: High Mass area. A and C: QFT (FT), QF3 (pH 5), QF4 (pH 3), QF5 (pH 2), QF6 (Org). B and D: QF1 (pH 9), QF2 (pH 7). 1: variant 1, 2: variant 2. Software used: Primer v6.

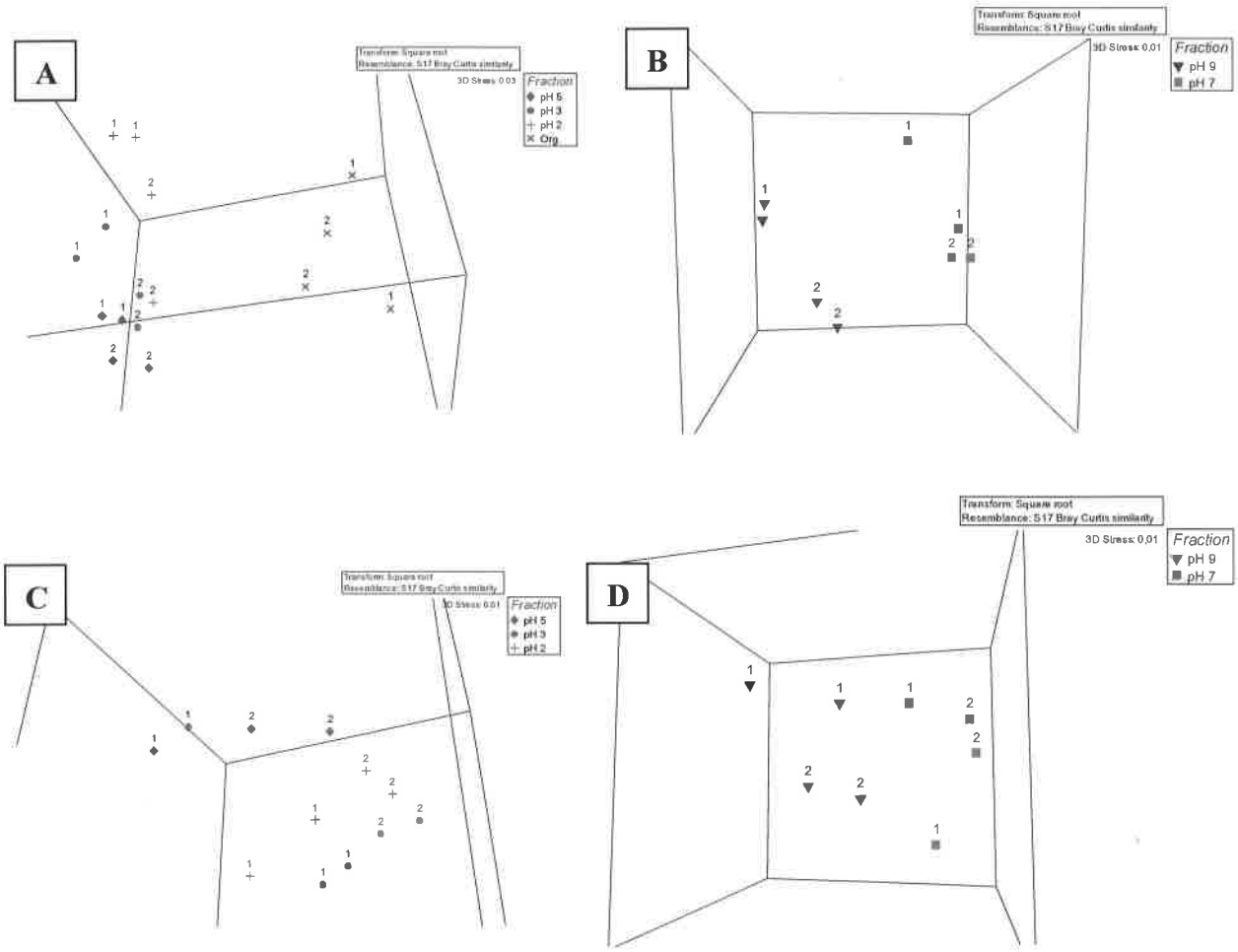


Figure 4.17: 3D MDS plot for lambs 4203 and 5029, CM10 chip. A and B: Low Mass area. C and D: High Mass area. A and C: QF3 (pH 5), QF4 (pH 3), QF5 (pH 2), QF6 (Org – not in C). B and D: QF1 (pH 9), QF2 (pH7). 1: variant 1, 2: variant 2. Software used: Primer v6.

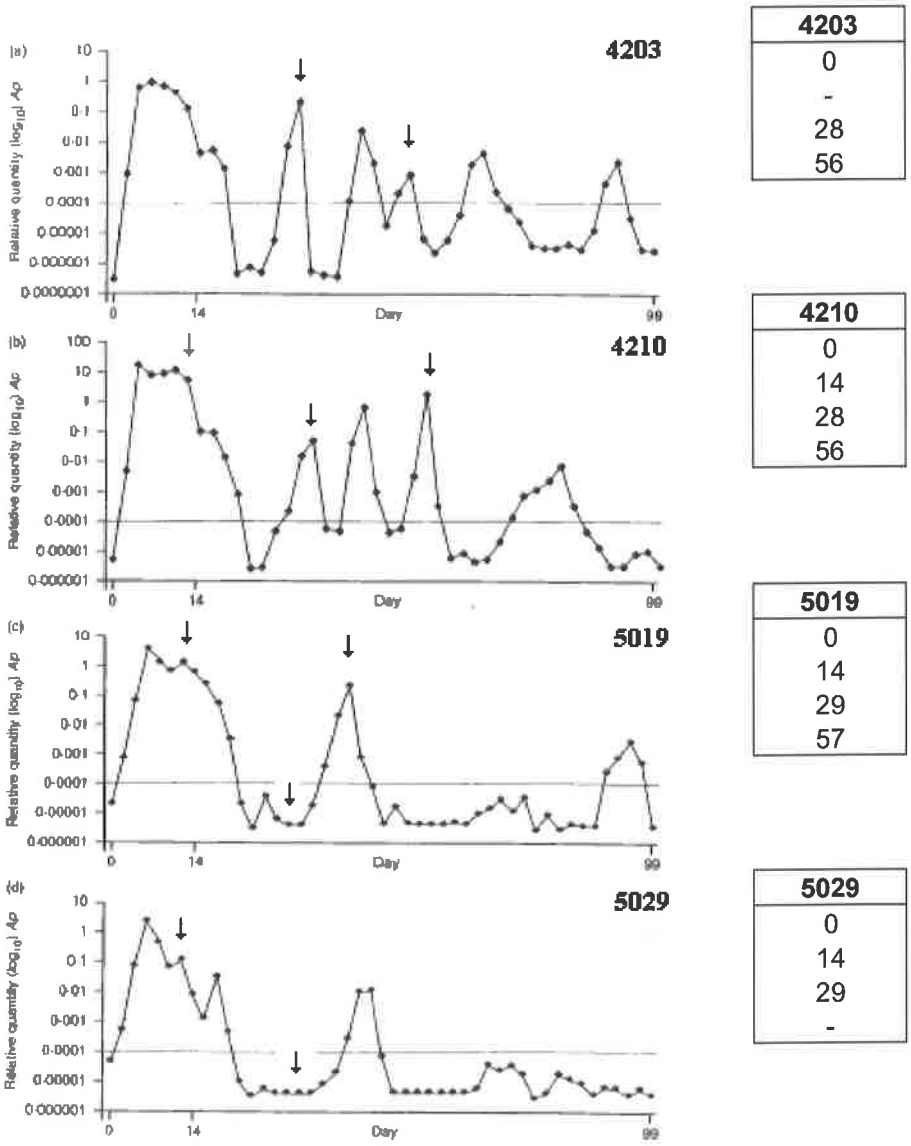
PRIMER v6 allows for making MDS subsets directly from a MDS-plot. In Figure 4.17 this is done with datasets from Q10, low mass (A and B) and Q10, high mass (C and D).

Clearly, there is a statistical argument for saying that variant 1 and 2 can be separated at the proteomic level. Within each fraction, both in the LM area and the HM area, variants are evidently separated and groups according to its respective parallel.

From a proteomic point of view this separation tells us that the serum proteome are affected by which variant of *A. phagocytophilum* the lambs are infected by. Three possibilities arises, firstly, the protein profile is altered due to immunoresponses in the lamb itself. Secondly, the protein profile is altered due to extracellular proteins secreted from the bacteria, thirdly, a combination of the two.

4.4 Discussion

The samples received from the Norwegian School of Veterinary Science were picked on the basis of the peaks of *Anaplasma phagocytophilum* in the lamb's peripheral blood (figure 4.18). It is likely to assume that the sampling for variant 2, in 2005, were based on the selected sampling dates from the experiment from 2004, performed on variant 1. The selections from variant 1, 4203 and 4210, seem to be chosen from peaks, while the selections from variant 2, 5019 and 5029, seem to be chosen to match the previous sample dates. The fact that the peaks are more numerous in the mass spectra for variant 1 compared to variant 2 correlates with the graph in figure 4.18.



4203
0
-
28
56

4210
0
14
28
56

5019
0
14
29
57

5029
0
14
29
-

Figure 4.18: Relative quantification of *Anaplasma phagocytophilum* (*Ap*) in the peripheral blood of four experimentally infected lambs. The lambs were inoculated on day 0, two with variant 1, 4203 and 4210, and two with variant 2, 5019 and 5029, of *Ap*. The infection was detected with real-time PCR. Samples with relative quantitation below 0-0001 were considered negative. Boxes to the right illustrates the days post inoculation the samplings were performed.

Proteins that seem to up-regulate with time are 8.5 kDa, 9.27 kDa, 11.6 kDa and 46.6 kDa. These peaks are recognized as abundant in the controls and concentration seems to increase as the infection progresses, all of them with highest abundance after eight weeks of infection.

These proteins origins from the lambs themselves (found in control samples) and they might be involved in an immunoresponse reaction triggered by the *A. phagocytophilum* infection.

Two protein peaks appear post-inoculation, 13.96 and 27.6 kDa. This is the same protein were 13.96 kDa is recognized as the double-charged ion of 27.6 kDa. This protein is not seen until after eight weeks of infection and might be an extracellular protein from *A. phagocytophilum*. This could be a possible biomarker for the disease, confirming the presence of the bacteria in the animals. However, the detection of the protein at this late stage of infection makes it unusable as a biomarker for early diagnosis.

Two other unique proteins appear post-inoculation, 31.2 kDa and 32.8 kDa, these are only seen in one parallel (31.2 kDa) or in a single individual (32.8 kDa) and cannot be considered as potential biomarkers.

Three proteins with transient behavior during the infection period are recognized, 3,8 kDa, 4.6 kDa and 9.3 kDa. The 4.6 kDa and 9.3 kDa is the same protein, with 4.6 kDa as the double-charged ion. These are all present in the controls, hence they origin from the lambs proteome and might be involved in immunoresponses. The transient behavior might indicate an alteration, at the proteomic level, caused by immunoresponses as the infection progresses.

The differences between the two variants of *A. phagocytophilum* might be found as unique peaks in the spectra, as shown in figure 4.15. There is however a rationale for believing that the two variants posses a very similar proteome and that the differences they express indirectly to an animal's blood, either with extracellular proteins originating from the bacteria itself or from immunoresponses in the infected animal, is neglectable. If any, we wanted to use multivariate methods to unveil these differences.

The multivariate analysis does indeed confirm differences between the two variants. This is seen in all fractions in both the low mass- and the high mass area, and also on both chip types. Which proteins that contributes to this separation is not known to this point, but it is possible to unveil this information by using statistical software.

4.5 Further research for the Sjodogg project

To increase the reliability on these possible biomarkers the experiment would have to be repeated several times. With several samples from a larger number of individuals within the two variants the results would be more trustworthy and representative. With several individuals there is a better basis for statistics and the variants might difference even more. Further analysis on the spectra in Ciphergen ProteinChip® Software might emerge the biomarkers. Spectrum treating and the best way of comparison holds a lot of time, the total amount of spectra exceeded 5000!

To identify the possible biomarkers the respected proteins would have to be purified and prepared prior to identification. This is a long and time-consuming process and was not possible in this project.

5 Conclusion

For serum samples, the ProteinChip® Serum Fractionation kit turned out to be the best method for protein peak discovery with SELDI-TOF-MS.

Up-regulated proteins were found eight weeks post inoculation at 8.5 kDa, 9.27 kDa, 11.6 kDa and 46.6 kDa, these proteins could be an immune response from the lamb. At 27.6 kDa a protein might confirm presence of extracellular proteins from the bacteria, A. phagocytophilum in the lambs, thus a potential biomarker.

The multivariate analysis confirms the differences between variant 1 and 2, and that it should be possible to distinguish between the two.

The research for biomarkers with Ciphergen ProteinChip® Software and multivariatate analysis with PRIMER 6 insinuates that it is possible to find a way to diagnose infected animals with certainty, though further research is needed. Further research might emerge unique biomarkers for the disease, Sjodogg, and the variants 1 and 2.

The methods used in this thesis might, in the future, be beneficial to diagnose tick borne diseases in humans.

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