CYTOKINES AND IMMUNOGLOBULIN GENETICS IN NEONATAL CALF DIARRHEA

INAUGURAL DISSERTATION
for the acquisition of the doctoral degree
Doctor medicinae veterinariae (Dr. med. vet.)
at the Faculty of Veterinary Medicine
Justus Liebig University Giessen, Germany

STEPHANI JOSEPHA FISCHER

Faculty of Veterinary Medicine, Institute of Hygiene and Infectious Diseases of Animals,
Justus Liebig University Giessen, Germany

Supervisor: Prof. Dr. Rolf Bauerfeind

Division of Microbiology and Animal Hygiene, Institute of Veterinary Medicine, Department of Animal Sciences, Faculty of Agricultural Sciences, Georg-August University Goettingen, Germany

Supervisor: Prof. Dr. Dr. Claus-Peter Czerny

Small Animal Clinic, Institute of Veterinary Medicine, Georg-August University Goettingen, Germany

Supervisor: Prof. Dr. Stephan Neumann

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submitted by

STEPHANI JOSEPHA FISCHER
Veterinarian from Hattingen, Germany

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For the acquisition of the doctoral degree at the Faculty of Veterinary Medicine, Justus Liebig-University Giessen, Germany

Dean: Prof. Dr. Dr. h.c. Martin Kramer

Referee 1: Prof. Dr. Rolf Bauerfeind
Referee 2: Prof. Dr. Dr. Claus-Peter Czerny, Prof. Dr. Stephan Neumann

Examiner: Prof. Dr. Klaus Doll

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To my parents and my beloved brother

"Knowledge is the only competitive advantage of our times,
it grows through open interaction with others."
Ronald Coase

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1 INTRODUCTION

Animal health is an important part of all scientifically recognized definitions of animal welfare. “Freedom from pain, injury or disease”, is one of the Five Freedoms, postulated by the Farm Animal Welfare Council in 1979. The World Organization for Animal Health (OIE) writes in its “Terrestrial Animal Health Code” that “an animal is in a good state of welfare if it is healthy [...]” (OIE, 2013). Early diagnosis and adequate therapeutics are crucial in improving animal health, and thus in making a contribution to animal welfare.

In cattle, enteric infectious diseases, such as neonatal calf diarrhea, still cause significant economic losses and pose a marked threat to animal welfare (Waltner-Toews et al., 1986; Warnick et al., 1995; Mohd Nor et al., 2012). The main pathogens responsible for neonatal calf diarrhea are *Bovine Coronavirus* (BCV), *Bovine Rotavirus* (BRV), enterotoxic *E. coli* equipped with F5 fimbriae (F5-ETEC) and *Cryptosporidium parvum* (*C. parvum*) (Kaske, 1993; Al Mawly et al., 2015a).

The diagnosis in the early stages of inflammation, already before clinical signs occur, can help to prevent a fatal course of the disease. Furthermore, an estimation of the prognosis enables the veterinarian to monitor critical patients more intensively. In several disorders, interleukins, and especially interleukin-6 (IL-6), appear to be such markers of early inflammation and prognosis (Hisaeda et al., 2011; Neumann et al., 2012; Schüttler et al., 2015; Mat-Nor et al., 2016). Interleukin-6 is a representative for the pro-inflammatory interleukins with a pleiotropic mode of action (Ataie-Kachoie et al., 2014). The role of IL-6 in inflammation includes induction of the acute phase response, triggering T-cell proliferation and stimulating the differentiation of B-cells (Banks et al., 1995; Yoshida et al., 2010; Rosser et al., 2014). In this dissertation, IL-6 will be proven as a prognostic marker in neonatal calf diarrhea. As very little information concerning IL-6 in newborn calves exists, the first study investigates the physiological development of IL-6 gene-expression and serum titers during the first four weeks of life. In a second study, IL-6 will be investigated in calves suffering from diarrhea to determine the prognostic reliability of this parameter in predicting the course of the disease.

Beside the early diagnosis, an effective therapeutic strategy is essential for animal health. Treatment of livestock becomes more and more critical due to the growing negative image of antibiotic use, the resistance situation in many bacterial pathogens

and the lack of adequate antiviral therapeutics (Bosman et al., 2014; Liu et al., 2016). To prevent viral and bacterial infections in the case of neonatal calf diarrhea, two main strategies are currently pursued. The first is dam vaccination with compounds containing antigen preparations of common diarrheagenic pathogens. The second is passive immunization of calves with concentrated immune sera, obtained by colostrum purification. However, these two options are tainted with several disadvantages. Effective dam vaccination requires significant logistical effort during immunization and colostrum management. Furthermore, colostrum quality is limited to the cow's individual immunological competence (Kuegler et al., 2015). The elaborate production of orthologous and homologous immune sera results in expensive compounds, whose application is limited to individual cases. It can be stated that species-specific immunoglobulins are important for prophylaxis and therapy. Unfortunately, no standardized economic solutions are available. One possibility to overcome this therapeutic shortfall, is the generation of species-specific high affine single-chain fragment (scFv) antibodies in immunized antibody libraries and their production by recombinant expression systems. In a scFv molecule, a peptide linker connects the variable domain of an antibody-heavy chain to the variable domain of an antibody-light chain (Farajnia et al., 2014). The limitation of the scFv antibody on structures crucial for antigen binding results in a very small but functional molecule (Sandhu, 1992). Regarding tissue penetration, this is particularly advantageous, especially for future clinical applications (Ahmad et al., 2012). Therefore, the third study of this dissertation aims at the generation of a bovine scFv antibody against BCV. Initially, a primer will be established for the amplification of the whole genomic antibody repertoire in cattle. This will be used to amplify antibody encoding mRNA from B-cells of a donor animal. The antibody sequences will be cloned and transformed into *E. coli* cells with the aim of generating a highly diverse bovine antibody library. Phage display will be used to screen the library for specific antibodies against BCV. Isolated antibodies will be further characterized concerning their specificity and affinity.

The present dissertation aims to improve the prognostic and therapeutic potential in neonatal calf diarrhea by establishing a reliable prognostic marker and by developing innovative antiviral drugs. This will contribute to an improvement of farm animal welfare. The development of a recombinant species-specific antibody library will

further promote the therapeutic application of recombinant antibodies in veterinary medicine, as they are already frequently used in human medicine (Reichert, 2015).

2 LITERATURE

2.1 Neonatal Calf Diarrhea

Neonatal calf diarrhea is described as being one of the most devastating diseases in rearing calves (Lorenz et al., 2011a). The disease has an important impact on the economic calculation in dairy farming as well as on the welfare of the affected animals (Donovan et al., 1998; Mohd Nor et al., 2012; Windeyer et al., 2014). Usually, mixed infections with one of the four most important pathogens (enterotoxic *E. coli* equipped with F5 fimbriae (F5-ETEC), *Bovine Rotavirus* (BRV), *Bovine Coronavirus* (BCV), *Cryptosporidium parvum* (*C. parvum*)) are detected (de la Fuente et al., 1999; Foster et al., 2009). Despite these infectious agents, several environmental factors, such as housing conditions and colostrum quality, impair the risk and outcome of diarrhea (Quigley et al., 1995; Al Mawly et al., 2015b). Neonatal calf diarrhea primarily affects calves in their first two weeks of life (Lorenz et al., 2011a). The animals suffer from secretory malabsorptive diarrhea (Foster et al., 2009). Depending on the respective pathogen responsible for the diarrhea, specific treatment options are either available or missing. In *E. coli* infections, the use of antibiotics is indicated, but the growing negative image of antibiotics and bacterial resistances require careful consideration of the application (Bosman et al., 2014; Liu et al., 2016). For *C. parvum* infections, drugs containing the active component Halofuginon are licensed in Germany, but bear the disadvantage of having a very small therapeutic interval with a high risk of intoxications (Silverlas et al., 2009). Regarding the viral pathogens (BCV, BRV), no specific anti-viral therapy is available. However, the most important therapeutic intervention is a rapid and sufficient fluid substitution (Meganck et al., 2014). In order to avoid outbreaks of neonatal calf diarrhea, several preventive strategies, such as dam vaccination, cautious colostrum management and effective disinfection strategies are recommended (Meganck et al., 2015).

2.2 Bovine Coronavirus

2.2.1 Taxonomy and Structure

Bovine Coronavirus (BCV) was first described in 1972 (Stair et al., 1972; Mebus et al., 1973a). Until 2008 the virus was taxonomically grouped in the order *Nidovirales,* family *Coronaviridae*, genus *Coronavirus*, species *Bovine Coronavirus* by the International Committee on Taxonomy of Viruses (ICTV; www.ictvonline.org).

In 2008, the committee assigned *Bovine Coronavirus* together with other coronaviruses, such as *Human Coronavirus OC43,* to a new species *Betacoronavirus 1*, due to the high percentage of sequence identity. Today, the correct taxonomic classification of *Bovine Coronavirus* is: order *Nidovirales,* family *Coronaviridae*, subfamiliy *Coronoavirinae*, genus *Betacoronavirus*, species *Betacoronavirus 1*.

The virus is composed of five major structural proteins, the spike (S) protein, the hemagglutinin-esterase (HE) protein, the transmembrane (M) protein, the small envelope (E) protein and the nucleocapsid (N) protein (Chouljenko et al., 2001). A structural model of a BCV is shown in figure 1. The S protein (190 kD) consists of two subunits S1 (105 kD) and S2 (90 kD) (Abraham et al., 1990a). The S1 subunit forms the characteristic outer projection of this viral surface protein and contains a highly polymorphic domain, which is assumed to contribute to pathogenicity (Yoo et al., 1991b; Rekik et al., 1994). Host-cell receptor binding is the main function of the S1 subunit (Kubo et al., 1994; Peng et al., 2012). Additionally, it agglutinates mouse, rat and chicken red blood cells (Schultze et al., 1991a). The S2 subunit is located in the virus membrane and is important for the fusion of virus and host cell membrane (Yoo et al., 1991a; Luo et al., 1998). The second surface protein with hemagglutination capability, is the like-named HE protein. This protein is as a 120-140 kD dimer, which is assembled by two 65 kD monomers connected by disulphide bridges (Hogue et al., 1989). In comparison to the S1 subunit of the S protein, it is less potent in hemagglutination, as it only agglutinates rat and mice red blood cells, but not chicken red blood cells (Schultze et al., 1991a). Despite that, the HE protein shows hemadsorbtion, receptor-binding and receptor-destroying functions (Kienzle et al., 1990; Schultze et al., 1991b). Another smaller surface protein is the so-called small envelope (E) protein (8.4-12 kD) (Abraham et al., 1990b; Ko et al., 2006; Liu et al., 2007a). It bears important functions for sufficient assembly and morphogenesis of virus particles (Fischer et al., 1998; Raamsman et al., 2000).

The M protein (24-26 kD) consists of three different domains (King et al., 1982; Lapps et al., 1987): the N-terminal domain, which lies on the outside of the virus, the membrane spanning domain and the carboxy-terminal domain, which lies in the inside of the virus particle and interacts with the nucleocapsid (Clark, 1993). The fifth major structural protein, is the N protein (50-52 kD), which is associated with the genome forming a helical nucleocapsid (King et al., 1982; Lapps et al., 1987).

All these proteins are encoded in the positive-sense single stranded RNA genome (27-30kb), in the 5'-3'- order HE, S, E, M, N (Guy et al., 1979; Spaan et al., 1988). At the 5'-end additional non-structural proteins, such as the RNA polymerase are encoded (Spaan et al., 1988).

Concerning the antigenicity, for long time, only one serotype had been suggested for BCV (Tsunemitsu et al., 1995; Bok et al., 2015). More recent studies, showed the existence of at least two clades of BCV, when strain comparison is based on the amino acid sequence of the S protein (Fulton et al., 2013; Kanno et al., 2013). Despite these observations of the overall antigenicity for virus strain comparisons, antigenic structures were studied in more detail by using monoclonal antibodies to investigate specific epitopes on the major structural proteins of BCV. Most neutralizing epitopes were found on the S1 subunit of the S protein (Deregt et al., 1987; Vautherot et al., 1990; Michaud et al., 1993). These investigations determined four antigenic domains (A-D) using monoclonal antibodies in competition ELISA tests (Vautherot et al., 1990). Domain A and C also contain non-neutralizing epitopes, which seemed to be highly conserved between BCV isolates (Michaud et al., 1993). In the S2 subunit two different antigenic domains (A, B) were shown (Vautherot et al., 1992b). In domain A a highly conserved linear epitope was found (Vautherot et al., 1992a). In western blotting, antibodies binding to the S2 subunit showed additional reactivity on a 200 kD glycoprotein (gp200) (Vautherot et al., 1992b). This gp200 was supposed to be an oligomer of some S2 subunits. Neutralizing epitopes were also found on the HE protein (Deregt et al., 1987; Parker et al., 1989; Parker et al., 1990; Vautherot et al., 1992b). Antibodies against the HE protein bound to conformational dependent epitopes on the HE dimer, as they did not react on the denatured protein (Vautherot et al., 1990; Vautherot et al., 1992b). Antibodies binding to the M protein were only able to neutralize the virus when complement was added (Saif, 1993). Epitopes found on
the nucleoprotein were shown to be continuous and non-neutralizing (Hussain et al., 1991).

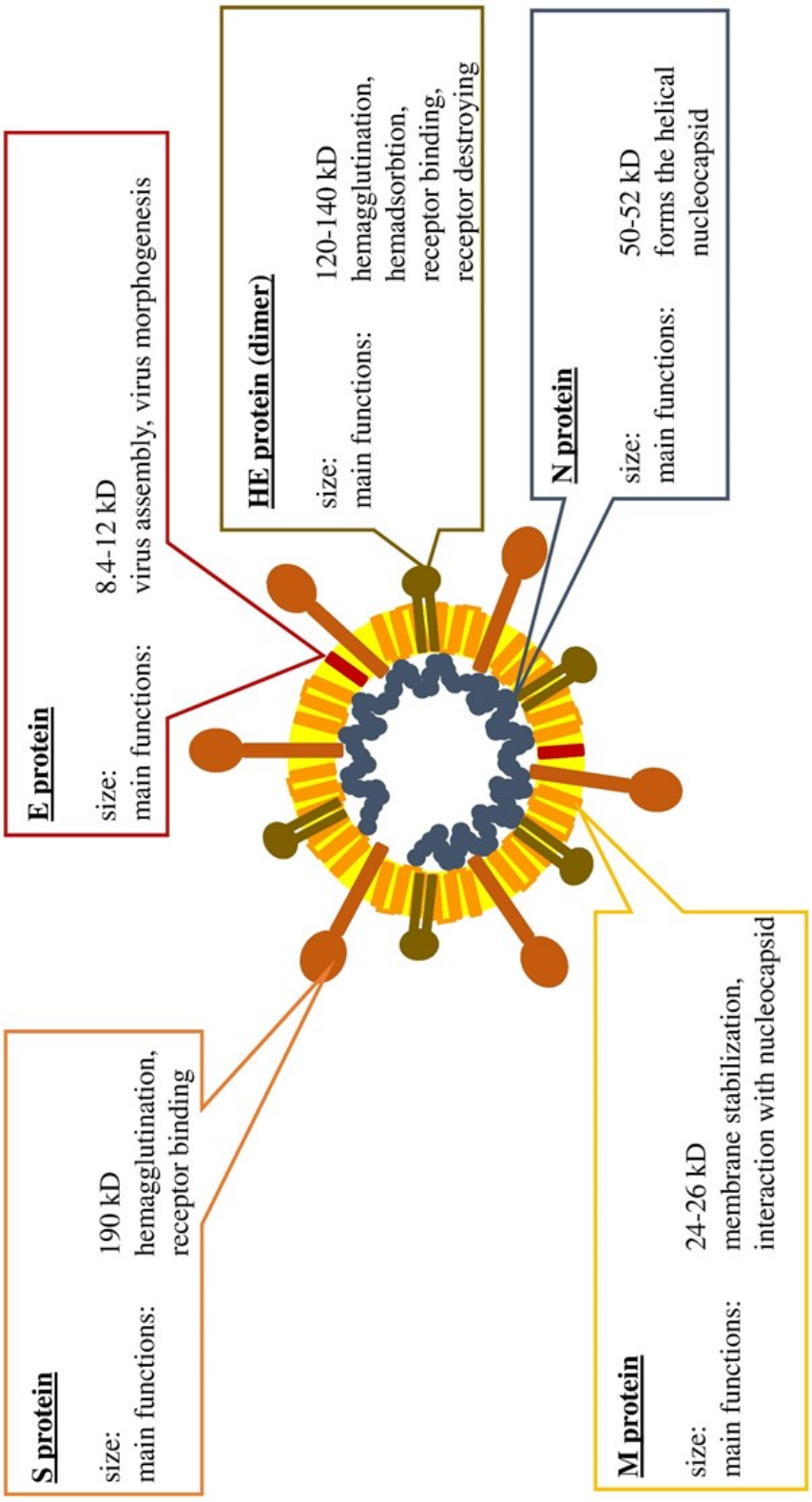


Fig. 1. *Bovine Coronavirus*. Structure of a BCV virus particle with the main structural proteins. S, spike-protein; E, small envelope protein; HE, hemagglutinin-esterase protein; N, nucleo-protein, M, membrane protein

2.2.2 Epidemiology and Pathogenesis of Diarrhea caused by BCV

Bovine Coronavirus is distributed worldwide and serum antibodies against BCV can be detected in most adult cattle (Abraham et al., 1992; Kapil et al., 1999; Park et al., 2006; Coura et al., 2015; Gunn et al., 2015; Moore et al., 2015). Adult cattle are the main reservoir for this virus and a consecutive occurrence on the same farm is common (Crouch et al., 1985; Clark, 1993). Virus excretion increases at birth and during the winter months, making calves born during winter seasons more prone to an infection (Bulgin et al., 1989; Gulliksen et al., 2009a). But carrier animals can also be found among healthy calves, where up to 24% carry the virus (Heckert et al., 1991).

Pathologic changes and clinical outcome has been intensively studied in the years after the first description of BCV in 1972 (Mebus et al., 1973b; Mebus et al., 1975; Lewis and Phillips, 1978; Langpap et al., 1979).

The infection of calves occurs via the oronasal route and initially affects the small intestine, where it leads to the stunting of the villi. It spreads to the large intestine, where an atrophy of colonic rides occurs. The virus replication in surface epithelia cells catalyzes cell death and the replacement of these cells with immature cells. These cells have a deficient absorptive and digestive capacity, whereas the secretory activity is functional. Therefore, these cell replacement causes severe loss of water and electrolytes in the intestine of infected calves. The infection is resolved by self-limitation, as the new immature cells are more resistant to an infection.

Despite the clinical picture of neonatal calf diarrhea, which mainly affects 1-2 week old calves, two other clinical pictures were related to BCV (Boileau et al., 2010; Cho et al., 2013; Ammar et al., 2014). The first is a respiratory tract infection, where the virus infects epithelia cells of the nasal cavity and tracheas, causing respiratory symptoms, such as rhinitis, sneezing and coughing (Saif, 2010). The second is winter dysentery, a bloody diarrheic disease in adult cattle (Saif, 1990). No reliable genetic marker was found to differentiate BCV strains, isolated from these three clinical syndromes (Zhang et al., 1994; Tsunemitsu et al., 1995; Traven et al., 2001).

2.3 Bovine Interleukin 6

2.3.1 Cytokines and the Immune System

Following the recommendations of the Nomenclature Committee of the International Union of Immunological Societies (IUIS), cytokines are defined as molecules which originate in immune cells and mediate important steps in an immune response (Paul et al., 1992). The main characteristics of cytokines are redundancy, ambiguity, pleiotropy and the biological activity in a nano- to picomolar range (Lunney, 1998). In the organism, cytokines form a complex network for intercellular communication (Subramanian et al., 2015). Three communication networks can be distinguished: one between immune cells, one between body cells and one at the interface of immune and body cells (Frankenstein et al., 2006). Focusing on the network between immune cells, cytokines connect the different levels of immune cells (Figure 2) (Iwasaki et al., 2015). A sensor cell (e.g. macrophages, dendritic cells) recognizes an antigen and starts to produce level 1 cytokines, which activate specific immune cells (e.g. lymphocytes). These immune cells then activate other immune effector cells, such as B-cells or macrophages, via the secretion of level 2 cytokines. During this pathway, the type (e.g. Th1, Th2) and the duration of an immune response is controlled by the secreted cytokine pattern (Lunney, 1998). In several medical contexts, the detailed understanding of cytokine pathways is important. Especially in autoimmune diseases and chronic inflammation, cytokines were supposed to play a major role in the pathogenesis (Barnes, 2009; Liu et al., 2013b; Landskron et al., 2014; Raphael et al., 2015). Furthermore, in animal genetic research, efforts were made to breed for disease resistance by focusing on markers which are related to specific cytokines (Maryam et al., 2012; Sato et al., 2015).

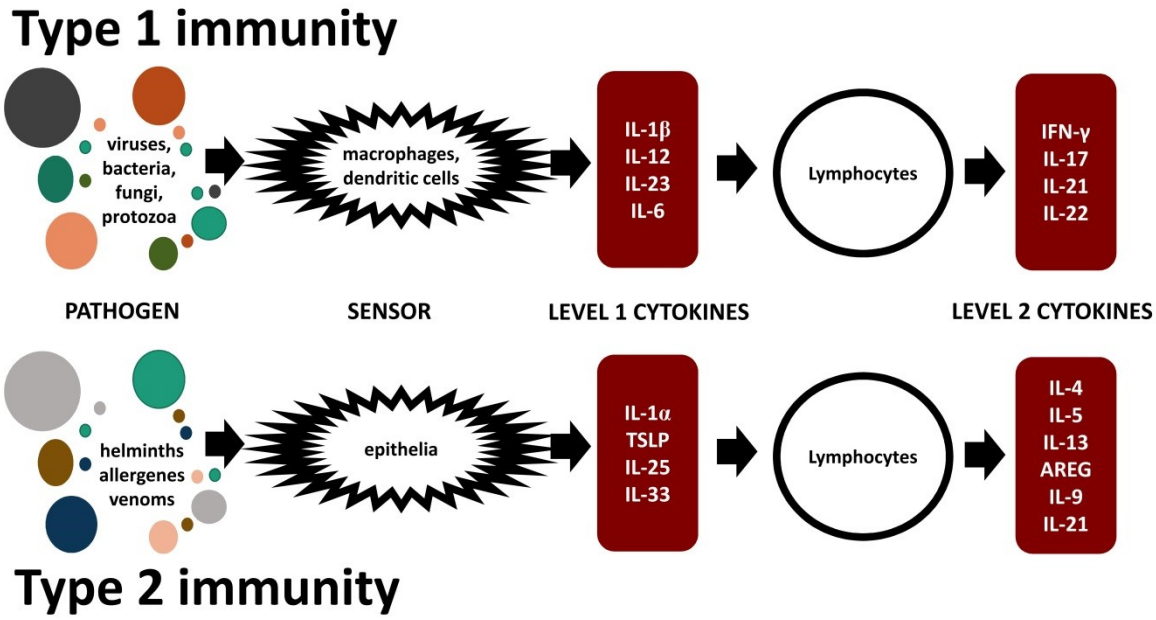


Fig. 2. Role of Level 1 and Level 2 cytokines in Type 1 and Type 2 immunity. Adapted by permission from Macmillan Publishers Ltd. [Nature Immunology] (Iwasaki A., Medzhitov R.; Control of adaptive immunity by the innate immune system.), copyright © (2015)

2.3.2 Interleukin-6

2.3.2.1 Protein

The first descriptions of Interleukin-6 (IL-6) were based on its biological function (Hirano, 2014). Before the cDNA of IL-6 was described in 1986, it was found in literature as T-cell replacing factor (TRF), B-cell growth factor II (BCGF II), B-cell stimulating factor (BSF2), TRF-like factor, B-cell differentiation factor II (BCDF II), Interferon beta 2 (IFNß2), plasmacytoma/ hybridoma/ myeloma growth factor or hepatocyte-stimulating factor (Schimpl et al., 1972; Howard et al., 1982; Swain et al., 1982; Teranishi et al., 1982; Hirano et al., 1986; Zilberstein et al., 1986; Gauldie et al., 1987; Van Damme et al., 1987; Kawano et al., 1988). In 1988 the molecule was finally renamed as Interleukin-6 by the New York Academy of Science (Hirano, 2014). The human IL-6 is a 212 amino acid protein with two potential N-glycosylation sites (Hirano et al., 1986; Droogmans et al., 1992). In comparison, the bovine IL-6 is a 208 amino acid protein with only one potential N-glycosylation site. It shows 76 %

similarity on the nucleotide and 53% similarity on the protein level to the human protein (Droogmans et al., 1992). In both species, the protein shows a relatively conserved middle part with four invariant cysteines, which is considered important for biological activity (Tanabe et al., 1988; Droogmans et al., 1992).

2.3.2.2 Receptor

The effect of IL-6 is transmitted via two different pathways. One pathway uses a membrane-bound form of the IL-6 receptor (classical pathway) and another pathway uses a soluble from of the IL-6 receptor (trans-signaling pathway).

The membrane-bound form of the IL-6 receptor (IL-6R) is mainly located on hepatocytes, leukocytes and megakaryocytes (Rose-John et al., 2006). It is a 80 kD protein, which is also known as Typ 1 cytokine α-receptor subunit or CD126 (Yamasaki et al., 1988). For successful signal transmission, the IL-6 receptor forms a heterodimer unit with the gp130 (Skiniotis et al., 2005). This 130 kD glycoprotein is also known as a signal transducing β-receptor unit or CD130 (Taga et al., 1989). It is universally expressed on cell surfaces and is not specific for the IL-6 signaling pathway (Xu et al., 2013). Neither the binding of IL-6 to the IL-6R nor to the gp130 alone leads to signal transmission; only the complex of IL-6, IL-6R and the gp130 is functional (Taga et al., 1997). The subsequent signal transmission in the cell either leads to the activation of a Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathway, mainly regulating cell development and homeostasis, or to the activation of a Src homology-2 protein tyrosine phosphatase (SHP)-2 driven Ras-Raf (**r**at **s**arcoma protein; **r**apidly **a**ccelerated **f**ibrosarcoma protein) mitogen-activated protein kinase (MAPK) pathway, mainly regulating cell growth and differentiation (Rawlings et al., 2004; McCubrey et al., 2007; Rose-John, 2012).

The trans-signaling pathway of IL-6 is mediated by a soluble form of the IL-6 receptor (sIL-6R) (Novick et al., 1989). The sIL-6R is generated by alternative splicing of the IL-6R mRNA or by proteolytic cleavage of the membrane bound IL-6R (Wolf et al., 2014). The complex of IL-6 and the sIL-6R has a significant higher affinity towards the gp130 than its counterpart, the complex of IL-6 and IL-6R (Rose-John et al., 1990; Rose-John, 2012). Therefore, the IL-6 effect differs between the classical and the trans-signaling pathway. The quality of cell response mediated by the trans-signaling

pathway is found to be stronger and prolonged (Peters et al., 1998). Aside from the sIL-6R, a soluble form of the gp130, called sgp130RAPS also exists (Narazaki et al., 1993). The sgp130RAPS is generated by alternative splicing of mRNA (Wolf et al., 2014). In a physiological state, the sgp130RAPS neutralizes the complex of IL-6/sIL-6R in the circulation, due to a balance of all three compounds (Jostock et al., 2001; Richards et al., 2006).

When taking together these findings and looking at the overall effect of IL-6, it is shown that the classical IL-6 pathway leads to anti-inflammatory processes, whereas the trans-signaling IL-6 pathway leads to pro-inflammatory processes (Grivennikov et al., 2009; Scheller et al., 2011).

2.3.2.3 Regulation

In healthy humans, the IL-6 serum concentrations are in the pg/ml range and IL-6 is neutralized by a complex of sIL-6R and sgp130 (Rose-John, 2012; Hou et al., 2016). Under septic conditions the serum concentrations increase up to µg/ml and cancel the neutralizing effect of sIL-6R and sgp130 (Lin et al., 2015). The regulation of IL-6 mainly occurs on the transcriptional level. Various microRNAs and transcription factors regulate the IL-6 gene expression (Table 1) (Baccam et al., 2003; Terasaka et al., 2010). Out of the promoting transcription factors, NF-κB seems to be the most important (Li et al., 2002). Besides, secondary messengers such as bacterial lipopolysaccharides, viruses, other cytokines and growth factors can enhance the IL-6 gene expression (Ray et al., 1988; Isshiki et al., 1990; Park et al., 2003). Additional regulation occurs on the post-transcriptional level via stabilization of the 3'UTR region of the IL-6 mRNA. Again, several different proteins and microRNAs act in a stabilizing or degrading manner on the IL-6 mRNA (Table 1) (Xu et al., 2011; Akira, 2013; Masuda et al., 2013). Finally, a genetic polymorphism was found in the IL-6 promotor region, which leads to a higher IL-6 transcription rate in the corresponding phenotype (Fishman et al., 1998; Ataie-Kachoie et al., 2014).

Table 1 Proteins and microRNAs regulating the IL-6 gene expression.

LEVEL	Upregulation/Stabilization		Suppression/Degradation	
Genomic	174 G/C genotype → high producer		174 C/C genotype → low producer	
	Protein	**miRNA**	**Protein**	**miRNA**
Transcription	***Primary messengers***			
	NF-κB		Ahr	miR-155
	SP-1		GR	miR-146a/b
	NF-IL-6		ER	miR-223
	AP-1		p53	
	IRF1		Rb	
	Mutantp53		PPARα	
	CREB			
	Secondary messengers			
	bacterial LPS viruses			
	IL-1			
	TNF-α			
	EGF			
	PDGF			
	TGF-β			
Post-Transcription	Arid5a		Regnase-1	miR-365
	P38α		BRF1	miR-608
			BRF2	

Adapted from Tanaka T., Narazaki M., Kishimoto T., 2014. Il-6 in inflammation, immunity, and disease. Cold Spring Harb Perspect Biol 6, a016295. Copyright © 2014 Cold Spring Harbor Laboratory Press

2.3.2.4 Physiology and Pathophysiology

The two main functions of IL-6 are the regulation of inflammatory processes and the induction of an immune response. During inflammation, IL-6 is produced in the local lesion and reaches the liver via blood stream (Heinrich et al., 1990). The hepatocytes respond to a IL-6 stimulation with the production of acute phase proteins (Gauldie et al., 1987; Banks et al., 1995).

Despite this systemic pro-inflammatory effect, IL-6 also acts on the local level. Resting T-cells are reactivated by an IL-6 mediated increase of IL-2 and prevented from apoptosis due to the activation of STAT3 and Bcl-2 by IL-6 (Teague et al., 1997; Atreya et al., 2000; Yoshida et al., 2010). Naïve CD^{4+} cells maturate to Th2 effector

cells under the influence of IL-6 and TGF-β and CD^{8+} cells develop into cytotoxic T-cells (Okada et al., 1988; Rincon et al., 1997; Chen et al., 2003). The combination of IL-6 and TGF-β is also responsible for the balance of T_{reg} and Th17 cells at the site of inflammation and thereby creates a pro-inflammatory milieu (Bettelli et al., 2006; Korn et al., 2009; Kimura et al., 2010). Taken together, these findings demonstrate that the local IL-6 concentration is responsible for the change of an acute neutrophil dominated to a sustained monocyte-dominated inflammation (Kaplanski et al., 2003). Beside the effects on the inflammatory cascade and the innate immune response, IL-6 also affects the adaptive immune response. It triggers the development of B_{reg} and plasma cells, and therefore leads to an augmented production of immunoglobulins (Hirano et al., 1986; Muraguchi et al., 1988; Rosser et al., 2014).

Furthermore, IL-6 is involved in several other metabolic processes, such as haematopoiesis, angiogenesis and cell growth (Hassan et al., 1995; Heike et al., 2002; Gopinathan et al., 2015).

Two different pathophysiological conditions are possible in the IL-6 metabolism, an excess or a lack of IL-6. An excessive production of IL-6 is observed in several autoimmune and IgG mediated diseases (Yamashita et al., 2011; Zhou et al., 2011; Ho et al., 2015). For example, in rheumatoid arthritis, IL-6 is responsible for the local inflammatory milieu in the affected joint by inducing chemokine and cell adhesion molecule expression (Suzuki et al., 2010). Furthermore, joint destruction is reinforced by IL-6, due to the activation of osteoclastogenesis (Kotake et al., 1996). In mice models of Crohn's Disease, IL-6 is proven to provoke the expression of leukocyte adhesion molecules and pro-inflammatory cytokines resulting in a chronic inflammation of the intestine (Yamamoto et al., 2000).

Finally, IL-6 is also described as playing a critical role in the activation of oncogenic pathways (Grivennikov et al., 2008; Atsumi et al., 2014).

A lack of IL-6 is linked to an exacerbated inflammation, due to the down-regulation of IL-10 production in B_{reg} cells and an increased production of pro-inflammatory proteins (Hurst et al., 2001; Rosser et al., 2014). For instance, Interleukin-6-deficient mice developed more severe lung damage than the wild-type strain after an infection with Influenza virus A strains (Lauder et al., 2013).

2.3.3 Clinical Relevance

The crucial influence of IL-6 on the regulation of inflammation and immune response make this cytokine an interesting target for diagnostic, prognostic and therapeutic questions in medicine.

2.3.3.1 Human Medicine

In human medicine, IL-6 shows an applicability for the diagnosis of bacterial infection in newborns. In newborns admitted to hospital, increased serum IL-6 levels revealed a sensitivity of 73% and a specificity of 78% for the detection of a bacterial infection directly on admission (Buck et al., 1994). Furthermore, additional studies demonstrated the potential of IL-6 as discriminator between viral and bacterial gastroenteritis in children. Children suffering from an acute gastroenteritis had significantly higher IL-6 serum concentrations than those suffering from a viral gastroenteritis (sensitivity 79%, specificity 86%) (Lin et al., 2006). Similar was shown in cases of acute gastroenteritis due to infections with norovirus, rotavirus and *Salmonella* spp., where children tested positive for *Salmonella* spp., showed significantly higher IL-6 serum concentrations than children tested positive for norovirus or rotavirus (Chen et al., 2012).

The utility of IL-6 as a prognostic marker is shown on the genomic and the protein level. A single-nucleotide-polymorphism (SNP) in the IL-6R gene is associated with a higher risk for asthma (Ferreira et al., 2011). The genotype was related to a phenotype, displaying higher sIL-6R concentrations in serum and airways, leading to a dysregulation of the inflammatory cascade (van Dongen et al., 2014). On the protein level, IL-6 serum concentrations could predict the relapse probability in Crohn's disease and the disease activity in ulcerative colitis (Louis et al., 1997; Wine et al., 2013). In a mice sepsis model, IL-6 concentrations early after induction of sepsis, were a reliable marker for death within five days (sensitivity 82%, specificity 86 %) (Osuchowski et al., 2006). A study on 239 systemic inflammatory response syndrome (SIRS) patients, confirmed IL-6 as a predictor for mortality in humans, too (Mat-Nor et al., 2016).

The therapeutic applications concerning the IL-6 pathway mainly focus on the generation of recombinant antibodies. Currently, two recombinant antibodies are approved for therapeutic use. The first is tocilizumab, a humanized IgG1 antibody directed to IL-6R (Venkiteshwaran, 2009). It used for the treatment of rheumatoid

arthritis (Nishimoto et al., 2000). In 2013, the global sales for this antibody were reported to be 1,119 million US dollars, underlining its clinical relevance (Ecker et al., 2015). The second is siltuximab, an anti-IL-6 chimeric IgG1, licensed for the treatment of multicentric Castelman's disease, which is a lymphoproliferative disorder, characterized by an excessive production of IL-6 (van Rhee et al., 2014; Chen et al., 2015). Two more antibodies are currently in phase III clinical studies. Sirukumab (human anti-IL-6 IgG1) for the treatment of rheumatoid arthritis and giant cell arthritis, and SA237 (humanized anti-IL-6R IgG2) for the treatment of neuromyelitis optica and neuromyelitis opticaspectrum disorders (Reichert, 2015).

2.3.3.2 Veterinary Medicine

In veterinary medicine, IL-6 is investigated for use as diagnostic and prognostic marker in several species. Concerning therapeutic applications, research primarily focuses on IL-6 as an adjuvant in vaccine development.

The diagnostic potential of IL-6 is proven for several diseases in dogs. Interleukin-6 plasma or serum levels were increased in cases of congenital portosystemic shunts, idiopathic immune mediated polyarthritis and steroid responsive meningitis-arteritis (Maiolini et al., 2013; Foster et al., 2014; Kilpatrick et al., 2014). In hepatic diseases, IL-6 was able to distinguish between acute and chronic hepatitis, and primary and secondary hepatic tumors (Neumann et al., 2012).

In horses, IL-6 in synovia was used to detect joint diseases even in cases were the diagnostic of lameness was insensitive (Bertone et al., 2001). During an infection with equine infectious anaemia virus (EIAV), IL-6 values in serum correlated with the RT activity of the virus and in foals IL-6 serum concentrations were a useful indicator for sepsis (Barton et al., 1998; Sellon et al., 1999; Burton et al., 2009). Furthermore, IL-6 in serum and peritoneal fluid was shown to differ between colic types (e.g. inflammation, strangulation, non-strangulating or non-inflammatory, open, ruptured viscus) (Barton et al., 1999).

In sheep suffering from encephalitis due to an infection with Listeria monocytogenes, IL-6 in cerebrospinal fluid (CSF) showed a diagnostic sensitivity of 83% and a specificity of 100% (Abdlla et al., 2015). Interleukin-6 measured in serum of camel, was successful in detecting animals with urinary tract infections (El-Deeb et al., 2015).

More frequently, IL-6 is investigated as prognostic marker. In the Chiari malformation of the Cavalier King Charles Spaniel, pain caused by the syringomyelia was related to IL-6 detection in CSF (Schmidt et al., 2013). Levels of IL-6 in CSF were also shown to indicate a short survival time in ischemic stroke in dogs (Jeon et al., 2015). Additionally, the prognostic quality of IL-6 in plasma or serum of dogs was shown in idiopathic immune mediated polyarthritis, where it was correlated to pain and in pituitary-dependent hyperadrenocorticism, where it correlated to the risk for vision loss (Cabrera Blatter et al., 2012; Foster et al., 2014). The survival of dogs in intensive care units and dogs suffering from systemic inflammatory response syndrome, can also be estimated by measuring IL-6 in serum (Rau et al., 2007; Schüttler et al., 2015). Similar effects were shown in species other than dog. Interleukin-6 as a marker for survival was confirmed in cases of acute abdomen in horses and encephalitic listeriosis in sheep (Barton et al., 1999; Abdlla et al., 2015).

Concerning the therapeutic application of IL-6 in veterinary medicine, several studies investigated IL-6 as an adjuvant in vaccine development. In most cases, a recombinant IL-6 protein and a immunogenic surface protein of the pathogen, against which the immunization is directed, are together encoded in a vector used for vaccination. For example, a recombinant fowlpox virus (FPV) vector system, bearing the chicken IL-6 gene and the hemagglutinin (HA) gene of the H5N1 subtype of highly pathogenic avian influenza (HPAI), was able to improve the vaccination response in ducks, when compared to the vector system without the IL-6 gene (Qian et al., 2012). In other studies, IL-6 is not included in the vector but administered separately. This approach, was also shown to increase the vaccination response in piglets vaccinated against Hog cholera, in mice vaccinated against equine influenza virus hemagglutinin (HA) and in rats immunized with dinitrophenylated Pneumococcus (Pockley et al., 1991; Larsen et al., 1998; Li et al., 2011). Despite the improvement of vaccines, IL-6 was tested in tumor treatment. The intratumoral application of a plasmid carrying the human IL-6 and the human IL-15 gene sequence was able to inhibit further growth of Canine transmissible venereal tumor (CTVT) in beagles (Chou et al., 2009). Other investigations used IL-6 as a carrier molecule. Multiple myeloma cells, which were shown to overexpress IL-6R, were attacked by a fusion protein of IL-6 and a truncated mutant form of Pseudomonas exotoxin (PE38KDEL) (Guo et al., 2012). Thereby,
tumor regression and survival time could be increased in mice.

In cattle research, the diagnostic, prognostic and therapeutic application of IL-6 mainly focuses on dairy cattle and in particular on mastitis and uterine diseases. Interleukin-6 gene expression was found to be elevated in endometrial cells in subclinical endometritis (Ghasemi et al., 2012). Serum IL-6 concentrations were also elevated in subclinical endometritis as well as in cases of pyometra (Brodzki et al., 2015a; Brodzki et al., 2015b). In mastitis, elevated IL-6 levels were found in serum and milk (Nakajima et al., 1997; Hagiwara et al., 2001). Subclinical mastitis could be diagnosed by measuring the IL-6 concentration in milk, which was even more sensitive than counting the number of somatic cells (Osman et al., 2010; Sakemi et al., 2011).

The prognostic value of IL-6 for the development of postpartum diseases, such as metritis, endometritis and retained placenta, was determined by gene expression analyses of uterine cells or peripheral blood mononuclear cells (PBMC) and by measuring the IL-6 concentration in serum samples. Gene expression levels for IL-6 were elevated in endometrial cells and PMBCs at calving, when cows develop endometritis in the postpartum period (Galvao et al., 2011; Galvao et al., 2012). Additionally, increased serum IL-6 levels prepartum, were also proven as a risk factor for developing postpartum diseases, such as endometritis (Ishikawa et al., 2004; Trevisi et al., 2012). In contrast, cows showing a decreased IL-6 serum concentration prepartum were more likely to develop a retained placenta (Ishikawa et al., 2004). Interleukin-6 was also successful in predicting survival in cases of severe mastitis, when measured already at the beginning of symptoms (Hagiwara et al., 2001; Hisaeda et al., 2011). Approaches to establish IL-6 as an adjuvant for vaccines, as described for other species, failed in cattle (Kumar et al., 2014).

2.4 Bovine scFv Antibody Library

2.4.1 Bovine Antibody Structure and Function

Antibodies are the main functional proteins of the adaptive immune system (Litman et al., 2010). They are produced by plasma cells, which develop from activated B-cells (Janeway et al., 2001). Five bovine antibodies isotypes are known, immunoglobulin D (IgD), immunoglobulin M (IgM), immunoglobulin G (IgG), immunoglobulin E (IgE) and immunoglobulin A (IgA) (Walther et al., 2013). The isotypes differ in structure and function. Typically, antibodies display a Y-shaped structure with two exceptions (Harris et al., 1998). IgM antibodies usually occur as a pentamer, comprising five single Y-

shaped proteins connected by joining peptides (Müller et al., 2013). The second exception is the secretory form of IgA, which occurs as a dimer, also connected by a joining peptide (Woof et al., 2011). The single Y-shaped protein is formed by four polypeptide chains of two different types: heavy and light chains (Schroeder et al., 2010). The light chains also appear in two forms: the lambda light chain and the kappa light chain. The lambda light chain is predominantly expressed in cattle (98% of circulating B-cells) (Butler, 1997). Each of the three different chains (heavy, lambda light, kappa light) consists of one variable domain and one to four constant domains (Padlan, 1994). Figure 3 displays the schematic conformation of each known antibody isotype with the exact number of domains for each chain. Two identical heavy (H) chains and two identical light (L) chains form one Y-shaped antibody molecule. The molecule can be divided into a constant and a variable domain. The variable domain is formed by the two entire light chains and the variable domains and the first constant domains of the two heavy chains. This part of the antibody molecule mainly contributes to antigen binding. More precisely, there are three specific segments in each variable domain of the respective chain, which are mainly responsible for antigen binding. These segments are called complementary determining regions 1-3 (CDR1-3). Within the CDRs a high amino acid diversity is present to allow for the recognition of a variety of different antigens. In cattle, an additional mechanism for diversification exists in the CDR3 region of the heavy chain. Exceptionally long CDR3 regions are generated by insertion of conserved short nucleotide sequences (CSNS) into the coding region of the CDR3 region (Koti et al., 2010b). Through intra-CDR3H disulphide bridges within those long CDR3s a unique ‘stalk and knob’ structure is created, which contributes to diversity via creation of mini-domains (Wang et al., 2013).

The remaining segments of the two heavy chains, which are not included in the variable antibody domain, form the constant domain of the antibody molecule, where the antibody isotype is determined (Padlan, 1994). This molecule part mediates the effector function of the antibody including binding to surface receptors of immune cells and binding of complement factors (Desjarlais et al., 2011).

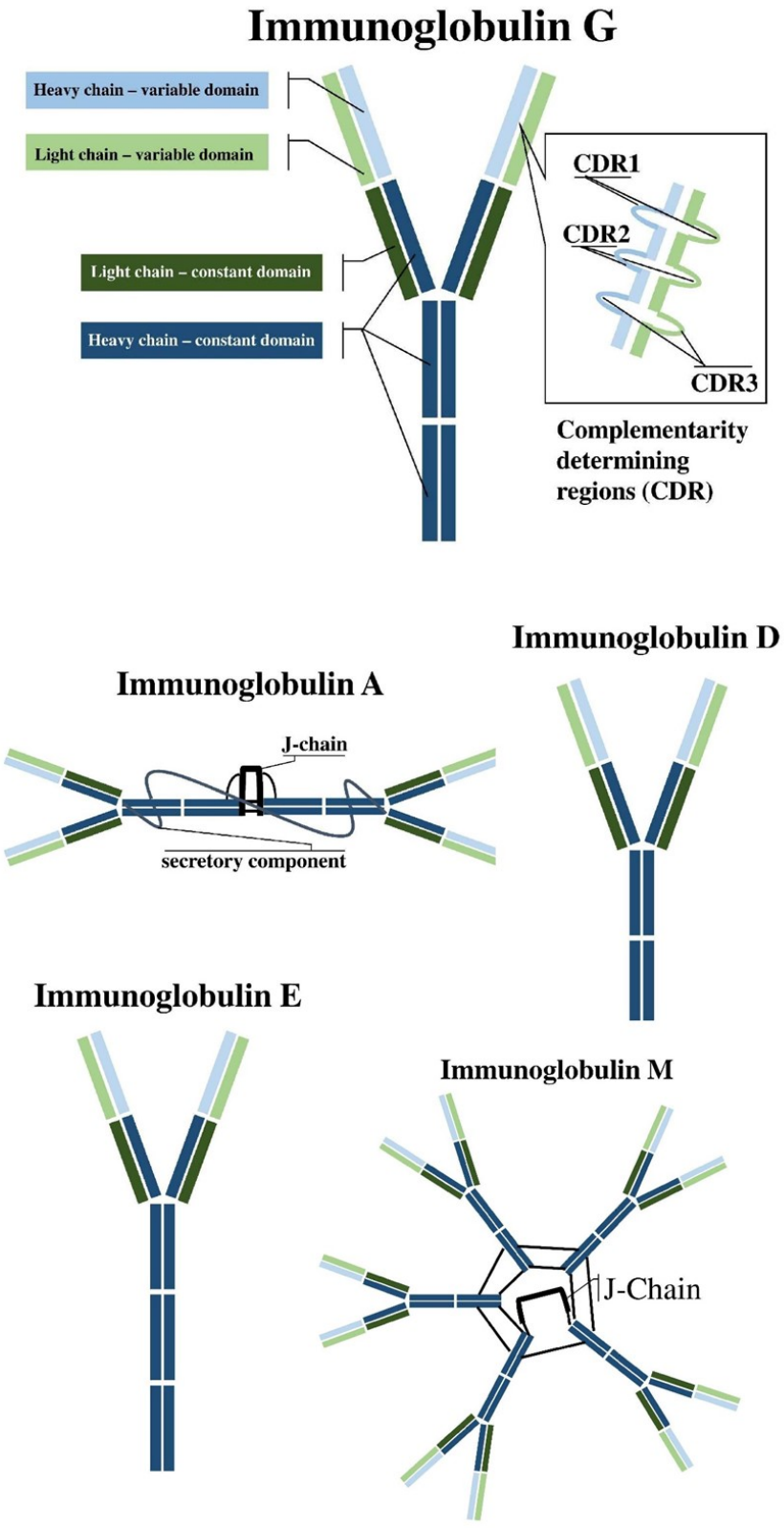


Fig. 3. Antibody Classes. Individual antibody domains are labeled as examples in the immunoglobulin G molecule.

2.4.2 Bovine Antibody Development and Diversification

The current assembly UMD 3.1 of the bovine genome allows the detailed analysis of bovine immunoglobulin genes (Zimin et al., 2009). Immunoglobulins are encoded by a set of germline genes. For each of the three different antibody chains (heavy chain, lambda light chain, kappa light chain) a separate gene locus exists (Aitken et al., 1999). Despite the main gene locus, some genes are localized elsewhere. Within the loci, several genes exist for the different parts of the respective antibody chain. A complete antibody chain is encoded by the recombination product of a variable (V) gene, a joining (J) gene and a constant (C) gene. In the heavy chain, an additional gene exists: the diversity (D) gene, which is inserted between the joining and the constant gene. The gene rearrangement in a variety of possible combinations leads to antibody diversity. Additionally, allotypes are described for the constant genes in all chains.

Twenty-two κ-light chain variable genes (IGKV) are localized on chromosome 11, with eight of them being functional genes (Ekman et al., 2009). They can be grouped into four subfamilies (IGKV1-4), from which members of the IGKV2 family are mainly expressed (Kaushik et al., 2014). All of the three κ-light chain joining genes (IGKJ) are functional. IGKJ1 is preferentially expressed (Stein et al., 2012). Only one κ-light chain constant gene (IGKC) exist, for which two allotypes are described (Stein et al., 2012).

Twenty-five λ-light chain variable genes (IGLV) (17 functional genes) are identified on chromosome 17 and can be grouped into three gene families (Kaushik et al., 2014). Genes from the IGLV1 family are predominantly expressed (Pasman et al., 2010). This gene family is further divided into five subfamilies (IGLVa/b/d/e/x). Genes of IGLV1d, IGLV1e and IGLVx code for λ-light chains, found together with heavy chains, expressing exceptionally long CDR3 regions (Saini et al., 1999; Saini et al., 2003). λ-light chain joining genes (IGLJ) and λ-light chain constant genes (IGLC) are organized into four cassettes of which two are functional (IGLJ2-IGLC2, IGLJ3-IGLC3) (Chen et al., 2008). IGLJ3-IGLC3 is preferentially expressed (Pasman et al., 2010). Five allotypes are described for the IGLC3 gene and three for the IGLC2 gene (Diesterbeck et al., 2012).

Thirty-six heavy chain variable genes (IGHV) genes are identified in the current bovine genome assembly. The ten functional ones are located on Chromosome 21 and 8 (Walther et al., 2013). No other mammalian species is known to have two functional heavy chain loci (Das et al., 2008). Data about the number of IGHD genes (10-15) differ,

probably due to imprecise genome assembly. They are spread over three chromosomes (BTA 7/8/21) and can be grouped into four gene families (IGHD-A/-B/-C/-D) (Niku et al., 2012; Walther et al., 2013). The heavy chain diversity genes (IGHD) are jointly responsible for the three CDR3 size-categories described (Walther et al., 2013).

The six heavy chain joining genes (IGHJ) are located on chromosome 21 (Zhao et al., 2003). Two of them are functional. A duplicate of this locus exists on chromosome 8 (Hosseini et al., 2004; Zimin et al., 2009).

Heavy chain constant genes (IGHC) are located on chromosomes 7, 8, 20 and 21 (Zhao et al., 2003; Walther et al., 2013). Genes for all immunoglobulin classes (IgD, IgM, IgG, IgA, IgE), already known in other mammals, also exist in cattle. The single IgD IGHC gene shows very low expression rates (Xu et al., 2012). Two functional IgM IGHC genes with three different allotypes are described (Saini et al., 2001). For IgG IGHC genes three subclasses are described (IgG1/2/3) (Knight et al., 1988). For IgG1 four allotypes are known (Symons et al., 1989; Saini et al., 2007), for IgG2 and IgG3 two allotypes each (Kacskovics et al., 1996; Rabbani et al., 1997). A single IGHC gene is described for IgA and IgE, respectively (Knight et al., 1988; Walther et al., 2013).

In comparison to other mammals, such as humans and mice, the total number of immunoglobulin genes in bovines is small, leading to limited combinatorial diversity ($0.15x10^5$) (Sun et al., 2013; Kaushik et al., 2014). In cattle other mechanisms have evolved to overcome this handicap.

The diversification of the antibody repertoire already begins in the embryo. Gene recombination of V-, D- and J- genes occurs as early as 125 days after gestation (Saini et al., 2002). During recombination, random N and P addition to the recombination sites as well as junctional flexibility increase antibody diversity (Kaushik et al., 2002). Junctional flexibility is defined as nucleotide additions and deletions at the junction sites because of imprecise joining (Schatz, 2004). Additionally, the immunoglobulin diversity in the neonate is the result of the size heterogeneity in the CDR3 region of the heavy chain and somatic hypermutation, without contact of the immune system to external antigen (Saini et al., 2002). Somatic hypermutation here means random changes in the coding sequence for the variable domain. Also after birth, this is a major mechanism in the maturation of the immune system (Verma et al., 2012).

Newborn calves are exposed to various environmental antigens. When the B-cell receptor (BCR) is brought into contact with antigen, the B-cells become activated and

undergo somatic hypermutation as well as class switch recombination (Saini et al., 2015). During class switch recombination, genes of the constant domains of the heavy chain 'switch', leading to the expression of different antibody isotypes (Estes, 1996). B-cells with strong antigen interaction will be selected and stimulated. They will replicate and survive more efficiently. This process is called affinity maturation (Saini et al., 2015). As another antigen-dependent feature in adult cattle, diversity of CDR3H regions is increased by insertion of conserved short nucleotide sequences (CSNS) into the CDR3 coding sequence, after contact of the B-cell to an antigen (Koti et al., 2010a). These exceptionally long CDR3H occur in 8-10% of circulating bovine B-cells (Kaushik et al., 2002).

The tissues involved in the diversification process differ in the embryo and the newborn. B lymphopoesis takes place in the foetal bone marrow and lymph nodes (Ekman et al., 2010). Additionally, VJλ recombination is detected in the foetal spleen (Lucier et al., 1998). The jejunal Peyer's patches (JPP) are important loci for antibody diversification in the embryo, but develop into secondary lymphoid tissue throughout life (Yasuda et al., 2004). In contrast, the ileal Peyer's patches (IPP) develop later during gestation, but become the important perinatal lymphoid tissue, with high B-cell proliferation around birth (Lucier et al., 1998; Yasuda et al., 2004; Yasuda et al., 2006). They involute at sexual maturity (Yasuda et al., 2004). Despite the diversification in the gut-associated lymphoid-tissue (GALT), diversity in peripheral lymphoid tissue increases 1-2 weeks after birth (Zhao et al., 2006).

2.4.3 Recombinant scFv Antibodies and Antibody Libraries

Various formats of recombinant antibodies exist. Figure 4 gives an overview of the most common molecule formats. The scFv molecule, first described in 1991, is reduced on the variable domain of a heavy chain (VH) and the variable domain of a light chain (VL) (Bird et al., 1991). The two domains are combined by a peptide linker. Several different peptide linkers were described (Chen et al., 2013). The length of the linker used in the scFv molecule mainly effects the characteristics of the molecule in solution. Linker peptides with 3 to 12 amino acids do not allow the correct folding into a functional scFv molecule and lead to the formation of multimers (Hudson et al., 1999). A length of 15 amino acids was reported to be optimal for the

construction of scFv molecules (Zuhaida et al., 2013). Despite the length, the amino acid composition also contributes to optimal linker performance. In scFv molecules a flexible linker is needed to ensure the optimal confirmation of the both variable domains (Chen et al., 2013). The most commonly used flexible linker peptides consist of a sequence of glycine and serine. Glycine as a small non-polar amino acid ensures the flexibility. Serine as a small polar amino-acid provides for stability of the linker in aqueous solutions (Chen et al., 2013). In scFv antibodies, the $(G_4S)_n$ linker format is widely used (Spahr et al., 2014). Besides the linker composition, the arrangement of the V-domains can also differ in scFv molecules (VH-linker-VL vs. VL-linker-VH). Recent investigations show that the optimal orientation is individual for each scFv molecule (Cheng et al., 2015; Liu et al., 2015).

ScFv molecules can be isolated from three different forms of antibody libraries (naïve, immunized, synthetic). Naïve libraries are formed from B-cells of donors which have not been exposed to the antigen of interest before. Therefore, B-cells, which provide the DNA for the library construction, have not undergone affinity maturation in the donor organism and provide a wide range of different antibody sequences (Rahumatullah et al., 2015). In immunized libraries, donors had been immunized with the antigen of interest. B-cells undergo affinity maturation in the vaccinated organism and therefore mRNA antibody sequences isolated from B-cells for library construction are shifted towards a higher specificity and neutralization ability. The probability of isolating a specific high affine antibody is higher in immunized libraries compared to naïve libraries (Ahmad et al., 2012). Additionally, the effort to isolate a high affine antibody is lesser in immunized libraries, because a lower library diversity and a fewer number of panning rounds are needed (Miller et al., 2008).

Synthetic libraries are made by *in-silico* mutations of antibody molecules (Farajnia et al., 2014). In a constant antibody framework, predominantly the CDR regions are diversified by the introduction of synthetic oligonucleotide sequences. These libraries are independent of a immunized donor and have the advantage of artificially improving the antibody molecule structure for favorable attributes such as a good expression rate in *E. coli* and a high stability (Hairul Bahara et al., 2013).

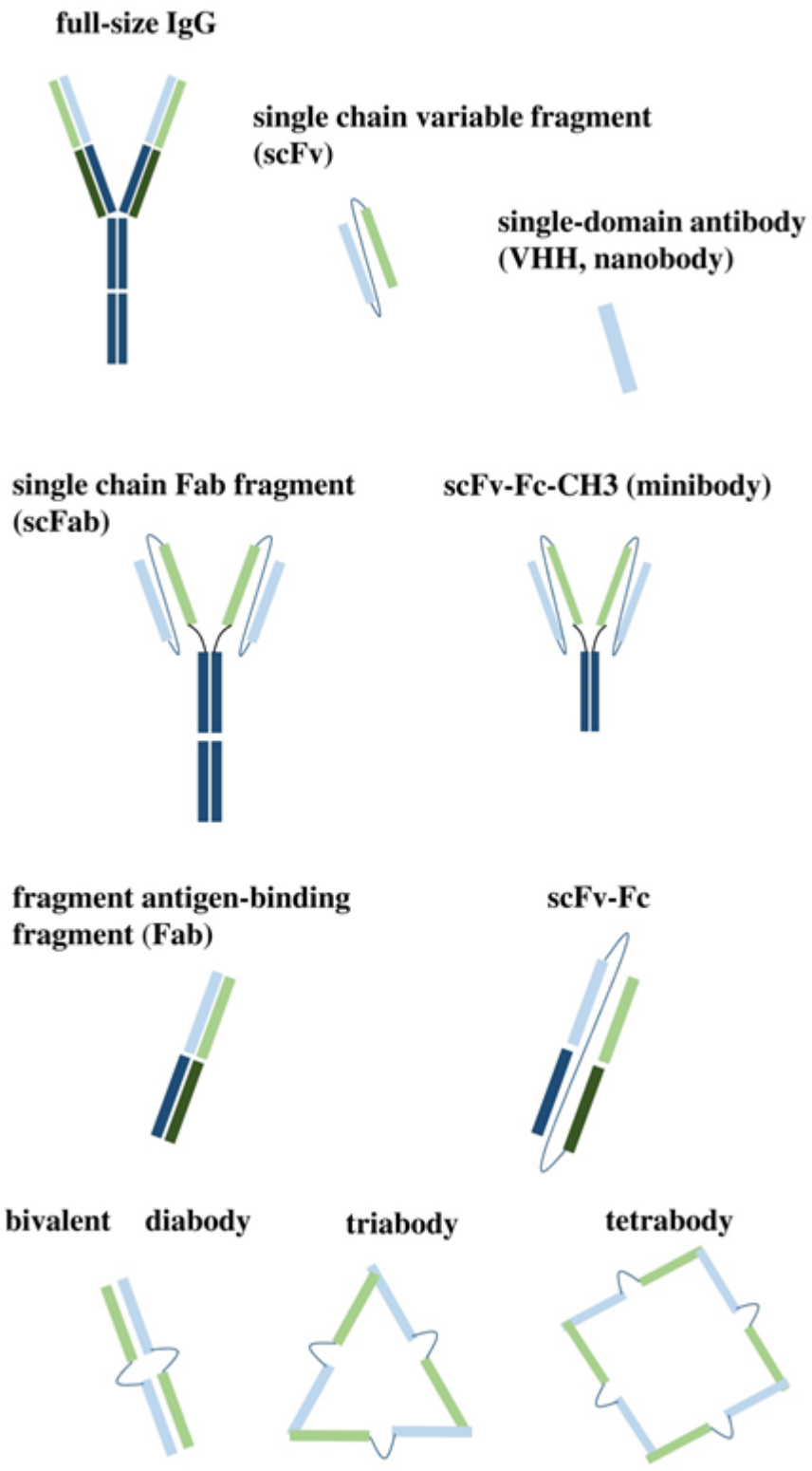


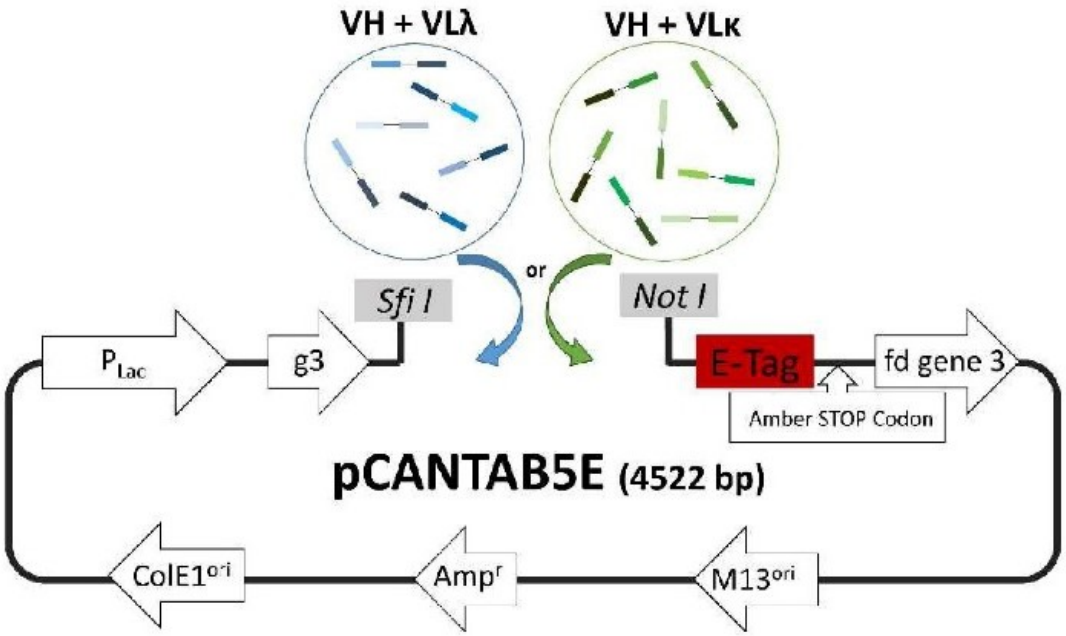
Fig. 4. Recombinant antibody formats. The picture shows commonly used recombinant antibody formats in comparison to full-size Immunoglobulin G (IgG)

2.4.4 Phage Display

The method of phage display was first described in 1985 as an innovative method of cloning (Smith, 1985). A DNA sequence was cloned into the sequence of one of the surface proteins of a filamentous bacteriophage, which then displays the protein structure as a part of the surface protein on its surface. This construct showed the advantage of simultaneously having the genotype and the phenotype of a targeted protein in the same organism. Initially, this technique was used for the rapid isolation of peptides from huge libraries, using an antibody directed against the specific peptide for the screening (panning) process. In 1990, the method of phage display was modified and now antibody sequences were cloned into the phage genome and screened against specific peptides (McCafferty et al., 1990). Figure 5 shows an example for one cycle during the process of phage display. Panning rounds are repeated with specific binders of the previous round to increase the specificity and the affinity of the antibody-phage construct stepwise, from round to round. Several modifications of the method have been described, concerning the specific phage and the surface protein used for expression (Ebrahimizadeh et al., 2014). The most common phage display procedure uses a phagemid carrying the antibody-surface protein sequence and a helper phage providing other proteins necessary for the phage assembly (Ponsel et al., 2011). Antibody DNA sequences are cloned into the phagemid vector and transformed into competent bacterial cells. The transformed bacteria are infected with the helper phage to accomplish the formation of complete

phage particles carrying the antibody DNA sequence and displaying the encoded antibody fragment on its surface (Breitling et al., 1991).

STEP 1

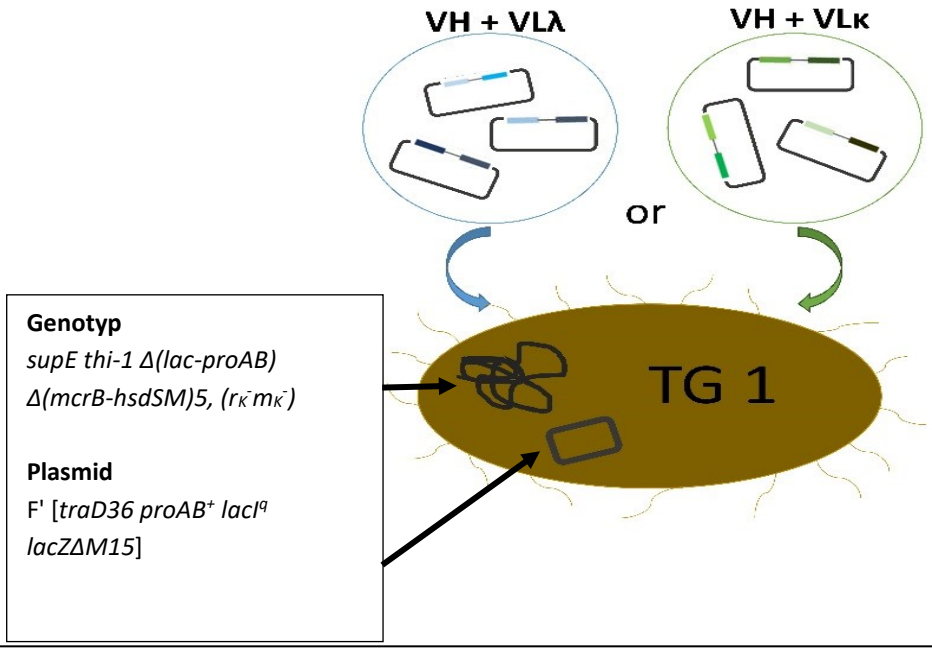


Construction of the scFv library in pCANTAB5E.
ScFv DNA and vector DNA were digested with *Sfi*I and *Not*I. For the library generation either a VH+VLλ sequence or a VH+VLκ sequence was ligated in a digested vector using T4 Ligase.

P_{Lac}	*lac* promotor region
g3	phage g3 signal (targets expression to the host bacterium periplasm)
E-Tag	peptide Tag
Fd gene 3	gene for phage coat protein 3
M13ori	origin of replication of the M13 genome
Amp^r	ampicillin resistance gene
ColE1 ori	origin of replication of the *E. coli* ColE1 plasmid

Fig. 5a. Phage display procedure according to Fischer et al. "Isolation of a high-affinity single-chain antibody fragment (scFv) from a bovine phage display library neutralizing bovine coronaviruses." (continued on the next page)

STEP 2



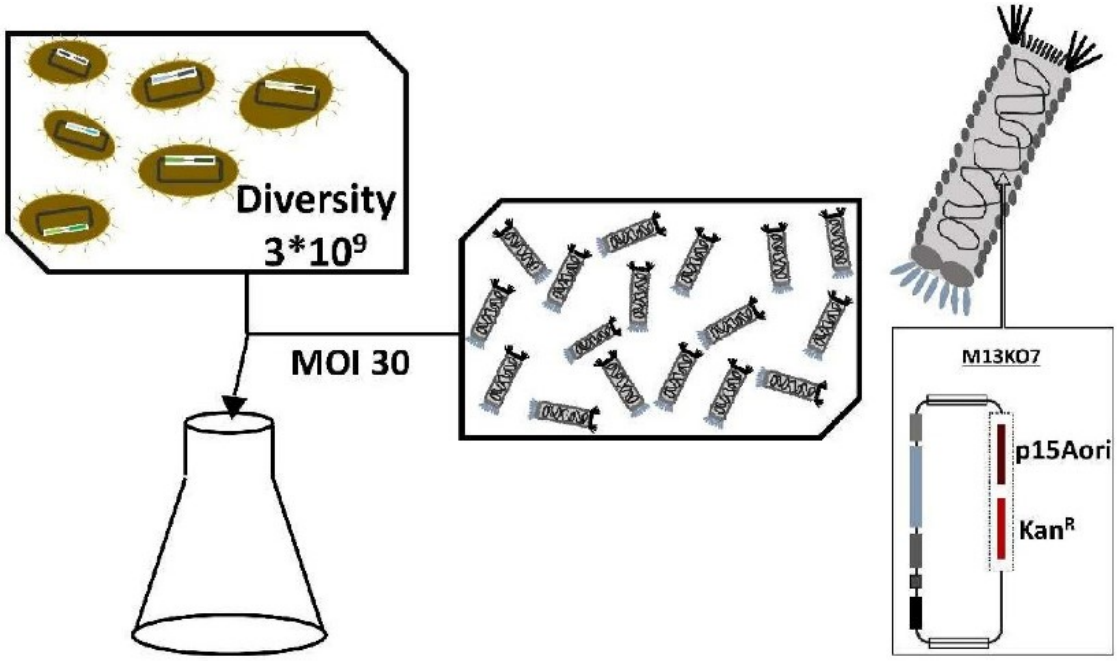
Cloning of ligated library in TG1 cells.

Electro-competent *E. coli* TG1 cells were transfected with scFv-vector constructs.

supE	mutant tRNA, suppresses UAG mutation (required for lytic growth of some phage mutants)
thi-1	mutation in thiamine metabolism
Δ(*lac-proAB*)	deletion from *lac* operon into the genes for proline synthesis (cannot be on lactose, requires proline)
Δ(*mcrB-hsdSM*)	deletion in mutation of methylcytosine restriction system and host specificity gene S and M
$r_k^- m_k^-$	restriction and methylation deficient
F’	F plasmid that picked up chromosomal DNA from E.coli
traD36	conjugation deficiency
$proAB^+$	mutations in proline metabolism (requires proline in minimal medium)
$lacI^Q$	overproduction of *lac* repressor gene
*lacZ*ΔM15	partial deletion in β-D-galactosidase gene (blue/white screening on X-Gal)

Fig. 5b. Phage display procedure according to Fischer et al. “Isolation of a high-affinity single-chain antibody fragment (scFv) from a bovine phage display library neutralizing bovine coronaviruses.”. (continued on the next page)

STEP 3



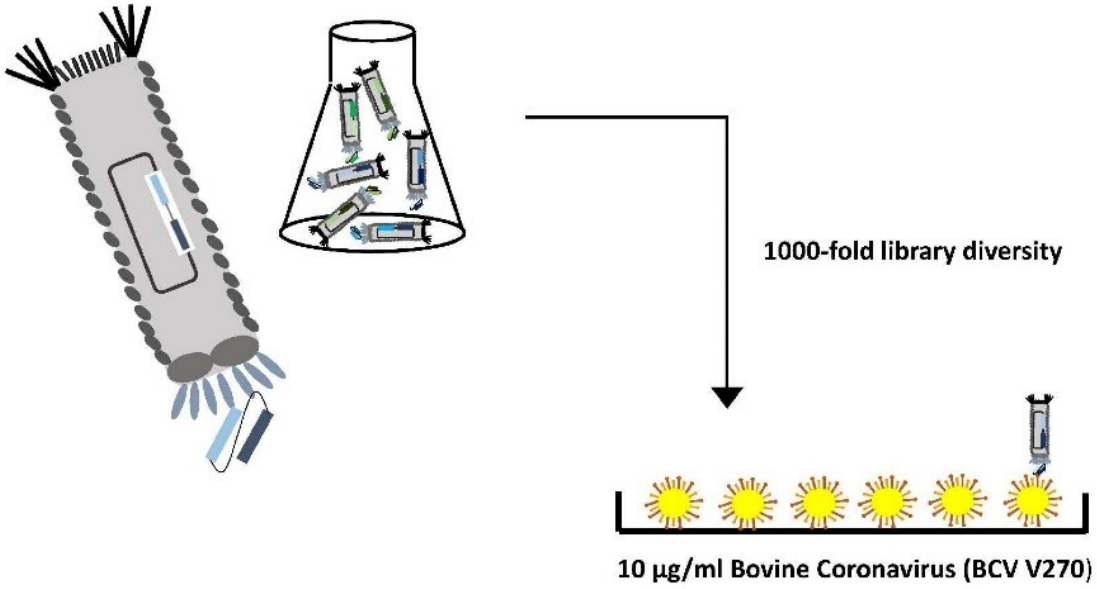
Infection of TG1 library with M13KO7 helper phages

Transformed TG1 cells were grown to reach log phase. The culture was supplemented with M13KO7 helper phages at MOI 30. The supplemented culture was grown for 16-20 hours. Phages were harvested from the bacterial culture by precipitation using 20% polyethylene glycol (PEG).

White, black, grey and blue boxes in the helper phage genome indicate genes for coat Proteins and other phage proteins important for replication.

Fig. 5c. Phage display procedure according to Fischer et al. "Isolation of a high-affinity single-chain antibody fragment (scFv) from a bovine phage display library neutralizing bovine coronaviruses." (continued on the next page)

STEP 4



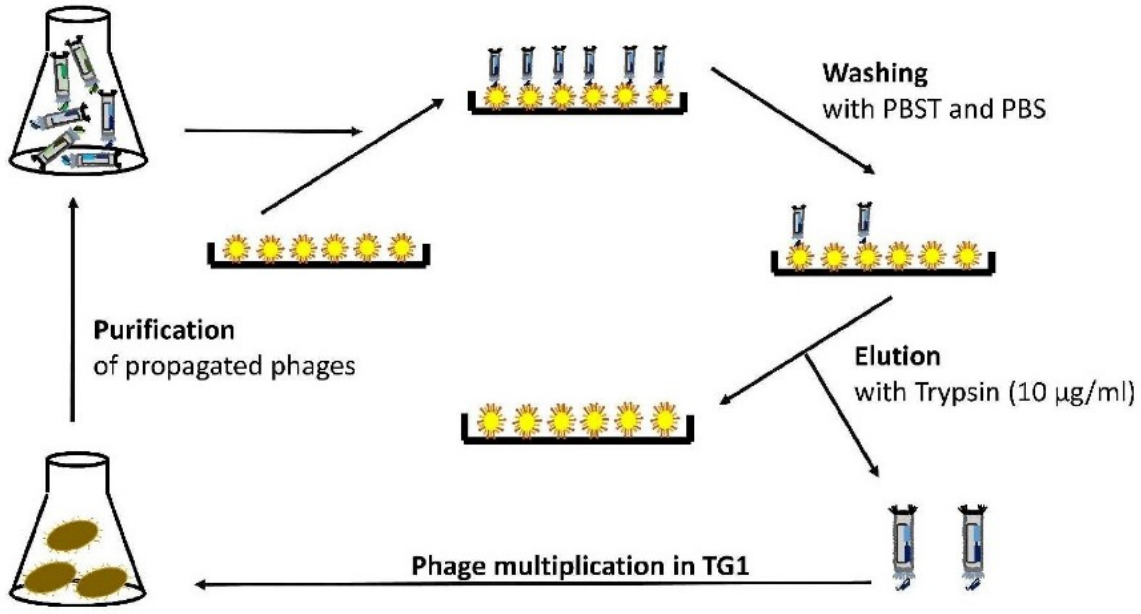
Panning.

The phages harvested from the TG1 library each carried a recombinant pCANTAB5E vector containing a scFv sequence. Simultaneously, the encoded scFv molecule is displayed on the phage surface as fusion protein with the phage's coat protein 3.

In the first panning round, the phage suspension representing 1000-fold library diversity ($3*10^{13}$ phages) was added to a 96-well plate coated with 10 µg/ml BCV V270.

Fig. 5d. Phage display procedure according to Fischer et al. "Isolation of a high-affinity single-chain antibody fragment (scFv) from a bovine phage display library neutralizing bovine coronaviruses." (continued on the next page)

STEP 5

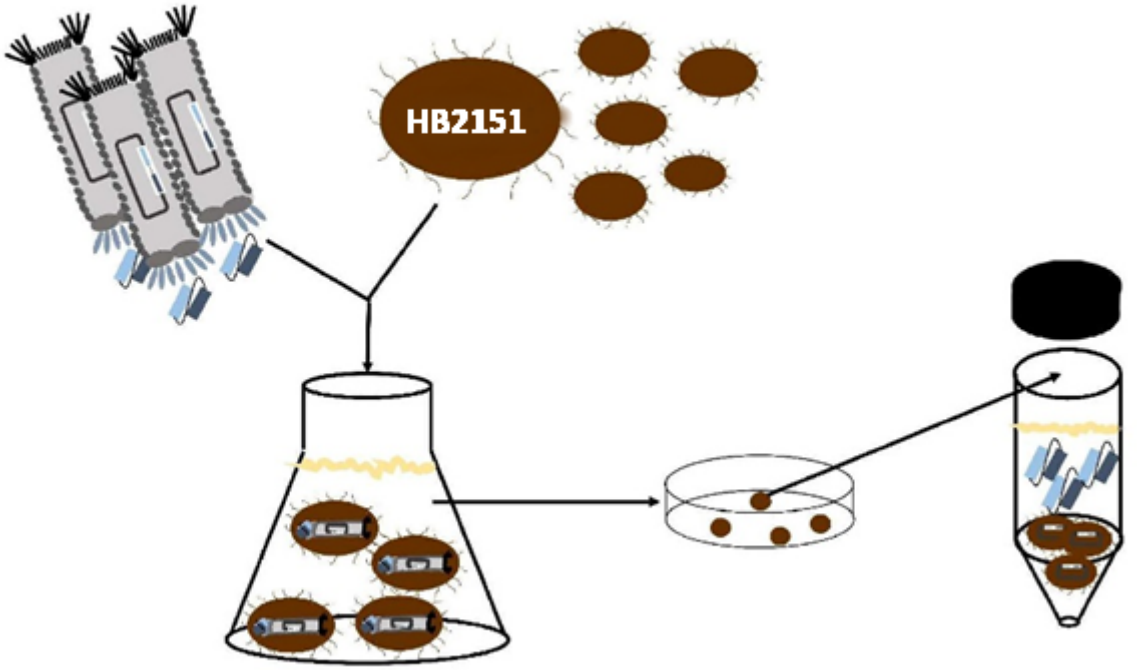


Panning.

In the first panning round, phages were incubated for 1 hour on BCV V270 coated ELISA plates. Afterwards unbound phages were washed away. Bound phages were eluted with 10 µg/ml trypsin. Eluted phages were propagated in TG1 cells overnight and harvested the next day by precipitation from the bacterial culture. These phages were used in the next panning round. Panning conditions were made more stringent from panning round to panning round, by decreasing virus coating concentration, by decreasing incubation time of phages and by increasing washing steps.

Fig. 5e. Phage display procedure according to Fischer et al. "Isolation of a high-affinity single-chain antibody fragment (scFv) from a bovine phage display library neutralizing bovine coronaviruses." (continued on the next page)

STEP 6



Expression of BCV V270 scfv antibodies.

After a sufficient number of panning rounds. Phages of the last elution were used for infection of *E.coli* HB2151 cells which will pick up the scFv encoding vector from the phages. The infected bacteria were grown for 1 hour before they were transferred onto agar plates and grown overnight. The next day, single colonies were picked and grown individually in liquid medium to reach log phase. Induction of scFv expression occurred by replacing the medium with medium containing no glucose but IPTG (2 mM). Expression was conducted overnight before soluble scFv proteins could be harvested from the culture supernatants.

Fig. 5f. Phage display procedure according to Fischer et al. "Isolation of a high-affinity single-chain antibody fragment (scFv) from a bovine phage display library neutralizing bovine coronaviruses".

2.4.5 Clinical Relevance

2.4.5.1 Advantages of scFv Molecules for Clinical Applications

Recombinant antibodies generated by phage display libraries overcome several disadvantages of the hybridoma technology, which was the standard method for generating recombinant antibodies for years (Loureiro et al., 2015). The isolation process of antibodies derived from phage display is faster, easier and more cost efficient (Pandey, 2010; Liu et al., 2013a). Additionally, the immunization of mice, which is necessary in hybridoma technology, can be avoided and the number of animal experiments can be reduced. These technique also eliminates the main drawback of mouse monoclonal antibodies in therapeutic use, which is the formation of human-anti-mouse-antibodies (HAMA) (Watkins et al., 2000). For the generation of antibody phage display libraries, B-cells of the target species for which a therapeutic antibody is developed are used. Therefore, the antibody encoding DNA sequences isolated from these cells are completely species-specific.

The scFv as one format of recombinant antibodies has several advantages, due to its small full-size. When compared to monoclonal antibodies, it shows a better tissue penetration ability and a more rapid blood clearance (Ahmad et al., 2012). The latter characteristic is of particular importance when, for therapeutic purposes, radionuclides are linked to the scFv (Oriuchi et al., 2005).

2.4.5.2 Human Medicine

The use of recombinant antibodies in therapy has become an important field in human medicine. Currently, more than 50 antibodies are licensed for therapeutic and diagnostic applications (http://www.imgt.org/mAb-DB/index#Approval_antibodies). They are predominantly developed for cancer therapy to avoid collateral damage in healthy tissue, owing to chemotherapy and radiation (Suryadevara et al., 2015). Another important field of application is intoxications. A very recent study describes the successful isolation of a neutralizing scFv-Fc antibody for use in Botulinum Neurotoxin E intoxications (Miethe et al., 2015). And finally, efforts are made to develop antiviral antibodies which bear the potential to generate specific antiviral drugs in the future. A scFv has been already developed against the Influenza (H1N1)

Virus, preventing an H1N1 virus infection in mice after intranasal application (Cho et al., 2015).

2.4.5.3 Veterinary Medicine

Antibody libraries for several species, such as mice, rats, chicken, sheep, swine and dogs, have already been published (Charlton et al., 2000; Li et al., 2004; Wieland et al., 2006; Sepulveda et al., 2008; Braganza et al., 2011; Wang et al., 2014). Despite these relatively common species, current immunoglobulin research focuses on two more exotic species, camel and shark. They are of special interest because of their unusual immunoglobulin structure (Figure 6). Camel antibodies are homodimers of two heavy chains. Their functional variable domain consists only of one variable domain (Hamers-Casterman et al., 1993). Recombinant antibodies, so called nanobodies or variable domain of the heavy chain of the heavy chain antibody (VHH), derived from camel antibody libraries show both an excellent tissue penetration ability due to their small size and a high stability and a high solubility (Harmsen et al., 2007; Fu et al., 2013). Shark antibodies display similar characteristics. The shark immunoglobulin isotype novel antigen receptor (IgNAR) was first described in 1995 and still is an important topic in antibody research (Greenberg et al., 1995; Krah et al., 2015; Zielonka et al., 2015). However, recombinant antibodies from animal derived libraries are usually developed for therapeutic purposes or research questions in human medicine (Ohtani et al., 2013; Caljon et al., 2015; Miethe et al., 2015). They are preferred to human antibodies because of their pharmacokinetic characteristics, as described for camel and shark antibodies, and in cases where animals, such as mice and rats, serve as model organisms for pathogenesis studies of human diseases (Sepulveda et al., 2008; Tamura et al., 2008). Studies which focus on the development of recombinant antibodies against pathogens relevant in veterinary medicine mostly use commercial available naïve human libraries (Blazek et al., 2004; Kim et al., 2004; Chang et al., 2006; Dezorzova-Tomanova et al., 2007; Golchin et al., 2008; Molinkova et al., 2008). Only a few studies are available which aim to generate species-specific antibodies for therapeutic applications in veterinary medicine. For sheep and swine, naïve antibody libraries were developed to show the possibility for species-specific library construction in general (Charlton et al., 2000; Li et al., 2004). A IgA-Fab fragment specific for *Eimeria acervulina* was successfully isolated from a chicken antibody library, and a canine scFv antibody against a capsid antigen of canine parvovirus was generated for dogs (Wieland

et al., 2006; Braganza et al., 2011). Concerning cattle, only one publication described the generation of a bovine antibody library (O'Brien et al., 1999; O'Brien et al., 2002). In these investigations only a limited number of primers was used for the amplification, with only one primer pair for each antibody chain (VH, VL). The authors aimed to develop a recombinant bovine antibody binding to the L4 protein of *Bovine Papillomavirus-4* (BPV-4). However, the study rather focuses on principles of library construction than on the isolation and characterization of a recombinant antibody.

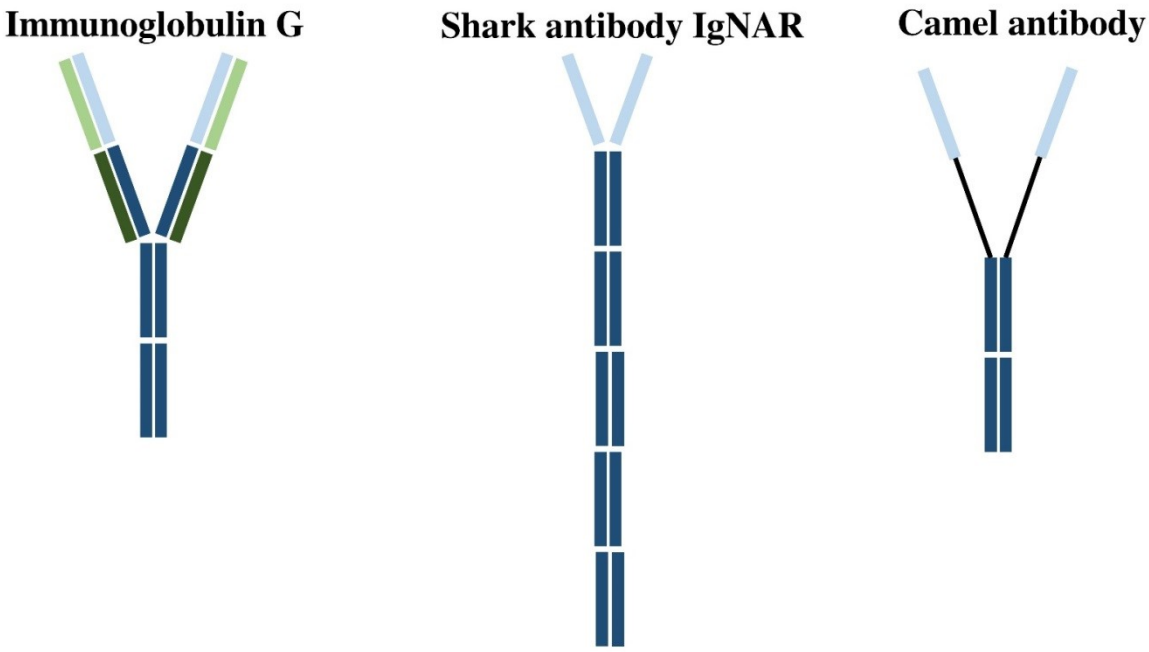


Fig. 6. Special antibody forms. Structure of a conventional immunoglobulin G molecule, a shark antibody and a camel antibody.

3 STUDIES PERFORMED

3.1 Chapter I

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DYNAMICS OF SALIVARY IMMUNOGLOBULIN A AND SERUM INTERLEUKIN 6 LEVELS IN NEWBORN CALVES

Stephani Fischer[b], Sophie Diers[b], Rolf Bauerfeind[c], Claus-Peter Czerny[b], Stephan Neumann[a*]

[a]Small Animal Clinic, Institute of Veterinary Medicine, Faculty of Agricultural Sciences, Georg-August-University of Goettingen, Burckhardtweg 2, 37077, Goettingen, Germany
[b]Department of Animal Sciences, Institute of Veterinary Medicine, Division of Microbiology and Animal Hygiene, Faculty of Agricultural Sciences, Georg-August-University of Goettingen, Burckhardtweg 2, 37077 Goettingen, Germany
[c]Institute of Hygiene and Infectious Diseases of Animals, Justus Liebig University of Giessen, Frankfurter Str. 85-89, 35392, Giessen, Germany

*Corresponding Author: Stephan Neumann, Small Animal Clinic, Institute of Veterinary Medicine, Georg-August-University of Goettingen, Burckhardtweg 2, 37077, Goettingen, Germany
E-mail: sneuman@gwdg.de

E-mail addresses:
Stephani Fischer (stephani.fischer@agr.uni-giessen.de)
Claus-Peter Czerny (cczerny@gwdg.de)

ABSTRACT

Mucosal immunoglobulin A (IgA) is an important component of the adaptive first line immune defense. Especially in newborn calves, where diarrheal pathogens can cause severe diseases, the adaptive mucosal immunity is crucial. Regulating local IgA production is complex. Cytokines and in particular, interleukin 6 (IL-6), are described to be main actors in this process. In vaccination trails, IL-6 is used to improve the immune response with ambiguous results. Therefore, this study investigated the influence of IL-6 on the physiological development of salivary IgA production in newborn calves. Ten clinically healthy female Holstein calves were sampled over a period of four weeks. Immunoglobulin A and IL-6 were measured in serum, colostrum, milk and saliva. In addition, to compare the mucosal immune response with the systemic immune response, immunoglobulin G was measured in serum, colostrum and milk, too, using a commercial enzyme-linked immunosorbent assay (ELISA). In order to determine the ability of newborn calves to produce IL-6 actively by themselves, the relative gene expression was analyzed in peripheral blood mononuclear cells (PBMC) using a quantitative reverse transcriptase polymerase chain reaction (qPCR). Interleukin 6 gene expression in PBMCs was detected directly after birth. A significant positive correlation between serum IL-6 and salivary IgA was found on day seven ($p = 0.043$). Furthermore, the IL-6 serum concentration peaked on day 14 and coincided with a remarkable IgA increase in saliva ($p < 0.01$). Therefore, IL-6 seemed to play a role during the first initialization phase of a local IgA response.

Keywords

time course, adaptive immunity, interleukin 6, immunoglobulin A, qPCR, ELISA

INTRODUCTION

Immunoglobulin A in saliva acts as a first specific barrier against oronasal infections (Marcotte et al., 1998). Especially in newborn calves where most pathogens invade into the body via an oronasal route, IgA is of particular importance. For example, neonatal diarrhea is one of the most important diseases in calf rearing. It can lead to high mortality and significantly impair animal welfare (Waltner-Toews et al., 1986; Hasoksuz et al., 2002).

In cattle, the ileal Peyer's patches (IPP) were described as the primary lymphoid organ for IgA-producing cells (Yasuda et al., 2004). The IgA-producing B cells migrate to the lamina propria of mucosal surfaces, where IgA production mainly takes place (Husband et al., 1999). In contrast, immunoglobulin G (IgG) is the predominant immunoglobulin in the blood and in the colostrum of ruminants (Butler, 1983). Synthesis of IgG in cattle occurs mainly in peripheral lymph nodes and in the spleen (Saini et al., 2002; Yasuda et al., 2006).

As calves are agammaglobulinemic at birth, colostrum and thereafter milk serve as an important source of both immunoglobulins before the active immunity develops (Hernandez-Castellano et al., 2014). The active humoral immune response was shown to be regulated to a large extent by various cytokines (Varzaneh et al., 2014). This was described for IgA precursor cell retention, proliferation and antibody secretion in detail (Husband et al., 1996). Several *in vitro* studies indicated a strong dependency especially between interleukin 6 (IL-6) and IgA production (Beagley et al., 1989; Fujihashi et al., 1991; McGhee et al., 1991).

Interleukin 6 is produced in both lymphoid and non-lymphoid cells, and the regulating factors are diverse (Ataie-Kachoie et al., 2014). The roles of IL-6 in inflammation include induction of the acute phase response (APR), triggering T-cell proliferation and stimulating the differentiation of B-cells into plasma cells (Barton, 1997). As for the immunoglobulins, colostrum is described to be the main source of IL-6 in newborn calves (Yamanaka et al., 2003b).

In-vitro, a positive influence of IL-6 on IgA production was proven for humans and mice (Beagley et al., 1989; McGhee et al., 1991). For mice and guinea pigs this was also found *in-vivo* (Ramsay et al., 1994; Wang et al., 2011). In cattle, the regulation of IgA production is still not understood completely. There are very few studies, which investigated the effect of IL-6 on bovine cells *in vitro* (Zhu et al., 1999; Yamanaka et al., 2003a). These investigations lead to the hypothesis that the correlation between IL-6 and IgA is evident in cattle, too. However, vaccination trials using recombinant IL-6 as adjuvants failed to confirm this assumption *in-vivo* (Kumar et al., 2014).

To sum up, a sufficient development of the active humoral immune response in newborn calves is crucial. In cattle, current research results show inconsistencies in the verification of *in-vitro* result. To improve the health management in newborn calves, a detailed understanding of the immune system is important.

Therefore, we were keen to investigate the physiological development of bovine IL-6 and IgA in clinically healthy calves. The first objective of this study was to observe the development of immunoglobulins and IL-6 over a period of four weeks after birth. The second objective was to characterize the source of interleukin 6 in detail by a relative gene expression analysis.

MATERIAL AND METHODS

Animals

The study was conducted with ten clinically healthy female Holstein calves, housed in a commercial dairy farm near Goettingen, Lower Saxony, Germany. Samples were taken during October and November 2014.

The management of newborn calves after birth included a disinfection of the umbilical cord, the insertion of ear tags and the administration of four liters colostrum via tube feeder. The colostrum was taken from the farm's own colostrum bank and given to the calves within the first hour after birth. Prior to the storage, the quality of colostrum had been determined using a colostrometer and colostrum showing a sufficient immunoglobulin content (140 to 50 mg/mL) was pooled. The colostrum administered to the calves in this investigation originated from different colostrum pools. Newborn calves were separated from their dams' directly after birth and brought to the calf pen. At the pen, calves were sheltered outdoors in igloos (CalfHouse PE, Albert Kerbl GmbH, Buchbach, Germany). After the one-time application of colostrum (day 0), calves were fed pasteurized waste milk *ad libitum*. In week two, the milk was replaced by a commercial milk replacer (50% skimmed milk). From week three on starter grain was offered and calves had free access to water. Calves were weaned completely four weeks after birth. All animals were monitored twice a day by the animal caretakers and underwent a complete clinical examination on each sampling time point to ensure healthiness. Thereby, heart rate, breathing rate, temperature and general condition were examined. Calves showing any abnormalities were excluded from the study.

Samples

All samples were collected with the owner's consent and the procedure was carried out in accordance with the German Protection of Animals Act under the supervision of the

Commissioner of Animal Welfare of the Faculty of Agriculture, University of Goettingen, Germany. For the purpose of laboratory analysis blood (20 mL) and saliva samples were taken from the animals directly after birth before the uptake of colostrum (day 0) as well as on days 1, 7, 14, 21, and 28 *post-partum*. Blood samples were collected by puncturing the Jugular vein (Strauß needle, 2 x 0.43 mm; Dispomed Witt oHG, Gelnhausen, Germany). Ten milliliters blood were collected in a 12 mL K3-EDTA tube (EDTA 95 PP, KABE laboratory technology, Nuembrecht-Elsenroth, Germany) and another 10 mL blood were collected in a 12 mL serum tube (SE 95 PP, KABE laboratory technology, Nuembrecht-Elsenroth, Germany). Immediately after collection, the tubes were placed in a cooling box. Saliva samples were taken using a Salivette (Sarstedt, Nuembrecht, Germany). Here, the absorbent cotton was fixed with a clamp and the calf was allowed to chew on it for at least one minute. Then, the cotton was placed back into the collection tube stored in a cooling box. Colostrum and milk samples of 50 mL each were collected in 50 mL centrifugation tubes (Sarstedt, Nuembrecht, Germany) on day 0, 1 and 7. The commercial milk replacer, fed to the calves after day 7, was not taken into consideration. All samples were transported to the laboratory within maximum three hours.

Sample preparation

Blood serum was collected by centrifugation of serum tubes (2500 x*g*, 5 min, 4 °C), split into 200 µL aliquots and stored at -80 °C. Salivettes were centrifuged (2500 x*g*, 25 min, 4 °C) and saliva supernatants were stored as 200 µL aliquots at -80 °C. Colostrum and milk samples were centrifuged (2500 x*g*, 25 min, 4 °C) and the fat layer was removed using a sterile pasteur pipette. Milk and colostrum aliquots of 500 µL were stored at -80 °C.

Bovine IgA ELISA

To determine the IgA concentration in serum, saliva, colostrum and milk, the commercial Bovine IgA ELISA Core Kit pink-ONE (KomaBiotech, Seoul, Korea) was used. Samples were measured according to the manufacturer's instructions. In brief, plates were coated with a sheep anti-bovine IgA. The standard dilutions were measured in duplicates, the sample dilutions in triplicates. As detection antibody, an HRP-conjugated sheep anti-bovine IgA was used. The optical density (OD) was determined at 450 nm with a Sunrise microplate reader (Tecan, Maennedorf, Switzerland). For data analysis, average optical densities (ODs) were calculated from duplicates and triplicates and blank reduction was performed. Standard curves were generated and IgA concentrations were determined using CurveExpert 1.4 software (CurveExpert Copyright © 1995–2007 Daniel Hyams). The detection limit of the test was

15.6 ng/mL and coefficients of variations (CV) were < 5% (intra-assay) and < 14% (inter-assay) as specified by the manufacturer.

Bovine IgG ELISA

For the measurement of IgG in bovine serum, colostrum and milk, the Bovine IgG ELISA Core Kit pink-ONE (KomaBiotech, Seoul, Korea) was used. The test procedure was the same as described for the Bovine IgA ELISA unless the Detection Antibody was diluted 1: 10,000 in Washing Solution. The test's detection limit was 7.8 ng/mL and CVs were < 7% (intra-assay) and < 14% (inter-assay) as specified by the manufacturer.

IL-6 ELISA

IL-6 concentrations in serum, saliva, colostrum and milk were determined by using the commercial High Sensitive ELISA Kit for bovine IL-6 (USCN Life Science, Wuhan, China). As stated by the manufacturer the detection range of the assay was 1.56–100 pg/mL and the CVs were < 10% (intra-assay) and < 12% (inter-assay), respectively. The assay was performed following the manufacturer's instructions. First, standards and samples were added to the pre-coated plate. The standard was added in duplicates and the samples in triplicates. The detection was carried out using a Biotin-conjugated antibody specific to IL-6 (Detection Reagent A) and an Avidin conjugated Horseradish Peroxidase (Detection Reagent B). All wells were measured at 450 nm in the Sunrise microplate reader (Tecan, Maennedorf, Switzerland). Results were calculated as described for the Bovine IgA ELISA.

PBMC isolation

Peripheral blood mononuclear cells (PBMCs) were isolated from approximately 10 mL of EDTA blood via density gradient centrifugation. The gradient was created using Ficoll Paque (GE Healthcare, Chalfont St. Giles, UK). Centrifugation was carried out at 400 x*g*, for 40 min at 18–20 °C. The cells were washed twice with PBS and contamination with red blood cells (RBCs) was removed by incubation in red blood lysis buffer (155 mM ammonium chloride (NH_4Cl), 12 mM sodium hydrogen carbonate ($NaHCO_3$), 0.1 mM EDTA). After isolation, viable PBMCs were counted using trypan blue (Sigma-Aldrich, St.Louis, MO, USA). PBMCs were stored in 300 µL RNAprotect Cell Reagent (Qiagen, Hilden, Germany) in liquid nitrogen.

RNA isolation and cDNA synthesis

Total RNA was extracted from bovine PBMCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). Quantity and purity of RNA were determined by UV absorbance at 260 and 280 nm in a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, USA). Reverse transcription was performed using the SuperScript III first-strand Synthesis Supermix (Invitrogen, Carlsbad, USA) and random hexamer primers. First, isolated RNA was denatured at 65 °C for 5 min. Afterward, cDNA was synthesized at 25 °C for 10 min, followed by 55 °C for 50 min. The reaction was terminated at 85 °C for 5 min. The cDNA was stored at –80 °C.

qPCR

Relative quantification of IL-6 gene transcription was performed as described previously (Alluwaimi et al., 2002). Messenger RNA of bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) served as a normalizer for the assay. IL-6 and GAPDH primers and probe sequences, shown in Table 1, were derived from the RTprimer database (Pattyn et al., 2003). The 25 µL PCR master mix contained 400 nM primers, 80 nM probe, 12.5 µL TaqMan® Universal PCR MM with UNG (Uracil N-glycosylase), 10 ng cDNA and was adjusted to 25 µL using Nuclease-free water (Qiagen, Hilden, Germany). qPCR was run in the Mx3005P Cycler (Applied Biosystems, ThermoScientific Inc., Waltham, USA) under the following cycle conditions: UNG incubation at 50 °C for 2 min, one cycle at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The UNG incubation step, before polymerase activation, allowed for the elimination of carry-over contamination by releasing uracil from DNA. All samples of a specific calf were run in triplicates in the same assay. For data analysis, triplicates were averaged and the efficiency (E) was calculated based on the absolute increase of fluorescence in the exponential phase. Therefore, a linear regression analysis was conducted. Cycle threshold (Ct) values were corrected for the determined efficiency (E) and the relative gene expression was calculated as $\frac{E_{GAPDH}{}^{CT_{GAPDH}}}{E_{IL6}{}^{CT_{IL6}}}$.

Table 1

Primers and probes used for qPCR

Gene	Accession no.	Designation	Function	Sequence (5´–3´)
GAPDH	AF022183	GAPDH.489p	probe	ATACCCTCAAGATTGTCAGCAATG CCTCCT
		GAPDH.463pf	forward primer	GGCGTGAACCACGAGAAGTATAA
		GAPDH.58spr	reverse primer	CCCTCCACGATGCCAAAGT
IL-6	X62501	IL-6.192p	probe	CGTCATTCTTCTCACATATCTCCTT TCTTATTGCAGAG
		IL-6.271f	forward primer	TCAGCTTATTTTCTGCCAGTCTCT
		IL-6.167r	reverse primer	TCATTAAGCACATCGTCGACAAA

qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-6, interleukin 6

Statistical analysis

Protein concentrations and gene expression data are displayed as mean values (MV) $\pm$ 2 standard errors (2SE). In order to compare the time course of IL-6 and the immunoglobulins, a two-way repeated measure analysis of variance (ANOVA) was applied using STATISTICA 12 software (StatSoft (Europe) GmbH, Hamburg, Germany). Here, IL-6 concentrations were correlated with IgA contents in serum and saliva, and IgG in serum over the time period from day seven till day 28. In order to minimize the initial influence of colostrum uptake, day 0 and 1 were excluded from this correlation. Using the same statistic method, the influence of the calves' own IL-6 gene expression on the serum concentrations was proven. Fold-changes of IL-6 in serum were correlated to the data revealed from the relative gene expression analysis. Fold-changes between the sampling time points were used for calculation in order to compare the relative data from the gene expression with the absolute protein concentrations in serum. In addition, correlations were calculated individually for each sampling time point. Here, the concentration of IL-6 in serum and IgA in serum, IgA in saliva and IgG in serum, as well as the

fold-changes of IL-6 in serum and fold-changes of the relative gene expression, were compared. Concerning colostrum, correlations were calculated between the colostrum concentrations of each protein (IL-6, IgA, IgG), between the colostrum concentration and the respective serum concentration on day one and between the IL-6 colostrum concentration and the concentration of IgA in serum, IgA in saliva and IgG in serum on day one. The same procedure applied to the milk. A *P*-value < 0.05 was considered to indicate statistical significance.

RESULTS

Immunoglobulin A

The IgA concentrations in colostrum, milk, serum and saliva are shown in Figure 1. The mean IgA concentration in colostrum was 10.8 ± 4 mg/mL. In serum collected from the calves, IgA showed a peak on day 1 with a mean concentration of 5.4 ± 2.4 mg/mL ($p < 0.01$). In the calves' saliva, IgA became detectable on day 14 (0.7 ± 0.06 mg/mL, $p < 0.01$).

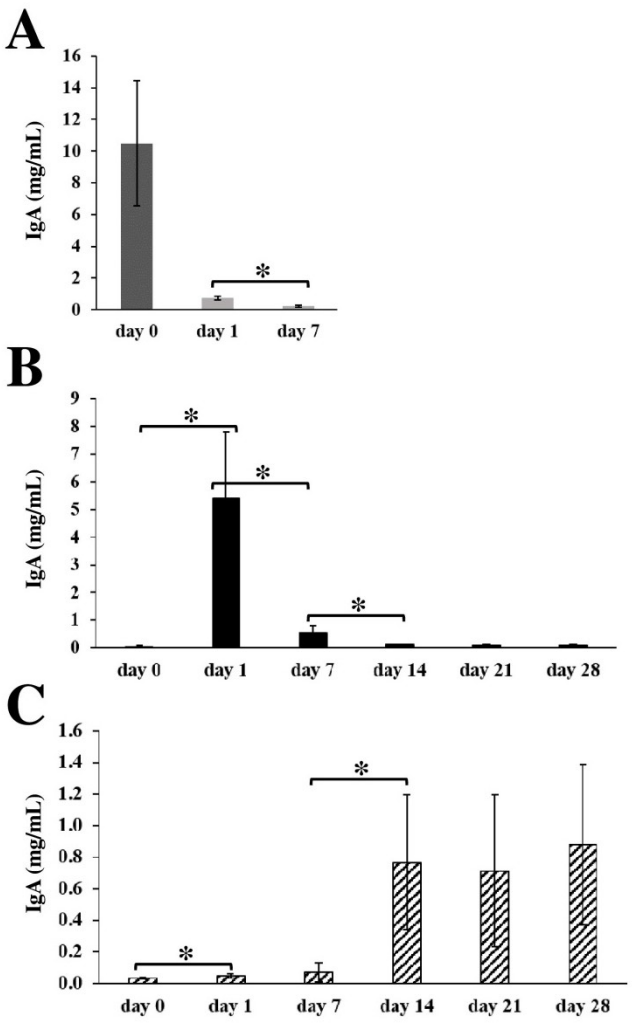

Fig. 1. Immunoglobulin A concentrations. (A) IgA concentrations in colostrum on day 0 (dark grey) and pasteurized waste milk on day 1 and 7 (light grey). (B) IgA concentrations in serum. (C) IgA concentrations in saliva. Data are shown as mean values (MV) ± 2 standard errors (n = 10). Day of birth is set as day 0. Significant changes are marked by asterisks (*). The significance level is set at $p < 0.05$.

Immunoglobulin G

The IgG concentrations in colostrum, milk, and serum are shown in Figure 2. Similar to IgA, the IgG concentration in the administered colostrum (41.9 ± 13.8 mg/mL) was high. In the calves' serum, the mean IgG concentration increased rapidly to a peak concentration of 3.7 ± 1 mg/mL on day 1.

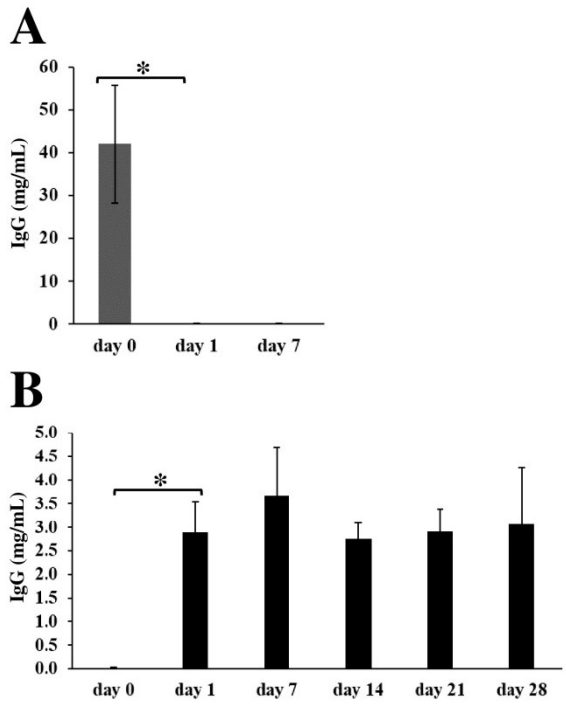

Fig. 2. Immunoglobulin G concentrations. (A) IgG concentrations in colostrum on day 0 (dark grey) and milk on day 1 and 7 (light grey). (B) IgG concentrations in serum. Data are shown as mean values (MV) ± 2 standard errors (n = 10). Day of birth is set as day 0. Significant changes are marked by asterisks (*). The significance level is set at $p < 0.05$.

Interleukin 6

The time course of mean IL-6 concentrations and the relative IL-6 gene expression is shown in Figure 3. The mean concentration in colostrum was 3.9 ± 1.3 mg/mL. The used ELISA system failed to detect any IL-6 concentration in saliva collected from the calves (data not shown). Serum concentrations of the calves' IL-6 and gene expression in PBMCs were detected from day 0 onwards. The ANOVA ($F(1, 36) = 2.73$, $p = 0.08$, $\eta^2p = 0.35$) revealed no evidence that the time course of fold-changes in serum concentration and relative gene expression was not similar. The fold-changes day-by-day correlate for each sampling time point, except day one ($p < 0.05$) (Table 2).

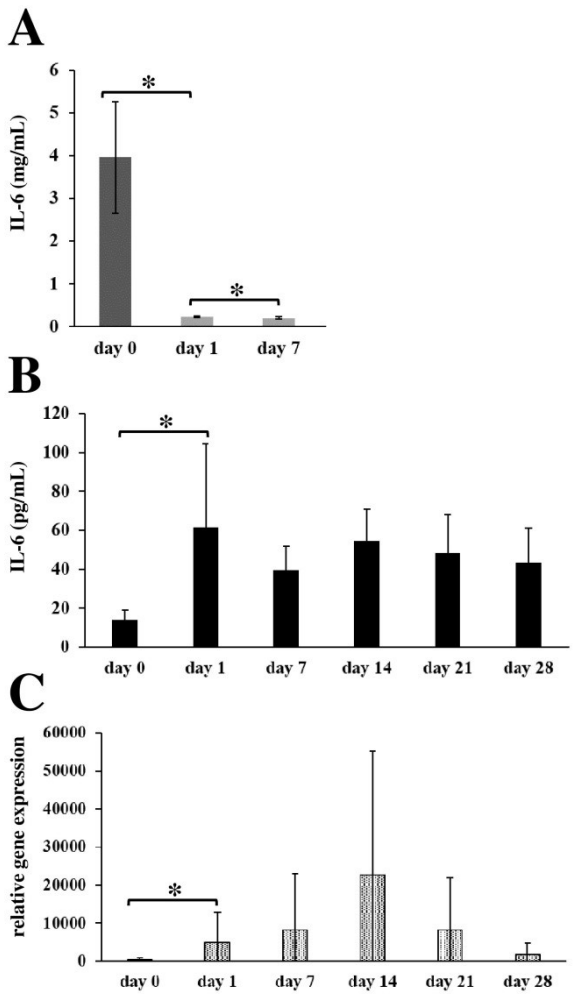

Fig. 3. Interleukin 6 gene expression in PBMC and interleukin 6 concentrations. (A) IL-6 concentrations in colostrum on day 0 (dark grey) and milk on day 1 and 7 (light grey). (B) IL-6 concentrations in serum. (C) IL-6 relative gene expression in PBMCs. Data are shown as mean values (MV) ± 2 standard errors (n = 10). Day of birth is set as day 0. Significant changes are marked by asterisks (*). The significance level is set at $p < 0.05$.

Table 2

Correlations between fold-changes of IL-6 gene expression in PBMCs and serum IL-6 concentrations from 10 clinically healthy female Holstein calves. The significance level is set at $p < 0.05$.

	r	***P***
fold-changes day 1-7	0.9286	< 0.001
fold-changes day 7-14	0.7586	0.011
fold-changes day 14-21	0.7535	0.012
fold-changes day 21-28	0.9435	< 0.001

r, R-value (correlation coefficient R); *P*, *P*-value

Correlations of IL-6, IgA and IgG

Comparisons between IgA, IgG and IL-6 concentrations in colostrum revealed a positive correlation between IgA and IgG concentrations ($p < 0.05$). When the colostrum concentration of IgA, IgG and IL-6 was compared to its respective concentration in serum on day 1, none of the three molecules showed a direct relationship between colostrum and serum concentration. Likewise, the colostrum IL-6 concentration showed no correlation with the concentrations of IgA in serum and saliva, and IgG in serum on day 1. Using a two-way ANOVA for repeated measures, the time course of IL-6 in serum was compared to the time courses of IgA concentrations in serum and saliva, of IgG concentrations in serum, and of IL-6 gene expression in PBMCs. The analysis was applied to data collected in the period from day 7 to day 28 in order to minimize the assumed effects of the colostral immunoglobulins. No differences on the time courses of IL-6, IgA and IgG were evident, as *P*-values were > 0.05 (Table 3). Regarding the statistical analysis of serum IL-6, serum and salivary IgA, and serum IgG concentrations on each individual sampling time point, a significantly positive correlation was shown for the concentrations of IL-6 in serum and IgA in saliva on day 7 ($p < 0.05$).

Table 3

Results for the two-way repeated measures analysis of variance (ANOVA) of TIME*parameter (day 7–28)

Parameter 1	Parameter 2	F	df, df	*P*	η^2_p
IL-6 (S)	IgA (S)	1.25	1, 54	0.3	0.06
IL-6 (S)	IgA (SA)	1.11	1, 54	0.35	0.06
IL-6 (S)	IgG (S)	1.32	1, 54	0.28	0.07
IL-6 (S)	IL-6 (GE)	2.73	1, 36	0.08	0.35

S, serum; SA, saliva; GE (relative gene expression in PBMC); F, F-value; df, degree of freedom; PA, parameter; p, *P*-value; η^2_p, partial eta squared

DISCUSSION

Beside others, IL-6 is an important cytokine in the regulation of the antibody development in mammals (Husband et al., 1996). Focusing on IgA, conflicting research results exist regarding the influence of IL-6 (Yamanaka et al., 2003a; Kumar et al., 2014). Therefore, this study investigated the natural development of IgA, IgG and IL-6 in newborn calves over a period of four weeks, paying special attention to the possible influence of IL-6 on the IgA development. As observed in figure 1, from day 14 on, IgA became detectable in saliva, what was probably the result of the calves' adaptive immune responses towards natural exposure to specific environmental microbes. This observation suggested that the calves' own mucosal immunity starts to develop considerably earlier than the systemic immunity (e.g. IgG), which is described to develop from the fourth week of life (Barrington and Parish, 2001). A descriptive analysis of the IgA and the IL-6 time courses revealed a coincidence of an IL-6 increase in serum and the occurrence of IgA in saliva on day 14. Regarding the serum and salivary IgA, IgG and IL-6 concentrations day-by-day, a significant correlation for IgA in saliva and IL-6 in serum was found on day 7. Interpreting this data with caution, IL-6 seems to play a role in the induction phase of IgA production, but it seems to have no influence on the actual IgA concentration in saliva. There are diverse factors, which can make it difficult to demonstrate a linear relation between IL-6 and immunoglobulins *in vivo.* The network of cytokines in a living organism is complex and there are plenty of interactions (Seillet et al., 2014). Concerning IL-6 in particular,

other cytokines were also discussed and considered to be the main trigger for the immunoglobulin production (e.g. IGIP, IL-2, IL-10) (Estes, 1996; Austin et al., 2003). Furthermore, IL-6 does not act directly on B-cells, but its effects are mediated by IL-21, released from $CD4^+$ T-cells upon their stimulation with IL-6 (Dienz et al., 2009; Gowane et al., 2014). In addition, the individual immunologic competence for producing antibodies and cytokines must be considered.

This individualism was also rediscovered in the present study, in the form of high standard errors (SE). Concerning colostrum and milk, the statistical analysis for each individual calf on day one revealed no correlation between colostrum and serum concentrations, neither for IgA nor for IgG. These findings show the disparity in any specific colostrum and in the calves' ability for intestinal absorption of immunoglobulins. These findings were confirmed by other authors, who could not show a positive correlation when colostrum and serum were individually analyzed for each animal of the study (Bender et al., 2009; Secor et al., 2012). However, there are also other studies which show significant correlations between colostrum and serum immunoglobulin concentrations (Osaka et al., 2014). It was suggested, that a high immunoglobulin concentration in colostrum is likely to result in a high immunoglobulin serum concentration in the respective calf, although individual factors in the calf can have an influence on these general assumption. Besides the individual ability for absorption, the colostrum administration plays an important role. Various factors, such as the volume administered, time point and application technique were discussed (Kruse, 1983; Besser et al., 1991; Rauprich et al., 2000). As the colostrum administration in this study follows a standardized protocol, differences between the calves could be neglected here.

Immunoglobulin A, as a primary mucosal immunoglobulin, rapidly declined in serum after day 1, whereas for IgG, the colostral immunity lasts at least for 2 to 4 weeks (Chase et al., 2008). During this time, the calves' own IgG production is negligible (Barrington et al., 2001). Therefore, in this study, the influence of IL-6 on the serum IgG concentration could not be examined, as serum IgG was mainly of colostral origin. In comparison to IgG concentrations reported in the literature, the amounts measured in this study were low, in both serum and colostrum (Wittum et al., 1995; Dewell et al., 2006; Meganck et al., 2014). Colostrum IgG levels are influenced by various factors. The individual immune competence of the dam as well as the number of gestations is important (Morrill et al., 2012). Colostrum of primiparous cows is shown to contain lower amounts of IgG than colostrum from multiparous cows (Gulliksen et al., 2008). There was a high number of heifers on the farm on which the sampling for this study was conducted. This might be one possible explanation for the low IgG concentrations in

colostrum. Furthermore, the collection of colostrum occurred not always directly after birth and a delayed collection is also described to negatively influence the amount of IgG (Moore et al., 2005).

The results obtained from the relative gene expression analysis proved that bovine PBMCs could produce IL-6 directly after birth. Even so, the serum concentration of IL-6 on day 1 appeared mainly influenced by the high concentrations of IL-6 in the colostrum. After day 7, a commercial milk replacer was fed to the calves so that a further transfer of IL-6 from the milk was excluded. From day 7 onwards, the data based on the relative gene expression and the serum concentration of IL-6 showed a similar time course. Moreover, correlations of fold-changes revealed significant coherence for each day. From these findings, we conclude that the peak of IL-6 in serum on day 14 was mainly due to the calves' own production of IL-6. The half-life time of IL-6 in serum was reported to be below 10 hours and therefore serum concentrations on day 14 were unlikely to result from colostrum uptake on day 1 (Banks et al., 2000). Our results differ from previous studies, where the IL-6 serum concentration until day 28 was exclusively represented by maternal IL-6 taken up via the colostrum (Yamanaka et al., 2003b). This discrepancy may be due to the different methodologies used. Instead of a conventional RT-PCR and detection of DNA by agarose gel electrophoresis, a qRT-PCR was used in this study. Concerning a reliable quantification of DNA, qPCR was described to be more accurate than quantification by agarose gel electrophoresis (VanGuilder et al., 2008).

In conclusion, it was proven that newborn calves could produce IL-6 by themselves, directly after birth. Regarding the influence of the calves' IL-6 on the physiological salivary IgA development, this study affirmed the hypothesis that IL-6 supports IgA production in saliva at specific time points during the development of an immune response in newborn calves. Later on, no subsequent relationship between IL-6 and IgA was established. The IgA-mediated mucosal immunity appeared as early as two weeks after birth and therefore much earlier than it is assumed for the systemic IgG.

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3.2 Chapter II

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IL- 6 AS A PROGNOSTIC MARKER IN NEONATAL CALF DIARRHEA

Serum Interleukin 6 as a Prognostic Marker in Neonatal Calf Diarrhea

Stephani Fischer, † Rolf Bauerfeind, ‡ Claus-Peter Czerny, † and Stephan Neumann*[1]

[*] Small Animal Clinic, Institute of Veterinary Medicine, Faculty of Agricultural Sciences, Georg-August University Goettingen, Goettingen 37077, Germany

† Department of Animal Sciences, Institute of Veterinary Medicine, Division of Microbiology and Animal Hygiene, Faculty of Agricultural Sciences, Georg-August University Goettingen, Goettingen 37077, Germany

‡ Institute of Hygiene and Infectious Diseases of Animals, Justus Liebig University, Giessen 35392, Germany

[1]Corresponding Author: sneuman@gwdg.de

Stephan Neumann, Small Animal Clinic, Institute of Veterinary Medicine, Faculty of Agricultural Sciences, Georg-August University Goettingen, Burckhardtweg 2, 37077 Goettingen, Germany

Telephone number: +495513913918

Fax: +495513913919

ABSTRACT

Neonatal calf diarrhea is still one of the most important diseases in calf rearing and severe diarrhea has a marked impact on animal welfare. Furthermore, significant economic losses can result from this disease due to high mortality rates, high medical costs and low weight gain. In order to avoid a fatal outcome of the disease, it is crucial that vulnerable calves are identified as early as possible. Interleukin 6 (IL-6) is described as an early and reliable prognostic marker in several diseases. In this study, 20 scouring calves were tested by ELISA for their IL-6 serum concentrations. Samples were collected twice, at the beginning of diarrhea and 7-10 days later. Regarding the clinical outcome after 7-10 days, calves were classified as recovered or non-recovered. A receiver operating characteristic (ROC) analysis was conducted to determine the prognostic value of IL-6 for the progress of clinical symptoms. At the beginning of diarrhea, the IL-6 concentration was significantly higher in non-recovering calves compared to those that recover 7-10 days after the onset of diarrhea. IL-6 proved to be a useful additional parameter in the clinical examination. High initial IL-6 values can support the decision for closer monitoring and an adapted therapeutic strategy for the respective calves. This may help to prevent unnecessary animal suffering and reduce economic losses.

Keywords

neonatal calf diarrhea, interleukin 6, ELISA, *Bovine Rotavirus, Cryptosporidium parvum*

INTRODUCTION

Neonatal calf diarrhea poses a marked threat to animal welfare and causes significant economic losses due to high mortality, higher medical costs and low weight gain (Waltner-Toews et al., 1986, Warnick et al., 1995, Mohd Nor et al., 2012). Infectious and non-infectious causes can lead to severe diarrhea in calves. In the first 4 weeks of life, infections with *Bovine Coronavirus* (BCV), *Bovine Rotavirus* (BRV), *Cryptosporidium* (*C.*) *parvum* and enterotoxic *E. coli* (ETEC) in particular can cause inflammatory and functional damage to the intestine (Kaske, 1993, Al Mawly et al., 2015). Insufficient management of the diseased calves can quickly result in a fatal outcome (Meganck et al., 2014). A reliable prognostic marker for the progress of diarrhea would enable dairy farmers and veterinarians to monitor critical patients more closely.

In several disorders, interleukins and especially interleukin 6 (IL-6), appear to be such a marker of early inflammation and prognosis (Rincon, 2012). The helical 208-AA glycoprotein IL-6 is produced by several cell types and mainly operates as an inducer of the acute phase response (Barton, 1997, Droogmans et al., 1992). Therefore, it is upregulated in the early stage of an inflammatory process and rapidly decreases after 12-24 h (Okabe et al., 1996, Volante et al., 2004). Thus, IL-6 could serve as an early marker for inflammation since serum titers increase before the inflammation becomes clinically apparent.

Several studies in human medicine investigated IL-6 as a prognostic marker related to a specific pathogen. Children displaying hemolytic uremic syndrome (HUS) due to an infection with enterohemorrhagic *E. coli* (EHEC) showed higher levels of IL-6 when the disease became more severe in nature (e.g. anuria, extrarenal manifestation, chronic renal sequelae) (Karpman et al., 1995). Likewise, high IL-6 values were present in children suffering from shigellosis when complications arose during illness (de Silva et al., 1993). For humans and pigs, it was shown that serum IL-6 values were elevated in individuals displaying diarrhea due to an infection with rotavirus (Jiang et al., 2003, Azevedo et al., 2006).

Despite the direct connection of IL-6 to a specific pathogen, IL-6 has also been established as a reliable marker for the clinical outcome of human patients, independent from the type of underlying disease. Interleukin 6 was prognostic for sepsis and mortality in adult humans suffering from a bacterial infection, regardless of what pathogen was causatively involved (Moscovitz et al., 1994). This was confirmed by another study among severely infected humans at an emergency department, where non-survivors had higher IL-6 serum concentrations at the initial examination than survivors (Wilhelm et al., 2012). In hospitalized human neonates, an increased IL-6 serum level was proven 100% sensitive in detecting individuals who developed a clinical sepsis at later stages of their disease (Buck et al., 1994). In veterinary medicine, IL-6

was also a reliable parameter to monitor dogs in intensive care units (Schüttler et al., 2015). The mean IL-6 concentration of these dogs was significantly higher in the non-survivor group compared to survivors.

Only a small number of studies covered the diagnostic and prognostic potential of IL-6 in cattle. For instance, IL-6 values in cows suffering from subclinical mastitis were elevated in the early stages of the disease, even before the number of somatic cells increased (Sakemi et al., 2011). Other investigators focused on the IL-6 serum values in pregnant cows pre-partum (Ishikawa et al., 2004). They found high IL-6 serum concentrations in those cows that developed endometritis post-partum. In the case of coliform mastitis, serum IL-6 was shown to be significantly higher in non-survivors when sampled at day 0-9 of the onset of clinical symptoms (Nakajima et al., 1997).

As investigations of serum IL-6 concentrations in the case of neonatal calf diarrhea are lacking and the improvement of therapy would benefit from a reliable prognostic marker, this study investigated the prognostic quality of IL-6 for the progress of clinical symptoms in neonatal calf diarrhea. Therefore, a controlled cohort study over 10 days was carried out on 20 scouring calves. Calves were monitored for clinical changes as well as for changes in the body homeostasis (e.g. hematology, clinical chemistry) related to diarrhea. For the determination of the prognostic potential, scouring calves were divided into two groups, regarding their classification at day 10 as ‘recovered’ or ‘non-recovered’. A ROC analysis with calculation of the AUC was carried out to prove the accuracy of IL-6 determination in serum for the estimation of the prognosis.

MATERIAL AND METHODS

Animals

Calves were housed on a commercial dairy farm near Goettingen, Lower Saxony, Germany. The farm is organized in terms of 3 locations. One location is for the 500 milk yielding and late pregnant cows, one for the calves and the last one for the offspring, older than 8 weeks. Biotechnical measures directly after birth included disinfection of the umbilicus and application of earmarks. Four liters colostrum were fed to the calves via tube feeder within the first hour of life. The colostrum came from the farm’s colostrum bank and was controlled for sufficient quality with a colostrometer. Within 24 hours after birth, newborn calves were brought to a special calf location, which was geographically separated from the barn for the milk-yielding cows. The newborn calves were sheltered outdoors in igloos individually and were fed pasteurized waste milk *ad libitum* in the first week after birth. Then, a commercial milk replacer

(50% skimmed milk) displaced the milk. From week 3 on, starter grain was offered and calves had free access to water. Calves were weaned 4 weeks after birth.

Sampling was conducted during January to July 2014. All Holstein Friesian calves displaying diarrhea were included in the study. Calves were excluded when showing any other diseases, such as respiratory tract infections or infections of the umbilicus. Finally, the group of scouring calves consisted of 20 animals (age 12.9 ± 2.0 days; 89 % female). Scouring calves were sampled once at the first day of evident symptoms and again 7-10 days later. At these time points, all animals passed a complete clinical examination with information on heart rate, breathing rate, temperature, dehydration, and general condition were gathered. Diarrhea scoring was undertaken using an already established scoring system, evaluating the fecal consistency in a 4-point scale from normal (0) to totally liquid (4) (Hasoksuz et al., 2002). All calves received an oral electrolyte solution from the beginning to the end of diarrhea and were initially treated with 0.5 mg/kg meloxicam. Throughout the study period, calves were monitored twice per day by the animal care takers.

After the sampling period, the calves were further divided into 2 subgroups; one subgroup (n = 11; age 15.0 ± 2.8 days) which included calves that clinically recovered 7-10 days after the first symptoms appeared and the other subgroup (n = 9; age 10.2 ± 1.7 days), which included calves evaluated as non-recovered, because they were still showing diarrhea, still being medically treated, or showing other abnormalities in the clinical examination (e.g. reduced general condition). All clinical examinations were performed by the same veterinarian to avoid an examiner bias.

Samples

All samples were acquired with the owner's consent and the procedures were carried out in accordance with the German Protection of Animals Act under the supervision of the Commissioner for Animal Welfare, Faculty of Agriculture, University of Goettingen. For the purpose of laboratory analysis, 5 mL EDTA-treated blood and 10 mL whole blood for serum harvest were collected by jugular venipuncture. Stool samples were collected from the rectum following digital stimulation. All samples were cooled immediately after collection and brought to the laboratory within 6 hours. Blood samples and feces were processed directly. Serum was stored at -20°C until further use in the IL-6 ELISA.

Hematology and Clinical Chemistry

The complete blood count and the serum clinical chemistry were analyzed in all calves, to ensure the healthiness of the control group and to determine pathological changes due to diarrhea. EDTA blood samples were utilized for a complete blood count using a CellDyn 3500 Analyzer (Abbott GmbH & Co KG, Wiesbaden, Germany). Because differentiation of bovine lymphocytes and monocytes can be difficult, the results of the CellDyn 3500 Analyzer were verified by a manual blood count. Serum samples were aliquoted to 500-µL aliquots and stored at -20°C. One aliquot was used directly for the clinical chemistry analysis with the Konelab 20i (Thermo Fisher Scientific Inc., Dreieich, Germany).

Stool Analysis

Stool samples were analyzed by a commercial lateral immunochromatography test (Bio-X Diagnostics, Rochefort, Belgium) to identify the causative pathogen for diarrhea, such as *Bovine Coronavirus*, *Bovine Rotavirus*, *Cryptosporidium parvum* and *E. coli* F5 (K 99).

IL-6 ELISA

IL-6 serum concentrations were determined using the commercial High Sensitive ELISA Kit for bovine IL-6 (USCN Life Science, Wuhan, China). First, standard and samples were added in duplicate to the pre-coated plate and incubated for 2 hours at 37°C. Subsequently, all liquid was removed and Detection Reagent A (Biotin-conjugated antibody specific to IL-6) was added for 1 hour at 37°C. The plate was washed 3 times with wash solution (1x) and Detection Reagent B (avidin-conjugated horseradish peroxidase) was added and incubated at 37°C for 30 min. The plate was washed 5 times with wash solution before substrate solution (TMB substrate) was added for 20 min at 37°C. Color development was stopped with stop solution (H_2SO_4) and optical densities were measured in all wells at 450 nm in the Sunrise microplate reader (Tecan, Maennedorf, Switzerland). For data analysis, average optical densities (ODs) were calculated and blank reduction was performed. Standard curves were generated and sample concentrations were determined using the CurveExpert 1.4 software (CurveExpert Copyright 1995-2007 Daniel Hyams). A detection limit of 1.56 pg IL-6/mL was reported by the kit manufacturer. The coefficient of variation (CV) for the intra-assay test precision was < 7%.

Statistical Analysis

Results were shown as mean values (MV) ± 2 standard errors (2SE). Data from the clinical examination, the hematology and the IL-6 ELISA were tested for normal distribution.

Data of recovering and non-recovering animals were compared at both sampling time points. Regarding the IL-6 values in these groups, further statistical tests were performed. A two-way analysis of variance (2-way ANOVA) was calculated to investigate the interaction of group (recovering, non-recovering) and sampling time point (first, second). In order to investigate a change in the odds of IL-6 concentrations at the onset of diarrhea (first sampling time point), to predict a prolonged course of diarrhea (non-recovering) a univariate logistic regression analysis was performed. The goodness-of-fit of the model was assessed by performing an Omnibus test of model coefficients and a Hosmer-Lemeshow test, as well as calculating the Nagelkerke R^2. The influence of the variable IL-6 was estimated using the Wald statistic.

As the age of calves is described to be a risk factor to develop diarrhea, this variable is analyzed in the same way regarding the age of calves at the first sampling time point (Pare et al., 1993; Gulliksen et al., 2009b). Subsequently, a multivariate logistic regression analysis was performed, with both variables (IL-6, age) and a potential interaction effect between these variables. The goodness-of-fit for this analysis was conducted as for the univariate analysis. Using forward model selection, a P-value of < 0.05 was chosen as the cut-off for the respective variable to stay in the model. In order to further evaluate the prognostic quality of IL-6, a receiver-operating characteristic (ROC) curve was generated with the IL-6 values of the recovering and the non-recovering animals at the first sampling time point. The sensitivity was defined as the percentage of samples which were classified correctly as non-recovering. The specificity was defined as the percentage of samples which were classified correctly as recovering. The area under the curve (AUC) was calculated by trapezoidal summation and its statistical significance was estimated. All analyses were carried out using STATISTICA 12 software (StatSoft (Europe) GmbH, Hamburg, Germany) and IBM SPSS Statistics 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.). The statistical significance level was set at $P < 0.05$.

RESULTS

Clinical, Biochemical, and Hematological Findings

The results of the clinical and laboratory examination are shown in Table 1. Most of the recovering calves showed mild symptoms of diarrhea (score 2, n = 9) and none of them displayed diarrhea at the second sampling time point. Severe diarrhea was only seen in the non-

recovering group (score 4, n = 2), in which 5 calves were still displaying diarrhea 7-10 days after the onset of symptoms. The clinical chemistry and the hematology revealed no significant differences between recovering and non-recovering animals, neither at the first nor at the second sampling time point (data not shown). Despite the number of white blood cells at the first sampling being within the reference values (5-10*10^9 cells/L) for recovering (RG) (6.5 ± 1.3*10^9 cells/L) and non-recovering calves (NRG) (10.3 ± 2.1*10^9 cells/L), animals from the NRG showed higher values than the RG animals ($P < 0.01$). This was caused by a higher number of neutrophils (RG: 2.6 ± 0.6*10^9 cells/L, NRG: 6.1 ± 2.4*10^9 cells/L, reference range: 1-3.5*10^9 cells/L). At the second sampling time point, no significant differences were detected.

Table 1 Results of the clinical examination and the leukogram of healthy (control) and scouring calves. All scouring calves were sampled a second time 7-10 days after the first sampling. Calves recovered from diarrhea at the 2. Sampling time point were compared to those that did not recover using a T-Test. Data are displayed as means ± 2 SE.

Parameter	**Ref. Range**	**1. Sampling**			**2. Sampling**		
		Recovering (n = 11)	Non-Recovering (n = 9)	*P*-value	Recovering (n = 11)	Non-Recovering (n = 9)	*P*-value
Age (days)		15.0 (± 2.8)	10.2 (± 1.7)	0.01	23.1 (± 3.3)	17.2 (± 1.7)	< 0.01
Diarrhea scoring[1]		0 (n = 0) 1 (n = 0) 2 (n = 9) 3 (n = 2) 4 (n = 0)	0 (n = 0) 1 (n = 0) 2 (n = 4) 3 (n = 3) 4 (n = 2)	-	0 (n = 11) 1 (n = 0) 2 (n = 0) 3 (n = 0) 4 (n = 0)	0 (n = 4) 1 (n = 0) 2 (n = 4) 3 (n = 1) 4 (n = 0)	-
Detected pathogen		BRV[8] (n = 3) C. parvum[9] (n = 5) BRV and C. parvum (n = 3)	BRV (n = 0) C. parvum (n = 7) BRV and C. parvum (n = 2)	-	BRV (n = 1) C. parvum (n = 4) BRV and C. parvum (n = 0)	BRV (n = 2) C. parvum (n = 4) BRV and C. parvum (n = 1)	-
Heart rate (min^{-1})	90-110	136.4 (± 7.2)	142.2 (± 10.1)	NS[10]	141.8 (± 9.3)	142.2 (± 6.9)	NS
WBC[2] **($*10^9$cells/L)**	5-10	6.5 (± 1.3)	10.3 (± 2.1)	< 0.01	8.2 (± 1.6)	7.4 (± 1.6)	NS
NEU[3] **($*10^9$cells/L)**	1-3.5	2.6 (± 0.6)	6.1 (± 2.4)	< 0.01	4.0 (± 1.1)	5.8 (± 5.7)	NS
LYM[4] **($*10^9$cells/L)**	0.6-3.4	2.0 (± 0.4)	2.5 (± 1.1)	NS	3.3 (± 0.6)	4.0 (± 1.8)	NS
MONO[5] **($*10^9$cells/L)**	≤ 0.9	0.4 (± 0.4)	1.5 (± 0.6)	< 0.01	0.9 (± 0.4)	2.5 (± 2.6)	NS
EOS[6] **($*10^9$cells/L)**	≤ 0.7	0.03 (± 0.05)	0.02 (± 0.03)	NS	0.01 (± 0.02)	0.02 (± 0.03)	NS
BASO[7] **($*10^9$cells/L)**	≤ 0.2	0.01 (± 0.02)	0.1 (± 0.1)	NS	0.07 (± 0.06)	0.04 (± 0.03)	NS

[1]Diarrhea scoring according to Hasoksuz et al., 2002, 0 = normal, 1 = pasty, 2 = semiliquid, 3 = liquid with some solid material, 4 = totally liquid;

[2]WBC, white blood cell count; [3]NEU, neutrophils; [4]LYM, lymphocytes; [5]MONO, monocytes; [6]EOS, eosinophils; [7]BASO, basophils; [8]BRV, *Bovine Rotavirus*; [9]C.parvum, *Cryptosporidium parvum*; [10]NS, not significant ($p > 0.05$).

Detection of Diarrheagenic Pathogens in Fecal Samples

Bovine Rotavirus (BRV) and *Cryptosporidium parvum* were identified as the diarrhea-causing pathogens in scouring calves (Table 1). Mono-infections with only 1 pathogen, as well as double-infections were detected. Pathogens changed from the first to the second sampling time points, in both the RG and the NRG. In the RG infections with BRV decreased from 3 to 1 positive samples, infections with *C. parvum* decreased from 5 to 4 positive samples and double infections were no longer detected at the second sampling. In the NRG infections with *C. parvum* decreased from 7 to 4 positive samples, double infections decreased from 2 to 1 and 2 new BRV infections were detected at the second sampling time point.

Interleukin 6 in Serum Samples

Interleukin 6 results are presented as means ± 2 SE (Fig. 1). There was no significant interaction between the effects of group (RG, NRG) and sampling time point (1, 2) on serum IL-6 (F = (1, 36) = 1.776, p = 0.191).

In terms of disease progression, initially the NRG calves showed a higher (p = 0.02) IL-6 mean value (42.3 ± 6 pg/mL) than the RG calves (31.8 ± 5.5 pg/mL).

A logistic regression was conducted to determine the effects of age and IL-6 at the onset of diarrhea (first sampling time point) on the odds for calves to develop a prolonged course of diarrhea (Table 2). The univariate logistic regression model for IL-6 (Model 1) was statistically significant, as the P-value for the chi-square statistic (Omnibus test of model coefficients) was 0.014 and the Hosmer-Lemeshow test showed no statistical significance (p = 0.640). The model explained 34% (Nagelkerke R^2) of variance. The Wald statistic revealed a significant influence of IL-6 in the model (p = 0.041). The odds for calves developing a prolonged course of the disease increase by 14% for each unit of IL-6 (Odds ratio (OR) 1.14; 95% CI 1.0-1.3). A T-test revealed the age of the RG and the NRG animals being significantly different (p = 0.01). A univariate logistic regression model was conducted to test for a significant influence of the variable “age” on the odds for a prolonged course of diarrhea. As the analysis revealed significance (p = 0.039) a multivariate logistic regression was performed including IL-6 and age (Model 2). In this model, none of the variables showed significance. Additionally, no confounding by age was noted as there was less than a 20 % change in the coefficient for IL-6 with the addition of age in the model. Furthermore, even the interaction term (age*IL-6; Model 3) showed no significance, when included in the model.

The value of IL-6 as a prognostic marker for the clinical outcome of scouring calves was further evaluated in a ROC analysis (Fig. 2). The ROC analysis for IL-6 values of the diarrhea group

at the beginning of diarrhea revealed a cut-off of 38.7 pg/mL, with a sensitivity of 77 % and specificity of 82 % for identifying calves with a prolonged recovery period. The area under the curve (AUC = 0.808, 95% confidence interval: 0.609-1, p = 0.02) showed a good predictive ability for IL-6 to discriminate between RG and NRG animals at the onset of diarrhea.

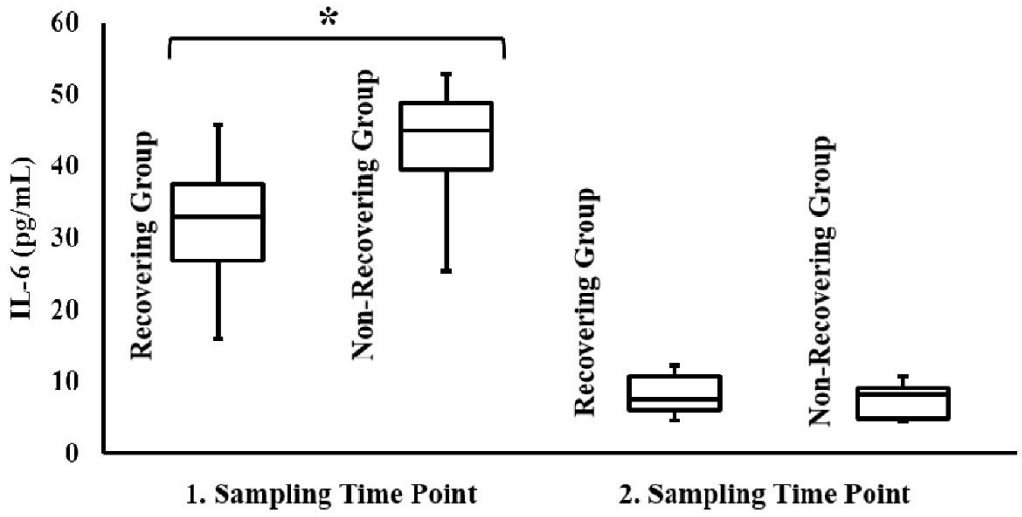


Fig. 1. Comparison of Interleukin 6 (IL-6) in scouring Holstein Friesian calves with different clinical outcome. The scouring calves were grouped by clinical recovery 7-10 days after (2. sampling time point) the onset of diarrhea (first sampling time point). IL-6 serum concentrations of the recovering group1 (n = 11) and the non-recovering group1 (n = 9) were compared on both sampling time points.

* $p < 0.05$;

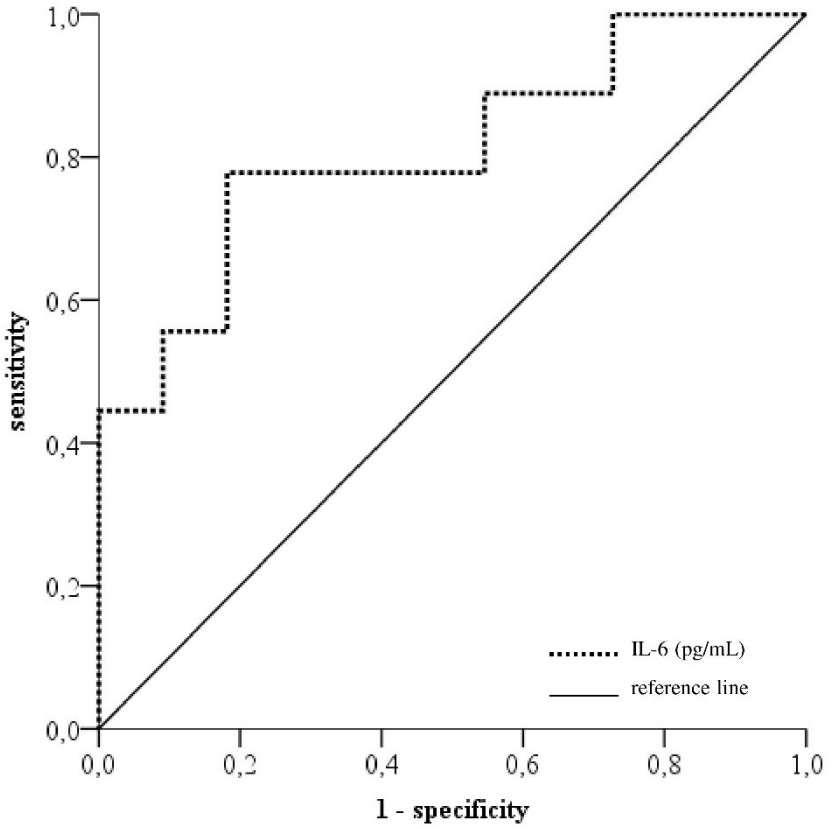
[1]Recovering group, calves were clinically recovered 7-10 days after the onset of diarrhea;

[2]Non-Recovering group, calves were not clinically recovered 7-10 days after the onset of diarrhea

Table 2 Results of the logistic regression analysis for model 1. IL-6 values at the onset of diarrhea (first sampling time point) were evaluated for their odds to predict a prolonged course of diarrhea (non-recovering).

		Model 1 "IL-6" (n = 20)
Variable		IL-6
B[1]		0.134
SE[2]		0.066
P*-value		0.041
Exp(B)		1.143
95% C.I.[3] **for Exp(B)**	**Lower**	1.005
	Upper	1.300
Omnibus test*		$p = 0.014$
Nagelkerke R[2]		0.346
Hosmer-Lemeshow test*		$p = 0.640$

[1]B, Regression coefficient B; [2]SE, standard error; [3]C.I., confidence interval; * P-value < 0.05 was regarded as significant



Test Variable	Standard Error[a]	Area under the curve	Asymptotic Significance[b]	Asymptotic 95% Confidence interval	
				Lower Bound	Upper Bound
IL-6 (pg/mL)	0.120	0.808	0.02	0.609	1.0

[a] under the nonparametric assumption
[b] Null hypothesis true area = 0.5

Fig. 2. ROC[1] curve analysis for the evaluation of the prognostic performance of interleukin 6 (IL-6) in scouring Holstein Friesian calves. Scouring calves (n =20) were grouped by their clinical recovery status 7-10 days after the onset of diarrhea (recovering group[2], n = 11; non-recovering group[3], n = 9). The IL-6 serum concentrations at the onset of diarrhea were evaluated for reliability in identifying calves with a prolonged recovery period.

[1]ROC, receiver operating curve;

[2]Recovering group, animals which were clinically recovered 7-10 days after the onset of diarrhea;

[3]Non-Recovering group, animals which were not clinically recovered 7-10 days after the onset of diarrhea

DISCUSSION

In this study the prognostic value of the IL-6 serum concentration was investigated over the course of neonatal calf diarrhea. The main result was that calves developing a prolonged course of the disease showed significantly higher IL-6 values at the onset of symptoms.

Initially, hematology, clinical chemistry were analyzed. The calves showed elevated levels of neutrophils while the total number of white blood cells was within the reference range. The neutrophilia was rather related to handling stress during sampling than to diarrhea, as it was similar in both groups. An increase of neutrophils due to stress was already described for cattle in cases of shipping and heat-stress (Kegley et al., 1997, Mitlohner et al., 2002). The differential blood count, measured by the CellDyn 3500 Analyzer, revealed increased numbers of monocytes when compared to the reference values. However, manual counting of blood smears could not confirm the monocytosis. The CellDyn 3500 Analyzer classifies cells by measuring volume and granularity. It is likely that the number of monocytes was overestimated by mistaking them for large lymphocytes (Stoeber et al., 1967).

The detection of diarrhea-causing pathogens revealed differences between the first and the second sampling time point in both groups (recovering, non-recovering). In the recovering group 5 calves still shed either BRV or *C. parvum* at the second sampling time point, although none of them showed diarrhea. This was described in other studies also, were BRV shedding occurred up to 11 days after infection, whereas diarrhea only lasts 7 days (Vega et al., 2015). Similarly, *C. parvum* detection in feces was shown to be not always connected to diarrhea in the corresponding animals (Bjorkman et al., 2015). In the non-recovering group 2 animals showed a BRV infection at the second sampling time point, which had not been apparent at the first sampling time point. It was suggested, that this was due to a secondary infection, which is frequently described in neonatal calf diarrhea (Bartels et al., 2010, Silverlas et al., 2010). In the classification of calves as recovered or non-recovered the pathogen detection was less important than the results of the clinical examination when focusing on the establishment of a prognostic marker.

Serum IL-6 concentrations were greater in the diarrhea group than in controls. This is consistent with findings of an increased IL-6 gene expression in peripheral blood mononuclear cells (PBMCs) and in intestinal epithelial cells from scouring calves *ex vivo* due to an infection with *Bovine Rotavirus* (Aich et al., 2007, Qadis et al., 2014).

Calves further investigated regarding the course of diarrhea and the ability of IL-6 to predict the clinical outcome after 7-10 days. Therefore, they were divided into recovering and non-recovering groups after collection of data. At the onset of diarrhea, IL-6 was significantly

greater in recovering and non-recovering animals. Because calf age differed between the two groups, age was evaluated additionally. In a multivariate logistic regression model none of the variables IL-6 and age and showed significance. A possible interaction between these two variables was not observed. However, the univariate logistic regression model for IL-6 showed a significance influence of this variable in predicting a prolonged course of diarrhea and was therefore regarded as the most reliable model in this investigation. The odds to develop a prolonged course of diarrhea increase by 14% for each unit of IL-6 and model could explain 34% of the variance. In literature, there are several other factors discussed increasing the risk for diarrhea (Bendali et al., 1999; Pare et al., 1993; Wiest et al., 1998). Birth weight and calf housing (e.g. open barn vs. closed barn) were identified as potential risk factors (Al Mawly et al., 2015; Pare et al., 1993), whereas immunoglobulin G serum concentrations seemed to have no effect (Meganck et al., 2015; Pare et al., 1993). These parameters were not assessed in the study presented here, but could have been different in recovering and non-recovering calves. Therefore, these parameters should be taken into consideration in future investigations, especially focusing on potential interactions between these parameters and IL-6.

The value of IL-6 as an initial parameter for the prognosis of scouring calves was further evaluated in a ROC analysis. The area under the curve (AUC) was used to measure the accuracy of IL-6 in distinguishing recovering from non-recovering animals. A value of 1 was regarded as a perfected test, whereas a value of 0.5 corresponds to random chance. The AUC value of 0.808 confirmed IL-6 as a good test for the estimation of the prognosis. Furthermore, the ROC analysis allowed the definition of a cut-off value. For the establishment of IL-6 as a prognostic marker, the sensitivity was given priority over the specificity, as a high number of false positive classifications are less important than a high number of false negative classifications. In practice, this means, that it is better to monitor a calf more closely, which is misclassified as non-recovering, as to overlook a calf, which is prone to develop a prolonged course of diarrhea. The cut-off was set at 38.7 pg/mL, with a sensitivity of 77% and specificity 82%, as this was the concentration with the most appropriate balance between sensitivity and specificity.

Taken together the results of the ROC analysis and the univariate logistic regression, the determination of IL-6 at the onset of diarrhea was a useful tool to identify calves developing a prolonged course of the disease. It is recommended that scouring calves displaying IL-6 values above 38.7 pg/mL should be given special attention, as they were prone to develop a prolonged course of diarrhea. These results underline the prognostic potential of IL-6, which was already shown for adult cattle in cases of postpartal endometritis and coliform mastitis (Ishikawa et al.,

2004, Nakajima et al., 1997). Therefore, IL-6 seems highly valuable to forecast complications and possibly life-threatening progression of an infectious disease in cattle.

In conclusion, this study showed IL-6 to be a useful supplemental parameter for the estimation of disease progression in neonatal calf diarrhea. Elevated IL-6 values may lead the veterinarian to the decision to monitor scouring calves more closely and to adapt the therapeutic strategy accordingly. Thereby animal suffering and economic losses could be avoided. As the animals in our study only showed infections with *Bovine Rotavirus* or *Cryptosporidium parvum*, further investigations are needed into all relevant pathogens for neonatal calf diarrhea (e.g. *Bovine Coronavirus*, enterotoxic *E. coli* encoding F5 (K99) fimbriae).

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Chapter III

Paper under review in Molecular Immunology (date of submission 29 February 2016)

Isolation of a high-affinity single-chain antibody fragment (scFv) from a bovine phage display library neutralizing Bovine Coronaviruses.

Stephani Fischer[a], Rolf Bauerfeind[c], Stephan Neumann[b,] Claus-Peter Czerny[a*]

[a] Division of Microbiology and Animal Hygiene, Institute of Veterinary Medicine, Department of Animal Sciences, Faculty of Agricultural Sciences, Georg-August University Goettingen, Burckhardtweg 2, 37077 Goettingen, Germany; stephani.fischer@agr.uni-goettingen.de

[b] Small Animal Clinic, Institute of Veterinary Medicine, Georg-August University Goettingen, Burckhardtweg 2, 37077, Goettingen, Germany; sneuman@gwdg.de

[c] Institute of Hygiene and Infectious Diseases of Animals, Veterinary Faculty, Justus Liebig University Giessen, Frankfurter Str. 85-89, 35392, Giessen, Germany; rolf.bauerfeind@vetmed.uni-giessen.de

*Corresponding Author: Claus-Peter Czerny, Department of Animal Sciences, Georg-August University, Burckhardtweg 2, 37077 Goettingen, Germany

E-mail: cczerny@gwdg.de

ABSTRACT

Bovine Coronavirus (BCV) is one of the main pathogens responsible for causing severe diarrhea in neonatal calves. High mortality rates and marked economic losses due to neonatal calf diarrhea indicate deficiencies in current preventive and therapeutic strategies. Species-specific antibodies against BCV were considered to address this therapeutic lack. In order to generate single chain antibody fragments (scFv) against BCV a bovine phage display library was constructed. Initially, a detailed analysis of bovine immunoglobulin genetics was conducted, based on already published data, own investigations and a BLAST analysis. A new bovine primer set covering the entire bovine immunoglobulin repertoire was designed. It was used to amplify the antibody repertoire of a Holstein Frisian bull immunized against BCV. The amplified antibody encoding cDNA from isolated peripheral blood mononuclear cells (PBMCs) provided the basis for an immunized single-chain variable fragment (scFv) phage display library of a diversity of $3*10^9$ independent clones. A panel of bovine scFv antibodies with identical sequence profiles was isolated from the library. The bovine scFv-SF-3E4 showed excellent binding kinetics (K_m = 595 pM) and neutralization ability (≥ 0.24 µg/ml) against BCV-V270. The epitope was investigated using western blotting and a competition ELISA, and found to be located in the S1 subunit of the BCV spike protein. The established recombinant scFv antibodies are promising new species-specific drug candidates for prevention and therapy of neonatal calf diarrhea caused by coronaviruses.

Keywords

Bovine Coronavirus, immunoglobulin library, phage display, single-chain variable fragment

INTRODUCTION

Neonatal diarrhea is still considered to be one of the most important diseases when rearing calves, and animal welfare is markedly affected by severe diarrhea. Furthermore, high mortality rates, drug costs and low weight gain may result in significant economic losses (Waltner-Toews et al., 1986; Warnick et al., 1995; Hasoksuz et al., 2002). Important etiologic pathogens include *Bovine Coronavirus*, *Bovine Rotavirus*, enterotoxic *E. coli* (F5 fimbriae) and *Cryptosporidium parvum* (Uetake, 2013). Effective treatment is essential to ensure animal health and welfare. Today, treatment of livestock is a key public and governmental concern. This can be attributed to increasing bacterial resistances and the lack of adequate antiviral therapeutics available. Currently, two preventive strategies for neonatal calf diarrhea are employed in the field. The first is dam vaccination. In Germany, presently four different vaccines are licensed by the Paul-Ehrlich-Institut (http:\\www.pei.de). The second strategy is the passive immunization of calves with orthologous horse serum or concentrated bovine colostral immune serum. Both options are subject to a number of disadvantages. Effective dam vaccination requires significant logistical effort during immunization and colostrum management. Furthermore, colostrum quality is limited to the cow's individual immunological competence (Kuegler et al., 2015). The elaborate production of orthologous and homologous immune sera results in expensive compounds, whose application is limited to individual cases. It can be stated that species-specific immunoglobulins are important for prophylaxis and therapy. Unfortunately, no standardized economic solutions are available. Alternatively, species-specific recombinant antibodies for passive immunization can be isolated from antibody phage display libraries. Currently, no comprehensive bovine antibody library exist. The single-chain variable fragment antibody (scFv) is the commonly used recombinant antibody format for these purposes (Ahmad et al., 2012; Wang et al., 2015). In an scFv molecule, a peptide linker connects the variable domain of an antibody-heavy chain to the variable domain of an antibody-light chain (Farajnia et al., 2014). The limitation on the antibody domains, which are crucial for antigen binding, results in a very small but functional molecule showing excellent tissue penetration (Sandhu, 1992). These properties make scFv antibodies advantageous for future clinical applications (Ahmad et al., 2012). However, in therapeutic applications, scFv antibodies show lower affinities, reduced serum half-life time and less neutralization ability compared to full-size antibodies, due to the lacking Fc part of the molecule (Chames et al., 2009; Jorgensen et al., 2014; Unverdorben et al., 2016). This handicap can be easily overcome by the reformation of a full-size antibody from a scFv molecule, which is a simple method due to the development of new plasmids containing the requested constant domain (Bujak et al., 2014; Rasetti-Escargueil

et al., 2015). Furthermore, compared to the common hybridoma technology for recombinant antibody generation, further advantages emerge. The library construction is based on the homologous immunoglobulin repertoire. Therefore, antibodies generated are completely species-specific and do not exhibit immunogenicity when administered to the respective host species (Frenzel et al., 2014). The *in-vitro* process also enables the generation of antibodies against high virulent pathogens, high toxic or endogenous substances (Hairul Bahara et al., 2013). Phage display technology allows for a rapid and easy library screening by mimicking the antibody affinity maturation *in-vivo*. In the animal, the native B-cell population initially produces a highly diverse antibody spectrum. Only those B-cell clones producing the most specific antibodies, with the greater affinity, multiply (Murphy, 2009; Paul, 2012). The repeated panning rounds during the phage display procedure imitate this process (Winter et al., 1994). Antibody specificity and affinity can be easily improved by adapting panning conditions, as well as by genetically engineering the selected antibody subsequently (Altshuler et al., 2010; Lee et al., 2015). To sum up, the construction of antibody phage display libraries is a straight forward and cost-effective method to generate species-specific high-affinity scFv fragments (Liu et al., 2013).

To construct a bovine scFv library, detailed knowledge of the bovine immunoglobulin genes is necessary. Currently two bovine genome assemblies are available. They provided the basis for recent insights into bovine immunoglobulin genetics. Contrary to humans and mice, 95% of antibody light chains in cattle express the lambda isotype (Arun et al., 1996; Chen et al., 2008). Furthermore, the number of bovine variable domain (V) genes is modest in comparison to other species, such as human and mice (Parng et al., 1996). To still ensure sufficient antibody diversity, cattle show individual strategies such as somatic mutation without antigen contact, gene conversion and exceptional long CDR3 regions (Meyer et al., 1997; Walther et al., 2013). Bovine immunoglobulin genes are organized into several genomic loci. The functional bovine heavy chain locus is located on chromosome 21. Additional loci were found on chromosomes 7, 8 and 20 (Walther et al., 2013). Sequence analysis revealed 36 different VH genes. Thirty of them were classified as functional genes. A leader sequence (LH) is connected upstream to each VH gene. This sequence is necessary for the transportation of the mRNA to the ribosomes during protein translation. Fifteen DH genes and six JH genes (2 functional) were identified (Walther et al., 2013; Liljavirta et al., 2014). Each constant region of the three immunoglobulin G (IgG) subclasses IgG1, IgG2 and IgG3 is encoded by an individual gene (Gu et al., 1992). The two light chain isotypes are located on different chromosomes; kappa on chromosome 11 and lambda on chromosome 17 (Arun et al., 1996). The lambda locus includes 63 VL_{λ} genes in

total and 25 functional genes. As described for the heavy chain, in the light chains each V gene is also connected to an upstream leader sequence. The JL_λ and CL_λ genes were organized in four cassettes, each cassette comprising one JL_λ and one CL_λ gene. Two cassettes were found to be functional (Ekman et al., 2009; Diesterbeck et al., 2012). For the kappa locus, 22 VL_κ genes are described and eight are considered to be functional. Three JL_κ genes and only one CL_κ are known and considered to be functional (Ekman et al., 2009; Stein et al., 2012). As usual, the D segment is lacking in the light chain (Paul, 2012). Based on this data, a universal primer set was generated to thoroughly amplify the bovine immunoglobulin gene repertoire. An immunized bovine immunoglobulin library was generated and screened using phage display technology. As proof-of-principle, a bovine anti-BCV scFv was isolated from the library and compared to a neutralizing monoclonal antibody (Czerny et al., 1989) concerning specificity, affinity and pharmacokinetics.

MATERIAL AND METHODS

Viruses, bacteria, cell line and antibodies

The *Bovine Coronavirus* strains BCV-V270 Munich (Czerny et al., 1989), BCV-L9 (derived from Dr. Werner Herbst, Justus Liebig University Giessen) and the human coronavirus strain OC43 (Tyrrell et al., 1978; Czerny et al., 1989) were propagated in VERO cells. VERO cells were grown in MEM containing 10% fetal calf serum (FCS). For virus propagation the FCS concentration was decreased to 5%. After a sufficient development of the cytopathogenic effect (CPE), the infected cell culture was freeze-thawed (-80°C/20°C) and centrifuged at 6,000 x g for 30 min at 4°C to remove cell debris. The supernatant was centrifuged in a SW 28-rotor (Beckman Coulter GmbH, Krefeld, Germany) for one hour (104,000 x g). Virus pellets were resuspended in TEN buffer (0.02M Tris-HCl, 0.15M NaCl, 0.001M EDTA, pH 7.6; all from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and layered on a continuous sucrose gradient (20-60%, w/w in TEN buffer; Carl Roth GmbH & Co.KG, Karlsruhe, Germany). Centrifugation was carried out in a SW 40-rotor (Beckman Coulter GmbH, Krefeld, Germany) for five hours at 4°C (114,000 x g). The virus band was collected by lateral puncture, pelleted in the SW 28-rotor for one hour (104,000 x g) and resuspended in TEN buffer. The purified virus was stored at -80°C. For comparison a sucrose gradient purified Vaccinia Virus strain Elstree, the murine monoclonal anti-BCV-V270 antibody 1/1 and an anti-BCV-V270 guinea pig immune serum were used (Czerny et al., 1989; Czerny et al., 1994). *E. coli* strains used in this study were purchased from commercial suppliers (TG1: Lucigen Corporation, Middelton, USA ; HB2151: GE Healthcare, Chalfont St. Giles, UK).

Primer design

The primer design was based on published bovine immunoglobulin gene sequences and database entries. Using the BLAST software (Altschul et al., 1990), the genome of *Bos taurus* (GCA_000003055.5 Bos_taurus_UMD_3.1.1) (Zimin et al., 2009) was screened using bovine heavy and light chain DNA sequences of the leader, the variable, the joining and the first constant region. The Lasergene MegAlign 12 software (DNAStar, Madison, USA) was used to align all sequences found by the ClustalW algorithm. Primer sets were generated incorporating both functional and pseudogenes. Similar sequences were covered by one single primer using wobble bases. The amplification was set up as nested PCR. In the first PCR (PCR 1), cDNA encoding the leader, variable, joining and first constant region of the respective chain was amplified. The second PCR (PCR 2) amplified a shorter segment, corresponding to the variable and joining region. Therefore, primers were generated for each leader, variable, joining and first constant region of the respective chains. VL_{λ}, VL_{κ} and JH primers were designed to contain an overlapping 30-nucleotide sequence encoding a $(G_4S)_3$ glycine-serine linker, connecting VH and VL chains in the scFv molecule.

Animal, blood collection and lymphocyte isolation

A four-year-old Holstein Friesian breeding bull was immunized three times with the dam vaccine Scourguard3 (Zoetis, Berlin, Germany), containing live attenuated *Bovine Coronavirus*, live attenuated *Bovine Rotavirus* and inactivated *E. coli* (K99). The intervals between the vaccinations were two weeks. 250 ml blood were taken from the *vena jugularis* three weeks after the last vaccination. The procedure was carried out in accordance with the German Protection of Animals Act under the supervision of the Commissioner for Animal Welfare from the Faculty of Agriculture at the University of Goettingen. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Ficoll Paque Plus (GE Healthcare, Chalfont St. Giles, UK). Cells were washed with PBS (137 mM NaCl, 12 mM Phosphate, 2.7 mM KCl, pH 7.4; all from Carl Roth GmbH&Co.KG, Karlsruhe, Germany). Red blood cell (RBC) contamination was removed by incubation in RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM EDTA; all from Carl Roth GmbH&Co.KG, Karlsruhe, Germany) for 5 min on ice. Isolated PBMCs were stored in liquid nitrogen until further use.

RNA isolation and cDNA synthesis

All RNA was extracted from PBMCs using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA quantity and purity were determined by UV absorbance at 260 and 280 nm in a NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, USA). Reverse transcription was performed using the SuperScript III first-strand Synthesis Supermix (Invitrogen, Carlsbad, USA) and random hexamer primers. First, isolated RNA was denatured at 65°C for 5 min. Afterwards cDNA was synthesized at 25°C for 10 min, followed by incubation at 55°C for 50 min. The reaction was terminated at 85°C for 5 min. cDNA was stored at -80°C.

Amplification of cDNA encoding bovine VH and VL immunoglobulin chains

The amplification was set up as nested PCR. First, all possible primer combinations for PCR 1 and PCR 2 were tested individually in a 12-temperature-step (50-70°C) gradient PCR using different DMSO concentrations (0%, 2.5%, 5%). In PCR 1, the master mix contained 5 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM), 0-5% DMSO (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 µl dNTPs (10 mM) (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 0.4 µl DNA Polymerase (1 U/µl; Biotools B&M Labs S.A., Madrid, Spain) and 500 ng cDNA. The volume was adjusted to 50 µl with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). The PCR reaction was initialized for 10 min at 95°C, followed by 35 cycles of 95°C for 1 min, 50-70°C for 1 min and 72°C for 2 min. The final elongation was set at 72°C for 10 min. As a control for the successful RNA isolation and the reverse transcription into cDNA, the bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – a housekeeping gene, constantly expressed in all cells – was amplified. The product of PCR 1 was purified by the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and used as template in PCR 2. The master mix for PCR 2 contained 10 µl 5x GC Reaction Buffer (Peqlab, Erlangen, Germany), 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM), 0-5% DMSO (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 µl dNTPs (10 mM) (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 µl KAPA Hifi polymerase (1 U/µl; Peqlab, Erlangen, Germany) and 25 ng DNA (purified product of PCR 1). The final volume was adjusted to 50 µl with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). Cycling conditions were set up as follows: 95°C for 5 min, 30 cycles at 98°C for 20 sec, 50-70°C for 15 sec and 72°C for 30 sec, and 72°C for 5 min. Gradient PCRs were run in a TProfessional Cycler (Biometra, Goettingen, Germany). All PCR products were analyzed in a 1% agarose gel and cloned using the Strata Clone PCR Cloning Kit (Agilent Technologies, Santa Clara, USA). At least three clones for

each primer combination were sequenced and proved to contain an open reading frame (ORF) and to display a bovine immunoglobulin DNA sequence. The software used included Lasergene SeqMan Pro, MegAlign (DNAStar, Madison, USA), ExPASy translate tool (Gasteiger et al., 2003) and the IMGT database (Lefranc, 2001). Afterwards the single primers were pooled, resulting in one forward and one reverse primer pool for each PCR and immunoglobulin chain. The primer amounts in the pools were adjusted, due to the number of genes the individual primer covers. Primer pools were tested as described above. The PCR 1 and PCR 2 were repeated under the defined optimal conditions in order to gain material for the recombination.

Generation of single chain antibody fragments

VH, VL_λ and VL_κ PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). For the recombination, 300 ng of the VH PCR product was combined with either 300 ng PCR product of VL_λ or VL_κ. The reaction mix contained 5 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 2.5% DMSO (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 µl dNTPs (10 mM) (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 0.4 µl DNA polymerase (1 U/µl; Biotools B&M Labs S.A., Madrid, Spain) and the volume was adjusted to 50 µl with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). The recombination took place at 95°C for 10 min, 10 cycles of 95°C for 1 min, 60°C for 1 min, 72°C for 2 min and a final elongation at 72°C for 10 min. The 800 bp recombination product was purified as described for the single chains and further amplified with primers containing the restriction enzyme cleavage sites for *Sfi*I and *Not*I. The amplification was set up with 5 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 2 µl forward primer (10 µM), 2 µl reverse primer (10 µM), 2.5% DMSO (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 1 µl dNTPs (10 mM) (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 0.4 µl DNA polymerase (1 U/µl; Biotools B&M Labs S.A., Madrid, Spain) and 25 ng of the recombination product. The final volume was adjusted to 50 µl with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). Cycling conditions were set at 95°C for 10 min, followed by 25 cycles at 95°C for 1 min, 67°C for 1 min, 72°C for 2 min, and 10 min at 72°C. Both recombination and amplification were run in a T3000 cycler (Biometra, Goettingen, Germany). The amplification products were evaluated by electrophoresis through a 1% agarose gel and DNA sequencing. Procedure and reagents were the same as described for VH and VL gene amplification.

Construction of the scFv library in pCANTAB5E

The phagemid pCANTAB5E was used to construct the immunized bovine IgG scFv library. This vector contains an insertion site flanked by the restriction enzyme cleavage sites of *Sfi*I and *Not*I. The insertion site is followed by an E-Tag sequence and an amber stop codon. The latter separates the insert-E-Tag sequence from the fd-phage-gene 3, which codes for the minor coat protein III of the phage M13. The phagemid was purified from dam$^-$/dcm$^-$ *E. coli* (New England Biolabs GmbH, Frankfurt, Germany) using the QIAGEN Plasmid *Plus* Midi Kit (Qiagen, Hilden, Germany). The scFv DNA and the vector DNA were digested with *Sfi*I (New England Biolabs GmbH, Frankfurt, Germany) and *Not*I (New England Biolabs GmbH, Frankfurt, Germany). Calf intestine phosphatase (CIP; New England Biolabs GmbH, Frankfurt, Germany) was used to dephosphorylate the vector DNA. Both digested scFv DNA and digested dephosphorylated vector DNA were purified using the QIAquick PCR purification Kit (Qiagen, Hilden, Germany). Ligation was facilitated with T4 Ligase (New England Biolabs GmbH, Frankfurt, Germany) overnight at 16°C. Ligated DNA was purified and concentrated using the DNA Clean&Concentrator^{TM}-5 Kit (Zymo Research Europe GmbH, Freiburg, Germany). 100 ng of the purified ligated DNA were transformed into Phage Display Electrocompetent TG1 cells (Lucigen Corporation, Middelton, USA), following the manufacturer's instructions. Electroporation was carried out in a BIO-RAD Gene Pulser® (Bio-Rad Laboratories GmbH, Munich, Germany). Bacteria were grown for 1 hour at 37°C with 250 rpm in Recovery Medium (Lucigen Corporation, Middelton, USA) before they were plated onto 2xTYGA agar plates (20 g glucose, 16 g tryptone, 10 g yeast extract, 5 g NaCl, 15 g agar-agar, 100 µg/ml ampicillin, ad 1 L sterile water; all from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and incubated overnight at 37°C. Bacteria were scraped from the plates with 6 ml 2xTYGA medium containing 15% glycerol (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). The bacterial suspension was aliquoted to 500 µl and stored at -80°C. The transformation efficiency was calculated based on a dilution series of the transformed bacteria on 2xTYGA agar plates. Clones were analyzed to contain full-length inserts by colony PCR and DNA sequencing of PCR products. For the colony PCR, single colonies were picked in a 20 µl master mix containing 2 µl 10x Standard Reaction Buffer with $MgCl_2$ (Biotools B&M Labs S.A., Madrid, Spain), 0.5 µl primer R1 (10 µM), 0.5 µl primer R2 (10 µM), 0.5 µl dNTPs (10 mM) (Carl Roth GmbH&Co.KG, Karlsruhe, Germany), 0.2 µl DNA polymerase (1 U/µl; Biotools B&M Labs S.A., Madrid, Spain) and adjusted to the final volume with HPLC water (Carl Roth GmbH&Co.KG, Karlsruhe, Germany). PCR was run in a T3000 cycler (Biometra, Goettingen, Germany) under the following conditions: 94°C for 10 min, 30 cycles at 94°C for 1 min, 55°C

for 1 min and 72°C for 2 min, followed by 72°C for 15 min. Ligations and transformations were repeated until a sufficient diversity of independent clones was achieved. Library diversity was tested in a fingerprint analysis with *BstN*I (New England Biolabs GmbH, Frankfurt, Germany). Digested colony PCR products were analyzed by electrophoresis through a 2% agarose gel. Individual clones were proven to contain an ORF and a DNA insert encoding displayed full-size scFv antibodies by sequencing, as described for the cloning during PCR establishment.

Phage Display

All library clones were pooled and a dilution series was cultured on 2xTYGA agar plates to determine the bacterial cell concentration per ml in the pooled library. Phage display was conducted based on current protocols (Shaw and Kane, 2009; Zhang and Dimitrov, 2009). A 500 µl bacterial library aliquot was thawed on ice and grown in 500 ml 2xTYGA medium at 37°C with 250 rpm to reach log phase (OD 0.4-0.6). The culture was infected with MOI 30 of M13KO7 Helper Phage (New England Biolabs GmbH, Frankfurt, Germany). The incubation occurred stationary at 37°C for 30 min, followed by 30 min at 37°C with gentle shaking. The infected culture was centrifuged at 1,800 x g for 15 min at 4°C (Dupont Sorvall RC-5B, Rotor GSA; London, UK). Cell pellets were resuspended in 2TY medium supplemented with ampicillin (100 µg/ml; Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and kanamycin (50 µg/ml; Carl Roth GmbH & Co.KG, Karlsruhe, Germany), and grown at 30°C for 16-20 hours with 250 rpm. Bacteria were pelleted at 1,800 x g for 10 min at 4°C (Dupont Sorvall RC-5B, Rotor GSA; London, UK). The phages in the supernatant were precipitated by adding 20% PEG with 2.5 M NaCl (both from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) at a volume ratio of 1:5. The solution was swayed for 45 min on ice followed by another 45 min stationary incubation on ice. Precipitates were pelleted by centrifugation at 1,800 x g for 1 h at 4°C (HERAEUS™ Multifuge™ 3 S-R, Rotor 7500 6445; Thermo Fisher Scientific Inc., Waltham, USA) and were resuspended subsequently in PBS without Ca^{2+} and Mg^{2+} (8 g NaCl, 0.2 g KCl, 2.89 g $Na_2PO_4x12H_2O$, 1 g glucose, ad 1 l sterile water; all from Carl Roth GmbH & Co.KG, Karlsruhe, Germany). The precipitation was repeated once. After resuspension, the phages were centrifuged again at 1,800 x g for 10 min at 4°C. The supernatant was stored short-term at 4°C or frozen at -80°C. The phage titer was determined by a dilution series in TG1 cells. TG1 cells were grown to log phase (OD 0.4-0.6) in an antibiotic-free 2TYG medium. From this culture a 900 µl-aliquot was mixed with 100 µl of the diluted phage suspension and grown at 37°C for 30 min stationary and 30 min at 37°C with 250 rpm. The dilution series was plated on 2xTYGA agar and grown at 30°C overnight. By counting the bacterial colonies, the phage titer was

calculated. In addition, the phage concentration was determined with the NanoDrop 1000 spectrophotometer (NanoDrop Technologies, Rockland, USA) at 280 nm, where an absorbance of 1 was assumed to display a concentration of $2.3*10^{12}$ cfu/ml (Zhang and Dimitrov, 2009).

For panning, a 96-well NUNC MaxiSorb plate (NUNC™, Langenselbold, Germany) was coated with 10 µg/ml BCV-V270 (field isolate, Munich, Germany) for 4 h at 37°C. The plate was washed three times with PBST (8 g NaCl, 0.2 g KCl, 0.12 g KH_2PO_4, 0.91 g Na_2HPO_4, 1 ml Tween-20, ad 1 l sterile water; all from Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and blocked with 2% skimmed milk powder (SMP) (Carl Roth GmbH & Co.KG, Karlsruhe, Germany) containing 10% FCS in PBS, for 2 hours at 37°C. The plate was washed three times and phage suspensions were added with a titer, representing 1000-fold the library diversity, and incubated for 2 hours at room temperature. Added phage suspensions had been preincubated for 1 hour in block solution (2% SMP, 10% FCS in PBS). The plate was washed ten times with PBST and bound phages were eluted with 10 µg/ml Trypsin (Carl Roth GmbH & Co.KG, Karlsruhe, Germany). TG1 and HB2151 cells, grown to log phase (OD 0.4-0.6) in 2TYG, were co-incubated with eluted phages for 30 min at 37°C (stationary), followed by 30 min at 37°C with 250 rpm. Infected bacteria were plated on 2xTYGA agar and grown at 30°C overnight.

To determine the titer of eluted phages, a dilution series was performed in TG1 cells. Following, overnight incubation, TG1 cells were scraped with 2xTYGA containing 15% glycerol. A 100 µl aliquot was used in the next panning round and the rest was stored at -80°C. Two additional panning rounds were performed with slight modifications. Initially only 100 ml bacterial culture was used and only one precipitation step was performed. The panning conditions were made more stringent with each panning round. The antigen concentration was reduced to 5 µg/ml BCV-V270 in the second round and 2.5 µg/ml in the third round. Additionally, the incubation time was reduced to one hour and washing steps were increased to 20 washing steps in the second round and 40 washing steps in the third round. The HB2151 cells were used to produce soluble scFvs for further tests in ELISA. Individual clones were picked and grown overnight at 37°C with 250 rpm in 2xTYGA medium. A 20 µl-aliquot of this overnight culture was added to fresh 180 µl 2xTYGA medium and grown at 37°C with 250 rpm to reach log phase (OD 0.4-0.6). The bacteria were pelleted at 1,800 x g for 15 min at 4°C, resuspended in 200 µl 2TYA containing 1 mM IPTG (Carl Roth GmbH & Co.KG, Karlsruhe, Germany) and incubated for 16-20 h at 30°C with 250 rpm. Cultures were centrifuged at 1,800 x g for 15 min at 4°C and the supernatants were tested in the ELISA. Therefore, a 96-well NUNC MaxiSorb plate (NUNC™, Langenselbold, Germany) was coated with 5 µg/ml BCV-V270, washed three times with PBST and blocked for 2 hours at 37°C with 2% SMP (Carl Roth GmbH & Co.KG, Karlsruhe,

Germany) containing 10% FCS in PBS. Wells were emptied and the culture supernatants of induced HB2151 clones were added. The incubation lasted for 2 hours at room temperature. After five washes with PBST, the detection antibody (polyclonal goat E-tag (HRP); abcam ®, Cambridge, UK) was added at 1: 2,000 dilution in block solution (2% SMP, 10% FCS in PBS), and incubated for 1.5 hours at room temperature. The plate was washed ten times with PBST, and TMB substrate (IDEXX GmbH, Ludwigsburg, Germany) was applied for 20 min at room temperature in the dark. The color reaction was stopped with HCl (IDEXX GmbH, Ludwigsburg, Germany) and the optical density was measured with the Sunrise microplate reader (Tecan, Maennedorf, Switzerland) at 450 nm. Alternatively, TG1 clones were tested to contain a specific anti-BCV-V270 scFv using the same procedure with some modifications. Individual TG1 clones were infected with M13KO7 phages, as described above. The supernatant of an overnight culture was used in the ELISA as described for the HB2151 cells, except that the detection antibody was HRP/Anti-M13 Monoclonal Conjugate (GE Healthcare UK Ltd, Buckinghamshire, UK). Clones displaying sufficient binding activity were sequenced. The protein structure of the respective anti-BCV-V270 scFv was analyzed and a 3D model was calculated based on protein homology using the Phyre^2 server (Kelley et al., 2009) and VMD 1.9.1 software (Humphrey et al., 1996).

Purification of scFvs

Soluble scFvs, produced in HB2151 cells, were purified by affinity chromatography using an ÄKTA start chromatography system (GE Healthcare UK Ltd, Buckinghamshire, UK). The scFvs were passed through an anti-E-Tag HiTrap column (GE Healthcare UK Ltd, Buckinghamshire, UK) with a flow rate of 1 ml/min. Elution was carried out with 0.1 M glycine (pH 3; Carl Roth GmbH & Co.KG, Karlsruhe, Germany). The flow-through was neutralized immediately using 1 M TRIS, 0.13 M NaN_3 (both from Carl Roth GmbH & Co.KG, Karlsruhe, Germany).

Western blotting

The scFv-SF-3E4 was loaded into a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel. Electrophoresis was carried out in a Bio-Rad Mini Trans Blot Cell electrophoresis system (Bio-Rad Laboratories GmbH, Munich, Germany) for 65 min at 150 V and 400 mA using a Bio-Rad Power Pac Basic (Bio-Rad Laboratories GmbH, Munich, Germany). Subsequently, separated proteins were transferred to a PROTRAN BA83 nitrocellulose membrane (GE Healthcare, Chalfont St. Giles, UK) by western blotting carried out in the same system as the electrophoresis

for 75 min at 150 V and 400 mA in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (w/v); all from Carl Roth GmbH&Co.KG, Karlsruhe, Germany). The membrane was blocked for 2 hours at room temperature with 5% SMP containing 10% FCS in PBST (all from Carl Roth GmbH&Co.KG, Karlsruhe, Germany). Afterwards, the membrane was washed twice with PBST and once with PBS. A polyclonal rabbit anti-E-Tag antibody (Novus Biologicals, Littleton, USA) was added in a 1:500 dilution and incubated at 4°C overnight. Next the membrane was washed three times with PBST and three times with PBS. Incubation with an HRP-conjugated monoclonal goat anti-rabbit antibody (Sigma-Aldrich Chemie GmbH, Munich, Germany) in a 1:500 dilution lasted for 2 hours at room temperature. After two washes with PBST and two washes with PBS, Bio-Rad HRP-conjugate substrate (Bio-Rad Laboratories GmbH, Munich, Germany) was added. The color reaction was stopped with distilled water.
In order to identify the scFv-SF-3E4 binding site, western blotting was conducted using 10 µg BCV-V270. The procedure was the same as described above. The scFv-SF-3E4 was added to the membrane at a concentration of 78 ng/ml. As controls, an anti-BCV-V270 guinea pig immune serum (1:500) and the anti-BCV-V270 mAb 1/1 (250µg/ml) were used. The membranes were incubated at 4°C overnight. Then, the membranes were washed three times with PBST and three times with PBS. Incubation with the respective conjugate lasted for one hour at room temperature (scFv-SF-3E4: HRP conjugated polyclonal goat anti-E-tag antibody (1:250) (abcam ®, Cambridge, UK); guinea pig anti-BCV-V270 immune serum: HRP conjugated polyclonal goat anti-guinea pig IgG (1:500) (Sigma-Aldrich Chemie GmbH, Munich, Germany); anti-BCV-V270 mAb 1/1: HRP conjugated polyclonal goat anti-mouse IgG (1:500) (Sigma-Aldrich Chemie GmbH, Munich, Germany)). After two washes with PBST, and two washes with PBS, Bio-Rad HRP conjugate substrate (Bio-Rad Laboratories GmbH, Munich, Germany) was added. The color reaction was stopped with distilled water.

Indirect and competition ELISAs

The ELISA procedure was the same as described for the phage display, despite using different virus concentrations for coating, and the detection antibody was adapted to the respective sample. All assays were run in triplicate.

Testing serum samples against 5 µg/ml BCV-V270 in triplicates proved the vaccination response. An HRP conjugated sheep anti-bovine IgG antibody (KOMA Biotec Inc., Seoul, Korea) was used for detection of serum IgG bound to the BCV test antigen. OD values were corrected for ELISA background noise and the cut-off was set at 0.07.

To evaluate the scFv-SF-3E4s specificity, the antibody was tested against different *E. coli* strains (TG1, HB2151; 10^6 cells/ml), against vaccinia virus strain Elstree (2 µg/ml) and against Bovine Serum Albumin (5 µg/ml; Carl Roth GmbH&Co.KG, Karlsruhe, Germany). Cross reactivity was tested against 5 µg/ml BCV-L9 and 5 µg/ml HCoV OC43. The scFv-SF-3E4 antibody was detected using an HRP conjugated polyclonal goat anti-E-tag antibody (abcam ®, Cambridge, UK).

In a competition, ELISA the anti-BCV-V270 mAb 1/1 and the scFv-SF-3E4, were evaluated for binding to the same virus epitope. Therefore, one of the antibodies was titrated on the ELISA plate coated with 1 µg/ml BCV-V270 and incubated for one hour at 37°C. The liquid was removed and the other antibody was added in a concentration, found in previous assays to give a sufficient signal with low background. The incubation lasted for one hour at 37°C. The assay proceeded as described above. The detection antibody was chosen according to the second antibody (scFv-SF-3E4: HRP conjugated polyclonal goat anti-E-tag antibody (abcam ®, Cambridge, UK); anti-BCV-V270 mAb 1/1: HRP conjugated goat anti-mouse IgG (Sigma-Aldrich Chemie GmbH, Munich, Germany). The test was repeated with the first and the second antibody being switched. As a control, each antibody was tested on virus alone, and both were titrated in a two-fold serial dilution and incubated in sextuplicate in the second antibody concentration.

Affinity measurement

Both the scFv-SF-3E4 and the mAB 1/1 were titrated on an ELISA plate coated with 5µg/ml BCV-V270. Similarly, the affinities of the scFv-SF-3E4 towards BCV-L9 and OC43 were determined. The affinity was calculated based on the Michealis-Menten and the Lineweaver-Burk equation using GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla California USA).

Neutralization assay

The purified scFv-SF-3E4 was titrated in triplicate in a two-fold serial dilution on a 96-well tissue culture plate (NUNC™; Thermo Fisher Scientific Inc., Waltham, USA), and 10^2 $TCID_{50}$ of BCV-V270 were added to each well. Antibody and virus were incubated for one hour at 37°C before 100 µl of VERO cells were added. The inhibition of the CPE was finally read out seven days post infection. As controls, an anti-BCV-V270 guinea pig immune serum, an anti-BCV-V270 mAb 1/1, a virus control on cells alone and a cell control without virus, were used.

RESULTS

Animal immunization

A Holstein Friesian bull was immunized with three Scourguard3 injections. The immune response was evaluated by indirect ELISA on each vaccination time point, as well as two and three weeks after the third vaccination (Fig. 1). A small amount of anti-BCV-V270 IgG was detected even before the first immunization (titer: 1:2,400). A seroconversion was apparent already 14 days after the first immunization. Titers increased after each vaccination with the highest titer three weeks after the third vaccination (titer: 1:18,900).

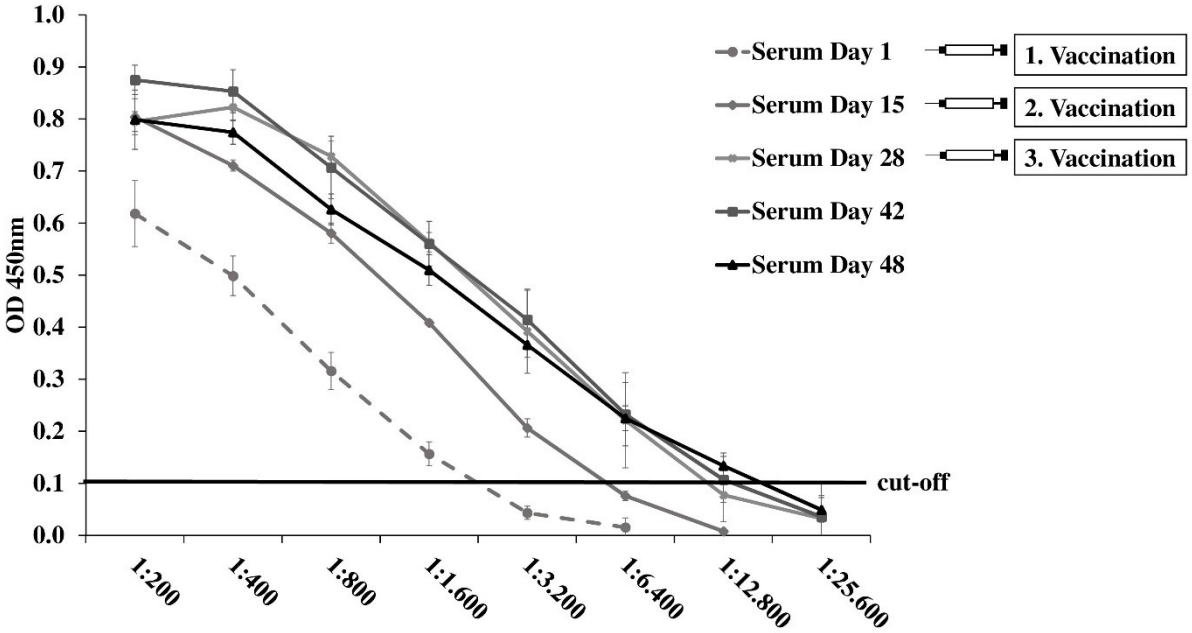

Fig. 1. Serum titer of anti-BCV-V270 IgG in the Holstein Frisian bull before and after vaccination with Scourguard3. The ELISA plate was coated with 1 µg/ml BCV-V270. Sera were diluted 1:100 and titrated in a two-fold dilution series (triplicates). The cut-off (horizontal black line) was set at an OD 450nm of 0.1.

Primer design

For the construction of the bovine antibody phage display library, a detailed analysis of the bovine immunoglobulin genetics was conducted. The analysis included current literature and database entries (Chen et al., 2008; Ekman et al., 2009; Koti et al., 2010; Pasman et al., 2010), data of our own group (Diesterbeck et al., 2012; Stein et al., 2012; Walther et al., 2013; Walther et al., 2016) and recent experimental research results. The primer set design aimed to cover the whole bovine immunoglobulin spectrum known so far. In order to reduce the total number of primers, wobble bases were used to create primers covering several genes simultaneously. All primers used were summarized in Table 1 and supplemental Table 1a. Primers for the lambda light chain leader sequences (LL_λ) were designed based on published sequences first (Pasman et al., 2010). Designed primers were proven in a BLAST analysis and additional primers were constructed covering the genes not yet covered. In total, nine different LL_λ primers were designed (BoLLFOR1-9). The U32263 sequence was used as a query in the BLAST analysis of the lambda light chain variable genes (VL_λ). Thirty-six different sequences were detected. They were distributed over chromosome 17, 19 and on a still unplaced contig (NW_003100754.1). Ten primers were created to amplify these genes (BoVL_linker_FOR1-10). Four different lambda light chain joining genes (JL_λ) were described in the literature available (Chen et al., 2008). A BLAST search with one of these published sequences provided confirmation. Hence, four JL_λ primers were constructed (BoJLBACK1-4). For both VL_λ and JL_λ primers, special attention was given to the binding site in order to amplify the whole variable domain. As for the joining segment, a BLAST analysis of the constant genes (CL_λ) (query sequence: HQ456943.1) confirmed the published number of four genes (Chen et al., 2008; Diesterbeck et al., 2012). Primers were set in a highly conserved region so that only one CL_λ primer (BoCLBACK) had to be designed. The bIgK_Leader primer (Stein et al., 2012) served as a query in the BLAST analysis of the κ light chain leader genes (LL_κ). Finally, three different primers were designed (BoLKFOR1-3). As no published sequences existed for the bovine kappa variable region (VL_κ), a human IGKV sequence (AJ512647.1) was used as a query sequence. BLAST hits were checked for plausibility with data for the bovine κ light chain locus organization (Ekman et al., 2009), leading to the generation of 11 VL_κ primers (BoVK_linker_FOR1-11). Once again, wobble bases were used to reduce the total number of primers. The human JK1 sequence (McBride et al., 1982) was used as a query sequence for bovine kappa joining (JL_κ) sequences. Obtained BLAST hits were also checked using published data (Ekman et al., 2009), as for the variable region. The number of three different κ joining genes was confirmed, resulting in an equal number of constructed primers (BoJKBACK1-3).

Due to only one κ constant gene being published (Ekman et al., 2009; Stein et al., 2012) and confirmed by our own BLAST analysis, one primer (BoCKBACK) was designed for the κ constant region (CL_κ). Primers for the VH gene amplification were based on the current description of the corresponding gene locus (Walther et al., 2013). In addition to the BoLHBACK primer used in this publication, three more primers (BoLHFOR1-3) for the leader segment were designed to cover even the pseudogenes. Five primers (BoVHFOR1-5) were synthesized to amplify all 36 VH genes. Since all six bovine joining gene sequences for the heavy chain (JH) differ notably, each gene was considered by its own primer (BoJH_linker_BACK1-6). The BoIgG1-3CH1_FOR primer was used to amplify the first constant region of IgG1-3 (Walther et al., 2013). VL_λ, VL_κ and JH primers were designed to contain an overlapping nucleotide sequence encoding a 15 amino acid glycine-serine $(G_4S)_3$ peptide linker in the scFv molecule. JL_λ-, JL_κ- and VH primers, used to amplify the scFv sequence, were extended for restriction enzyme cleaving sites for *Sfi*I and *Not*I.

Table 1 Primers used for the construction of the bovine scFv library.

Primer Name	Sequence (5'-3')
PCR 1 - heavy chain forward primers	
BoLHBACK	ACCCACTGTGGACCCTCCTC
BoLHFOR2	TGGGGTYYTCTCCTCTGCCTGG
BoLHFOR3	TGCAGTCTTCTCCTCTGCCTKG
BoLHFOR4	GTCTGGTCCTTCCCCATCCTGC
PCR 1 - heavy chain reverse primer	
BoIgG1-3Ch1_forb	GGCACCCGAGTTCCAGGTCA
PCR 2 - heavy chain forward primers	
BoVHFOR1	CAGGTGMAGSTGCRGGAGTCG
BoVHFOR2	AAGGTGCAGCTGCRGGAGTSG
BoVHFOR3	CAGATGMRCTGCWGCAGTCG
BoVHFOR4	CAGGTGCTGGGGCGGGAGTCR
BoVHFOR5	CMGRTGCAGCTGCAGGARTCA
PCR 2 - heavy chain reverse primers	
BoJH_linker_BACK1	*AGAACCACCTCCGCCTGAACCGCCTCCACC*CAAGGACACGGTG ACCGGGGTGC
BoJH_linker_BACK2	*AGAACCACCTCCGCCTGAACCGCCTCCACC*TGAGGAGACGGTG ACCYYGAKCC
BoJH_linker_BACK3	*AGAACCACCTCCGCCTGAACCGCCTCCACC*TGAGGAGACGGTG CCCAGGGCAG
BoJH_linker_BACK4	*AGAACCACCTCCGCCTGAACCGCCTCCACC*TGAGGAGATGGAG AYSGGGGYGC

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Table 1 continued from previous page

BoJH_linker_BACK5	*AGAACCACCTCCGCCTGAACCGCCTCCACC*TGAGCAGACAGTG CCCAGGCTTC
BoJH_linker_BACK6	*AGAACCACCTCCGCCTGAACCGCCTCCACC*TGAGGAGACGGTG TTTTGGATTC

PCR 1 - lambda light chain forward primers

BoLLFOR1	AR GGC WCA GGT GAG GCC TCC
BoLLFOR2	TC TWC ACA GGT GAC TCG ATC
BoLLFOR3	TC TGC ACA GGT GAC TCG ATS
BoLLFOR4	TC TGC RCA GGT GCG GCC CCC
BoLLFOR5	CCTCACTTACGGCTCAGGTCA
BoLLFOR6	YCTCKCTCACTGCWYAGGTAG
BoLLFOR7	CCTYGCCCACYSCACAGGTCA
BoLLFOR8	CCTCTCTCWCTRCWCAGGTAG
BoLLFOR9	GGTCGCTCTSTGCACAGRTGA

PCR 1 - lambda light chain reverse primer

BoCLBACK	AC GGT CAC GCT ACC CGG GT

PCR 2 - lambda light chain forward primers

BoVL_linker_FOR1	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGGCTGT GCTGACYCAGCCR
BoVL_linker_FOR2	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGGCTRT GCTGACTCAGCYG
BoVL_linker_FOR3	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGGMTG TGCTGACTCAGCMG
BoVL_linker_FOR4	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGTCTRG CCTGACTCAGCCT

Continued on the next page

Table 1 continued from previous page

BoVL_linker_FOR5	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGGCTGGGCTAACTCAGCCG
BoVL_linker_FOR6	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*TCCTATGAACTGACMCAGYYGA
BoVL_linker_FOR7	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGGTGTTGTGGGCTGCATCC
BoVL_linker_FOR8	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*TCTTCTCAGCTGACTCAGCCG
BoVL_linker_FOR9	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGCSTGTGCTGACTCAGYCRG
BoVL_linker_FOR10	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*CAGACTGTGGTCCAGGAGCCAG

PCR 2 - lambda light chain reverse primers

BoJLBACK1	CAGGACGGTCAGTGTGGTCCCGCT
BoJLBACK2	CAGGACGGTCACTCTGGTCCCGCC
BoJLBACK3	CAGGACGGTGACCCAGGTCCCGCC
BoJLBACK4	CAGGACAGTCAGCCTGGTCCTGCC

PCR 1 - kappa light chain forward primers

BoLKFOR1	GRGYTCCTCCTGCTCTGGGT
BoLKFOR2	TTCCTCCTGTTACTGTGGTT
BoLKFOR3	RGTCTCCTGCTSCTCTGGCT

PCR 1 - kappa light chain reverse primer

BoCKBACK	TGGTTTGAAGAGAAGACGGATG

PCR 2 - kappa light chain forward primers

BoVK_linker_FOR1	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GATGTWGTGCTGACCCAGACTCC

Continued on the next page

Table 1 continued from previous page

BoVK_linker_FOR2	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GACATTGTGCTGACCCAGACTCC
BoVK_linker_FOR3	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GACATCCAGGTRACCCAGTCTCC
BoVK_linker_FOR4	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GACCTCCAGATGAYYCAGTCTCY
BoVK_linker_FOR5	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GACCTCCAGATGACCCAGTCTCC
BoVK_linker_FOR6	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GACCTCCAGATGATTCAGTCTCC
BoVK_linker_FOR7	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GCTATTGTTCTGACCCAGACTCC
BoVK_linker_FOR8	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GGCATCCAGATGACTCAGTCTCC
BoVK_linker_FOR9	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GCTAYWAKGCAGACCCAGACTCT
BoVK_linker_FOR10	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GCTATTATGCAGAACCAACTATA
BoVK_linker_FOR11	*GTTCAGGCGGAGGTGGTTCTGGCGGTGGCGGATCG*GAAATTATGTTAACGCAGTCTCC

PCR 2 - kappa light chain reverse primers

BoJKBACK1	ATTGATTTCCACCTTGGTCCCG
BoJKBACK2	TTTGATCTCTACCTTGGTTCCT
BoJKBACK3	TTTGACTTCCAGCTTGGTTCCT

Amplification of the recombined product (VH + Vλ) - forward primers

BoVH_SfiI_FOR1	GTCCTCGCAACTGCG**GCCCAGCCGGCC**CAGGTGMAGSTGCRGGAGTCG

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Table 1 continued from previous page

BoVH_SfiI_FOR2	GTCCTCGCAACTGCG**GCCCAGCCGGCC**AAGGTGCAGCTGCRGGAGTSG
BoVH_SfiI_FOR3	GTCCTCGCAACTGCG**GCCCAGCCGGCC**CAGATGMRCTGCWGCAGTCG
BoVH_SfiI_FOR4	GTCCTCGCAACTGCG**GCCCAGCCGGCC**CAGGTGCTGGGGCGGGAGTCR
BoVH_SfiI_FOR5	GTCCTCGCAACTGCG**GCCCAGCCGGCC**CCMGRTGCAGCTGCAGGARTCA

Amplification of the recombined product (VH + Vλ) - reverse primers

BoJL_NotI_BACK1	GAGTCATTCTCGACTT**GCGGCCGC**CAGGACGGTCAGTGTGGTCCCGCT
BoJL_NotI_BACK2	GAGTCATTCTCGACTT**GCGGCCGC**CAGGACGGTCACTCTGGTCCCGCC
BoJL_NotI_BACK3	GAGTCATTCTCGACTT**GCGGCCGC**CAGGACGGTCACTCTGGTCCCGCC
BoJL_NotI_BACK4	GAGTCATTCTCGACTT**GCGGCCGC**CAGGACAGTCAGCCTGGTCCTGCC

Amplification of the recombined product (VH + Vκ) - reverse primers

BoJK_NotI_BACK1	GAGTCATTCTCGACTT**GCGGCCGC**ATTGATTTCCACCTTGGTCCCG
BoJK_NotI_BACK2	GAGTCATTCTCGACTT**GCGGCCGC**TTTGATCTCTACCTTGGTTCCT
BoJK_NotI_BACK3	GAGTCATTCTCGACTT**GCGGCCGC**TTTGACTTCCAGCTTGGTTCCT

Amplification of GAPDH

GAPDH_for	TGGTCACCAGGGCTGCT
GAPDH_rev	GGAGGGGCCATCCACAGTCT

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Table 1 continued from previous page

Colony PCR	
R1	CCATGATTACGCCAAGCTTTGGAGCC
R2	CGATCTAAAGTTTTGTCGTCTTTCCA

Underlined letters show wobble bases. Wobbles are defined as: K – G/T, M – A/C, R – A/G, S – G/C, W – A/T, Y – C/T. The *italic* letters indicate the nucleotide sequence for the peptide linker. The **bold** nucleotides represent the restriction enzyme recognition sites for *Sfi*I and *Not*I

Amplification of VH and VL chains genes from cDNA prepared from PBMCs

In the first PCR (PCR 1) of the designed nested PCR, all lambda light chain primer combinations (n = 9) – except the primer pair BoLLFOR2 and BoCLBACK – resulted in products detectable in gel electrophoresis. Primer combinations differed in intensity and number of bands. A PCR product of 578 bp was proven by sequencing to represent functional antibody sequences. PCR products of other sizes displayed antibody sequences containing no ORF or truncated ORFs. In the PCR 1 for the kappa light chain, only the BoLKFORk2 and BovCKBACK primer pair gave no result, when a total number of three different combinations were tested. As for the lambda light chain, band intensity in agarose gels differed notably. Products with functional antibody sequences were about 414 bp in size. PCR 1 for the heavy chain revealed at least three products ranging between 600 and 800 bp in size with all possible primer combinations tested (n = 4). Size distribution was confirmed in sequence analysis and was attributed to length differences in the CDR3 regions. Afterwards, primers were pooled including all designed primers. The PCRs with the pooled primer sets for all three chains yielded products of the expected size, as evaluated in the individual PCRs (Fig. 2). Optimal annealing temperature and DMSO concentration were defined as 65°C and 2.5%. Concerning PCR 2, 36 of all possible 40 lambda light chain primers combinations yielded reasonable PCR products. Sizes ranged from 200 to 800 bp. Sequence analysis revealed functional antibody sequences in products with 329 bp. Depending on size, some PCR products displayed sequences without a functional ORF or unrelated sequences possibly from miss-priming. For the kappa light chain, all possible primer combinations (n = 33) resulted in PCR products. Size distribution in agarose gel electrophoresis was similar as with the lambda light chain primer sets. Five of the 33 possible primer combinations yielded functional antibody sequences of 337 bp. The PCR product size distribution in PCR 1 for the heavy chain was re-discovered in PCR 2 for all possible primer combinations (n = 30) tested. Some double bands appeared at 300-500 bp, due to the size variances in bovine CDR3 regions as proven by sequencing. As with PCR 1, PCR 2 was repeated for all three chains with pooled primer sets (Fig. 3). Amplification failures, because of primer inhibition, were not detected. An annealing temperature of 67°C and 2.5% DMSO were set as optimal conditions for PCR 2.

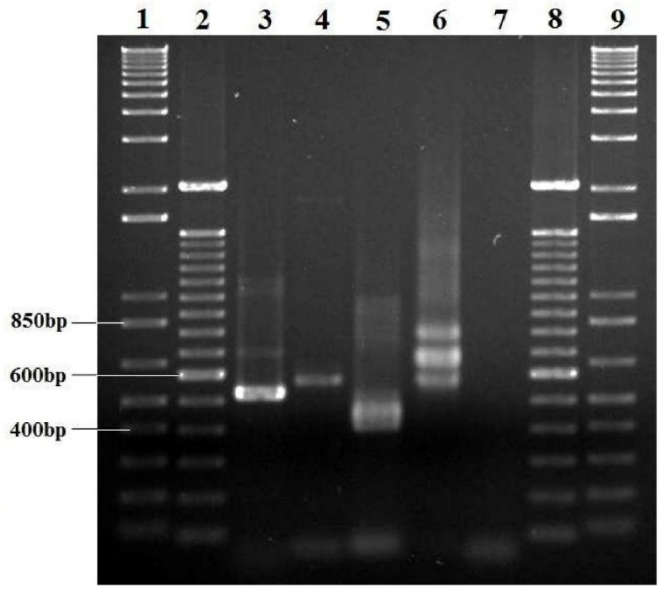

Fig. 2. Amplifications products from bovine cDNA by PCR 1 using pooled primer sets.
Lanes 1 and 9: 1 kb ladder. Lanes 2 and 8: 100 bp ladder. Lane 3: bovine GAPDH (positive control). Lane 4: lambda light chain. Lane 5: kappa light chain. Lane 6: heavy chain. Lane 7: no template negative control (H_20).

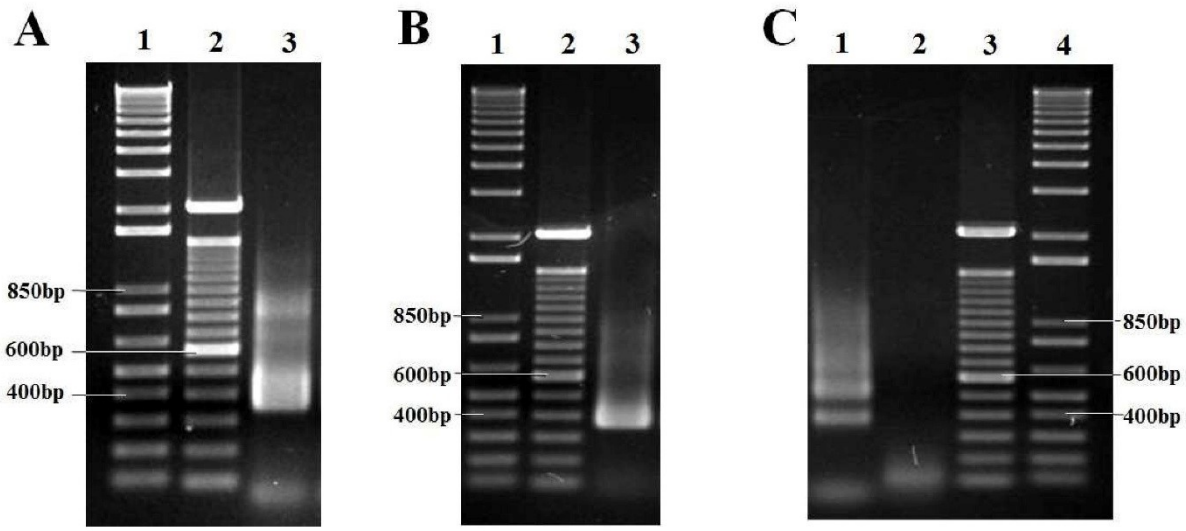

Fig. 3. Amplifications products from bovine cDNA by PCR 2 for lambda light chain (A), kappa light chain (B) and heavy chain genes (C) using pooled primer sets. (A): Lane 1: 1 kb ladder. Lane 2: 100 bp ladder. Lane 3: lambda light chain. (B): Lane 1: 1 kb ladder. Lane 2: 100 bp ladder. Lane 3: kappa light chain. (C): Lane 1: heavy chain. Lane 2: no template negative control. Lane 3: 100 bp ladder. Lane 4: 1 kb ladder.

Generation of scFv sequences

For the generation of scFv encoding DNA sequences, the PCR 2 products encoding heavy chain variable domains (VH) were either recombined to lambda light chain variable domain products (VL_λ) or to kappa light chain variable domain products (VL_κ). Analysis of the recombination in a 1% agarose gel revealed a weak band at 800 bp and a more distinct band at 400 bp (Fig. 4). Sequence analysis confirmed the 800 bp band to represent complete functional scFv sequences. This meant that genes for both chains (VH, VL), recombined to a single ORF, contained typical bovine antibody sequence features and conserved nucleotides positions as published recently (Lefranc et al., 2003; Walther et al., 2013). The recombination product amplification – with primers containing the restriction enzyme cleavage sites for *Sfi*I and *Not*I – resulted in a distinct band at 800 bp (Fig. 5). During the amplification of the VH-VL_λ recombination product, an additional band appeared at 600 bp, which was found to be a product of miss-priming. Repeated sequencing of random 800 bp products showed functional scFvs with the restriction enzyme cleavage sites being introduced correctly (data not shown).

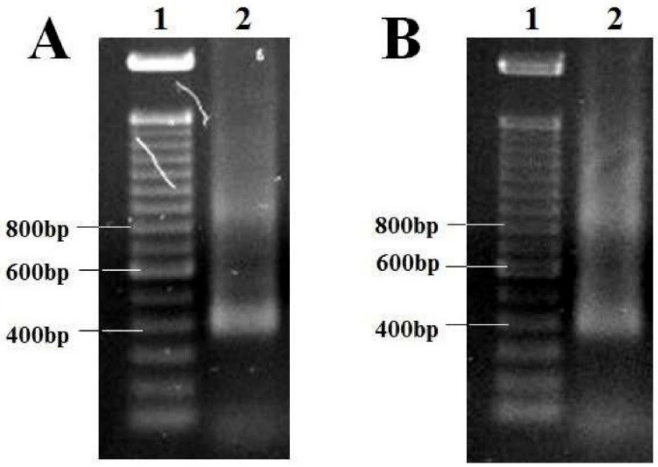

Fig. 4. Recombination of VH and VL_κ (A) as well as VH and VL_λ (B) encoding DNA sequences (recombination control by agarose gel electrophoresis). (A) Lane 1: 100 bp ladder. Lane 2: VH-VL_κ recombination product. (B) Lane 1: 100 bp ladder. Lane 2: VH-VL_λ recombination product.

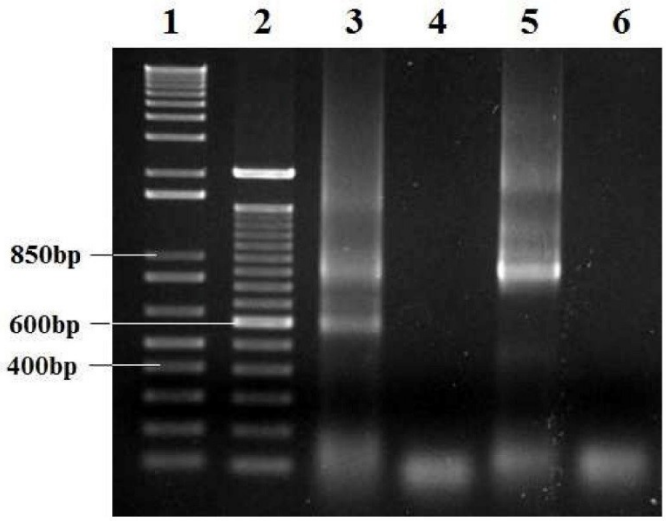

Fig. 5. Control of PCR amplification of recombination products. Lane 1: 1 kb ladder. Lane 2: 100 bp ladder. Lane 3: amplification of the VH-VL$_\lambda$ recombination products. Lane 4: no template control of recombination reaction. Lane 5: amplification of the VH-VL$_\kappa$ recombination products. Lane 6: no template control of amplification reaction.

Construction of the scFv library in pCANTAB5E

Two sublibraries were constructed. The lambda sublibrary contained scFv sequences comprising a VH sequence and a VL$_\lambda$ sequence. Twelve transformations with an average transformation efficiency of $1.3*10^9$ cfu/µg were needed to reach a library diversity of $1.5*10^9$ independent clones. The second (kappa) sublibrary included scFv sequences, where the VH sequence was recombined to a VL$_\kappa$ sequence. To achieve a library diversity equal to that of the lambda sublibrary, six transformations with an average transformation efficiency of $2.6*10^9$ cfu/µg, were performed. Eighteen individual clones of each transformation were proven by colony PCR to contain a full-size scFv sequence (800 bp) (data not shown). Size estimation of colony PCR products in 1% agarose gels proved both sublibraries to contain 98% full-size scFv sequences. Fifty clones from each sublibrary were further evaluated in a fingerprint analysis using *BstN*I. Both libraries showed good diversity as multiple repetitions of restriction patterns were absent (Fig. 6). For phage display and further characterization of the scFv molecules, it was important to confirm functional vector-insert constructs. Functionality was given when the construct contained a complete scFv sequence as insert, a correct E-Tag sequence, and an amber stop codon. The latter two are part of the vector, lying downstream of the insertion site. Therefore, a random clone selection from both sublibraries was sequenced (data not shown).

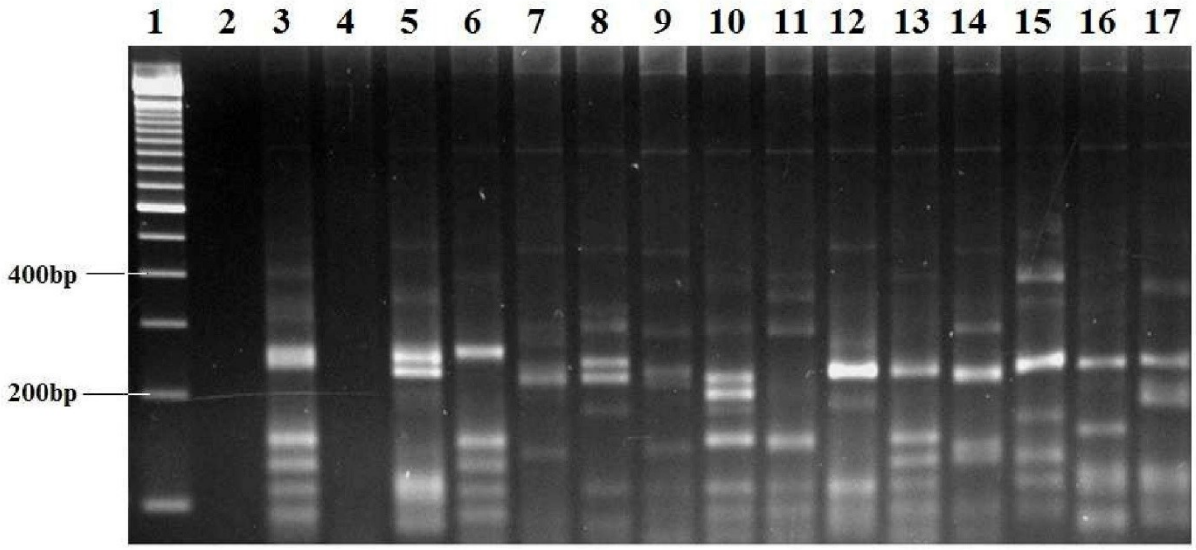

Fig. 6. Fingerprint analysis (*BstN*I) of colony PCR products from 15 bacterial clones of the lambda sublibrary. Lane 1: 100 bp ladder. Lane 2: no template negative control. Lane 3-17: products of the *BstN*I digest of 15 independent clones.

Identification of a BCV-specific scFv by phage display

To control the correct process of the phage display procedure, phage input and output titers were determined in each panning round. An interplay of depletion (output titer) and enrichment (input titer) was expected to ensure correctness (Fig. 7). For the first panning round, the input titer was estimated to be $1.5*10^{11}$ cfu/ml, when calculated with the TG1 dilution series, and $3.4*10^{14}$ cfu/ml when measured with the NanoDrop spectrophotometer. To ensure a phage concentration representing at least 1,000-fold the library diversity (Kotlan et al., 2009), one milliliter of the phage solution was used in the first panning round. This represented 50-fold to 10,000-fold of the library diversity, according to the calculation method used. The output titer was $3.7*10^{3}$ cfu/ml, based on a TG1 dilution series only. In the second panning round, the input phage titer was between $1*10^{11}$ cfu/ml (TG1 dilution series) and $1.9*10^{13}$ cfu/ml (NanoDrop). The concentration of eluted phages was $1.3*10^{2}$ cfu/ml. In the last panning round, a concentration between $3*10^{11}$ cfu/ml (TG1 dilution series) and $2.7*10^{13}$ cfu/ml (NanoDrop) was measured for the input phages. Finally, a total of 26 TG1 clones and seven HB2151 clones were obtained after the last panning round.

The specificity test for BCV-V270 in an ELISA revealed a total of nine well-reacting clones, which remained negative when tested on a non-coated ELISA plate. Sequence analysis indicated all nine clones contained the same scFv sequence. The sequences encoding the VH domain and the VLλ domain of the scFv-SF-3E4 are available from GenBank (submission ID:

1895283, final accession numbers will be added once received). The amino-acid sequence and the 3D prediction of the scFv-SF-3E4 showed a compact molecule (281aa) with the structures crucial for antigen binding (CDR1-3) lying on the surface close to each other (Fig. 8B and C).

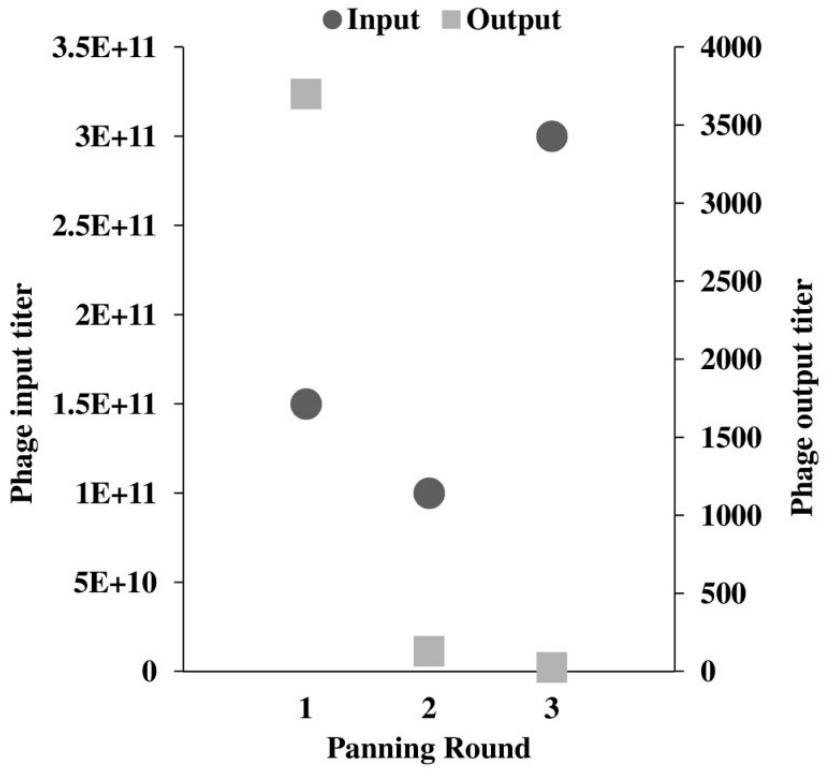

Fig. 7. Phage titers during phage display. Input and output titers were determined in TG1 dilution series. The left vertical axis shows the phage input titers. The right vertical axis shows the phage output titer.

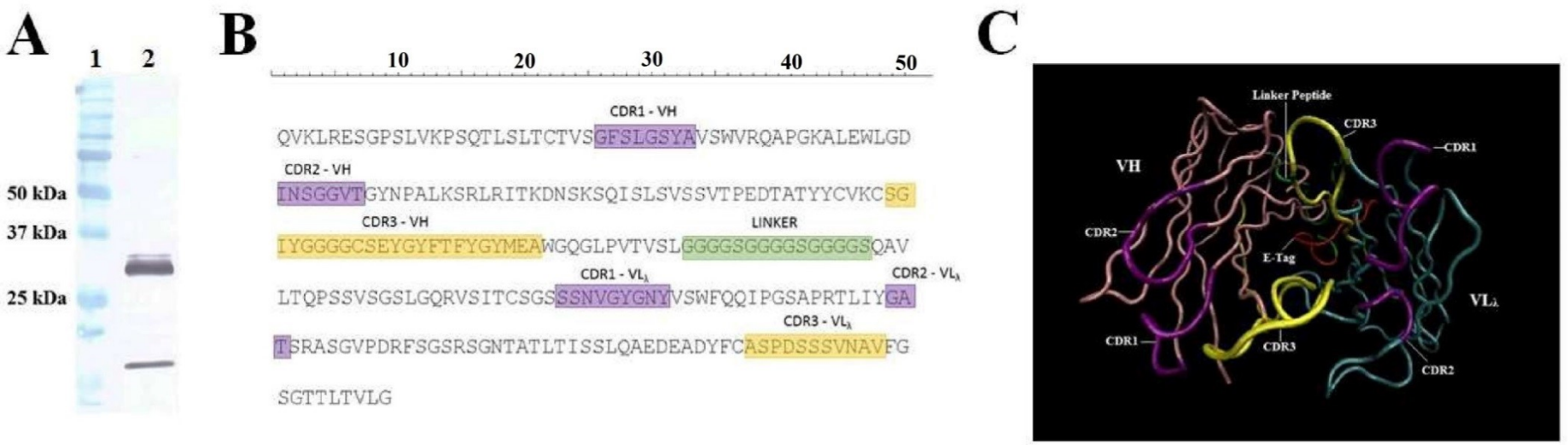

Fig. 8. Anti-BCV-V270 scFv-SF-3E4 molecule. Western blotting (A), amino acid sequence (B) and 3D model (C) of. (A): The purified scFv-SF-3E4 was run in a gel electrophoresis on a 12% SDS gel and was then blotted on a nitrocellulose membrane. Detection was carried out using a polyclonal rabbit anti-E-Tag antibody and an HRP conjugated detection antibody (monoclonal goat anti-rabbit). Lane 1: Precision Plus Protein Standard (Bio-Rad). Lane 2: anti-BCV-V270 scFv-SF-3E4. (B): Colored boxes show the CDR regions of the respective chain (VH, VL_λ) and the $(G_4S)_3$ linker. (C): 3D model, predicted by protein homology modeling (VMD 1.9.1).

Western blotting

When the scFv-SF-3E4 was analyzed in a western blot using an anti-E-Tag antibody for detection, a sharp band at approximately 31 kD was detected, typical for single chain antibody fragments (Fig. 8A). The target protein of the scFv-SF-3E4 was investigated with BCV-V270 under reducing and non-reducing conditions. For comparisons, the murine mAB 1/1 and the guinea pig immune serum were used (Fig. 9). The anti-BCV-V270 guinea pig serum, used as a positive control, revealed the expected multiple band patterns with obvious differences between reducing and non-reducing conditions. The main immunogens were detected with bands at 50 kD and 100 kD. The scFv-SF-3E4 failed to react under reducing conditions, whereas a sharp band at approximately 100 kD was observed under non-reducing conditions. The murine mAb 1/1 showed no reaction in western blotting, when standard concentrations were used (data not shown). Only in very high concentrations, weak bands were observed. A double band at 50 kD and single band at 10 kD were apparent under reducing conditions. The 50 kD band could be also observed under non-reducing conditions together with a band >250
kD (data not shown).

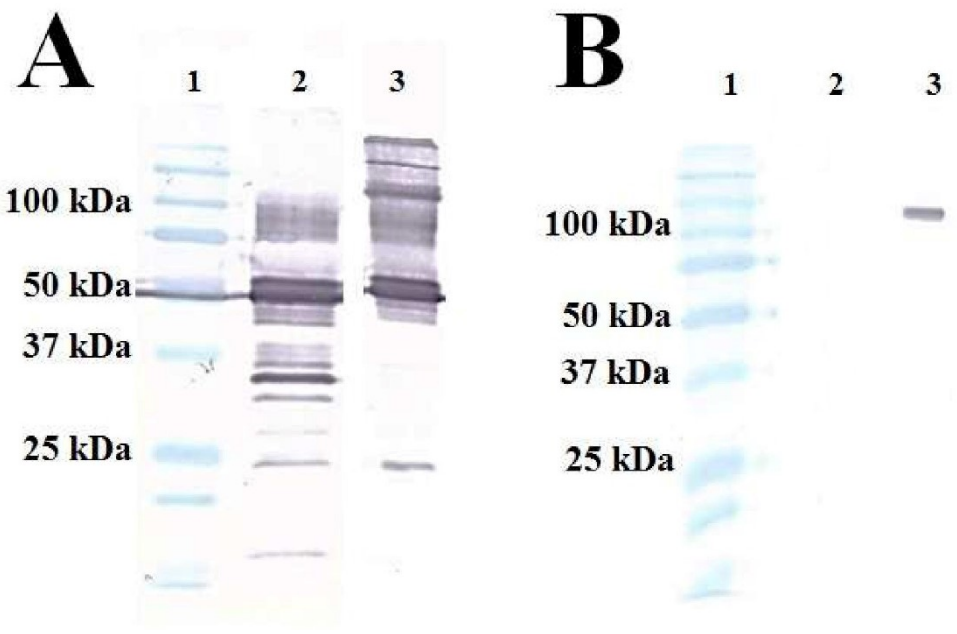

Fig. 9. Western blot of guinea pig immune serum (A) and scFv-SF-3E4 (B) under reducing and non-reducing conditions. 10 µg of BCV-V270 was loaded on a 12% SDS gel and blotted on a nitrocellulose membrane. Antibody and serum were incubated on the membrane overnight before the respective HRP conjugated detection antibodies were added. Lane 1: Precision Plus Protein Standard (Bio-Rad). Lane 2: reducing conditions. Lane 3: non-reducing conditions.

Specificity, affinity and competition study

Specificity of scFv-SF-3E4 was evaluated in an ELISA using different test antigens. As presented in Fig. 10, cross reactivity was evident for both bovine coronaviruses tested (BCV-L9, BCV-V270) as well as for the human coronavirus strain OC43. The scFv-SF-3E4 did not bind to BSA, two different *E. coli* strains (TG1, HB2151) or the Vaccinia virus strain Elstree used as negative controls. The kinetic was calculated by preparing a Michaelis-Menten and Lineweaver-Burk plot for the scFv-SF-3E4 and the murine mAB 1/1 (Fig. 11). For the scFv-SF-3E4, a Michealis-Menten constant (K_m) of 595.2 pM was calculated for the affinity towards BCV-V270. The constants calculated for the other coronaviruses investigated, were K_m = 831.4 pM for the OC43 and K_m = 687.9 pM for the BCV-L9, respectively. For the affinity of the murine mAb 1/1 towards BCV-V270 a K_m of 206.5 pM was calculated. In the competition assay, no specific inhibition was observed between both antibodies. The scFv-SF-3E4 did not inhibit the mAb 1/1. Vice versa the mAb 1/1 inhibited the scFv-SF-3E4 at an average of only 20%, which was far below a specific inhibition of 50%.

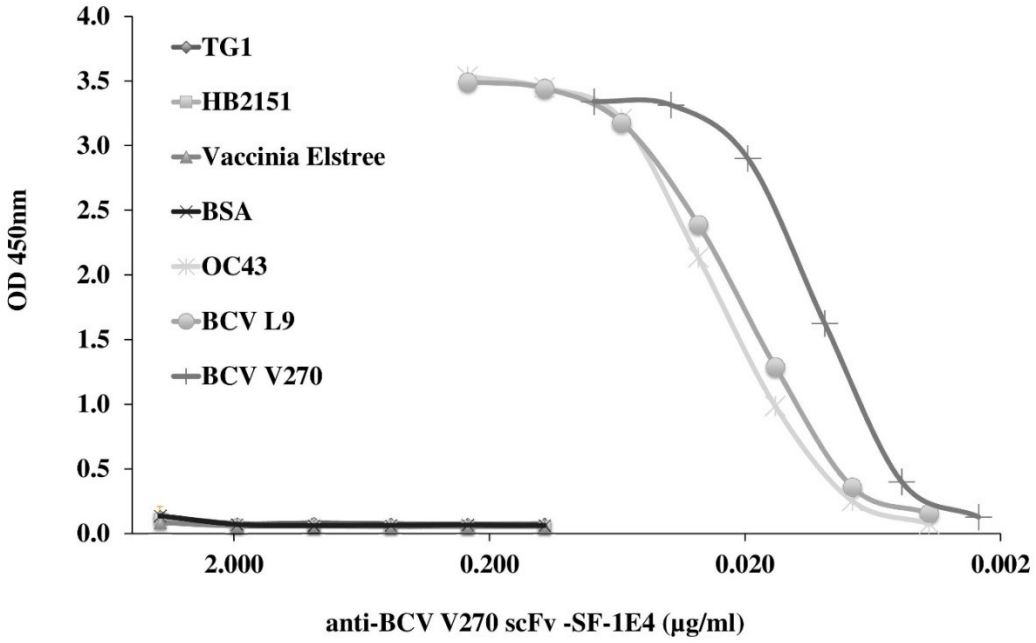

Fig. 10. Specificity and cross reactivity of the scFv-SF-3E4. ELISA plates were coated with different *E.coli* strains (TG1, HB2151; 10^6 cells/ml), Vaccinia Virus strain Elstree (2 µg/ml), BSA (5 µg/ml), BCV-L9 (5 µg/ml), HCoV OC43 (5 µg/ml) and BCV-V270 (5 µg/ml). The scFv-SF-3E4 was titrated in a serial (log2) dilution series and detected using an HRP conjugated polyclonal goat anti-E-tag-antibody.

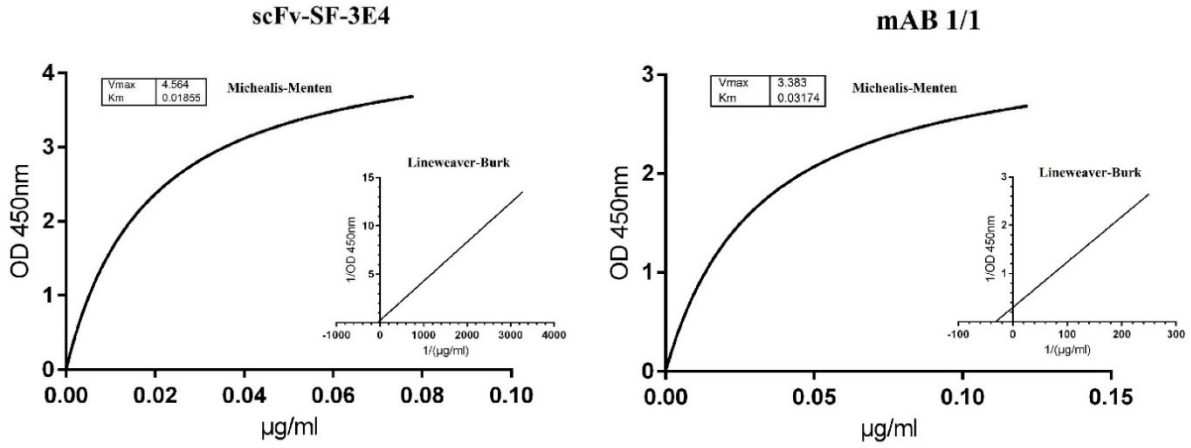

Fig. 11. Binding kinetics of the scFv-SF-3E4 and the mAB 1/1. The kinetics were determined by calculating a Michealis-Menten Plot and Lineweaver-Burk Plot.

Neutralizing activity against BCV-V270

The virus-neutralizing capacity of the scFv-SF- 3E4 was tested in VERO cell cultures infected with 10^2 $TCID_{50}$ of BCV-V270. A distinct CPE with rounded and detached cells was observed for the virus control. The cell layer of the cell control remained confluent, until the end of the assay. Both positive controls – the guinea pig immune serum (1:3200) and the murine mAb 1/1 (15.6 mg/ml) – neutralized the BCV-V270 completely. The scFv-SF-3E4 was also sufficient to neutralize the virus completely until a concentration of ≥ 0.24 µg/ml.

DISCUSSION

Here the design of a complex bovine primer set, suitable for amplifying the comprehensive bovine antibody gene repertoire, was established. It was used to generate a highly diverse bovine IgG scFv phage display library allowing the isolation of a bovine anti-BCV scFv.

For species other than cattle (e.g. dog, sheep, rat, mice), complex primer sets for the immunoglobulin gene repertoire amplification are already available (Charlton et al., 2000; Sepulveda and Shoemaker, 2008; Braganza et al., 2011; Yuan et al., 2015). However, only limited primer sets, comprising only one primer combination for each antibody chain, were published for cattle immunoglobulin gene amplification so far (O'Brien et al., 1999). Meanwhile, a deeper understanding of cattle antibodies exists due to intensive research on bovine immunoglobulin genetics (Chen et al., 2008; Ekman et al., 2009; Pasman et al., 2010; Diesterbeck et al., 2012; Stein et al., 2012; Walther et al., 2013). Therefore, a primer set was designed to cover all bovine genes known so far, coding for the antibody variable domains,

including pseudogenes. The usage of pseudogenes during a gene conversion is a mechanism to diversify the antibody repertoire (Wysocki et al., 1989; Das et al., 2011). It has already been described for other species such as chicken (Reynaud et al., 1987; Thompson et al., 1987). The decision to include pseudogenes was based on investigations, where gene conservation was also suggested for the bovine lambda light chain (Parng et al., 1996). Although this was questioned by other authors, there is still no experimental evidence available disproving this (Lucier et al., 1998).

Our results from BLAST analysis differed from published data concerning the number and location of VL_λ and VL_κ genes (Ekman et al., 2009). It was assumed that these differences may have been generated by using different updated versions of the bovine genome assembly. The evaluation of the individual primer combinations for the VH and VL gene amplification revealed differences in the amount of PCR products. The preferential usage of specific V, D, J and C genes in V(D)J recombination and expression can be a possible explanation (Hosseini et al., 2004; Chen et al., 2008; Pasman et al., 2010; Stein et al., 2012). In the light chain gene amplification, several primer combinations showed multiple bands in agarose gel electrophoresis. Cloning and sequencing of PCR products revealed no functional ORFs in products, differing from the expected size of 329 bp for the lambda light chain and 337 bp for the kappa light chain. Mutations, resulting in non-functional sequences, may arise during somatic hypermutations, which are proven to be one of the main factors to generate antibody diversity in cattle (Kaushik et al., 2002). The second important factor for bovine immunoglobulin diversity is the occurrence of exceptionally long CDR3H regions (Kaushik et al., 2002). These can be categorized into three groups according to the size (Walther et al., 2013). This offers a plausible explanation for bands of multiple sizes observed during the VH gene amplification. The primer set was established to be universal for all cattle breeds. In horse breeds, recent investigations revealed differences in VL_λ gene transcription for at least two breeds (Hara et al., 2012; Walther et al., 2015). Data from other authors indicated the same for cattle (Saini et al., 1997). Therefore, primer combinations, which remain negative in one breed, can result in a positive amplification in another breed due to differences in gene transcription. Hence, it was decided to include all primers in the final primer pools, even those primers which gave no PCR results.

For the scFv construction, a VH sequence was recombined to either a VL_λ or a VL_κ sequence by an overlapping linker sequence. The encoded linker peptide $(G_4S)_3$ is widely used in the construction of scFv molecules (Hudson et al., 1999; Chen et al., 2013). The small and polar amino acids (glycine (G) and serine (S)) ensure flexibility, which is essential for the correct

orientation of VH and VL domains in the scFv molecule (Huston et al., 1988). A length of 15 amino acids guarantees single scFv molecules, whereas linker peptides smaller than 12 amino acids, result in multimer formation (Hudson et al., 1999). The recombination reaction resulted in an 800 bp scFv sequence and some non-recombined VH and VL chains (400 bp), as proven by cloning and sequencing. The following amplification revealed a complete scFv sequence, containing the desired restriction enzyme cleavage sites. To generate the scFv library, pCANTAB5E was chosen as a phagemid. The restriction enzyme cleavage sites in this vector allowed for directed cloning of scFv sequences in the right orientation. An E-tag sequence, following the vector insertion site, simplified thorough purification and detection of the soluble scFv protein, using an anti-E-Tag antibody. The scFv-E-tag sequence was separated from the fd-phage-gene 3 by an amber stop codon. Using suppressor (e.g. TG1) or non-suppressor strains (e.g. HB2151) of *E. coli*, the scFv-E-tag molecule could either be expressed fused or non-fused to the phage's pIII coat protein. As a result, laborious recloning after phage display was unnecessary. The library diversity was estimated by a fingerprint analysis using the restriction enzyme *BstN*I for digestion of the colony PCR products (Sotelo et al., 2012). The estimated library diversity of $3*10^9$ independent clones was considered to be good, when compared to published libraries with diversities ranging from $5*10^8$ (Dooley et al., 2003) to $2.9*10^9$ (Tamura et al., 2008). For the phagemid rescue, the M13KO7 helper phage was used. M13KO7 phages were constructed to preferentially incorporate single-stranded phagemids. After the phagemid intake, a fusion protein of the scFv and the M13 minor coat protein pIII was expressed on the phage's surface. The pIII protein was used for antibody display, as it allowed for the surface expression of large peptides. The expression of the fusion protein was controlled by a *lacZ* promotor and could be easily regulated by the glucose concentration in the growth medium. Without glucose supplementation, packaged phages were secreted in the cell culture medium, ensuring an easy screening procedure towards immobilized antigen (Carmen et al., 2002).

During the panning rounds, a decreasing phage titer after panning and an increasing titer in the following enrichment step was observed, ensuring the method's correctness. Phage input titers were measured using two different methods to guarantee a sufficient phage amount in the respective panning round. Titers measured using a NanoDrop spectrophotometer were $3.4*10^{14}$ cfu/ml (1. round), $1.9*10^{13}$ cfu/ml (2. round) and $2.7*10^{13}$ cfu/ml (3. round). The titers determined by a dilution series in *E. coli* TG1 cells were approximately two decimal powers lower. Several factors can affect an efficient phage infection. The bacterial F' pilus, being essential for the infection, is a fragile structure. Its expression depends on temperature and careful handling of the bacterial culture (Kotlan et al., 2009). The ratio of bacteria to phages is

also critical for the infection. It is assumed that one out of ten phages accomplishes a successful infection (Kasman et al., 2002). Therefore, deficiencies in the infection process were thought to cause the lower titers determined in the TG1 dilution series. The phage output titers decreased from $3.7*10^3$ cfu/ml in the first round to 26 cfu/ml after the last panning round due to increasingly stringent panning conditions in each round. In order to generate a high-affinity bovine scFv, the antigen concentration used for panning and the binding time for phages were reduced, whereas washing steps were increased. All nine clones from the last panning round, which reacted positively in the phage ELISA, carried the same scFv DNA sequence. This is not unusual in immunized libraries and has also been observed by other authors (Ruelker et al., 2012). Immunization leads to an enrichment of antigen-specific antibody sequences in the vaccinated animal. The ELISA, carried out with phages displaying the scFv on the surface, is said to give false positive results, where the scFv-phage-complex binds to the antigen but not to the scFv as an individual molecule (Frenzel et al., 2014). Therefore, the scFv-SF-3E4 was expressed as a soluble protein in *E. coli* HB2151 cells and purified from the bacterial culture supernatants using affinity chromatography. The purified scFv-SF-3E4 was characterized further. A 3D model (Phyre^2 server, VMD 1.9.1 software), predicted by a protein homology analysis of the amino acid sequence, displayed a compact molecule. Structures relevant to antigen binding (CDR1-3) were displayed on the surface, enabling antigen binding according to the lock-and-key model. In the Western blotting analysis, the scFv-SF-3E4 showed a sharp band at approximately 31 kD. This corresponds to sizes reported for other monomeric scFv molecules in literature (Liu et al., 2013; Zuhaida et al., 2013; Wang et al., 2014). Cross reactivity was found for both *Bovine Coronavirus* strains (BCV-L9, BCV-V270) and the HCoV OC43. The HCoV OC43 is the prototype for the former group II human coronaviruses, and is now grouped into the *Betacoronavirus* genus by the International Committee for Taxonomy Viruses (ICTV) (McIntosh et al., 1967; van der Hoek, 2007; Woo et al., 2009). Therefore, cross reactivity appears plausible. BCV-L9 is a cell-culture adapted apathogen variant from BCV strain Mebus (Cyr-Coats et al., 1988). Its ability to bind to BCV-L9 makes the scFv-SF-3E4 a promising candidate for future diagnostic and therapeutic applications, where a broad cross reactivity to all field virus variants is important. No unspecific binding was observed towards *E. coli* (strains TG1, and HB2141), Vaccinia virus strain Elstree or BSA.

The scFv-SF-3E4 neutralized BCV-V270. Neutralization ability was also described for other scFvs derived from immunized libraries (Rasetti-Escargueil et al., 2015). Immunized libraries contain antibody sequences, which already have passed natural affinity maturation, and are

therefore preselected for their high affinity and neutralizing ability (Miller et al., 2008; Nixon et al., 2014).

The calculation of the dissociation constants revealed an approximately three times higher affinity towards BCV-V270 of the murine mAb 1/1 (K_m = 206.5 pM) in comparison to the scFv-SF-3E4 (K_m = 595.2 pM). However, it must be taken into account that only one third of the mAb 1/1 molecule contributes to antigen binding and that the molecule contains two antigen binding sites, whereas the scFv-SF-3E4 only contains a single binding site. When compared to the literature, the affinity of the scFv-SF-3E4 is similar to most recently published scFv molecules (Lai et al., 2016; Lim et al., 2016; Moradi-Kalbolandi et al., 2016).

The interference between the bovine scFv-SF-3E4 and the murine anti-BCV-V270 full-size mAb 1/1 was tested in a competition ELISA. Criteria for a reliable competition were (I) a reciprocal occurrence of competition, (II) the occurrence of competition over a range of several dilutions and (III) a percentage of competition > 50% (Michaud et al., 1993; Crawford et al., 2015). There was no reciprocal competition, as the scFv-SF-3E4 failed to inhibit the binding of the mAb 1/1. The inhibition of the scFv-SF-3E4 by the mAb 1/1, was at 18%, far below the level of significance. A clear concentration dependency was absent and the inhibition remained at the same level over the whole dilution series of the mAb 1/1. The inhibition was considered to be an allosteric inhibition caused by the molecule size of the full-size mAb, rather than a specific inhibition of an epitope. Western blotting with BCV-V270 confirmed this assumption, since a distinct band pattern for both antibodies was observed. By the scFv-SF-3E4 an antigen was detected under non-reducing conditions only. Due to its size of approximately 100 kD, the detected antigen was considered to represent one of the two spike protein subunits (S1, S2) of BCV. The 190 kD spike protein is one of the four surface proteins of BCV and other coronaviruses, which is cleaved into two subunits S1 (gp105) and S2 (gp90) (Abraham et al., 1990; Clark, 1993; Walls et al., 2016). Neutralizing BCV epitopes are mainly located on the S1 subunit and were usually described to be discontinuous epitopes, which lose antigenicity under reducing conditions (Vautherot et al., 1992b; Yoo et al., 2001). As the scFv-SF-3E4 displayed a strong neutralizing ability and did not react in western blotting under reducing conditions (100 kD), the corresponding epitope was assumed to be located within the spike protein subunit S1. Alternatively, the 100 kD band could represent the hemagglutinin/esterase protein (HE) of BCV. Neutralizing epitopes were described for this surface protein, too (Vautherot et al., 1990; Vautherot et al., 1992b). As the HE protein is a dimer of two identical 65 kD monomers, most antibodies binding this protein show a two band pattern (Parker et al., 1990; Nguyen et al., 1998). One band under non-reducing conditions representing the 120 kD dimer and another

band under reducing conditions representing the 65 kD monomers. The scFv-SF-3E4 did not show this pattern in western botting. Therefore, binding to the S1 subunit was assumed to be more likely than binding to the HE protein. The mAb 1/1 remained negative in western blot when used in standard concentrations (data not shown). It was considered, that the main epitope detected by the mAb 1/1 is conformation-dependent and, therefore, did not react in the western blot under denaturating conditions. Only in very high concentration (250 µg/ml) a weak positive reaction could be observed, with bands of 50 kD, 100 kD and >250 kD. Therefore, the staining of these bands was assumed to be unspecific binding and did not represent the functional epitope of the mAB 1/1. This was also obvious against the background that published epitopes on the nucleoprotein (50 kD) and the S2 subunit (100 kD and >250 kD) were described to be non-neutralizing (Deregt et al., 1987; Hussain et al., 1991; Vautherot et al., 1992a). All these hypotheses about the epitopes detected by the scFv-SF-3E4 and the mAb 1/1 must be proven in further investigations using defined recombinant proteins and considering even non-denaturating conditions.

CONCLUSION

A novel large bovine primer set was developed to amplify the whole bovine antibody repertoire known so far. It was used to construct an immunized bovine scFv phage display library showing high diversity ($3*10^9$ independent clones). A high-affinity neutralizing scFv against BCV, was isolated from the library. Based on published research data, this was the first time that a bovine scFv has been successfully isolated from a phage display library. The excellent binding and neutralization capabilities make scFv-SF-3E4 a promising candidate for further therapeutic use.

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Supplemental Table 1a

Primers used for the construction of the bovine scFv library.

Primer Name	Sequence (5'-3')	Ref. Seq.	Binding Site
PCR 1 – heavy chain forward primers			
BoLHBACK	ACCCACTGTGGACCCTCCTC	AC_000164.1 (Bos taurus breed Hereford chromosome 7, Bos_taurus_UMD_3.1, whole genome sequence)	15655287-15655306 15679520-15679539 ***15692083-15692102*** ***15709804-15709823*** 15721891-15721910
		AC_000178.1 *(Bos taurus breed Hereford chromosome 21,* *Bos_taurus_UMD_3.1, whole genome sequence)*	***71507550-71507569*** ***71530064-71530083*** ***6102453-6102472*** ***71596318-71596337***
		NW_003100762.1 *(Bos taurus breed Hereford unplaced,* *Bos_taurus_UMD_3.1, whole genome sequence)*	***1590-1609***
BoLHFOR1	TGGGGTYYTCTCCTCTGCCTGG	AC_000164.1 *(Bos taurus breed Hereford chromosome 7,* *Bos_taurus_UMD_3.1, whole genome sequence)*	15580592-15580613 15677729-15677751

		AC_000178.1 *(Bos taurus breed Hereford chromosome 21, Bos_taurus_UMD_3.1, whole genome sequence)*	6100686-61007071 71563465-71563486 71509614-71509635
BoLHFOR2	TGCAGTCTTCTCCTCTGCCTKG	AC_000164.1 *(Bos taurus breed Hereford chromosome 7, Bos_taurus_UMD_3.1, whole genome sequence)*	15720093-15720095
		AC_000178.1 *(Bos taurus breed Hereford chromosome 21, Bos_taurus_UMD_3.1, whole genome sequence)*	71594533-71594554
BoLHFOR3	GTCTGGTCCTTCCCCATCCTGC	AC_000178.1 *(Bos taurus breed Hereford chromosome 21, Bos_taurus_UMD_3.1, whole genome sequence)*	71511566-71511587
PCR 1 – heavy chain reverse primers			
BoIgG1-3Ch1_forb	GGCACCCGAGTTCCAGGTCA	AC_000177.1 *(Bos taurus breed Hereford chromosome 20, Bos_taurus_UMD_3.1, whole genome sequence)*	***71902586-71902605***
		NW_003099305.1 *(Bos taurus breed Hereford unplaced, Bos_taurus_UMD_3.1, whole genome sequence)*	***1218-1237***

		NW_003100065.1 *(Bos taurus breed Hereford unplaced, Bos_taurus_UMD_3.1, whole genome sequence)*	***4197-4217***
PCR 2 – heavy chain forward primers			
BoVHFOR1	CAGGTGMAGSTGCRGGAGTCG	AC_000178.1 *(Bos taurus breed Hereford chromosome 21, Bos_taurus_UMD_3.1, whole genome sequence)*	6100575-6100554 ***6102337-6102316*** ***71507685-71507706*** 71509746-71509767 ***71530199-71530220*** 71594423-71594402 ***71596202-71596181*** ***15709688-15709667*** 15719983-15719962 15721775-15721775
		NW_003100762.1 *(Bos taurus breed Hereford unplaced, Bos_taurus_UMD_3.1, whole genome sequence)*	***1725-1746***
BoVHFOR2	AAGGTGCAGCTGCRGGAGTSG	AC_000164.1 *(Bos taurus breed Hereford chromosome 7, Bos_taurus_UMD_3.1, whole genome sequence)*	15655422-15655443 ***15691967-15691946***
BoVHFOR3	CAGATGMRCTGCWGCAGTCG	AC_000164.1 *(Bos taurus breed Hereford chromosome 7, Bos_taurus_UMD_3.1, whole genome sequence)*	15578331-15578311 15580721-15580741

		AC_000178.1 *(Bos taurus breed Hereford chromosome 21, Bos_taurus_UMD_3.1, whole genome sequence)*	71563356-71563336
BoVHFOR4	CAGGTGCTGGGGCGGGAGTCR	AC_000178.1 *(Bos taurus breed Hereford chromosome 21, Bos_taurus_UMD_3.1, whole genome sequence)*	71592485-71592464 71511693-71511714
BoVHFOR5	CMGRTGCAGCTGCAGGARTCA	AC_000164.1 *(Bos taurus breed Hereford chromosome 7, Bos_taurus_UMD_3.1, whole genome sequence)*	15677618-15677597 15679440-156794382

PCR 2 – heavy chain reverse primers

BoJH_linker_BACK1	*AGAACCACCTCCGCCTGAACCGCCTCCA CC*CAAGGACACGGTGACCGGGGTGC	AC_000165.1 (Bos taurus breed Hereford chromosome 8, Bos_taurus_UMD_3.1, whole genome sequence)	73095150-73095173
BoJH_linker_BACK2	*AGAACCACCTCCGCCTGAACCGCCTCCA CC*TGAGGAGACGGTGACCYYGAKCC	AC_000165.1 (Bos taurus breed Hereford chromosome 8, Bos_taurus_UMD_3.1, whole genome sequence)	***73093575-73093598***
BoJH_linker_BACK3	*AGAACCACCTCCGCCTGAACCGCCTCCA CC*TGAGGAGACGGTGCCCAGGGCAG	AC_000165.1 (Bos taurus breed Hereford chromosome 8, Bos_taurus_UMD_3.1, whole genome sequence)	***73095330-73095353***

BoJH_linker_BACK4	*AGAACCACCTCCGCCTGAACCGCCTCCA CC*TGAGGAGATGGAGAYSGGGGYGC	AC_000165.1 (Bos taurus breed Hereford chromosome 8, Bos_taurus_UMD_3.1, whole genome sequence)	73094804-73094827
BoJH_linker_BACK5	*AGAACCACCTCCGCCTGAACCGCCTCCA CC*TGAGCAGACAGTGCCCAGGCTTC	AC_000165.1 (Bos taurus breed Hereford chromosome 8, Bos_taurus_UMD_3.1, whole genome sequence)	73094124-73094147
BoJH_linker_BACK6	*AGAACCACCTCCGCCTGAACCGCCTCCA CC*TGAGGAGACGGTGTTTTGGATTC	AC_000165.1 (Bos taurus breed Hereford chromosome 8, Bos_taurus_UMD_3.1, whole genome sequence)	***73094494-73094517***
PCR 1 – lambda light chain forward primers			
BoLLFOR1	AR GGC WCA GGT GAG GCC TCC	AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	***73053239-73053258*** ***73056105-73056124*** 73059158-73059171 ***73062412-73062431*** ***73078894-73078913***
BoLLFOR2	TC TWC ACA GGT GAC TCG ATC	AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	72823465-72823479

BoLLFOR3	TC TGC ACA GGT GAC TCG ATS	AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	***72805951-72805965*** ***72811755-72811769*** ***72815184-72815198*** 72854174-72854189 ***72862254-72862269*** 72873794-72873809 72875948-72875963 ***72910218-72910232*** ***72929381-72929395*** 72943264-72943278 ***72951258-72951272*** 72962856-72962870 ***72969423-72969437*** ***75141676-75141690*** ***75145136-75145148***
		NW_003100754.1 *(Bos taurus breed Hereford unplaced, Bos_taurus_UMD_3.1, whole genome sequence)*	2016-2031
BoLLFOR4	TC TGC RCA GGT GCG GCC CCC	AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	***73089701-73089720***
		AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	***73113594-73113613***

		AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	***73096091-73096110***
		AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	***73108865-73108884***
BoLLFOR5	CCTCACTTACGGCTCAGGTCA	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	72940430-72940451 ***72881184-72881205***
BoLLFOR6	YCTCKCTCACTGCWYAGGTAG	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	72801103-72801124
		AC_000174.1 (Bos taurus breed Hereford chromosome 19, Bos_taurus_UMD_3.1, whole genome sequence)	***3428200-3428221***
BoLLFOR7	CCTYGCCCACYSCACAGGTCA	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	***72894854-72894875***

BoLLFOR8	CCTCTCTCWCTRCWCAGGTAG	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	***72808732-72808753*** 72967037-72967057 72772202-72972222
BoLLFOR9	GGTCGCTCTSTGCACAGRTGA	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	72947679-72947700 ***72826767-72826788*** 72846580-72846601 ***72870467-72870488*** 72889157-72889178
		NW_003100754.1 (Bos taurus breed Hereford unplaced, Bos_taurus_UMD_3.1, whole genome sequence)	2008-2029

PCR 1 – lambda light chain reverse primer

BoCLBACK	AC GGT CAC GCT ACC CGG GT	AC_000174.1 *(Bos taurus breed Hereford chromosome 17,* *Bos_taurus_UMD_3.1, whole genome sequence)*	***73139883-73139901*** ***73146182-13146200*** ***73152485-73152503*** 73158754-73158772

PCR 2 - lambda light chain forward primers

BoVL_linker_FOR1	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGGCTGTGCTGACYCAGC CR	AC_000174.1 *(Bos taurus breed Hereford chromosome 17,* *Bos_taurus_UMD_3.1, whole genome sequence)*	***72815313-72815333*** ***75145211-75145231*** ***72806080-72806100*** ***72826903-72826923*** ***72910347-72910367***

			72929510-72929530 72947815-72947835 ***72969552-72969572*** ***72849669-72849690*** 72854303-72854324 ***72862381-72862402*** ***72870604-72870625*** 72876077-72876098
BoVL_linker_FOR2	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGGCTRTGCTGACTCAGC YG	AC_000174.1 *(Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)*	72823594-72823614 ***72806080-72806100*** ***72811885-72811905*** ***72826903-72826923*** ***72910347-72910367*** ***72929510-72929530*** 72943393-72943413 ***72969552-72969572*** ***72849669-72849690*** 72854303-72854324 ***72862381-72862402*** ***72870604-72870625*** 72876077-72876098 72846717-72846738 72873924-72873945 72889295-72889316
BoVL_linker_FOR3	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGGMTGTGCTGACTCAGC MG	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	***72951385-72951405*** 72962985-72963005

BoVL_linker_FOR4	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGTCTRGCCTGACTCAGC CT	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	***73062549-73062569*** ***73056241-73056261*** ***73079029-73079049*** 73059287-73059307
BoVL_linker_FOR5	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGGCTGGGCTAACTCAGC CG	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome sequence)	***75141805-75141825***
BoVL_linker_FOR6	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*TCCTATGAACTGACMCAGY YGA	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***73096266-73096286*** ***73109046-73109063***
BoVL_linker_FOR7	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGGTGTTGTGGGCTGCAT CC	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***73201781-73201801***
BoVL_linker_FOR8	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*TCTTCTCAGCTGACTCAGC CG	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***73089860-73089880*** ***73113752-73113772***
BoVL_linker_FOR9	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGCSTGTGCTGACTCAGY CRG	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	72967178-72967199 ***72801244-72801265*** ***72808875-72808896*** 72972346-72972367

		AC_000174.1 (Bos taurus breed Hereford chromosome 19, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	3428596-3428617
BoVL_linker_FOR10	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*CAGACTGTGGTCCAGGAGC CAG	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	72940556-72940577 72881310-72881331

PCR 2 - lambda light chain reverse primers

BoJLBACK1	CAGGACGGTCAGTGTGGTCCCGCT	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***73151066-73151088***
BoJLBACK2	CAGGACGGTCACTCTGGTCCCGCC	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***73144755-73144777***
BoJLBACK3	CAGGACGGTGACCCAGGTCCCGCC	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	73138485-73138507
BoJLBACK4	CAGGACAGTCAGCCTGGTCCTGCC	AC_000174.1 (Bos taurus breed Hereford chromosome 17, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	73157343-73157365

PCR 1 – kappa light chain forward primers

BoLKFOR1	GRGYTCCTCCTGCTCTGGGT	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47008052-47008075 47027042-47027065 47041509-47041532 ***47052789-47052812*** 47059865-47059884 ***47071099-47071122*** 47077829-47077840 ***47089202-47089225*** 47097124-47097143 ***47104488-47104511*** 47011090-470110920 47121693-47121716 47148194-47148217
BoLKFOR2	TTCCTCCTGTTACTGTGGTT	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	46986812-46986832
BoLKFOR3	RGTCTCCTGCTSCTCTGGCT	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	46982672-46982692 47032941-47032960 47126244-47126263 ***47137392-47137411*** 47160154-47160173

PCR 1 – kappa light chain reverse primer

BoCKBACK	TGGTTTGAAGAGAAGACGGATG	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***47174110-47174132***

PCR 2 – kappa light chain forward primers

BoVK_linker_FOR1	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GATGTWGTGCTGACCCAG ACTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47027501-4702724 ***47053242-47053265*** ***47071540-4707163*** ***47089656-47089679*** ***47104941-47104964*** ***47122133-47122156***
BoVK_linker_FOR2	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GACATTGTGCTGACCCAGA CTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47008475-47008498
BoVK_linker_FOR3	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GACATCCAGGTRACCCAGT CTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***47137554-47137577*** 47160315-47160338
BoVK_linker_FOR4	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GACCTCCAGATGAYYCAGT CTCY	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	46982832-46982855 47033102-47033125

BoVK_linker_FOR5	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GACCTCCAGATGACCCAGT CTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	46982832-46982855
BoVK_linker_FOR6	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GACCTCCAGATGATTCAGT CTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47033102-47033123
BoVK_linker_FOR7	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GCTATTGTTCTGACCCAGA CTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47041937-47041960
BoVK_linker_FOR8	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GGCATCCAGATGACTCAGT CTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47126398-47126421
BoVK_linker_FOR9	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GCTAYWAKGCAGACCCAG ACTCT	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47078246-47078268
BoVK_linker_FOR10	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GCTATTATGCAGAACCAAC TATA	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	47097548-47097570

BoVK_linker_FOR11	*GTTCAGGCGGAGGTGGTTCTGGCGGTG GCGGATCG*GAAATTATGTTAACGCAGT CTCC	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	46987521-46987543

PCR 2 – kappa light chain reverse primers

BoJKBACK1	ATTGATTTCCACCTTGGTCCCG	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***47170260-47170283***
BoJKBACK2	TTTGATCTCTACCTTGGTTCCT	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***47169610-47169633***
BoJKBACK3	TTTGACTTCCAGCTTGGTTCCT	AC_000168.1 (Bos taurus breed Hereford chromosome 11, Bos_taurus_UMD_3.1, whole genome shotgun sequence)	***47169249-4716972***

Amplification of the recombination product (VH + Vλ) – forward primers

BoVH_SfiI_FOR1	GTCCTCGCAACTGCG**GCCCAGCCGGC** **C**CAGGTGMAGSTGCRGGAGTCG
BoVH_SfiI_FOR2	GTCCTCGCAACTGCG**GCCCAGCCGGC** **C**AAGGTGCAGCTGCRGGAGTSG
BoVH_SfiI_FOR3	GTCCTCGCAACTGCG**GCCCAGCCGGC** **C**CAGATGMRCTGCWGCAGTCG

BoVH_SfiI_FOR4	GTCCTCGCAACTGCG**GCCCAGCCGGC** **C**CAGGTGCTGGGGCGGGAGTCR
BoVH_SfiI_FOR5	GTCCTCGCAACTGCG**GCCCAGCCGGC** **C**CMGRTGCAGCTGCAGGARTCA

Amplification of the recombination product (VH + Vλ) – reverse primers

BoJL_Not1_BACK1	GAGTCATTCTCGACTT**GCGGCCGC**CA GGACGGTCAGTGTGGTCCCGCT
BoJL_Not1_BACK2	GAGTCATTCTCGACTT**GCGGCCGC**CA GGACGGTCACTCTGGTCCCGCC
BoJL_Not1_BACK3	GAGTCATTCTCGACTT**GCGGCCGC**CA GGACGGTCACTCTGGTCCCGCC
BoJL_Not1_BACK4	GAGTCATTCTCGACTT**GCGGCCGC**CA GGACAGTCAGCCTGGTCCTGCC

Amplification of the recombination product (VH + Vκ) – reverse primers

BoJK_Not1_BACK1	GAGTCATTCTCGACTT**GCGGCCGC**AT TGATTTCCACCTTGGTCCCG
BoJK_Not1_BACK2	GAGTCATTCTCGACTT**GCGGCCGC**TTT GATCTCTACCTTGGTTCCT
BoJK_Not1_BACK3	GAGTCATTCTCGACTT**GCGGCCGC**TTT GACTTCCAGCTTGGTTCCT

Amplification of GAPDH	
GAPDH_for	TGGTCACCAGGGCTGCT
GAPDH_rev	GGAGGGGCCATCCACAGTCT
Colony PCR	
R1	CCATGATTACGCCAAGCTTTGGAGCC
R2	CGATCTAAAGTTTTGTCGTCTTTCCA

Underlined letters indicate wobble bases. Wobbles are defined as: K – G/T, M – A/C, R – A/G, S – G/C, W – A/T, Y – C/T. The *italic* letters indicate the nucleotide sequence for the peptide linker. The **bold** nucleotides represent the restriction enzyme recognition sites for *Sfi*I and *Not*I. ***Bold and italic*** numbers functional genes.

4 GENERAL DISCUSSION

The aim of this dissertation was to investigate the relevance of Interleukin-6 as a prognostic marker in livestock diseases and to establish a technique of generating species-specific single-chain antibody fragments (scFv) in veterinary medicine. Therefore, neonatal calf diarrhea was chosen as a model for proof-of-principle.

Interleukin-6 is already established as a reliable prognostic marker in human medicine (Wilhelm et al., 2012; Khanna et al., 2013; Naffaa et al., 2013). Especially in newborns suffering from sepsis, this parameter is used to assess the prognosis in earlier stages of the infection (Buck et al., 1994; Küster et al., 1998; Meem et al., 2011). A commercial test is already available, with the indication to support diagnosis and management of patients suffering from sepsis (Elecsys® IL-6, Roche Diagnostics GmbH, Mannheim, Germany). In veterinary medicine, the prognostic value of IL-6 is investigated especially in laboratory animals, serving as models for humans, and in dogs, as one of the most popular companion animals (Rau et al., 2007; Huang et al., 2014; de Oliveira et al., 2015; Schüttler et al., 2015). Few investigations concerning IL-6 focus on livestock species, such as sheep, swine and camel (Tambuyzer et al., 2014; Abdlla et al., 2015; El-Deeb et al., 2015). In cattle, only a few studies looked into the prognostic value of IL-6. They concentrate on dairy cattle suffering from mastitis or postpartum diseases (Hagiwara et al., 2001; Ishikawa et al., 2004; Hisaeda et al., 2011; Trevisi et al., 2012).

The studies presented in this dissertation aim at transferring the prognostic potential of IL-6, found in dairy cattle, to calves suffering from neonatal calf diarrhea, as this is an important disease in livestock farming (Lorenz et al., 2011a; Lorenz et al., 2011b). Therefore, in the first instance, the physiologic development of IL-6 in newborn calves was investigated over a period of four weeks, considering the simultaneous development of the local immunity (mucosal IgA). In the second instance, the pathophysiology of IL-6 was examined in the course of neonatal calf diarrhea.

The information concerning the physiologic development of IL-6 in newborn calves *in vivo* is rare and describes that IL-6 in serum of newborn calves is mainly of colostral origin (Yamanaka et al., 2003). Furthermore, there are no data available which describe the influence of IL-6 on the development of the mucosal immunity, especially IgA, in calves, whereas in mice a strong positive influence has already been demonstrated (Ramsay et al., 1994). Instead, these findings in mice were transferred without evaluation to calves in therapeutic approaches, where IL-6 was tested as an adjuvant in

mucosal vaccination (Kumar et al., 2014). In this study, IL-6 failed to improve the mucosal IgA response and for this reason the connection of IL-6 and mucosal IgA in calves could be questioned. The author's own investigations showed that IL-6 gene expression in PBMCs of newborn calves was apparent directly after birth. The disagreement with the study of Yamanaka et al. (1993) is assumed to arise from the different methodology used. Quantitative real-time PCR, used in the own investigation, is described as being more sensitive when compared to the detection of PCR products in agarose gel electrophoresis, as used by Yamanaka et al. (1993) (VanGuilder et al., 2008). A linear connection of IL-6 and mucosal IgA was not verifiable in the author's own study. A significant correlation ($p \leq 0.05$) of IL-6 and IgA was apparent only on day 7. It was assumed that IL-6 is important in the initialization of IgA production, but is not the main regulator of the IgA concentration on mucosal surfaces. This can explain the failure of IL-6 as adjuvant in mucosal vaccination (Kumar et al., 2014). There are several factors impeding the demonstration of a linear connection between amounts of IL-6 and IgA *in vivo.* Interleukin-6 does not directly act on B-cells, but its effect is mediated via Interleukin-21. In detail, IL-6 from macrophages and dendritic cells triggers the IL-21 secretion in $CD4^+$ T-cells (Dienz et al., 2009). Subsequently, IL-21 induces the maturation of antibody secreting plasma cells (Ettinger et al., 2005). There is evidence that the same cascade is apparent in cattle, too (Gowane et al., 2014). Despite that, the detailed mechanisms regulating the IgA production seem to be unsolved and species-specific differences are confirmed (Pabst, 2012). In order to elucidate the exact mechanisms in cattle, further research is needed. The local IL-6 concentration in tissue underlying mucosal surfaces should be considered, as the development of IgA producing plasma cells is located in the local lymph nodes and the lymphatic sites in the lamina propria (e.g. isolated lymphoid follicles) (Kang et al., 2002; Lorenz et al., 2004; Macpherson et al., 2004). Therefore, more invasive studies are needed using bovine oral mucosa for gene expression analyses. The attempt to measure IL-6 gene expression in buccal mucosa cells obtained from mucosal swabs failed in the author's own investigations, because isolated RNA amounts were too small for further analysis. It was likely, that the aggressive milieu of saliva, containing lots of proteolytic enzymes, contributed to a rapid degradation of cells and therefore RNA (de Almeida Pdel et al., 2008; Chiang et al., 2015). Even, a direct transfer of the cytobrush (servoprax GmbH, Wesel, Germany), used for the mucosal swab, into RNAprotect Cell Reagent (QIAGEN, Hilden, Germany), did not improve the RNA output. Concluding from these findings,

future investigations ought to use mucosal biopsies rather than mucosal swabs to gain material for gene expression analyses.

Concerning the practical application, IL-6 remains an important key factor in the regulation of IgA production, although it seemed to have no linear relation to mucosal IgA concentrations. Latest hypotheses regarding the generation of IgA presume that IL-6 is an important supportive factor for persistence of plasma cells in the mucosal tissue (Pabst, 2012). Thus, the use of IL-6 as adjuvant in vaccinations still seems to be an interesting approach.

After the confirmation that newborn calves produce IL-6 by themselves, this parameter was investigated under pathophysiologic conditions (e.g. neonatal calves diarrhea) in order to prove its prognostic potential. The idea to establish a prognostic marker for calf diseases had already been followed in the 1980s (Bielefeldt Ohmann et al., 1985; Naylor et al., 1987; Bielefeldt Ohmann et al., 1989). Since then, the research has mainly focused on respiratory disease in calves. Clinical and pathologic parameters, such as rectal temperature and lung biopsies, failed to be reliable prognostic markers for disease outcome (Burgess et al., 2013; Theurer et al., 2014), whereas acute phase proteins (e.g. serum amyloid A (SAA), haptoglobulin (Hp)) were useful in predicting surveillance and treatment frequency (Godson et al., 1996; Berry et al., 2004; Tothova et al., 2010). Studies searching for a prognostic marker in the course of diarrhea in newborn calves are rare. Urea serum concentrations were shown to have some prognostic potential, when elevated day by day in scouring calves (Wiest and Klee., 1998).

Based on these findings and considering the aim of this dissertation to establish an early prognostic marker in neonatal calf diarrhea, serum interleukin 6 was chosen as a parameter of interest. It is described as a main inducer of acute phase proteins (Werling et al., 1996; Brock et al., 2011). Therefore, this parameter was assumed to be elevated even before the acute phase proteins, which have already been proven to be prognostic in respiratory disease in calves (Godson et al., 1996; Berry et al., 2004; Tothova et al., 2010). Recently, this assumption was confirmed in a lipopolysaccharide inflammation model in calves (Plessers et al., 2015).

The study conducted in the context of this dissertation, found interleukin 6 serum concentrations on the onset of diarrhea being significantly higher in calves which develop a prolonged course of the disease. Consequently, IL-6 was thought to be a helpful parameter in predicting the course of diarrhea in newborn calves. This statement has to be restricted to diarrhea, caused by BRV and *C. parvum*, as calves proved in this

study were infected only with these pathogens. These findings support published data, which show elevated levels of IL-6 gene expression in peripheral blood mononuclear cells (PBMCs) and in intestinal cells of calves suffering from diarrhea, due to an infection with BRV or *C. parvum* (Aich et al., 2007; Qadis et al., 2014). However, concerning infections with *C. parvum,* opposite findings were also described, where no elevated IL-6 gene expression in PBMCs was detectable (Takeda et al., 2003). Two reasons could explain these different findings. First, a broad range of different cells produce IL-6 and therefore gene expression analyses in a certain assortment of cells (e.g. PBMCs) may lead to false negative results, when compared with serum IL-6 concentrations (Nagahata et al., 2002). Second, the method by which the gene expression is analyzed determines the sensitivity of the respective investigation. The study, which found IL-6 gene expression to be absent in *C. parvum* infections, used gel electrophoresis for the detection of PCR product amounts (Takeda et al., 2003), whereas the other working group used real-time PCR for detection of DNA amplification (Qadis et al., 2014), which today is considered to be the gold standard for gene expression analyses, because of sensitivity and accuracy (Derveaux et al., 2010).

Neonatal calf diarrhea, is not exclusively caused by BRV and *C. parvum*, rather other pathogens, such as *Bovine Coronavirus* and enterotoxic *E. coli* (F5-ETEC), are important, too (Meganck et al., 2015). Accordingly, further research is needed to prove the prognostic potential of IL-6 in diarrhea caused by these pathogens as well. Furthermore, in order to use IL-6 determination as an on-farm prognostic tool, detection methods have to be improved. An attempt could be the development of lateral-flow-based immunoassays, which were already in use for the rapid detection of some diarrhea causing pathogens (Trotz-Williams et al., 2005). In human medicine, this technique was currently established to measure IL-6 in amniotic fluids, in order to detect intra-amniotic inflammation in pregnant women (Chaemsaithong et al., 2016).

In order to develop innovative drugs in the field of veterinary medicine for disease, such as viral infections, where specific therapeutics are still missing, a bovine single-chain fragment library was established. As proof-of-principle, a neutralizing bovine anti-BCV scFv was isolated from this library.

In a first step, a bovine primer set was needed to cover the whole bovine immunoglobulin spectrum. Until now, only one primer set was described for the generation of a bovine immunoglobulin library, consisting of four primer pairs (O'Brien et al., 1999). Two primer pairs were described to amplify the variable domain of the

heavy chain and two primer pairs were described to cover the variable domain of the light chain. In the latter, no difference was made between the lambda and the kappa light chain. It was likely, that these primers were not able to cover the whole variable domain immunoglobulin spectrum in cattle, with currently 36 VH genes, 63 VL_{λ} genes and 22 VL_{κ} genes (Ekman et al., 2009; Pasman et al., 2010; Diesterbeck et al., 2012; Stein et al., 2012; Walther et al., 2013).

Therefore, a new primer set was designed based on current knowledge and the author's own BLAST analyses. These were limited to the immunoglobulin spectrum, which is currently known from genetic research in cattle (Elsik et al., 2009; Elsik et al., 2016). When the results of the analyses were compared to current literature, differences arose concerning the number of different genes found for the respective immunoglobulin genes (Ekman et al., 2009; Pasman et al., 2010). It was assumed, that the differences originated from different genome assemblies used for the analyses. This finding underlines the important fact that a primer set can only be as comprehensive as the actual knowledge about the genomic basis. Primer sets have to be carefully adapted as soon as new genomic information is available. This is frequently done for antibody primer sets in other species (Sblattero et al., 1998; Sun et al., 2012). In the present work, it was decided to design a primer set, which also covered pseudogenes. Other species, such as chicken and rabbit, intensively use immunoglobulin pseudogenes to increase their antibody diversity (Weill et al., 1996; Lanning et al., 2015). In cattle, this mechanism is hypothesized for the bovine lambda light chain genes (Parng et al., 1996; Lucier et al., 1998). Until now, stringent proof neither for nor against the usage of bovine pseudogenes in gene conversion exists. As a consequence, pseudogenes were incorporated into the author's own study, in order not to miss any diversity during the construction of the antibody library.

The established primer sets were used for the amplification of bovine immunoglobulin cDNA. After the individual evaluation of each possible primer combination, the primers were pooled for the subsequent library construction. This means that one primer pool for PCR1 and one primer pool for PCR2 were made for each immunoglobulin variable domain (VH, VL_{λ},VL_{κ}). This procedure was successfully used in other studies (Schaefer et al., 2010; Meng et al., 2015; Rahumatullah et al., 2015). However, other authors avoid the usage of primer pools, because of the possible amplification bias (Gao et al., 2015). Meaning some primer combinations within the pool may work more efficient than others, and therefore some variable domains may be overrepresented in the generated

mixture of PCR products. The decision for or against using a primer pool should be based on the particular aim of the investigation. If the frequency of particular gene or gene family usage is of interest, than primer pools should be avoided. However, this project focused on innovative drug development. Therefore, special attention was given to the simplification and standardization of the methods used. The procedure should be easily transferred to other diseases and bear potential for production in industrial scales. Concerning the format of the recombinant bovine antibodies, the scFv format was chosen. Here again, this decision was made focusing on a simple and cost effective production, as well as on good pharmacokinetic characteristics. Single-chain antibody fragment libraries can be easily screened using phage display technology and the selected scFv fragment is suitable for production in simple expression systems like *E. coli* cells (Humphreys et al., 2000; Jurado et al., 2002; Ahmad et al., 2012; Wang et al., 2014). Additionally, these very small molecules show good pharmacologic properties, such as excellent tissue penetration ability (Sandhu, 1992; Xi et al., 2015). A possible disadvantage of these molecules is the absent Fc part, which in full-size antibodies mediate effector functions, such as complement activation and binding to other immune cells (Janda et al., 2016). In therapeutic applications, full-size antibodies or scFv-Fc fusion proteins mostly show higher affinities, prolonged serum half-life time and greater neutralization ability, due to the Fc part of the molecule (Chames et al., 2009; Jorgensen et al., 2014; Unverdorben et al., 2016). This handicap can be easily overcome by the reformation of a scFv-Fc or a full-size antibody from a scFv molecule, which is a simple method due to the development of new plasmids containing the requested constant domain (Bujak et al., 2014; Rasetti-Escargueil et al., 2015).

After designing the primer set for the construction of the bovine scFv antibody library, the initial step to generate the library was the immunization of a Holstein Frisian bull and the isolation of peripheral blood mononuclear cells after sufficient seroconversion. Immunized antibody libraries show several advantages for the selection of potential therapeutic antibodies in comparison to naïve libraries. During the immunization process, the B-cell population of the donor animal is preselected for antibodies binding to the antigen of interest (Lim et al., 2014). Subsequently, a fewer number of cells was suitable for DNA isolation and antibodies selected from the library often show higher affinity, when compared to those from naïve libraries (Liu et al., 2007b). However, the establishment of immunized libraries is not possible for toxic or highly virulent antigens, and in cases where antibodies directed against self-antigens are needed (Hairul

Bahara et al., 2013). Furthermore, a new library has to be generated for each antigen of interest (Shukra et al., 2014). The latter limitation can be overcome by the huge size of the immunized library, which allows for the identification of antibodies directed against antigens, other than the one used for immunization (Moon et al., 2011). The library constructed in this dissertation project used peripheral blood mononuclear cells for the initial RNA isolation step. These cells were isolated from the immunized bull after a sufficient seroconversion had been verified in the serum of the vaccinated animal. Although this approach worked well, further improvements of this step are possible. Instead of monitoring the seroconversion by serum antibody titres, an alternative could be the monitoring of antibody mRNA transcription by PCR, in order to determine the time-point, where an optimal antigen-specific immune response occurs (Rasetti-Escargueil et al., 2015). Another improvement for the simplification of antibody panning is the possibility of preselecting B-cells, producing the antibody of interest by flow-cytometry and cell sorting (Miller et al., 2008; Meng et al., 2015; Cox et al., 2016). Because this approach currently suffers from technical challenges, the library in this dissertation was screened by the widely-used phage display technique (Nixon et al., 2014; Cox et al., 2016). The phage display in combination with *E. coli* cells as expression system for selected antibodies is described as being a simple and cost effective method for the assembly of scFv fragments (Liu et al., 2013a; Wang et al., 2014; Lee et al., 2015). The facile and stable storage of phage particles at 4°C offers another advantage (Burritt et al., 1996). Limitations of this system were the occurrence of false-positive binders during panning, where binding capability is apparent only for a scFv molecule displayed on the phage surface but not when it is tested as purified protein (Nixon et al., 2014). Therefore, in the author's own study, for all binding phage-scFv combinations, the scFv was also tested as a purified molecule before it was classified as a specific binder. When thinking about future applications of the generated library, the screening against other antigens is of interest. Primarily, the library can be screened against the other antigens included in the vaccine used for immunization (BRV, *E. coli* (F5-ETEC), *C. parvum*). Furthermore, other antigens, not included in the vaccine, can also be tested. Therefore, some adaptions of the procedure are advisable, because it has to be assumed that these antibodies were underrepresented in the library. First, a negative selection can be performed against the antigens used during vaccination, which is commonly used in approaches where cross-reactivity should be excluded (Pande et al., 2010; Costa et al., 2014). Second, a hyperphage can be used in

the first panning round. This phage is made for a multivalent display of scFvs and therefore can lead to higher panning efficiencies (Rondot et al., 2001; Hust et al., 2011). Further possible improvements of the methods used in the author's own study concern the expression system for the scFv. Although *E. coli* cells are frequently used for the expression of recombinant antibodies, especially scFvs, their application has to be carefully considered due to the respective study aim (Ahmad et al., 2012). The most important aspects for the widespread usage are the convenient culture conditions and the easy handling (Verma et al., 1998). Furthermore, *E. coli* cells can be adapted to the study needs, as they can be genetically modified without much effort (Mahalik et al., 2014; Tas et al., 2015). However, the characteristics of this expression system in which most intensive attempts are made for improvement are simultaneously the most important disadvantages of this system. The first one is the limited expression capacity of *E. coli* cells, which can result in the formation of intra-cytoplasmic inclusion bodies, from which it is difficult and laborious to recover a soluble antibody (Martineau et al., 1998). Extensive protein expression can also overburden the metabolic capacity of *E. coli*, leading to decreased protein expression and cell death (Spadiut et al., 2014). In current research, several strategies were evaluated to overcome this problem (Mairhofer et al., 2010; Striedner et al., 2010). A second limitation of *E. coli* cells is the size of the protein to be expressed. Until now only scFv fragments and Fab fragments can be routinely expressed in *E. coli* cells (Harrison et al., 1996). Although, there are some descriptions of successful expression of full-size IgG molecules in *E. coli*, a large-scale production of functional full-size antibodies in this organism remains unlikely (Mazor et al., 2007; Frenzel et al., 2013). Other important deficiency of *E. coli* cells are their reducing cytoplasm, which prohibits a proper development of disulfide-bridges, and the lack of glycosylation ability (Verma et al., 1998). The problem of proper disulfide-bridge formation could be solved by directing the expressed proteins into the periplasmic compartment, where more oxidizing conditions are apparent (Dow et al., 2015). However, the glycosylation insufficiency disqualifies the *E. coli* system for the production of recombinant antibody formats containing a functional Fc part in the molecule. In this molecule part, glycans are of special importance for various functions of the antibody, such as activation of immune cells, extravasation and other pharmacokinetic properties (Hodoniczky et al., 2005; Kaneko et al., 2006; Jefferis, 2009; Venetz et al., 2015). Regarding this deficiency of *E. coli*, research focuses on other expression systems. The alternatives considered were yeast, fungi and plants

(Scheller et al., 2006; Decker et al., 2012; Goncalves et al., 2013; Sarkari et al., 2014). Unfortunately, these systems do not provide sufficient transformation efficiency needed for the construction of large antibody libraries (Verma et al., 1998).

In the future, two aspects will be important for the implementation of recombinant antibodies in the drug market. The first is the possibility for a safe, standardized and economical production, which is intensively investigated in current research (Gion et al., 2013; Meier et al., 2014). The second is the development of sufficient application systems, which ensure easy uptake by the patient (e.g. oral application) and excellent pharmaco-chemical stability for a sufficient half-life time in the patient. Although reports of a successful oral application of IgG molecules exist, most research approaches focus on the stable expression of therapeutic antibodies on the surface of bacterial cells (Krüger et al., 2002; Giomarelli et al., 2004; Monedero et al., 2004; Gunaydin et al., 2014; Jasion et al., 2015). Predominantly, bacterial species known as probiotics such as *Lactobacillus spp.* and *Streptococcus spp.* were studied. Despite the therapeutic potential, recombinant antibody formats were also used for the development for fast and simple on farm diagnostics (Wang et al., 2015; Tarasov et al., 2016). By coating them on carriers, such as nitrocellulose membranes and immunosensor chips, and using detection systems, which can be evaluated by the naked eye (e.g. gold nanoparticle labeling), an on-farm diagnosis within 10 min was
demonstrated in these studies.

5 SUMMARY

The aim of the present dissertation was to improve the prognostic possibilities and therapeutic strategies in neonatal calf diarrhea. Thereby, new techniques in drug development should be established in the field of veterinary medicine.

Therefore, three individual studies were conducted. Two of them focused on IL-6 as a potential prognostic marker in neonatal calf diarrhea. The third study aimed to generate a bovine scFv antibody specific for BCV, which would show the pharmacokinetic potential for a prospective therapeutic application.

In the initial investigation, ten healthy Holstein Friesian calves were observed from birth until day 28 after birth, regarding their IL-6 gene expression in PBMCs and IL-6 serum concentrations. Additionally, IgA concentrations in serum and saliva, as well as IgG concentrations in serum were monitored. These immunoglobulins were chosen as representatives for the local (IgA in saliva) and the systemic (IgG in serum, IgA in serum) adaptive immunity. The milk fed to the calves could have been a possible source for all these three molecules and therefore was investigated, too. The aim of this initial study was to look into the physiological development of the immune system after birth with special emphasis to the development of IL-6. The data showed that calves were able to produce IL-6 by themselves directly after birth. However, IL-6, as well as IgA and IgG were present in the colostrum and therefore the uptake of colostrum contributed to serum concentrations in calves during the first days. Immunoglobulin A concentrations in saliva started to increase around day 7 after birth. At this time point IL-6 serum concentrations and IgA concentrations in saliva showed a significant positive correlation. At the following sampling time points, a positive correlation could not be observed between these two parameters. It was assumed that, unlike in mice, IL-6 does not have a linear influence on IgA concentrations in saliva, but is an important factor in the initialization of the IgA development (Ramsay et al., 1994). Further investigations under controlled conditions are needed to confirm these preliminary findings in the field.

Referring to these findings, which confirmed IL-6 to be produced by calves even in the first days of life, the second study proved IL-6 as a prognostic marker in neonatal calf diarrhea. Twenty scouring calves were sampled two times, a first time at the beginning of diarrhea and a second time 7-10 days after the occurrence of symptoms. Data collected were clinical parameters, hematologic parameters, IL-6 serum concentrations and the diarrhea causing pathogen. For comparison, a control group of nine calves was

sampled once to determine the IL-6 basis concentration in healthy calves. At the first sampling time point, the scouring calves showed significantly higher IL-6 serum concentrations than the control calves. Furthermore, calves developing a prolonged course of the disease had significantly higher IL-6 serum concentrations compared to calves that were clinically recovered after 7-10 days. Interleukin-6 was assumed as a useful prognostic tool in neonatal calf diarrhea, which could help to prevent fatal courses of the disease by the early detection of critical patients. As BRV and *C. parvum* were the only pathogens detected in these study, the assumption should be proven for the other main pathogens in neonatal calf diarrhea (e.g. *E. coli* (F5-ETEC), BCV).

The third study of this dissertation aimed at developing a bovine antibody library as an innovative drug development strategy in veterinary medicine. As proof-of principle, a bovine scFv antibody against BCV should be screened from this library.

In preparation of the antibody library, a bovine primer set was designed with the intention of being able to amplify the whole genomic immunoglobulin diversity in cattle. Therefore intensive research was done regarding bovine immunoglobulin genetics, including literature research and the author's own BLAST analyses. The designed primers were intensively tested for functionality before they were used in library construction. For the library construction, isolated PBMCs from a vaccinated Holstein Friesian bull served as basic material. The bull had been immunized with the dam vaccine Scourguard3 (Zoetis, Berlin, Germany) containing the antigens BCV, BRV and *E. coli*. The total RNA isolated out of these PBMCs was reverse transcribed in cDNA and antibody sequences were amplified by use of the established primer set. Antibody sequences were cloned into the phagemid pCANTAB5E and transformed in TG1 *E. coli* cells. Thereby, a library with a diversity of approximately 3x 10^9 individual clones was build. Phage display was used to screen this library for antibodies against BCV. After three panning rounds a specific scFv fragment was isolated and further tested for specificity and affinity. The scFv-SF-3E4 showed excellent binding kinetics (K_m = 595 pM) and neutralization ability in cell culture until a concentration of 24 µg/ml. It was therefore considered to be an interesting candidate for further clinical applications.

The results of the studies conducted in this dissertation contribute to the improvement of prognostic measures and therapeutic strategies in neonatal calf diarrhea. The successful development of a recombinant bovine scFv antibody from a bovine antibody library could be a starting point for the broader establishment of these methods in

veterinary drug development. Overall, the advancement of animal health is an important factor for animal welfare and the improvement of animal welfare, especially in livestock species, will be an important issue during the next decades.

6 ZUSAMMENFASSUNG

Das Ziel der vorliegenden Dissertation war die Verbesserung der prognostischen und therapeutischen Möglichkeiten bei der neonatalen Diarrhoe des Kalbes. Dabei sollten zusätzlich neue Techniken bezüglich der Entwicklung innovativer Medikamente in der Veterinärmedizin etabliert werden.

Hierfür wurden drei Studien durchgeführt. Zwei der Studien untersuchten die prognostische Eignung von IL-6 und eine weitere Studie diente der Entwicklung eines rekombinanten bovinen scFv Antikörpers gegen BCV.

Die erste der IL-6 Studien untersuchte die post-partale Entwicklung der Parameter IL-6, IgA und IgG in zehn gesunden Holstein Friesian Kälbern. Dabei wurde die Genexpression von IL-6 in den peripheren mononuklearen Blutzellen und die IL-6 Konzentration im Serum der Kälber erfasst. Zusätzlich wurden die Ausbildung der lokalen und systemischen adaptiven Immunität in Form von IgA und IgG untersucht. Hierfür wurden IgA Konzentration in Speichel und im Serum sowie IgG Konzentration im Serum gemessen. Alle Parameter wurden zudem auch in der Milch erhoben, welche den Kälber während des Untersuchungszeitraumes verfüttert wurde. Die Studie erstreckte sich über einen Zeitraum von vier Wochen, in welchem die Kälber wöchentlich beprobt wurden. Das Ziel der Studie war es, die physiologische Entwicklung des Immunsystems neugeborener Kälber unter besonderer Berücksichtigung von Interleukin-6 zu erfassen. Es konnte gezeigt werden, dass Kälber, bereits direkt nach der Geburt, in der Lage sind selbstständig Interleukin-6 zu bilden. In den ersten Tagen nach der Geburt ist ein großer Anteil der gemessenen Serum Konzentration von IL-6, IgA und IgG jedoch bedingt durch die Aufnahme der Stoffe mit dem Kolostrum. Ab Tag 7 konnte ein Anstieg der IgA Konzentrationen im Speichel der Kälber beobachtet werden. Die IgA Speichelkonzentration und die IL-6 Serumkonzentrationen waren an diesem Beprobungstag signifikant positiv korreliert. An den nachfolgenden Beprobungstagen konnte keine signifikante Korrelation mehr nachgewiesen werden. Anders als in Mäusen scheint es in neugeborenen Kälbern keinen linearen Zusammenhang zwischen IL-6 und IgA zu geben (Ramsay et al., 1994). Jedoch kann ein entscheidender Einfluss von IL-6 in der Initialisierung der IgA Entwicklung angenommen werden. Weitere Untersuchungen unter kontrollierten Bedingungen sind notwendig um diesen im Feld gezeigten Zusammenhang zu bestätigen.

Aufgrund dieser Studie war davon auszugehen, dass neugeborene Kälber in der Lage sind, selbstständig IL-6 zu bilden. Somit war es möglich, IL-6 als potentiellen prognostischen Marker in Fällen von neonataler Diarrhoe, detaillierter zu untersuchen. Hierfür wurden 20 an Durchfall erkrankte Kälber in einem Abstand von 7-10 Tagen zweimalig beprobt. Die erste Beprobung fand beim Auftreten erster Symptome statt. Es wurden Daten zur klinischen Untersuchung, zur Hämatologie sowie die IL-6 Serum Konzentration erfasst und die Diarrhoe-Erreger bestimmt. Zu Vergleichszwecken wurden in einer Kontrollgruppe bestehend aus neun Tieren die basalen IL-6 Konzentrationen gesunder Kälber bestimmt. Die IL-6 Konzentrationen zu Beginn der Diarrhoe waren signifikant höher als in die der gesunden Kontrollgruppe. Des Weiteren, zeigten Tiere mit einem protrahierten Krankheitsverlauf signifikant höhere Interleukin-6 Serum Konzentrationen, als Tiere die nach 7-10 Tagen klinisch genesen waren. Somit kann die Bestimmung von Interleukin-6 im Serum von an Durchfall erkrankten Kälbern einen wertvollen Hinweis auf den klinischen Verlauf der Erkrankungen geben. Tiere, mit kritischen Interleukin-6 Werten, können intensiver beobachtet werden, um einem schwerwiegenden Verlauf der Diarrhoe vorzubeugen. Da die Tiere der Studie ausschließlich mit BRV und *C. parvum* infiziert waren, sollte diese Schlussfolgerung in weiteren Untersuchung auch für die anderen Haupterreger der neonatalen Diarrhoe des Kalbes (*E. coli* (F5-ETEC), BCV) bestätigt werden.

Das Ziel der dritten Studie war die Entwicklung einer bovinen Antikörper Bibliothek. Diese Technik kann zukünftig als Plattform für die Entwicklung neuartiger Medikamente dienen. Als proof-of-principle sollte ein boviner scFv Antikörper gegen BCV aus der Bibliothek isoliert werden.

Im Vorfeld der Bibliothekskonstruktion wurde ein bovines Primer Set entwickelt, mit welchem es möglich war, das ganze genetische bovine Antikörper Repertoire zu vervielfältigen. Hierfür wurden intensive Literaturrecherchen sowie eigene BLAST Analysen durchgeführt. Die erstellten Primer wurden vor dem Einsatz in der Bibliothekskonstruktion intensiv bezüglich ihrer Funktionalität evaluiert. Für die Bibliothek selbst dienten periphere mononukleare Zellen (PBMCs) eines immunisierten Holstein Friesian Bullen als Ausgangsmaterial. Der Bulle war zuvor mit der Muttertiervakzine Scourguard3 (Zoetis, Berlin, Deutschland) immunisiert worden. Diese enthält als Impfantigene BCV, BRV sowie *E. coli* (F5-ETEC). Die Gesamt-RNA der PBMCs wurde isoliert und mittels reverser Transkription in cDNA umgeschrieben.

Mit Hilfe des erstellten Primer Sets wurden die Antikörper Sequenzen aus diesem cDNA Pool amplifiziert. Diese Sequenzen wurden in einem nächsten Schritt in das Phagemid pCANTAB5E kloniert und in TG1 *E. coli* Zellen transformiert, bis eine Diversität von 3x 10^9 Klonen erreicht war. Die Phage Display Technik wurde genutzt um einen BCV spezifischen scFv Antikörper aus der Bibliothek zu isolieren. Nach insgesamt drei Panning Runden wurde ein BCV spezifischer scFv Antikörper aus der Bibliothek isoliert und weiter hinsichtlich seiner Spezifität und Affinität charakterisiert. Der Antikörper scFv-SF-3E4 zeigte eine exzellente Bindungskinetik (K_m = 596 pM) und war in der Lage bis zu einer Konzentration von 24 µg/ml das Virus in Zellkultur zu neutralisieren. Diese Eigenschaften machen den Antikörper zu einem interessanten Kandidaten für eine zukünftige therapeutische Anwendung.

Die Ergebnisse aller drei Studien, welche im Rahmen der vorliegenden Dissertation durchgeführt wurden, tragen zu einer Verbesserung von prognostischen und therapeutischen Möglichkeiten in Falle der neonatalen Diarrhoe des Kalbes bei. Die erfolgreiche Isolation eines spezies-spezifischen Antikörpers aus einer Antikörperbibliothek kann außerdem dazu beitragen, diese Methodik weiter in der Veterinärmedizin zu etablieren und zur Entwicklung innovativer Therapeutika zu nutzen.

Insgesamt ist die Verbesserung der Tiergesundheit ein entscheidender Baustein im Gesamtkomplex „Tierwohl“, welches auch in den nächsten Jahrzehenten ein wichtiges Thema in der Veterinärmedizin bleiben wird.

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8 APPENDIX

Abbreviations

Ahr	aryl hydrocarbon receptor
AP	activator protein 1
Arid5α	AT rich interactive domain 5A
BCDF	B-cell differentiation factor
BCGF	B-cell growth factor
Bcl	B-cell lymphoma protein
BCR	B-cell receptor
BCV	*Bovine Coronavirus*
BLAST	Basic Local Alignment Search Tool
BPV	*Bovine Papillomavirus*
Breg	regulatory B-cell
BRF	Transcription factor IIIB
BRV	*Bovine Rotavirus*
BSF	B-cell stimulating factor
CD	cluster of differentiation
cDNA	complementary desoxyribonucleic acid
CDR	complementary determining region
CDR3H	complementary determining region 3 of the antibody heavy chain
CREB	cyclic adenosine 3',5'-monophosphate response element binding protein
CSF	cerebrospinal fluid
CSNS	conserved short nucleotide sequences
DNA	desoxyribonucleic acid
E	small envelope protein
EGF	epidermal growth factor
EIAV	*Equine Infectious Anemia Virus*
ELISA	Enzyme-Linked Immunosorbent Assay
ER	estrogen receptor
Fab	fragment antigen-binding
Fc	fragment crystallizable
GALT	gut-associated lymphoid tissue
gp	glycoprotein

GR	glucocorticoid receptor
HAMA	human-anti-mouse-antibodies
HE	hemagglutinin-esterase
Hp	haptoglobulin
ICTV	International Committee on Taxonomy of Viruses
IFN	Interferon
IgA/D/E/G/M	Immunoglobulin A/D/E/G/M
IGHC	heavy chain constant gene
IGHD	heavy chain diversity gene
IGHJ	heavy chain joining gene
IGHV	heavy chain variable gene
IGKC	kappa light chain constant gene
IGKJ	kappa light chain joining gene
IGKV	kappa light chain variable gene
IGLC	lambda light chain variable gene
IGLJ	lambda light chain variable gene
IGLV	lambda light chain variable gene
IgNAR	immunoglobulin isotype novel antigen receptor
IL	interleukin
IL-6R	interleukin-6 receptor
IPP	ileal Peyer’s patches
IRF	interferon regulatory factor
IUIS	International Union of Immunological Societies
Jak	Janus kinase
JPP	jejunal Peyer’s patches
kD	kilodalton
M	transmembrane protein
MAPK	mitogen-activated protein kinase
miR	micro ribonucleic acid
mRNA	messenger ribonucleic acid
N	nucleocapsid protein
NF-IL-6	nuclear factor for IL-6 expression
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
P38α	mitogen-activated protein kinase p38a

p53	cellular tumor antigen p53
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PDFG	platelet-derived growth factor
PPARα	peroxisome proliferator-activated receptor alpha
Raf	rapidly accelerated fibrosarcoma protein
Ras	rat sarcoma protein
Rb	retinoblastoma protein
RNA	ribonucleic acid
S	spike protein
SAA	serum amyloid A
scFv	single-chain variable fragment
sgp130RAPS	gp130 of the rheumatoid arthritis antigenic peptide-bearing soluble form
sIL-6R	soluble form of interleukin-6 receptor
SIRS	systemic inflammatory response syndrome
SNP	single- nucleotide-polymorphism
SP-1	specificity protein 1
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
Th	T helper cell
TNF-α	tumor necrosis factor alpha
Treg	regulatory T-cell
TRF	T-cell replacing factor
VH	variable domain of the heavy chain
VHH	variable domain of the heavy chain of the heavy chain antibody
VLκ	variable domain of the kappa light chain
VLλ	variable domain of the lambda light chain

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Animal Welfare Declaration

During the animal experiments all measures were taken to avoid unnecessary pain, suffering and harm.

All animal experiments were done in accordance with the animal welfare officer of the Georg-August-University of Goettingen, Germany.

The Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Oldenburg, Germany, as responsible German authority, has been informed about all animal experiments performed in this dissertation:

Fischer S., Diers S., Bauerfeind R., Czerny C. P., Neumann S. (2016): Dynamics of salivary immunoglobulin a and serum interleukin 6 levels in newborn calves. Livest. Sci. 189, 1-7
(LAVES reference number 33.9-42502-04)

Fischer S., Bauerfeind R., Czerny C. P., Neumann S. (2016): Serum Interleukin 6 as a Prognostic Marker in Neonatal Calf Diarrhea. J Dairy Sci. 2016 Aug; 99 (8)
(LAVES reference number 33.9-42502-04)

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Declaration

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der Promotionsordnung des Fachbereichs Veterinärmedizin 06.11.2012 7.40.10 Nr. 1 S. 8 der "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten."

“I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.”

Goettingen, 23.06.2016

Signed: Stephani Fischer