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Gene expression and BSE progression in beef cattle

by

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To my late father Jan Bartusiak

Abstract

Bovine Spongiform Encephalopathy (BSE) belongs to a group of neurodegenerative diseases known as transmissible spongiform encephalopathies (TSEs) which affect many species. From 1986 more than 184,000 cattle in the UK have been confirmed to be infected with this disease, and in Canada total losses to the economy reached $6 billion.

This study examines the gene expression in three major innate immunity components: complement system, toll-like receptors, interleukins, and selected proteins of their signaling pathways. Quantitative real time polymerase chain reaction analyses were performed on *caudal medulla* samples to identify differentially expressed genes between non-exposed and orally challenged animals.

In general, immune genes were down-regulated in comparison to non-challenged animals during first 12 months of disease with a tendency to be up-regulated at terminal stage of BSE.

The results from this study provide a basis for further research on the mechanisms modifying immune responses and altering progression of the disease.

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List of Abbreviations

AD – Alzheimer's disease

Ag-Ab – Antigen-Antibody Complex

Ag-CS – Antigen-Complement System Protein Complex

APC – Antigen Presenting Cell

BASE – Bovine Amyloidotic Spongiform Encephalopathy

BBB – Blood Brain Barrier

BSE – Bovine Spongiform Encephalopathy

C1 – Complement Component 1

C1q – Complement Component 1, subcomponent q

C1qsrrs – C1 complex

C3 – Complement Component 3

C3b – Complement Component 3, subcomponent b

C3c – Complement Component 3, subcomponent c

C3d – Complement Component 3, subcomponent d

C4 – Complement Component 4

C5 – Complement Component 5

C6 – Complement Component 6

C7 – Complement Component 7

C8 – Complement Component 8

C9 – Complement Component 9

CD14 – Cluster of Differentiation 14

CD21 – Cluster of Differentiation 21

CD35 – Cluster of Differentiation 35

cDNA – Cellular Deoxyribonucleic Acid

CFB – Complement Factor B, factor B

CJD – Creutzfeldt - Jakob disease

CNS – Central Nervous System

CpG – Phosphate Linked Cytosine and Guanine

CR1 – Complement Receptor 1

CR2 – Complement Receptor 2

CRP – C-reactive Protein

CS – Complement System

CV – Coefficient of Variation

CWD – Chronic Wasting Disease

DNA – Deoxyribonucleic Acid

EUE – Exotic Ungulate Encephalopathy

FDC – Follicular Dendritic Cells

FFI – Familial Fatal Insomnia

FSE – Feline Spongiform Encephalopathy

GAPDh – Glyceraldehyde 3-phosphate Dehydrogenase

GM-CSF – Granulocyte Macrophage Colony Stimulating Factor

GSS – Gerstmann–Straussler–Scheinker Syndrome

HD – Huntington's disease

HK genes – Housekeeping Genes

iC3b – Inactive Complement Component 3, subcomponent b

IFN-β – Interferon β

IFN-γ – Interferon-γ

IL-1 – Interleukin 1

IL-10 – Interleukin 10

IL-12 – Interleukin 12

IL-1β – Interleukin 1, β chain

IL-2 – Interleukin 2

IL-6 – Interleukin 6

IL-8 – Interleukin 8

ILs – Interleukins

ISH – *In Situ* Hybrydysation

LPS – Lipopolysaccharides

LRS – Lymphoreticular System

MAC – Membrane Attacking Complex

MBL – Mannose-Binding Lectin

MBP – Myelin Basic Protein

mCD14 – Membrane Bound CD14

MHC – Major Histocompatibility Complex

MHC II – Major Histocompatibility Complex II

MIP1 – Macrophage Inflammatory Protein 1

MIP2 – Macrophage Inflammatory Protein 2

MOG – Myelin Oligodendrocyte Glycoprotein

mRNA – Messenger Ribonucleic Acid

MyD88 – Myeloid differentiation primary response gene-88

NF-kB – Nuclear Factor Kappa B

ODN – Synthetic Oligodeoxynucleotides

PAMPs – Pathogen Associated Molecular Patterns

PD – Parkinson's disease

PNS – Peripheral Nervous System

PrP^{BSE} – BSE Prion Particle

PrP^{C} – Cellular Form of Prion Protein

PrP^{CJD} – CJD Prion Particle

PrP^{CWD} – CWD Prion Particle

PrP^{RES} – Partially Resistant, Misfolded Form of Prion Protein

PrP^{Sc} – Scrapie Prion Particle

PSMB2 – proteasome, subunit beta, type 2

PSMD2 – proteasome, 26S non-ATPase, subunit 2

QRT-PCR – Quantitative Real-Time Polymerase Chain Reaction

RNA – Ribonucleic Acid

RT-PCR – Reverse Transcriptase Polymerase Chain Reaction

SAP – Serum Amyloid P

sCD14 – Soluble CD14

SD – Standard Deviation

SFI – Sporadic Fatal Insomnia

SSE – Subacute Spongiform Encephalopathy

TGF-β – Transforming Growth Factor β

TLR1 – Toll-Like Receptor 1

TLR2 – Toll-Like Receptor 2

TLR3 – Toll-Like Receptor 3

TLR4 – Toll-Like Receptor 4

TLR5 – Toll-Like Receptor 5

TLR6 – Toll-Like Receptor 6

TLR9 – Toll-Like Receptor 9

TLRs – Toll-Like Receptors

TME – Transmissible Mink Encephalopathy

TNF-α – Tumor Necrosis Factor

TSE – Transmissible Spongiform Encephalopathy

Chapter 1

General introduction

1.1. Introduction

Transmissible Spongiform Encephalopathies (TSEs) are group of diseases characterized by various neurological dysfunctions common to all affected species. Disease associated neuropathological changes are mainly observed in the central nervous system (CNS) and include: activation of glial cells, accumulation of abnormally folded host prion protein, neurodegeneration and neuronal loss with subsequent spongiform changes that lead to deterioration within the brain. Clinically the disease manifests itself with a long incubation period followed by severe and rapid neurodegeneration leading always to death of affected individuals.

Examples of TSE's in humans include: Creutzfeldt–Jakob disease (CJD) (Creutzfeldt 1920; Jakob 1921), Gerstmann–Straussler–Scheinker syndrome (GSS) (Gerstmann 1957), Familial Fatal Insomnia (FFI) (Lugaresi et al. 1986), Sporadic Fatal Insomnia (SFI) (Manetto et al. 1992) and kuru (Gajdusek and Zigas 1957; Zigas and Gajdusek 1957). TSEs affecting animals include Bovine Spongiform Encephalopathy (BSE) in

cattle (Wells et al. 1987), scrapie in sheep and goats (Leopoldt 1750), Chronic Wasting Disease (CWD) in white-tailed and mule deer, elk and moose (Williams and Young 1980), Feline Spongiform Encephalopathy (FSE) in cats (Leggett et al. 1990; Wyatt et al. 1991), Transmissible Mink Encephalopathy (TME) (Burger and Hartsough 1965; Hartsough and Burger 1965), and Exotic Ungulate Encephalopathy (EUE) in exotic ungulates (Jeffrey and Wells 1988; Kirkwood and Cunningham 1994). A list of TSEs can be found in Table 1.1.

Many different theories on the nature of the TSE infective agent have been proposed but in the course of time most of them were found groundless. The most popular hypotheses for TSEs' causative agent that survived confrontation with research are the virus and prion hypotheses.

Similarities in heat resistance as well as size between scrapie agent and viruses of Subacute Spongiform Encephalopathies (SSE) led Rohwer to the conclusion that scrapie could be caused by a virus (Rohwer 1984). The evidence for a viral cause was at the time persuasive, and this theory has since been supported by other researchers (Manuelidis 2007).

The second and most widely accepted theory states, that the infectious particle responsible for TSEs is a pathogenic

isoform of host cellular protein known as prion protein. The physiological prion protein known as PrP^C is a glycoprotein present in various tissues of the body with particularly high concentrations in lymphoreticular system (LRS) (Bruce et al. 2001) and nervous tissues (Beekes et al. 1996). The main role of LRS is to survey all entering or circulating antigens and mobilize an immune response against foreign microorganisms upon their discovery. The term "prion" was first proposed by Stanley Prusiner to denote a small proteinaceous infectious particle which was resistant to inactivation by most procedures that modify nucleic acids (Prusiner 1982). Despite being contradictory to the doctrine, that proteins cannot replicate and thus be infectious and cause diseases without nucleic acids, the prion hypothesis is now accepted by most researchers.

Historically, scrapie in sheep and goats was the first described TSE with the first publication dating back to the year 1750 (Leopoldt 1750). It took until the end of 1930s when it was undoubtedly demonstrated that scrapie is a transmissible disease (Cuillé and Chelle 1936). Certain clinical symptoms of scrapie are common to all TSE's and are characterized by slow, continuing development of neurologic anomalies: behavioral changes, tremors, lack of coordination, ataxia, recumbency and

sometimes blindness (Williams 2003). Clinical signs are accompanied by substantial weight loss that leads to general wasting of the infected animals. The dermatologic symptom pruritus (skin irritation) may develop in the course the disease forcing sheep to rub, scrape themselves against objects and that is where the genesis of the disease term "scrapie" lies.

Bovine Spongiform Encephalopathy commonly known as mad cow disease is a TSE of cattle. Since 1986 when the first case of BSE was reported more than 186000 cattle had the disease confirmed. The most probable cause of the disease was feeding cattle with recycled BSE contaminated ruminant tissue scraps such as meat-and-bone meal (Wilesmith et al. 1988; Wilesmith et al. 1991), commonly used feed in calves (Anderson et al. 1996). Due to such feeding practices it is estimated that from 1-3 million of cattle might have been exposed to the BSE agent (Anderson et al. 1996; Donnelly et al. 2002) creating the risk of BSE outbreak in the future. The oral transmission in calves was established in 1994 (Wells et al. 1994) and Foster successfully transmitted BSE to sheep a year earlier (Foster et al. 1993).

In 1987, one year from detection of first case of BSE, the UK Ministry of Agriculture officially reported that the disease was

found to be a new form of TSE, with clinical and histopathologic features similar to scrapie in sheep. Shortly after the disease was identified, important BSE control measures were implemented to stop BSE from spreading. Those included a ban on feeding animals with meat-and-bone meal and using bovine brains and spinal cords in feeding poultry and other nonruminant species (Schonberger 1998). In addition, in 1989, a ban on some high risk materials was introduced to protect human food supply. Implemented bans resulted in a dramatic decline of BSE occurrence in the UK, confirming indirectly the hypothesis that BSE contaminated feed was the likely cause of the outbreak. Measures so effective in the UK did not stop BSE from spreading to other countries, as the long incubation period meant that undetected infected animals, or animal byproducts, were transported to other countries in the early stages of the epidemic. Practically all European countries had confirmed cases of BSE. By the end of 2001 similar bans were in effect across the entire European Union, as new cases of BSE were being confirmed in many countries. The list of countries with confirmed BSE cases can be found in Table 1.2.

Recently, two atypical forms of BSE have been identified. H- and L-types can only be distinguished based on their different

molecular weight of prion protein in western blot analysis (Capobianco et al. 2007). The L-type of BSE is a form of bovine amyloidotic spongiform encephalopathy (BASE) which was first recognized in 2 cows in Italy (Beringue et al. 2006). In this particular atypical form of BSE the amyloid (insoluble fibrous protein) is distributed mostly in cerebrum (higher part of the brain), whereas in typical BSE amyloid tends to accumulate in cerebellum (the lower part of the brain). One experiment has shown the possible similarity of BASE with sporadic CJD in humans suggesting that some yet unidentified BSE strains could cause TSEs in other species and humans (Casalone et al. 2004). H-type atypical BSE can be differentiated from L-type by slightly higher molecular weight of PrP protein. In about 85% of cases atypical BSE affects cows older than 10 years of age, whereas classic BSE is typically recognized in much younger animals.

Coincidental occurrence of variant CJD and the outbreak of BSE in the UK suggested BSE as a probable cause of TSEs in humans. Some laboratory experiments and epidemiologic data supported the close relation between those two diseases (Will et al. 1996). Although the incidence of BSE and other TSEs seems to be on the decline, many millions of people and farmed

animals might have been exposed to BSE, which poses a serious threat of BSE related outbreak in the future.

Association of BSE with human TSEs has raised concerns about the health of farmed and free-ranging animals including deer, elk and moose. CWD was first identified among farmed mule deer in northern Colorado in the mid 1960s (Belay et al. 2004). It took a decade for CWD to be identified as a form of spongiform encephalopathy (Miller and Conner 2005). Year 1981 brought first CWD case in free-ranging elk in Colorado and several years later the disease was detected in various regions of Colorado and Wyoming (Miller et al. 2006). By the end of 2007 CWD in free-ranging and farmed herds has been identified in many western and eastern states of the US, 2 provinces in Canada: Alberta and Saskatchewan. Epidemiologic and experimental data indicated that CWD can be both transmitted by animal-animal contact, and saliva and feces contaminated environment (Lubick 2007; Miller et al. 2004). Such a rapid spread posed an enormous pressure on farmed animal industry with the real possibility of similar outbreak of CWD in US and Canada.

1.2. Research hypothesis

The present study tested the following research hypothesis:

"Oral administration of BSE infected material perturbs innate immunity gene expression in brain tissue both quantitatively and temporally in a dose dependent manner"

Table 1.1. Transmissible spongiform encephalopathies in humans and animals.

TSE disease	Host species	Transmission route
Bovine spongiform encephalopathy (BSE)	Cattle	Ingestion of BSE contaminated food
Chronic wasting disease	Deer, elk	Oral, horizontal, vertical transmission
Feline spongiform encephalopathy (FSE)	Cats	Oral
Transmissible mink encephalopathy	Farmed mink	Oral
Exotic ungulate encephalopathy	Kudu, nyala, oryx	Oral
Scrapie	Sheep, goats	Oral, horizontal, vertical transmission
Variant Creutzfeldt-Jakob disease (vCJD)	Humans	Ingestion of BSE contaminated food, blood transfusion
Sporadic Creutzfeldt-Jakob disease (sCJD)	Humans	Unknown. Spontaneous mutation in PRNP gene.
Iatrogenic Creutzfeldt-Jakob disease	Humans	Accidental medical exposure
Familial Creutzfeldt-Jakob disease	Humans	Germline mutation in PRNP gene
Gerstman-Sträussler-Scheinker syndrome	Humans	Germline mutation in PRNP gene
Fatal familial insomnia	Humans	Germline mutation in PRNP gene
Kuru	Humans	Ritualistic cannibalism

Adapted from Mabbott and MacPherson (2006).

Table 1.2. Approximate number of cases of BSE by country and year of detection.

Country	First detected (year)	Number of Cases (1986-2006)
United Kingdom	1986	184,533
Ireland	1989	1,593
Portugal	1990	1,021
Switzerland	1990	464
France	1991	984
Belgium	1997	133
Luxembourg	1997	3
The Netherlands	1997	82
Liechtenstein	1998	2
Denmark	2000	14
Germany	2000	409
Spain	2000	681
Austria	2001	5
Czech Republic	2001	26
Finland	2001	1
Greece	2001	1
Italy	2001	135
Japan	2001	33
Slovakia	2001	23
Slovenia	2001	7
Israel	2002	1
Poland	2002	55
Canada	2003	11
United States	2005	2
Sweden	2006	1

Adapted from www.oie.int/Eng/Info/en_esbru.htm

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Chapter 2

Literature review

2.1. Introduction

The immune system is crucial for host defense against invading pathogens. Although no known pathogen was shown to cause TSEs, overwhelming scientific evidence suggests that this group of diseases induces immunological reaction. It is clear that the immune system plays an important role in modulating susceptibility and progression of various TSEs including BSE. Even though the immune system was shown to be involved in pathogenesis of prion diseases, so far there were no signs of inflammation reported in diseased tissues.

The literature cites three major, coexisting divisions of immunological mechanisms. The first division, based on structural features, divides immunity into specific and nonspecific (humoral and cellular). The specific reaction always comprises antibodies synthesized, or cells selected to recognize particular antigen, whereas nonspecific reaction includes general response with non-antigen oriented mobilization of immune cells and secretion of soluble mediators. The second division is based on cellular function with autonomous or rapid first line defense

on one side, and central regulated immunity on the other. In this case autonomous immunity would comprise phagocytes, mast cells, natural killer T lymphocytes and the complement system (CS) which all act non-specifically. Central immunity would contain highly specific antibodies and lymphocytes. The third division, put forward on the basis of development, would distinguish innate (inborn) immunity and adaptive (acquired) immunity. Innate immunity would include the complement system, toll-like receptors and soluble inflammation mediators – interleukins (IL); adaptive would comprise specific cellular response. The most important role of immunity is to recognize invading microorganisms as non-self and trigger immune reactions to eliminate them.

2.2. Pathobiology of TSEs

A typical feature of all TSEs is the accumulation of an abnormally folded isoform of normal cellular PrP^{C} (Bolton et al. 1982), called PrP^{RES} or prion. The abbreviation PrP^{RES} denotes its partial resistance to digestion with enzyme proteinase K (McKinley et al. 1983) whereas prion is a general term for infectious agent. In the literature quite often other nomenclature for the infectious prion particle is in use, e.g. PrP^{BSE}, PrP^{Sc}, PrP^{CWD}, PrP^{CJD} to describe prion particle isolated from animals

infected with BSE, scrapie or CWD, or detected in CJD patients. Cellular PrP^C was shown to be widely expressed not only in the CNS (Kretzschmar et al. 1986), but also in developing and adult peripheral tissues (Bendheim et al. 1992; Caughey et al. 1988; Oesch et al. 1985). Although the nervous system seems to be the ultimate target for prion invasion, the accumulation of prions has been also shown in some extraneural tissues such as spleen and lymph nodes (Beekes and McBride 2000; Bruce et al. 1989; Farquhar et al. 1994; Foster et al. 1996; McBride et al. 1992; McBride et al. 1998).

In orally challenged animals significant amounts of prion infectivity can be detected in the lymphoreticular system (LRS). The common feature of experiments with animals peripherally challenge with TSE agent, is that LRS infectivity arises well before it can be detected in the central nervous system (CNS) (Aguzzi and Polymenidou 2004; Bruce et al. 2001; Glatzel et al. 2003; Sigurdson et al. 2001).

From the LRS the infectious agent is propagated to the mesenteric lymph nodes by the lymphatic route, and then can enter the bloodstream, however recent studies show that the alternative route, via the nervous system is possible (Hoffmann et al. 2007; McBride et al. 2001; Sigurdson et al. 2001). Indeed

both splanchnic and vagus nerves were shown to spread the prion infectivity from peripheral nervous system (PNS) to the CNS (McBride and Beekes 1999; McBride et al. 2001). As the parasympathetic nerves enter the CNS directly omitting the spinal cord it is possible even within the CNS to have several routes of prion trafficking (Baldauf et al. 1997; McBride et al. 2001). Propagation of TSEs' from the gut through the enteric nervous system (ENS) and the PNS was confirmed by cases of natural (Groschup et al. 1996; van Keulen et al. 1999; van Keulen et al. 2000), and experimental scrapie (Baldauf et al. 1997; Beekes et al. 1998; Beekes and McBride 2000; Groschup et al. 1999; Kimberlin et al. 1983; McBride and Beekes 1999) and BSE (Wells et al. 1998). Once PrP^{RES} enters the spinal cord it can spread in both anterograde and retrograde directions (Beekes et al. 1996). Involvement of the PNS in the spread of infectivity was confirmed by experiments showing severe delays in neuroinvasion after depletion of sympathetic nerves (Glatzel et al. 2001).

On reaching the CNS progression of disease evokes characteristic neurological changes including spongiform vacuolisation, lesions, significant activation and proliferation of microglial cells and loss of neurons, leading to

neurodegeneration. These changes in turn result in clinical symptoms including changes in temperament, aggressiveness, light and/or sound sensitivity. Cattle with BSE demonstrate difficulties of raising, abnormal posture and un-coordinated movement with a swaying gait.

The brain and spinal cord response to infection differs from that of other organs. The CNS is equipped with a blood-brain barrier (BBB), which is created by the tight arrangement of endothelial cells lining the blood vessels in the brain and spinal cord, forming a barrier between the circulation and the brain parenchyma (astrocytes, microglia). This physical barrier limits the entry of blood-borne immune cells: lymphocytes, monocytes and neutrophils; pathogens and large molecules (Petty and Lo 2002). It was demonstrated on a rodent model that bacterial lipopolisacharide (LPS) induced very potent inflammation reaction in the skin and very limited and delayed response in the brain (Matyszak 1998; Perry et al. 1995). In addition to a well defined morphological "wall" (tight junction), an immunological barrier exists. It manifests itself in the reduced expression of adhesion molecules and major histocompatibility complexes (MHC), as well as in an immunosuppressive microenvironment mediated by astrocytes and microglia. Those cells express large

levels of tumour necrosis factor that can induce apoptosis of infiltrating cells (Gasque et al. 2000). All these mechanisms together with a generally muted immune environment within the brain itself protect the fragile neuronal network from the risk of damage that could arise from a full-blown immune response. A local innate immune response is built up in order to limit the spread of infectious agents, destroy pathogens and remove cell debris.

The blood-brain barrier is very effective, but on rare occasions this barrier can be breached. In such cases there are two major strategies for defense systems to take action. The first mechanism is when stimulated glial cells produce cytotoxic and cytolitic immune molecules such as proteins of the complement system and perforin which destroys the pathogens' cell membrane. The second mechanism involves the phagocytic properties of microglial cells (Perry 1998). There is increasing evidence for astrocytes, oligodendrocytes and endothelial cells not specialized in phagocytosis, to posses some phagocytic properties (Flugel et al. 2000).

Due to the mechanisms mentioned above, the CNS is less accessible for cells of the adaptive immune response. Thus, the major response in the CNS in early stage of TSE progression is

due to three major components of innate immunity: proteins of the complement system (CS), toll-like receptors (TLR) and interleukins (IL).

2.3. Proteins of the complement system

The complement system, an essential component of the innate immunity, consists of at least 30 known soluble and cellular proteins that provide an immediate and unspecific defense against various microorganisms. Several experiments have shown CS proteins to be highly conserved between invertebrates and vertebrates which imply a common origin and crucial role in immunity (Dodds and Law 1998; Sahu and Lambris 2001; Smith et al. 2001). In mammals the major source of CS proteins is the liver, but many other cell types and tissues synthesize them locally (Morgan and Gasque 1997).

After a peripheral challenge the follicular dendritic cells (FDCs) are thought to be the cells responsible for PrP^{RES} accumulation in lymphoid tissue (Mabbott et al. 2000; Mabbott et al. 2003; Montrasio et al. 2000). In various models, the exact localization of prion buildup was proven to occur on FDCs membrane (Brown et al. 1999; Hill et al. 1999; Sigurdson et al. 2002; Van Keulen et al. 1996). In comparison to other tissues and cells, FDCs express high levels of cellular PrP^{C} (Brown et al.

1999; McBride et al. 1992), which is necessary for susceptibility to TSEs. The physiological role of FDCs is to trap and retain antigen-antibody (Ag-Ab) complexes as well as Ag-Ab-complement proteins macromolecules, and Ag-CS proteins (Yoshida et al. 1993). Subsequently those complexes are presented on the cell membrane to specific lymphocytes B to initiate the production of highly specific antibodies.

Complement protein C3 is essential for follicular localization of antigens (Papamichail et al. 1975), which suggests its crucial role in TSE pathogenesis. This was confirmed by the ablation of the C3 gene and complement receptors (CR1 and CR2) which resulted in significant delays of PrP^{RES} accumulation in spleen as well as in longer lifetime of infected animals (Klein et al. 2001; Mabbott et al. 2001).

There are three major routes of complement activation. The classical pathway starts with the C1 complex (C1qsrrs) binding to Ag-Ab complexes and subsequent activation of downstream cascades. Alternatively, CS activation is initiated by spontaneous activation and inactivation of C3 protein. The last mechanism is the lectin pathway initiated by mannose-binding lectin molecule (MBL) attaching to pathogens' carbohydrates (Fujita 2002).

Although, the classical pathway is primarily initiated in antibody dependent manner, antibody independent ways of activation is also possible. Molecules such as LPS, DNA, RNA, serum amyloid P (SAP); viral, bacterial, fungal membranes and C-reactive protein (CRP) have been shown to activate this complement pathway, reviewed in Gewurz et al. (1993).

C1q complement component was recently shown to be crucial for clearance of apoptotic cells. Independent studies demonstrated its binding to membranes of apoptotic keratinocytes and T lymphocytes (Korb and Ahearn 1997; Mevorach et al. 1998; Taylor et al. 2000), apoptotic neurons, myelin oligodendrocyte glycoprotein (MOG) and myelin basic protein (MBP) reviewed in Gasque et al. (2000). Binding was subsequently accompanied by complement activation. The finding was confirmed in a C1q-knockout mice study in which the clearance of apoptotic cells was severely impaired (Botto et al. 1998).

In 1987, Levi-Strauss and Mallat demonstrated in a rodent model that astrocytes, the most abundant cell type in the brain, were capable of producing molecules of the complement cascade (Levi-Strauss and Mallat 1987). Later, the complement proteins were also shown to be produced in microglia, oligodendrocytes

and neurons (Gasque et al. 2000). From these studies it became apparent that many brain cell types can actively generate complement as a defence against pathogens. The increased expression of CS proteins as a response to bacterial infection was shown in patients with meningitis (Stahel and Barnum 1997), as well as experimental models of encephalitis and scrapie (Dandoy-Dron et al. 1998; Dietzschold et al. 1995).

A "pathogen absent" example of activation of complement was shown in 1990's in human brain. Employment of reverse transcriptase polymerase chain reaction (RT-PCR), *in situ* hybridization (ISH) and northern blot analysis revealed the involvement of this component of innate immunity in Alzheimer's disease (Shen et al. 1997; Walker and McGeer 1992; Yasojima et al. 1999).

It is clear that CNS glial cells and neurons can synthesize complement molecules. Although the complement cascade is crucial for innate immunity, the impairment of regulation resulting in increased apoptosis can lead to neurodegeneration (Gasque et al. 2002). Involvement of complement proteins in brain immune responses was shown in many disorders. Accumulation of aggregated amyloid β peptide in brains of Alzheimer's disease (AD) patients' actively stimulates

complement cascade by both classical and alternative pathways (Emmerling et al. 2000), and activates glial cells (Meda et al. 2001). Activated microglia and complement proteins have been present in *substantia nigra* of patients with Parkinson's disease (PD) (Członkowska et al. 2002), as well as in animal models of this disease (McGeer and McGeer 2004). In brains with Huntington's disease neurons and astrocytes express higher levels of complement proteins: C1q, C4, C3, iC3b and C9 in comparison to normal brains (Singhrao et al. 1999).

Many independent experiments confirmed the important role of complement in TSEs. Proteins C1q, C3b, C3c, C3d and C4 were detected in the brains of humans affected with Creutzfeldt-Jakob disease (CJD) and Gerstmann-Straussler-Scheinker (GSS) disease (Ishii et al. 1984). Kovacs et al. (2004) confirmed the presence of active CS proteins (C1q and C3b) in extracellular PrP^{RES} deposits accompanied by formation of membrane attacking complexes (MAC), composed of proteins C5, C6, C7, C8 and C9, in neurons. The prion-infected brain was also shown to activate and recruit microglia to the site of immune responses. The cells were always co-localized with PrP^{RES} deposits (Williams et al. 1994). Since complement system proteins can also be synthesized in most of the peripheral tissues

and organs that prions utilize to invade CNS, complement is suspected to play an important role in early stages of TSEs. In particular this occurs during prion accumulation on FDCs, which are antibody- and complement-dependent antigen recognition sites (Yoshida et al. 1993). In the case of TSEs, PrP^{RES} is thought to be trapped and retained on the surface of FDCs as an interaction between complement cascade proteins and specific cellular complement receptors (Nielsen et al. 2000; Pepys 1976). The conformational change of normal PrP^{C} into PrP^{RES} was shown to provoke recognition by the C1q molecule or the globular part of C1q with subsequent binding to PrP^{RES} (Blanquet-Grossard et al. 2005). It was also shown that knock-down mice deficient in C3, C1q, Factor B, or C2 complement proteins were partially or fully protected against TSE, which manifested itself in significant delays in onset of the disease and reduced accumulation of PrP^{RES} in spleen (Klein et al. 2001; Mabbott et al. 2001). Klein's experiment has interesting outcomes regarding the dosage response in complement deficient mice. Low dosage of prion inocula combined with ablation of C1q, C3, C4, CD21/CD35, complement factor B (CFB), or C2 significantly delayed prion internalization and neuroinvasion after intraperitoneal inoculation. This might suggest that the complement system

actually promotes the spread of prions. High titres prion inocula appear to override the necessity of complement in prion pathogenesis (Klein et al. 2001).

2.4. Toll-like receptors

Since the CNS is guarded by the blood-brain-barrier even from the host's own immunological cells, the toll-like receptors, as a part of innate on site immunity, may play an even more important role in mediating responses in the CNS. A number of experiments into the role of TLRs in the CNS have emerged over past 13 years.

The history of toll and toll-like receptors (a family of pattern recognition receptors) begins in 1996. At that time *toll* was demonstrated to be an essential receptor in *Drosophila*'s defence against fungi (Lemaitre et al. 1996). Only one year later the first human receptor was identified and shown to be necessary for induction of the inflammatory response. The human receptor was closely related to the toll receptor and was termed toll-like receptor 4 (TLR4) (Medzhitov et al. 1997). This receptor was shown to specifically recognise lipopolysaccharides (LPS) as mice with a point mutation in TLR4 gene were unresponsive to LPS (Poltorak et al. 1998). Soon after TLR4 was described, other structurally related members of the toll-like

receptor family were discovered (Rock et al. 1998). It became obvious that individual TLRs were responsible for recognizing specific pathogen associated molecular patterns (PAMPs) present in various microbes including protozoa, bacteria, fungi and viruses (Alexopoulou et al. 2001; Hemmi et al. 2000; Lien et al. 1999; Takeuchi et al. 2002; Werts et al. 2001). In humans TLRs comprise at least eleven members, only some of which are conserved in other mammalian species (Zhang et al. 2004). Mammals including beef cattle share at least ten toll-like receptors highly conserved between species (Jann et al. 2009)

Stimulation of TLRs by various microbial components facilitates their dimerization. Recently, Ozinsky et al. (2000) demonstrated that recognition of PAMPs can be achieved by cooperation of two different TLRs. For example, in the case of peptidoglycan recognition, TLR2 and TLR6 were shown to form heterodimers. The cooperation between different receptors is a common mechanism. Bacteria from *Streptococci* family are processed by three receptors: CD14, TLR2 and TLR6 (Henneke et al. 2001). Receptors TLR1 and TLR2 were indicated to be crucial for recognition of lipoproteins derived from *Mycobacterium* (Takeuchi et al. 2002). Activation of specific TLR generates quite distinct outcomes in terms of gene expression.

Cooperation of TLR3 and TLR4 causes induction of interferon-β (IFN-β), whereas TRL2 and TLR5 were shown not to have any effect on IFN-β expression (Doyle et al. 2002; Toshchakov et al. 2003).

In the CNS all members of toll-like receptor families were shown to be abundantly expressed in microglial cells, astrocytes, and oligodendrocytes (Bsibsi et al. 2002; Kielian 2006). Microglia have been also reported to express high levels of TRL3, and respond to PAMPs by synthesizing IFN-β, interleukin-1β (IL-1β) and interleukin-6 (IL-6) (Olson and Miller 2004). Recently astrocytes were shown to express TLR3 (Farina et al. 2005; Scumpia et al. 2005). Abundant expression of this receptor in oligodendrocytes and astrocytes was confirmed by experiments in response to viral challenge (Prehaud et al. 2005). In addition to that several studies have demonstrated that TLR 2 expression in astrocytes can be elevated by various pathogens (Bowman et al. 2003; Carpentier et al. 2005; Esen et al. 2004).

TLR 9 mediates responses to synthetic oligodeoxynucleotides (ODN), unmethylated CpG dinucleotides present in bacterial and viral DNA (Takeda and Akira 2007). Although mammalian DNA contains CpG sequences as well, those are methylated, and as such do not trigger TLR 9. To date

several studies have shown expression of this particular receptor in microglia, its response to CpG and subsequent production of numerous proinflammatory mediators including immune receptor molecules (Dalpke et al. 2002; Iliev et al. 2004; Olson and Miller 2004; Takeshita et al. 2001). Microglia challenged with synthetic CpG ODN were found to induce neuron cell death (Iliev et al. 2004) suggesting a link between TLR's and neurodegeneration. Recently Lotz et al. (2005) showed CpG ODN ability to suppress β-amyloid-dependant production of TNF-α in microglia. This might suggest an important role of TLR 9 in Alzheimer disease and perhaps in BSE associated neurodegeneration (Perry et al. 2003). Astrocytes, another subset of brain cells, are also activated by exposure to CpG ODN and as a result express TLR 9 receptor (Bowman et al. 2003; Hosoi et al. 2004; Lee et al. 2004; Takeshita et al. 2001). However, not all data support TLR 9 expression and activation in astrocytes after CpG challenging. Farina et al. (2005) were unable to detect this receptor in human astrocytes. The discrepancy could be due to species differences, as studies reporting TLR 9 involvement in signal transduction were performed on rodent models.

CD14 is another molecule involved in innate immune responses. This receptor exists in two forms: cell membrane

associated, attached by a phosphatidylinositol anchor (mCD14) and soluble (sCD14) (Haziot et al. 1988; Kirkland and Viriyakosol 1998). CD14 was demonstrated to be expressed by various cells including microglia (Becher et al. 1996; Nadeau and Rivest 2000; Saito et al. 2000). As a part of the defence system CD14 requires interaction with TLR4 to transduce innate immune responses (Dobrovolskaia and Vogel 2002; Palsson-McDermott and O'Neill 2004).

Myeloid differentiation factor 88 (MyD88) is the common adaptor protein that most TLRs utilize to induce immune responses (Fitzgerald et al. 2001; Hemmi et al. 2002; Hemmi et al. 2003). Although most TLRs transduce their signal through this protein, MyD88 independent pathways were also identified (Kawai et al. 2001). Analysis of MyD88-deficient mice revealed its essential role in TLR signalling as well as in both the innate and the adaptive immune response (Hayashi et al. 2001). One of the steps in signalling is activation of the nuclear factor kappa B (NF-kB) which belongs to a family of transcription factors. This leads to transcription of genes of anti-inflammatory and pro-inflammatory cytokines (e.g. IL-1, IL-10) and chemokines (Takeda and Akira 2004; Takeda and Akira 2005). Experiments involving MyD88-deficient mice demonstrated that MyD88 plays

an important role in modulating interleukins synthesis, especially IL-1 (Adachi et al. 1998; Kawai et al. 1999) and IL-6 (Hayashi et al. 2001). Those results suggest that MyD-88 is a key protein mediating production of inflammatory cytokines induced by TLR receptors.

2.5. Interleukins

The immune system is regulated by a complex network of activators and inhibitors whose roles are to control the immunological response and ensure short- and long-distance communication between immune cells. In the normal physiologic state activators and inhibitors are balanced, limiting potentially harmful effects of an excess of inflammatory response. Various diseases or inborn (inherited) immune deficiencies can cause unbalanced presence or absence of immune activators and inhibitors. Lack of them may lead to the development of serious systemic infections (Kasai et al. 1997; Munoz et al. 1991). The balance between pro- and anti-inflammatory cytokines is constantly shifting and is dynamic rather than static. In addition to that, all anti-inflammatory molecules have some pro-inflammatory properties. Therefore, the net effect of immune response is an average of the presence or absence of many elements: kinetics of cytokine release and receptors density,

which both modify tissue responsiveness to cytokines (Dinarello 1998).

The interleukins are a family of cytokines (chemical messengers) that are very important regulators of both innate and adaptive immunity. They are able to activate antigen presenting cells (APCs), induce proliferation and promote migration of many types of immune cells to the site of inflammation. The first interleukin to be described was IL-2, found in white blood cells and first reported by Gordon & McLean (Gordon and MacLean 1965). It has since been found that interleukins are produced by a wide variety of cells (reviewed in Opal and DePalo 2000). The function of the immune system depends in a large part on interleukins, and rare deficiencies have been described, all featuring autoimmune diseases or immune deficiencies (Geha et al. 1991).

IL-6 expression is induced by LPS in the presence of TNF and IL1. This important interleukin acts through systemic activation of pro-inflammatory cytokines (Barton 1997). As IL-6 primarily induces acute-phase immune response, for a long time IL-6 was thought to have only pro-inflammatory effects. In 1996, Barton et al. (1996) showed for the first time in a murine model its anti-inflammatory potential. Anti-inflammatory

properties of IL-6 were confirmed by two additional experiments showing that IL-6 effectively down-regulates the synthesis of pro-inflammatory cytokines: IL-1 and TNF (Libert et al. 1994; Xing et al. 1998). It was shown in the same study that IL-6 had no effect on anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF- β). IL-6 was shown to promote synthesis of glucocorticoid hormones (Libert et al. 1994) and induce interleukin 1 receptor antagonist (IL-1ra), and TNF receptor p55 release in humans (Tilg et al. 1994). IL-6 has also a potential to inhibit pro-inflammatory cytokines granulocyte macrophage colony stimulating factor (GM-CSF), interferon-γ (IFN-γ) and macrophage inflammatory protein 2 (MIP-2) (Barton 1997).

The most important anti-inflammatory interleukin is IL-10. Initially due to its strong anti-inflammatory properties, this cytokine was designated as cytokine synthesis inhibition factor (Howard and O'Garra 1992; Lalani et al. 1997). IL-10 is a strong inhibitor of many cytokines: TNF-α, IL-1, IL-6, IL-8, IL-12, GM-CSF, MIP-1, and MIP2 (Clarke et al. 1998; Marchant et al. 1994). Moreover it was demonstrated to significantly decrease surface expression of major histocompatibility complex class II (MHC II) and CD 14 signaling (Opal et al. 1998). The importance of IL-10

in homeostasis was shown in mice knockout experiments. Mice lacking the IL-10 gene developed chronic enteritis which indicates its essential role in controlling gut-associated bacteria (Kuhn et al. 1993).

There is evidence suggesting IL-10 has a role in modulating prion diseases in mice. Comparisons of IL-10 knock-out to wild-type mice revealed that the genetically engineered animals are more susceptible to TSEs with much shorter incubation times (Thackray et al. 2004). It was demonstrated in the same experiment that in wild-type mice, TNF-α expression occurs at a later time during the infection, and this correlates with longer incubation period. Conversely, IL-10 deficient mice have much shorter incubation time and TNF-α is overexpressed earlier. But not all studies support the role of IL-10 and TNF-α in prion disease. No association between expression of both cytokines and development of prion disease was shown in mice after intracerebral inoculation (Cunningham et al. 2002; Mabbott et al. 2000).

2.6. Conclusions

It is clear that innate immunity plays an important role not only in haemostasis but also in a response to various pathogens. The complement system, toll-like receptors and interleukins were shown to modulate diseases, including TSE, on various stages of infection. Dynamic balance between various components of immune system can be either beneficial or detrimental to the host. It is very important to explain the basic mechanisms of TSEs from an immunological point of view as the insight into immune mechanisms would shed light on many various aspects of this new family of diseases.

Identifying immunological mechanisms modifying prion diseases will facilitate discovery of the key proteins in TSE pathogenesis. Targeting of the proteins directly involved in immune responses should become a priority in research on TSEs. Identification of the new proteins involved in defense against prion diseases would make them to be a potential target in the development of new drugs.

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Chapter 3

Gene expression analysis in cattle orally challenged with different doses of BSE agent

3.1. Introduction

The immune system and its elements have been known for many years. However, only recently has innate immunity, and its role in infection and tolerance mechanisms to self-antigens, been investigated intensively (Thornton and Morgan 2009). The host defense system can be divided into two components; innate and adaptive immunity. Innate immunity directs inborn, immediate and non-specific response to pathogens whereas adaptive is responsible for specific response. While TSE infectivity migrates through various tissues, a set of genes of both immunological components become down- or up-regulated (Huang and MacPherson 2004). It is certain, that the triggered genes are the host response for antigen stimuli.

Although there are numerous experiments showing gene expression changes in various animal models with many TSE strains (Baker et al. 1999; Booth et al. 2004; Brown et al. 2004; Brown et al. 2005; Cosseddu et al. 2007; Sawiris et al. 2007), the gene expression data of the genes involved in immune

response is partial. The great majority of research in this area comes from experiments on mice infected with PrP^{SC} (Skinner et al. 2006) or PrP^{CJD} (Lu et al. 2004). The experimental administrations of scrapie agent are mostly intracerebral (Xiang et al. 2004) and intraperitoneal (Riemer et al. 2004) or oral (Jeffrey et al. 2001).

Usually experiments utilizing microarrays for gene expression analyses validate and discuss only highly up regulated or down regulated genes, paying little attention to genes with lower changes in expression. In some of the microarray experiments the cut-off values for genes to be considered up regulated are set to the n-fold change value of 2 (Brown et al. 2004). As a result of such approaches the genes with lower than 2 up regulation could be easily missed. It might be that the most highly up regulated or down regulated genes, although being undoubtedly altered in the course of the disease, are not always the most important players in the pathobiology of diseases. More attention should be paid to subtle changes in key genes as they might be important but missed using microarray technique.

So far no major emphasis has been paid to the immunological mechanisms and their contribution in modulating

prion diseases (Dandoy-Dron et al. 1998). However, recent studies seem to accept the role of immune system proteins in TSEs' pathogenesis (Lu et al. 2004; Skinner et al. 2006; Xiang et al. 2004). Examining immune gene expression, grouped in functional networks as one of the parameters of experimental design, seems to be a must. Improved knowledge about the exact mechanisms responsible for modifying the course of prion diseases will allow for better understanding of what happens at all stages of prion disease and its progression. The present study tested the performance of selected immune genes grouped in functional networks of the complement system, toll-like receptors and interleukins.

3.2. Materials and methods

3.2.1. Experimental design and BSE agent administration

Total RNA samples used in this study were obtained from Roslin Institute, UK. In order to assess gene expression, eight cows at the age of six months, were given feed containing 1g or 100g BSE agent (four cows in each dosage group) and left to be sacrificed at time-points: 6, 9, 12 and 45 months after challenge. Four control animals (non-challenged with BSE agent) were slaughtered at the corresponding time-points to ensure

adequate age matching. All animals were Holstein dairy cows. *Caudal medulla* tissue samples were collected at slaughter, immediately immersed in liquid nitrogen to prevent RNA degradation. Total RNA was subsequently isolated from all twelve animals and stored in -80 degrees Celsius, under 100% ethanol to maximize its stability.

3.2.2. Sample preparation for quantitative real-time polymerase chain reaction

Total RNA extracted from *caudal medulla* tissues was prepared according to the protocol accompanying the Amino Allyl MessageAmp™ II aRNA amplification kit (Ambion, Foster City, USA). In brief, total RNA was reverse transcribed to single strand cDNA which was subsequently used as a template for cDNA second strand synthesis. The second strand was then purified and used for the synthesis of a single strand aRNA (antisense-RNA), which is complementary to mRNA. Newly synthesized aRNA was purified and quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Delaware, USA). All samples were diluted to the final concentration of 100ng/µl and used as a template for quantitative real time PCR experiment.

3.2.3. Selection of housekeeping genes

Most cellular processes, (e.g. proliferation, growth, differentiation, metabolism etc.) depend on constantly changing gene expression levels. Quantification of mRNA has always been important for genetic research and so far five methods are commonly used for this purpose: *in situ* hybridisation and northern blotting (Parker and Barnes 1999), RNAse protection assays (Hod 1992; Saccomanno et al. 1992), cDNA microarrays (Bucher 1999) and quantitative reverse transcription polymerase chain reaction (RT-PCR) (Weis et al. 1992).

The quantitative reverse transcription polymerase chain reaction (RT-PCR) is the most sensitive method for detection small quantities of messenger RNA (mRNA). Errors in quantification specific to RT-PCR are unavoidable since there is considerable variation between starting amount of mRNA taken from different individuals, and even between two different handling events of the same sample. To minimize variation and sample handling errors internal standards (housekeeping genes, HK genes) are in use against which values of tested genes can be normalised. The use of housekeeping genes as standards raises the question what would be the ideal internal control gene? From the experimental point of view the best internal

standard is the one which is expressed at a constant level between different tissues of an organism as well as within the same tissue between different organisms. It is obvious that such gene should not be affected by the experimental treatment (Karge et al. 1998). So far there is no single gene that satisfies those criteria. Therefore, researchers' effort focuses on selecting the best gene for specific experimental settings rather than applying one gene to all experiments regardless of their design. Since using a single housekeeping gene for normalisation can introduce additional errors related to higher variation between measured mRNA levels of single HK gene (Thellin et al. 1999), utilizing two or more internal standards has become a common practice. Subsequently a geometric mean can be taken and used for normalisation, which was shown to reduce experimental error considerably (Vandesompele et al. 2002). In the same experiment Vandesompele et al. showed a stable expression of GAPDh gene in many human tissues including brain, as well as its stability in expression between various tissues.

In 2001 Hsiao et al. tested around 7000 genes in 19 distinct tissue types identifying 451 HK genes that were expressed in all tissues. The experiment revealed that the most stably expressed HK genes were found among genes belonging

to a family of proteosome (multicatalytic proteinase complexes). Among 451 genes, PSMD2 and PSMB2 were found to be the most stable genes, having the lowest standard deviation (SD) and coefficient of variation (CV) values (Hsiao et al. 2001). As there were some difficulties with primers and probe design for the PSMD2 gene, the GAPDh gene along with PSMB2 was used as HK genes in this experiment.

3.2.4. Quantitative real-time polymerase chain reaction (QRT-PCR)

In this study, QRT-PCR was performed according to the TaqMan® methodology (Applied Biosystems, Foster City, USA). To summarize, aRNA samples are combined with TaqMan® master mix, forward primer, reverse primer, as well as highly specific fluorescent probe, and amplified (Appendix 2). In real-time PCR technology, a fluorogenic probe and both primers hybridize to the target sequence. As polymerase starts to synthesize an amplification product, it moves toward the probe, cleaving it. This reaction causes the probe to emit fluorescent light that can be directly and quantitatively measured.

The most important variable in real-time PCR is threshold cycle (Ct) which is defined as the number of amplification cycles it takes for fluorescence to reach the threshold intensity. Ct

value of tested genes compared to Ct values of internal standards (housekeeping genes), and Ct values of calibrators (non-treated animals) determines down regulation or up regulation of genes of interest in relation to the internal standard and the calibrator. When comparing gene expression changes between 100g and 1g challenged animals, 1g exposed animals can be used as calibrators to assess the relative change in 100g exposed cows.

In this study gene expression analysis of fourteen innate immunity genes was performed. Amplification was done on a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, USA) in triplicates for each sample, and each gene. Primers and probes (Table 3.1 and 3.2) were designed with PrimerExpress® software (Applied Biosystems, Foster City, USA).

The bovine sequences for primers and probe design were obtained from the Ensemble.org website (http://www.ensembl.org/Bos_taurus/Info/Index) and subsequently blasted against bovine sequences on NCBI (http://www.ncbi.nlm.nih.gov/genome/seq/BlastGen/BlastGen.cgi?taxid=9913) for a cross-reference. The forward or reverse primers were designed in different exons to avoid amplification of genomic DNA. Designed primers were uploaded onto UCSC In-Silico PCR (bovine) website

(http://genome.ucsc.edu) to check the length of amplicons (around 60-70 base pairs). The sequences obtained from this website were cross-referenced on Ensemble website to ensure the amplification of the specific gene.

The HK genes and tested genes were selected based on literature review of physiology and pathophysiology of immune responses. Housekeeping genes used in this study were: proteasome macropain, subunit beta, type 2 (PSMB2) and glyceraldehyde 3-phosphate dehydrogenase (GAPDh).

N-fold change was calculated according to ΔΔCt methodology. Ct values for two internal standard genes were averaged and subtracted from Ct value for gene of interest producing ΔCt value. ΔCt values were calculated for all animals and all genes (including housekeeping genes). ΔCt value for the calibrator sample (non-exposed animals) was then subtracted from ΔCt value for 1g or 100g exposed animals to calculate ΔΔCt value. Subsequently, n-fold change was calculated as follows:

$$\text{N-fold change} = 2^{-\Delta\Delta Ct}$$

In some instances, a 1g challenged animal was used as the calibrator for 100g exposed animal. In that case the difference in gene expression between 100g and 1g animals was calculated.

The rationale behind Ct values is that higher numbers of transcripts in a sample will result in faster amplification and faster generation of fluorescent signal. In that case threshold intensity value will be reached sooner with lower Ct values. In the opposite, low concentrations of transcripts will result in slower amplification and higher Ct values. The differences in Ct values for genes of interest between non-exposed and exposed animals normalized to housekeeping genes are directly related to the difference in transcripts' abundance.

3.3. Results

Histopathology of *medulla*, immunohistochemistry in *obex* and *medulla*, as well as clinical signs observations were performed in all animals exposed to BSE agent. For all animals at 6, 9, 12 months after challenge, the tests were negative with no apparent signs of BSE. The animals at 45 months time-point, challenged with 1g and 100g BSE contaminated feed exhibited possible BSE signs. However only 100g exposed dairy cow had immunohistochemistry positive in *obex* and *medulla*.

3.3.1. Genes of complement system

In general, all twelve genes tested in the present study showed moderate down regulation during the course of BSE.

There were some exceptions from that rule with regard to the time-point after challenge, dosage, and particular genes.

All CS genes except for C4 and CFB demonstrated the highest expression differences at 6 months after exposure. C4 showed the maximum difference at 45 months. When comparing the 1g exposed animals to non-exposed controls, genes C1q and C2 were highly up regulated at 6 months after the exposure, with n-fold change values 3.11 and 5.95 respectively (Figures 3.1 and 3.2). C3 expression was 49% higher than in the control animal (Figure 3.3). For genes C4 and CFB, 1g animals showed reduction in mRNA level by 57% and 20% respectively (Figures 3.4 and 3.5). The major change in gene expression in 1g animal occurred between 6 and 9 months after exposure for all genes except for C4. Starting at 9 months after challenge 1g treated animal show decrease in mRNA levels as compared to controls, with a tendency to approach normal expression levels at the 45 months time-point. For the 1g exposed animals, genes C1q and C2 show noticeably similar expression pattern throughout the course of BSE (Figures 3.1 and 3.2).

In the animals fed with 100g BSE contaminated feed only C1q was up regulated after 6 and 9 months (n-fold change value 1.35 and 1.38, respectively). C4 gene showed high up regulation

at the 45 months time-point. The rest of the genes were down regulated with the highest reduction in mRNA levels (by 93% and 89%) at the 6 months time-point, for C4 and CFB genes respectively. The 100g exposed cows behaved quite differently at 6 months after exposure. The mRNA levels for genes C3, C4, and CFB were reduced significantly with moderate reduction for C2. Gene C1q was the only exception. With few exceptions the animals exposed to higher dosage of BSE agent seem to have mRNA levels closer to non-exposed animals (Figures 3.1, 3.2, 3.3, 3.4 and 3.5).

3.3.2. Toll-like receptors.

Four genes: TLR2, TLR3, TLR4 and TLR9 were tested in the present study. In general toll-like receptor genes were down regulated at all time-points with a tendency to approach normal expression levels or show slight up regulation at 45 months. The maximum reduction in mRNA levels for TLR3 and TLR4 in the 1g exposed animals occurred at 12 months, and in the 100g exposed animals at 6 months after exposure. Both the 1g and 100g exposed animals show differences in a gene expression pattern in gene dependant manner. In the 100g exposed animals TLR4 were significantly down regulated at the 6 months time-point, whereas TLR3 showed significant down regulation 9 and

12 months after challenge. The 1g dose induced moderate changes in gene expression. Down regulation for the 1g exposed animals seems to be more apparent 9 and 12 months after challenge (Figures 3.6, 3.7).

At 6 months after the challenge, the TLR9 gene expression seems to be opposite to the genes TLR3 and TLR4. For the TLR9 gene, the 1g exposed animals show significant reduction in expression level, whereas for genes TLR3 and TLR4 significant reduction of expression is characteristic for the 100g challenged animals (Figures 3.6, 3.7, 3.8).

3.3.3. Interleukins, CD14 and MyD88

The time dependent expression pattern for both IL-6 and IL-10 was similar to that of TLR 3 and TLR4 genes. At time-points 6, 9 and 12 months after challenge moderate to significant reduction in relevant mRNA transcripts can be seen with a tendency to reach physiological levels at 45 months. However at this time-point, the IL-6 gene for the 100g dosage shows an increase in mRNA level by 86% as compared to non-exposed control. IL-10 follows the same trend, but the mRNA level is elevated by only 22% (Figures 3.9 and 3.10). CD14 expression is similar to TLRs, especially TLR4 with the 100g exposed animals showing significant down regulation at 6 and 12

months and minor reduction at 45 months post challenge. In regard to 1g dosage, an insignificant down regulation occurs at 6 months after challenge and reaches maximum reduction at 12 months (Figure 3.11).

MyD88 is generally down regulated and the levels of mRNA are reduced significantly at the 6, 9 and 12 time-points for both 1g and 100g exposed animals (Figure 3.12). Noticeably, time- and dosage-dependent expression pattern of MyD88 is almost identical with both interleukins, TLR3, TLR4, CD14 and CFB. It is characteristic for all of those genes to have a lower expression levels for the 100g exposed animals at 6 months time-points. For the time-points 9, 12 and 45 months post challenge, the 1g animals show lower expression levels in comparison to control animals.

3.3.4. Gene expression comparison of 100g to 1g BSE challenged animals (Table 3.3.)

At 6 months post challenge all the genes of the complement system analyzed in the present study were down regulated significantly. Starting from 9 months after challenge the CS genes show significant up regulation for animals challenged with higher dose in comparison to animal treated with

1g of BSE agent. The elevated, relative (100g to 1g exposed animals) levels of mRNA seem to return to normal values at 12 months time-point for CFB gene. At 45 months both C4 and CFB show high up regulation in 100g challenged cows as compared to the 1g exposed animals.

The toll-like receptor family shows a tendency to be differentially expressed with the course of disease reaching maximum up regulation at 12 months but returning to normal values at 45 months. TLR9 is highly up regulated at 6 months in the 100g exposed animals, showing a tendency to stabilize later in the course of BSE. In the 100g challenged animals, TLR3 gene showed lower expression levels in comparison to the 1g exposed animal at 6 months time-point, whereas the 100g animal was highly up regulated in comparison to the 1g animals at 12 months post challenge.

For the interleukin family only IL-6 gene was differentially expressed having a maximum up regulation at 6 and 45 months after exposure for the 100g challenged animals. Animals exposed to higher dose of BSE show elevated levels of mRNA for genes CD14, MyD88 and TNF-α at later stages of the disease, with the exception of CD14 and TNF-α which were down regulated at 6 and 12 months respectively.

3.4. Discussion

Although the experiments reporting expression of immune genes are limited, there are a small number of studies attempting to explain the possible role of immunity in prion diseases. In 1998 Dandoy-Dron et al. reported C1q (β-chain) up regulation in mouse intracerebrally inoculated with scrapie. The protein was highly up regulated at 120 days post inoculation, which is the terminal phase of the disease for mice (Dandoy-Dron et al. 1998). The up regulation of CS genes: C1q, C3 and C4 was confirmed in mice inoculated intraperitoneally and sacrificed at the terminal stage of scrapie (Riemer et al. 2004). Since in the present study C1q (α-chain) gene was down regulated at terminal phase, the results are quite different and discrepancies could be due to differences in route of antigen administration, dosage and the agent itself (scrapie versus BSE). Lu et al. (2004), using mice intracerebrally inoculated with CJD did not show any significant up regulation of C1q protein in contrast to this study and that of Riemer et al. (2004). Although the down regulation of CS genes at early stages of TSE reported in this study was confirmed by (Xiang et al. 2004), not all the genes reported here display similar expression patterns. For

example the C1q (α-chain) gene behaves quite differently especially early in the course of disease and in its terminal stage (Xiang et al. 2004). In addition to that CD14 also shows a quite different expression (down regulated throughout BSE) from that reported by Xiang et al. The complement system proteins are the first line defense molecules modifying inflammation from the first minutes, hours or days after infection. It is quite intriguing why there is down regulation of C2, C3, C4 and CFB genes in 100g challenged cows, whereas C1q, C2 and C3 are up regulated in 1g exposed animal at 6 months after challenge. Having only one time-point at the early stage of BSE it is hard to draw the kinetics of CS gene expression changes. To explain this phenomenon in dosage response additional experiments should be designed with additional time-points in early stages of BSE. The experiment would provide data on time dependent changes in CS genes expression levels and shed some light on the role of CS genes in BSE modulation.

Toll-like receptors are thought to be the most important sensors for recognition of various microorganisms. Their role is to sense PAMPs and send the message to highly specialized immunological cells (reviewed in Takeda and Akira 2005). In the present study the expression patterns of toll-like receptors,

TLR3, TLR4 and TLR9 do not confirm previous report by (Lu et al. 2004).

This study was unable to find TLR9 transcripts in whole brain homogenates, which might be due to generally high down regulation of TLR family genes confirmed not only in the present study. Although TLR2 and TLR3 were detected in the brains of mice, the levels remained constant over the course of CJD showing no involvement in the disease. It is possible that different strains of prions trigger particular TLRs and what we observe here is a specific for CJD signaling pathways or rather lack of signaling through TLR2 and TLR3. As a contrast, levels of TLR4 transcripts reached 10 and 15 fold change at 40 and 90 days post inoculation, respectively. This finding shows involvement of TLR4 in CJD in mice. In our experiment this gene was also shown to be involved in immune response. However, TLR4 was down regulated over the course of BSE, and up regulation occurred at 45 months after challenge, and only for the 100g exposed animal.

On the contrary, Xiang et al. results fully confirm findings of the present study. Although there is substantial difference between experimental designs (scrapie versus BSE, mice versus

cows), both experiments show low mRNA levels at early, and up regulation at late stage of prion disease (Xiang et al. 2004).

In 1997 for the first time an immunoreactivity study demonstrated IL6 to be associated with astrocytes during scrapie infection (Williams et al. 1997). Although the experiment provided no evidence of IL6 down regulation in early stage of scrapie, the presence of IL6 protein was shown later in the course of disease. These findings along with results of present study confirm involvement of IL6 in pathogenesis of TSEs at terminal stages of the disease. The novel finding reported here is the high down regulation of IL10, the most potent anti-inflammatory interleukin. The low levels of this protein and other anti-inflammatory interleukins might be responsible for the generally fast progression of TSEs. It would be very informative to know why interleukins mediating immune responses are up regulated in late TSE disease rather that in the early stages, where by logic their expression should occur.

CD14 is known to bind bacterial lipopolysaccharides. Those complexes subsequently bind to TLR4 and trigger neurodegeneration (Lehnardt et al. 2003; Wright 1999). Although in the present study the animals did not display the

evident signs of neurodegeneration, the synergistic action of CD14 and TLR4 can be seen from their expression profiles.

We report the expression of MyD88 gene in BSE for the first time, so it is impossible to compare results to other experiments. However Prinz et al. reported that MyD88 and most likely TLRs signalling (especially TLR9) is not involved in prion pathogenesis, since MyD88 knockout mice displayed the same disease incubation times as control mice (Prinz et al. 2003). Here, MyD88 was shown to be involved in pathogenesis of BSE with expression profiles similar to those of toll-like receptors and interleukins. Furthermore it is quite possible that MyD88 can cooperate with other TLRs in signal transduction in the course of BSE, so Prinz et al. conclusions about the lack of involvement of MyD-88 and TLR9 in prion diseases seem to be untimely.

Results from the present study show significant differences in gene expression between animals challenged with different doses of BSE agent. The small number of animals used in this study combined with no biological replicates available for tested animals makes it difficult to draw definitive conclusions as to the role of particular gene or even gene networks (CS, toll-like receptors or interleukins) in modulating BSE.

Another possible drawback of the present study testing gene expression in beef cattle is the different genetic background of all animals. Much more precise gene expression analyses are provided by rodent models as mice used in gene expression experiments are genetically identical. In this case the genetic background responsible for animal-to-animal variation can be easily avoided.

Future gene expression studies should be expanded to include biological replicates, to minimize the variation in gene expression occurring between animals or include only genetically identical ones. It is also very important to study mRNA levels in tissues at many more time points as this would give a valuable insight into kinetics of immunological responses. The way the kinetics changes in time is much more important in regard to immune processes in diseased tissue, than one single, static observation. It needs to be mentioned that immune networks comprise hundreds of constantly interacting proteins, thus the search for key molecules should be expanded to select new candidate genes that could be modifying BSE.

This is the first study focusing on selected innate immunity genes from BSE challenged cows. It is hard to compare these results to other results done mostly on mice and sheep as there

are considerable differences in gene expression levels between species. Those differences are detected even for the same gene but different prion strains within the same animal species (Skinner et al. 2006). Furthermore most of gene expression experiments utilize oligo-microarrays, and genes validated with QRT-PCR hardly ever include the innate immunity genes. Furthermore, microarrays testing thousands of genes simultaneously generate hundreds of genes showing changes in expression levels in the course of TSEs. As a result, gene expression experiments are likely to concentrate and subsequently validate very highly up regulated or down regulated genes regardless of their ontology. With such an approach there is constantly growing number of studies reporting molecules that seem to have little in common with immune response to the disease. In such a crowd, it is easy to overlook the importance of immune system genes, a potential key group of players in TSE progression.

Comparing the accumulation of prions in beef cattle and mice brains, it needs to be noted that mice start to accumulate PrP^{RES} at 90 days post intracerebral inoculation (Baker et al. 1999). As to the signs of prion disease, in the present study

accumulation can be detected at 45 months after challenge and only in the 100g BSE exposed animal.

3.5. Conclusions

The involvement of immune system in TSEs pathogenesis was confirmed by many researchers. The present study tested the expression of innate immunity genes in BSE exposed cows and confirmed participation of CS, TLRs and ILs in progression of the disease. It is intriguing that the coincidental expression pattern of almost all tested genes showed down regulation at all time-points with some minor exceptions. It suggests that the immune system is being shut down or disabled in response to BSE infection. As it seems that TSEs generally trigger different sets of genes depending on animal model, strain and route of administration, the experiments should focus on explaining prion diseases in a particular model, and not in the context of other TSEs. As to BSE itself further research should be conducted on greater number of animals in order to establish the role of particular genes in BSE progression. In addition to that, expression of immune genes should be tested at more time-points, as it would shed some light on kinetics of immune transcripts and processes they are responsible for. It is also absolutely crucial to test more genes of immunological ontology

to fully understand the complex process host response to BSE infection. Gene expression experiments should be supplemented with analysis of CS, TLR and IL proteins and their metabolites as this is the only way to understand real mechanisms modifying innate and adaptive immunity in BSE progression.

Table 3.1. List of primers used in QRT-PCR.

Gene ID	Forward Primer	Reverse Primer
Complement component 1, q subcomponent, A chain	GACCAGGTCTGGATTGAAAAAGA	GGCCTCTGAGCCGTGGTAA
Complement component 2	TCCAGTAAGAACTCCCGCAAA	GGAAGAGGTTGATGTGGAAATCTC
Complement component 3	CACACTCATCATCTACCTGGACAAG	TGGTGAACTTTGAAGGACAGACA
Complement component 4	GAGCGGCCAAGTCATCGT	GAGAGCTGCACTTCTGAGATGGT
Complement factor B	CCCCGACTCTGCCTGGTA	TCAGGCAATGGCGTCATG
Toll-like receptor 2	CTGGCCCTTCCTTCAAACC	TCCCGTTTTTTCTAGTGATTTCAAA
Toll-like receptor 3	CGCTGGACCTTTCCCGTAA	GAAGCCAGGCAAAGGAGTCA
Toll-like receptor 4	GTTTCCACAAAAGCCGTAAGGT	ACCATCGGCTCTGGATGAAG
Toll-like receptor 9	CAGTGGCCAGGGTAGTTTCTG	CCGGTTATAGAAGTGACGGTTGT
Cluster of differentiation 14	GGCTGATGGCAGCTCTCTGT	TTGCGTAGCGCTAGATATTGGA
Myeloid differentiation primary response gene-88	GGCAGCTGGAACAGACAAACTA	CAGGACGTCACGGTCAGACA
Tumor necrosis factor α	CCCAGGGCTCCAGAAGTTG	CGTGGTGGCTCCTGCAA
Interleukin 6	CCACCCCAGGCAGACTACTTC	CCATGCGCTTAATGAGAGCTT
Interleukin 10	TGCCACAGGCTGAGAACCA	CAGCTTCTCCCCCAGTGAGTT

Table 3.2. List of probes used in QRT-PCR.

Gene ID	Probe
Complement component 1, q subcomponent, A chain	CCCAGTAAGGGCCG
Complement component 2	TGCGCCCCATGGC
Complement component 3	TCTCACACACAGTGGAGG
Complement component 4	TCCATTGGTGTTCCTC
Complement factor B	TTGATCCTGGGCCTCT
Toll-like receptor 2	TGGTTTTAAGGCAGAATC
Toll-like receptor 3	TCCTTACGTGTGATGGGT
Toll-like receptor 4	ATTGTCGTGGTGTCCCAG
Toll-like receptor 9	AACCTGGGCATAGCC
Cluster of differentiation 14	CGAACAAGTTCCCGGCC
Myeloid differentiation primary response gene-88	CGGCTGAAGTTGTGC
Tumor necrosis factor α	TTGTGCCTCAGCCTC
Interleukin 6	ACTCCAGAGAAAACC
Interleukin 10	CCTGACATCAAGGAACA

Table 3.3. Comparison of 100g to 1g BSE exposed cows at four different time-points after challenge (6, 9, 12, 45 months). Values in the table denote n-fold change.

GENE	6	9	12	45
C1	0.44 ↓	2.82 ↑	2.77 ↑	0.72
C2	0.11 ↓	3.63 ↑	8.11 ↑	1.02
C3	0.31 ↓	1.55 ↑	2.45 ↑	1.42
C4	0.15 ↓	1.81 ↑	0.38 ↓	6.96 ↑
CFB	0.14 ↓	4.00 ↑	1.33	1.79 ↑
TLR2	0.60	1.03	1.57 ↑	1.06
TLR3	0.42 ↓	0.81	2.22 ↑	1.31
TLR4	0.57	1.18	2.55 ↑	1.98 ↑
TLR9	2.92 ↑	0.61	1.44	0.58
IL6	1.56 ↑	1.32	1.07	2.30 ↑
IL10	0.74	1.26	1.41	1.30
CD14	0.35 ↓	4.97 ↑	2.53 ↑	2.14 ↑
MyD-88	0.63	1.18	1.58 ↑	1.41
TNF-α	1.31	0.59	0.47 ↓	1.52 ↑

↑ Indicates relative up regulation. Values 1.5 and higher.

↓ Indicates relative down regulation. Values 0.5 and lower.

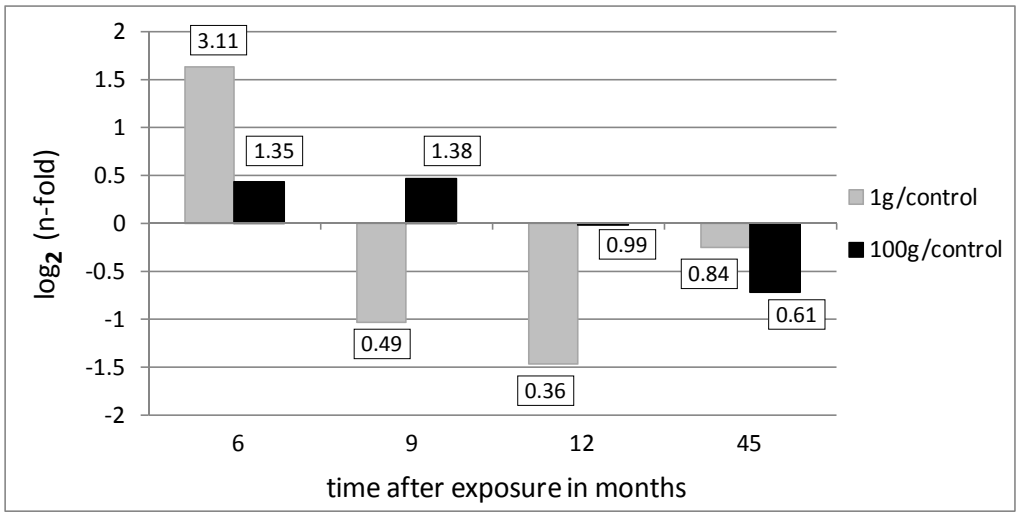

Figure 3.1. Expression of **C1q** (α-chain) gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

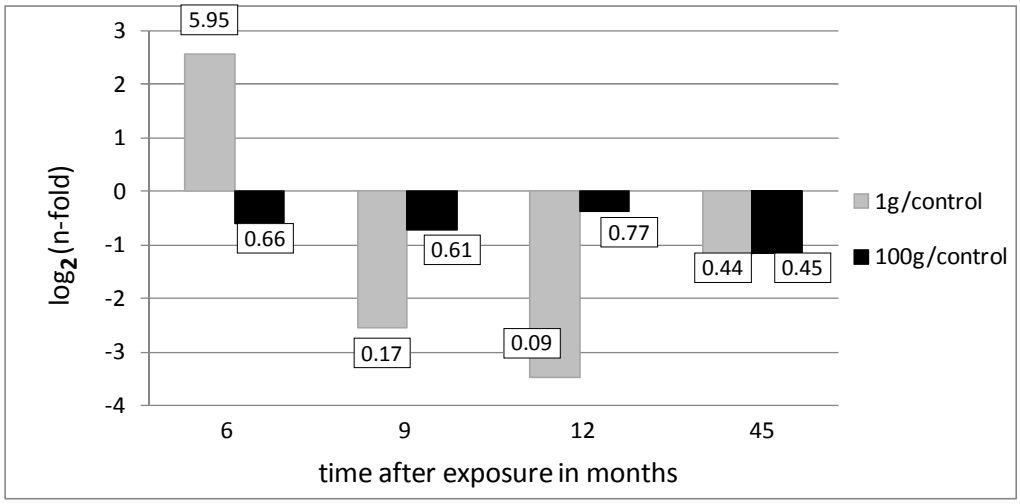

Figure 3.2. Expression of **C2** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

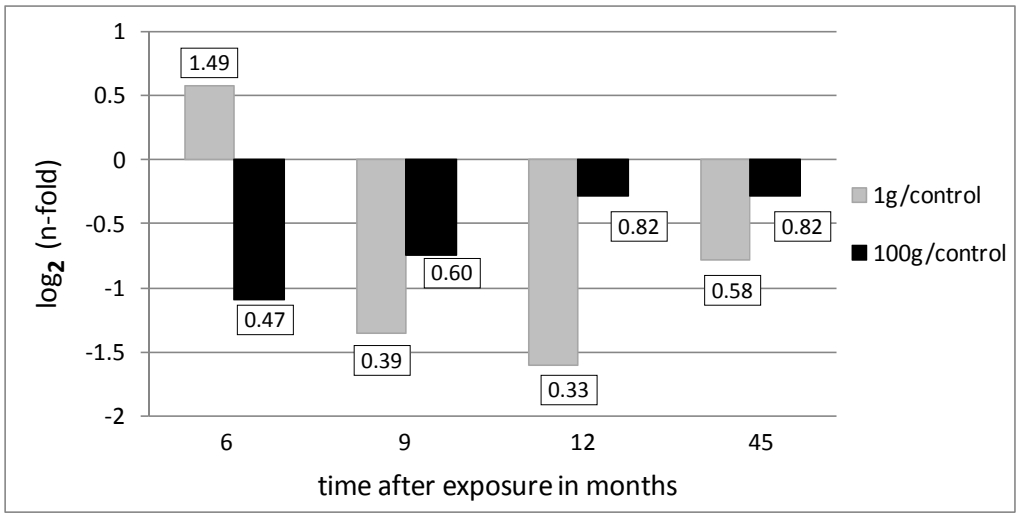

Figure 3.3. Expression of **C3** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

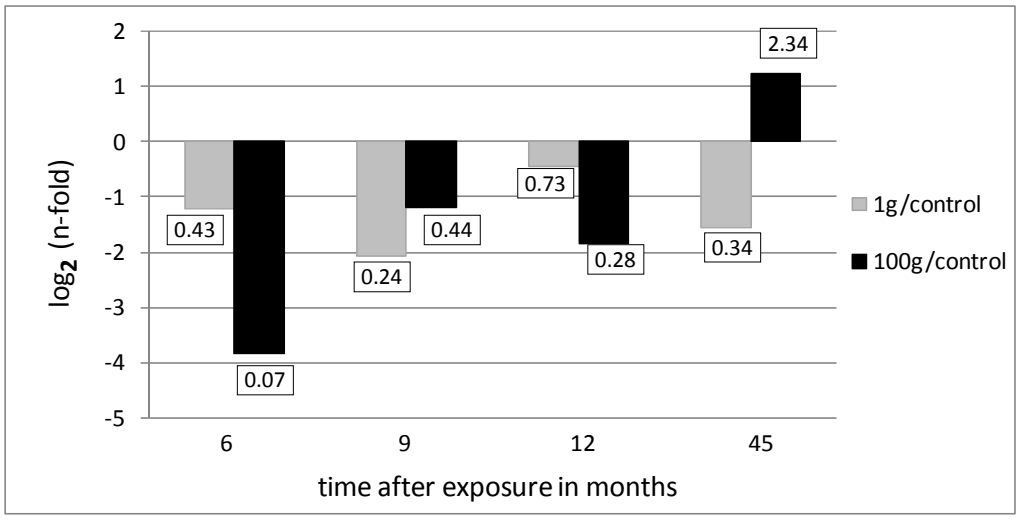

Figure 3.4. Expression of **C4** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

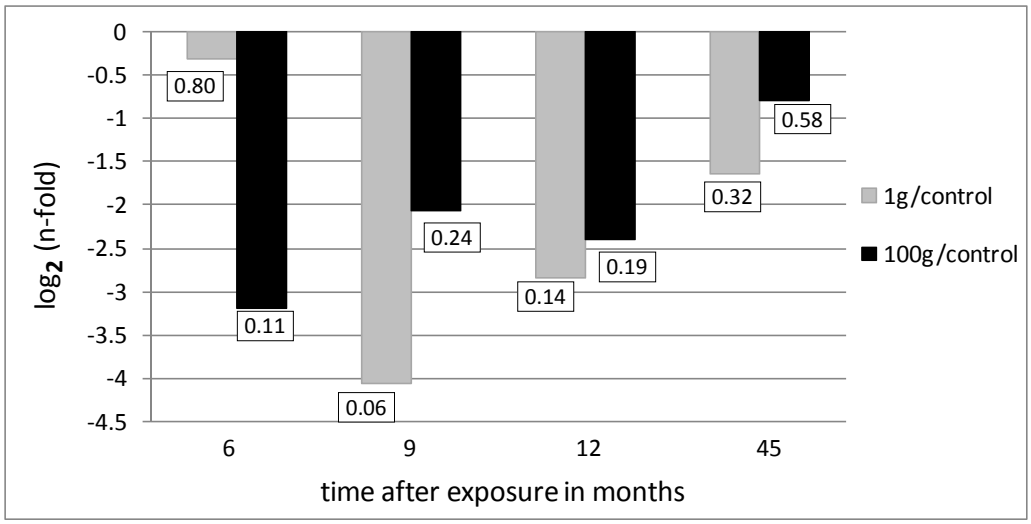

Figure 3.5. Expression of **CFB** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

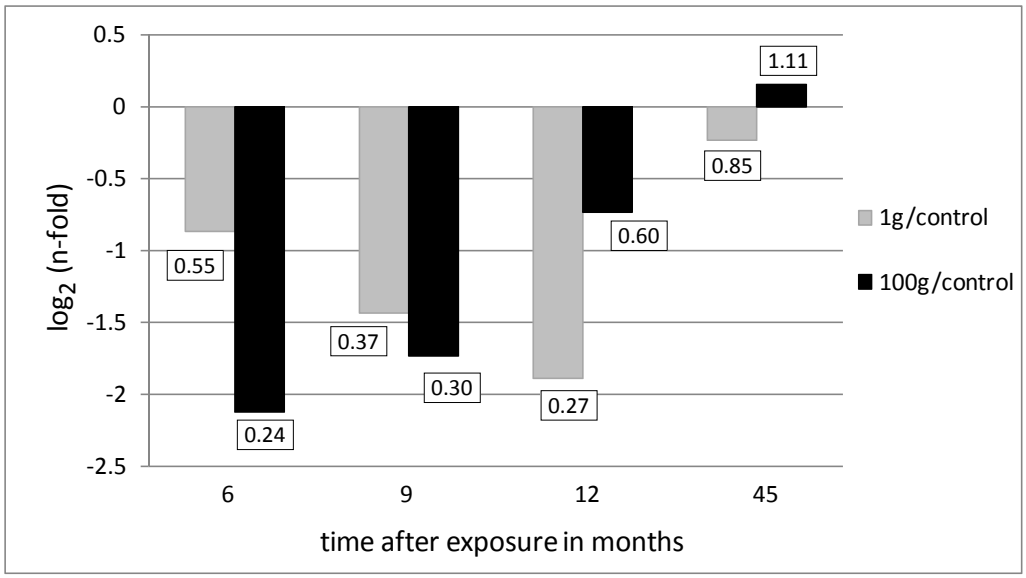

Figure 3.6. Expression of **TLR3** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

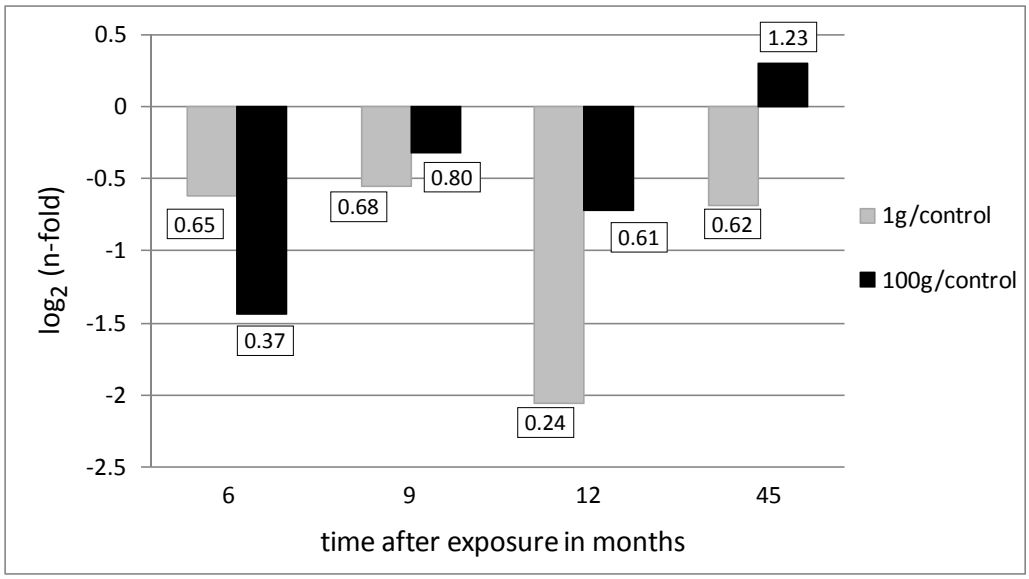

Figure 3.7. Expression of **TLR4** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

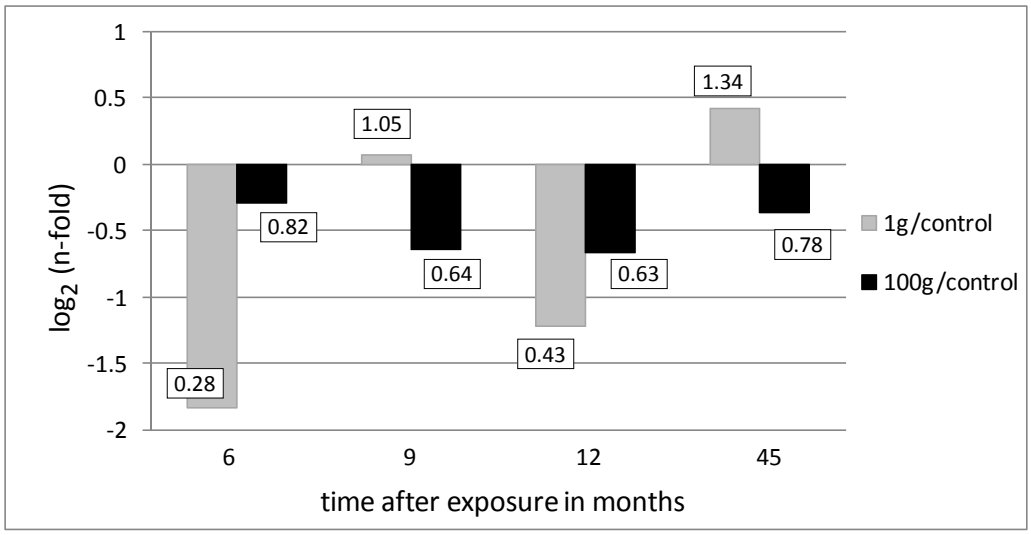

Figure 3.8. Expression of **TLR9** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

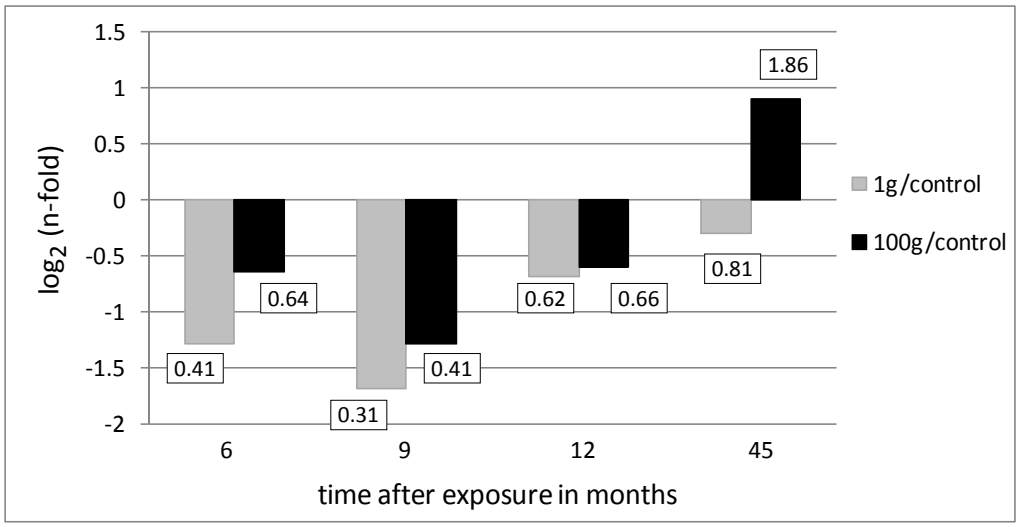

Figure 3.9. Expression of **IL-6** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

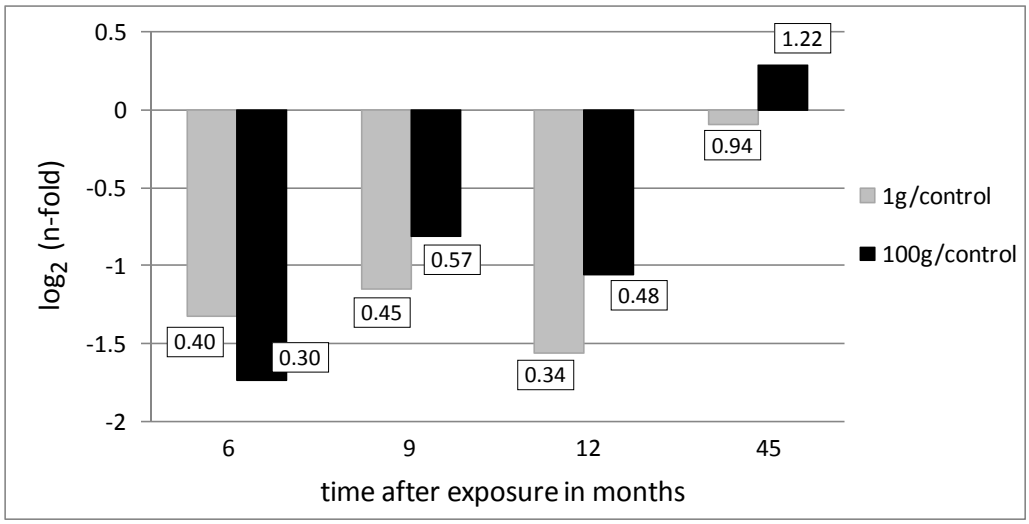

Figure 3.10. Expression of **IL-10** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

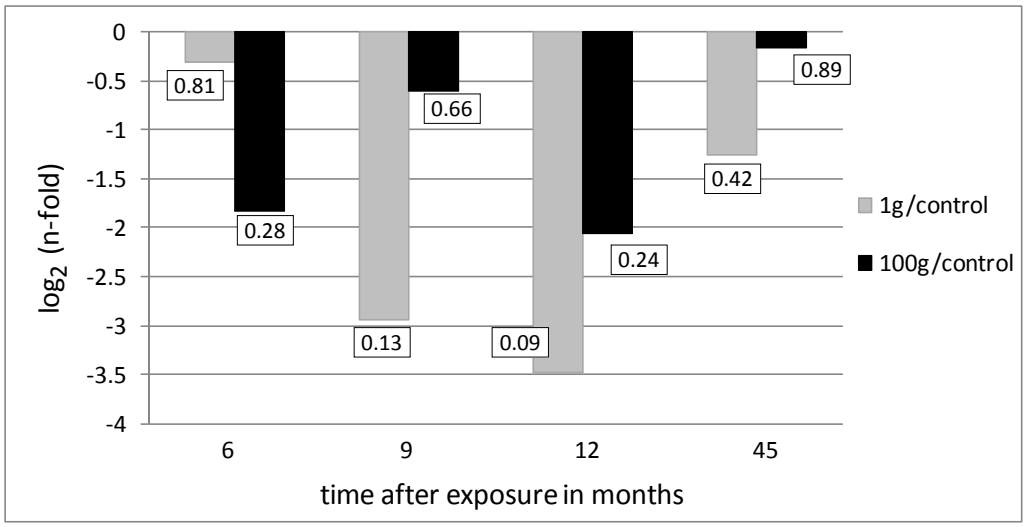

Figure 3.11. Expression of **CD14** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

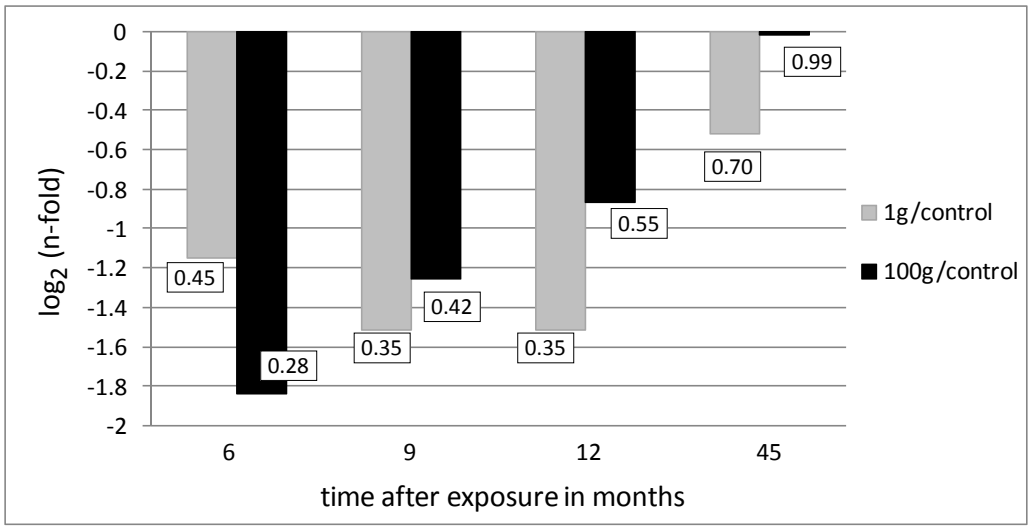

Figure 3.12. Expression of **MyD88** gene in 100g and 1g exposed cows in comparison to control (non-exposed) animals. Quantitative real-time polymerase chain reaction (QRT-PCR) results against time after challenge.

Numbers in boxes denote corresponding n-fold change.

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Chapter 4

General discussion and conclusions

4.1. General discussion

All diseases induce the immune system in response to invading microorganisms. The now widely accepted prion hypothesis (Prusiner 1982), in which the causative agent is said to be a misfolded version of a normal host derived cellular protein, raises the question of how the immune system responds to a self made protein. Although, no particular pathogen causing TSEs was yet identified, and the most accepted hypothesis seems to be the prion hypothesis, there is still growing evidence supporting involvement of the immune system in the modulating of TSEs.

Levels of expression of all three major components of immunity (CS, TLRs and interleukins) have been confirmed to be highly perturbated in various TSEs (Kitamoto et al. 1991; Lu et al. 2004; Mabbott et al. 2001), but even with the half of century of research in prion diseases, there is still little data explaining the role of immunity itself in pathogenesis of TSEs. The objective of this study was to examine the expression of selected

immunity genes, and perhaps provide a preliminary starting point for systematic research in this particular branch of science.

This thesis evaluated the gene expression in cattle orally challenged with two different doses of BSE contaminated feed at four time points. It was hypothesized that BSE exposed animals would demonstrate gene expression differences in both a dosage- and time dependent manner. Although in general, the results from this study were consistent with published literature, there are some time point related discrepancies reported in chapter 3. Results of Dandoy-Dron et al. (1998) and Riemer et al. (2004) are in conflict with results of this study, whereas Xiang et al. (2004) confirms the outcomes of our experiment. The great majority of tested genes showed a tendency to be down-regulated rather than up-regulated, which is surprising because an organism in a state of defense should up-regulate and synthesize immune proteins to tackle infection. The down-regulation itself can be considered surprising too, especially when a self protein is blamed to cause TSEs. As a matter of fact, from immunological point of view there should be no response to PrP^{RES} since TSEs are not presumed to be the autoimmune diseases.

The down regulation or up regulation of immune genes seems to depend on many factors. Firstly, the type of TSEs and whether the gene expression is examined in experimental or naturally identified cases of the disease. Secondly, the route of administration could have an impact on *in situ* mRNA synthesis, and thirdly dosage as well as various TSEs' strains themselves can trigger particular genes preferentially (Skinner et al. 2006).

The plethora of conflicting data makes it difficult to draw definite conclusions as to the importance of particular genes in pathogenesis of TSEs. Oligo-microarrays provide enormous amount of data, making it easy to overlook important genes that may not be highly expressed but more subtly modulated in response to infection. Researchers may focus only on very highly down- or up-regulated transcripts, as these are the ones that are evident using microarray analysis.

Therefore, it is beneficial to pursue systematic experiments that analyze particular biological systems such as the immune system, as this will shed some light on the role of immunity in pathogenesis of prion diseases. The experiments should not only focus on mRNA levels, as not all mRNA molecules are translated into proteins, but test proteins and their metabolites. Such an approach would ensure a better understanding of complex

immunological processes influencing susceptibility and progression of TSEs, including BSE. More time points should be analyzed to support systematic experiments. Four time points, tested in the present study are not sufficient to understand the possible role of particular genes in modulating prion diseases. Since experiments conducted on cows are very expensive and time consuming (feeding, maintenance cost and relatively long life span) the future experiments might focus on rodent models. It is also very important to test more genes coding proteins involved in immune responses in order to better understand the complex interaction between the host immune response and the infectious agent.

More complex experiments will require the best possible selection of housekeeping genes as internal standards including possible use of multiple standards to ensure accurate and optimized normalization.

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Appendices

Appendix 1 – Sample preparation for Quantitative Real-Time Polymerase Chain Reaction.

PART I. cDNA first strand synthesis. Reverse transcription reaction.

1. Pipette 1µg of total RNA sample into sterile, RNase-free 0.5 ml tube. Add 1µl T7 Oligo(dT) primer and adjust the total volume of reaction to 12µl with nuclease-free water. Mix with pipette and centrifuge briefly to collect the mixture at bottom of tube.
2. Incubate reaction mixture in 70°C for 10 min in a thermal cycler.
3. After incubation centrifuge briefly and place the tube on ice.
4. Prepare Reverse Transcription Master Mix: 2µl 10X first strand buffer, 4µl dNTP mix, 1µl RNase inhibitor, 1µl array script. Mix thoroughly with pipette and centrifuge briefly.
5. Add 8µl Reverse Transcription Master Mix to each sample. Pipette several times to mix and centrifuge briefly.
6. Incubate sample(s) in hybridization oven at 42°C for 2 hours.

7. After incubation centrifuge briefly. Place tubes on ice and proceed to Part II.

PART II. cDNA second strand synthesis.

1. Prepare Second Strand Master Mix: 63µl nuclease-free water, 10µl 10X second strand buffer, 4µl dNTP mix, 2µl DNA polymerase, 1µl RNase H. Mix thoroughly with pipette and centrifuge briefly.
2. Add 80µl Second Strand Master Mix to each sample. Pipette several times to mix and centrifuge briefly.
3. Incubate sample(s) in thermal cycler at 16°C for 2 hours.
4. After incubation centrifuge briefly. Place tubes on ice and proceed to Part III.

PART III. cDNA purification.

1. Preheat nuclease-free water in 55°C for at least 15 min.
2. Add 250µl cDNA binding buffer to your sample(s) mixing quickly with pipette. Transfer the whole mixture onto the center of cDNA cartridge filter in collection tube.
3. Centrifuge at 10000 RPM for 1 min in RT. Discard liquid from collection tube.

4. Place cDNA cartridge filter in the same collection tube. Add 500µl wash buffer onto the cartridge. Centrifuge at 10000 RPM for 1 min in RT. Discard liquid from collection tube.
5. Centrifuge again, as in step 4, to remove excess fluid from the filter. Transfer cartridge filter to a new elution tube.
6. Add 9µl nuclease-free water (55°C) onto the center of filter and incubate for 2 min in RT. Centrifuge at 10000 RPM for 1.5 min in RT.
7. Repeat step 6 and discard cartridge filter.

PART IV. aRNA synthesis.

1. Prepare aRNA Synthesis Master Mix: 3µl 50mM aaUTP, 12µl 25mM ATP, CTP, GTP mix, 3µl 50mM UTP solution, 4µl T7 10X reaction buffer, 4µl T7 enzyme mix. Mix thoroughly with pipette and centrifuge briefly.
2. Add 26µl aRNA Synthesis Master Mix to each sample. Pipette several times to mix and centrifuge briefly.
3. Incubate sample(s) in hybridization oven at 37°C for 16 hours.
4. After incubation add 60µl nuclease-free water to each sample and vortex gently to mix.

PART V. aRNA purification.

1. Preheat nuclease-free water in 55°C for at least 15 min.
2. Add 350µl aRNA binding buffer to each sample.
3. Add 250µl 100% (v/v) ethanol and quickly pipette 1-2 times to mix.
4. Transfer the whole mixture onto the center of aRNA cartridge filter in collection tube.
5. Centrifuge at 10000 RPM for 1 min in RT. Discard liquid from collection tube.
6. Place aRNA cartridge filter in the same collection tube. Add 650µl wash buffer onto the cartridge. Centrifuge at 10000 RPM for 1 min in RT. Discard liquid from collection tube.
7. Centrifuge again, as in step 6, to remove excess fluid from the filter. Transfer cartridge to a new aRNA elution tube.
8. Pipette 100µl nuclease-free water (55°C) onto the center of the cartridge filter and incubate for 2 min in RT. Centrifuge at 10000 RPM for 1.5 min in RT.
9. Place tube(s) with eluted aRNA on ice and proceed to Part VI.

PART VI. aRNA samples dilutions.

1. Measure the concentration of synthesized aRNA.
2. Dilute aRNA sample(s) to concentration of 100ng/µl.

3. The sample(s) are now ready to be used for quantitative real-time PCR reaction.

Appendix 2 – Quantitative Real-Time Polymerase Chain Reaction.

PART I. Quantitative real-time PCR.

1. Prepare Real-Time PCR Master Mix: 10µl TaqMan Fast Universal PCR Master Mix (2X), 0.18µl specific forward primer (100µM), 0.18µl specific reverse primer (100µM), 0.04µl specific probe, 4.6µl nuclease-free water.
2. Add the following to each in 96 well PCR plate: 15µl of master mix from step 1, 5µl aRNA (100ng/µl) from appendix 1, part VI, step 2. For each set of specific primers and probes, and each sample do reaction in triplicates.
3. Leave three wells empty, for negative control, for each set of primers and probes, and each sample.
4. Pipette only 20µl of master mix (step 1) to wells assigned for negative control.
5. Prepare TaqMan Positive Control Master Mix: 13.7µl TaqMan Fast Universal PCR Master Mix (2X), 2.6µl 10X Exo IPC Mix, 10X Exo IPC DNA, 7.7µl nuclease-free water.
6. Pipette 20µl of positive control master mix (step 5) to wells assigned for positive control.

7. Seal plate with optical adhesive cover, centrifuge at 2000 RPM for 1 min to remove bubbles.
8. Place the plate in Applied Biosystems StepOnePlus PCR thermal cycler. Close the drawer.
9. Thermal cycler conditions:

    Hold at 50°C for 2 min.

    Hold at 95°C for 20 sec.

    Cycle (each of 40) 95°C for 1 sec.

    Cycle (each of 40) 60°C for 20 sec.