On the aetiology of Bovine Neonatal Pancytopenia (BNP)

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List of Abbreviations

a-BoLA-I	Anti-BoLA class I
AEC	3- Amino-9- Ethylcarbazol
BHV	Bovines Herpes Virus
BNP	Bovine Neonatal Pancytopenia
BoLA	Bovine Leukocyte Antigen
BRDC	Bovine Respiratory Disease Complex
BRSV	Bovine Respiratory Syncytial Virus
BSA	Bovine Serum Albumin
BVD	Bovine Virus Diarrhoea
BVDV	Bovine Viral Diarrhoea Virus
CFU	Colony Forming Unit
CP	Cytopathic
ECL	Enhanced Chemiluminescence
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme Linked Immunosorbent Assay
EMA	European Medicines Agency
EU	European Union
FACS	Fluorescent Activated Cell Sorting
FAIT	Foetal Alloimmune Thrombocytopenia
FcR_n	Neonatal Fc receptor
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
FPLC	Fast Protein Liquid Chromatography
FSC	Forward light scatter
GEMM	Granulocyte/erythroid/macrophage/megakaryocyte
GIT	Gastrointestinal tract
HCP	Host Cell Proteins
HD	Haemorrhagic Diathesis
HLA	Human Leukocyte Antigen
HNA	Human Neutrophil Antigen
HPA	Human Platelet Antigen
HRP	Horseradish Peroxidase
HS	Haemorrhagic Syndrome
ICTV	International Committee on Taxonomy of Viruses
IgG	Immunoglobulin G
IL	Interleukin
IP	Immunoprecipitation
IRES	Internal Ribosomal Entry Site
kDa	Kilo Dalton
LC-MS-MS	Liquid Chromatography- Tandem Mass spectrometry
Ltd.	Limited
LTR	Long Terminal Repeat
mAb	Monoclonal Antibody
MCS	Multiple Cloning Site
MD	Mucosal Disease
MDBK	Madin-Darby bovine kidney
MFI	Median Fluorescence Intensity
MHC	Major Histocompatibility
MLV	Modified Live Vaccines

MoMLV	Moloney Murine Leukaemia Virus
NADL	National Animal Disease Laboratory
NAIT	Neonatal Alloimmune Thrombocytopenia
NCP	Non- Cytopathic
NRW	North Rhine Westphalia
NS	Non-structural peptides
NZ	New Zealand
OIE	World Organisation for Animal Health (Organisation Mondiale de la Santé Animale)
ORF	Open Reading Frame
Ori	Origin of replication
PAGE	Polyacrylamide Gel Electrophoresis
PBMC	Peripheral Blood Monoclonal Cell
PBS	Phosphate Buffered Saline
PE-Cy5.5	Phycoerythrin- Cyanine 5.5
PEI	Paul- Ehrlich- Institute
PHA	Phytohaemagglutinin
PHE	Plate Heat Exchanger
PI	Persistently Infected
Plat- E	Platinum- E
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SARSS	Suspected Adverse Reactions Surveillance Scheme
SDS	Sodium Dodecyl Sulphate
SNT	Serum Neutralisation Test
SSC	Side light scatter
Th cells	T helper cells
TRALI	Transfusion Related Lung Injury
UTR	Untranslated Region

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I owe my deepest gratitude to my family and my friends.

Zusammenfassung

Die Bovine Neonatale Panzytopenie (BNP) ist ein neuartiges hämorrhagisches Krankheitsbild bei Saugkälbern, das mit Blutungsneigung, hämatologischen Veränderungen und einer hohen Letalität einhergeht. Mutterkühen erkrankter Kälber wurden mit PregSure® BVD, ein stark adjuvantierter Impfstoff gegen Bovine Virusdiarrhoe (BVD) immunisiert. Der Impfstoff enthält Zellbestandteile von der Zellinie die zur Virusproduktion eingesetzt wird. Diese Zellbestandteile, führen bei geimpften Kühen zu der Bildung alloreaktiver BNP assoziierte Antikörper. Mittels Durchflusszytometrie und Immunpräzipitation konnten wir zeigen, dass PregSure® BVD Immunisierung zu einer BNP Alloantikörper Produktion führt. BNP Alloantikörper sind gegen hoch polymorphe Rinder MHC-I-Moleküle gerichtet (BoLA I). Acht BoLA I-Varianten aus der Produktionszelllinie wurden isoliert und davon wurden drei Allele identifiziert, die für die Mehrheit der PregSure® BVD induzierte BoLA I Reaktivität verantwortlich sind. Die BoLA I-Varianten von gesunden Kälbern werden nicht von den BNP assoziierten Alloantikörpern ihrer jeweiligen Muttertiere erkannt. Weiterhin haben wir untersucht ob BNP Alloantikörper mit menschlichen Zellen kreuz reagieren, um eine potenzielle Gefahr für Verbraucher von Rinderkolostrum auszuschließen. Wir konnten nachweisen, dass BNP Alloantikörper auch menschliche MHC-I Moleküle binden. BNP assoziierte Alloantikörper befinden sich auch in kommerziell hergestelltem Kolostrum Pulver, produziert aus Kolostrum von PregSure® BVD immunisierten Kühen. Zusammenfassend können wir zeigen, dass BNP ein Impfstoff induziert alloimmune Krankheit ist.

Abstract

Bovine Neonatal Pancytopenia (BNP) is a novel haemorrhagic disease in sucking calves, characterised by bleeding, haematological changes and high mortality. Dams that gave birth to BNP affected calves were immunized with PregSure® BVD, a highly adjuvanted vaccine against Bovine Viral Diarrhoea (BVD). We can show that bioprocess impurities in the vaccine, originating from the cell line used for vaccine production induces alloantibodies in vaccinated cattle. Via flow cytometry and immunoprecipitation we can demonstrate that PregSure® BVD immunization leads to BNP alloantibody production. BNP alloantibodies target highly polymorphic bovine MHC-I molecules (BoLA I). We sequenced eight BoLA I variants expressed by the production cell line and identified three alleles which are responsible for the majority of PregSure® BVD induced BoLA I reactivity. The BoLA I alleles of BNP unaffected calves are not recognized by the BNP associated alloantibodies of their respective dams. We also examined whether BNP alloantibodies cross-react with human cells, thus being a potential hazard for human colostrum consumers and could show that BNP alloantibodies are cross-reactive to human MHC-I and can even be found in commercial colostrum powder manufactured from cows immunized with PregSure® BVD. Overall we can demonstrate that BNP is a vaccine induced alloimmune disease.

1. Introduction

1.1 Bovine Viral Diarrhoea Virus (BVDV)

Bovine Viral Diarrhoea Virus (BVDV) is an enveloped positive-sensed single stranded RNA virus, classified as a member of the family *Flaviviridae* and belongs to genus Pestivirus, which consists only of viruses that do not infect humans (Rümenapf T. and Thiel, 2008). The other members of the genus Pestivirus are Classical Swine Fever Virus (CSFV) and Border Disease Virus (BDV).

The genome of BVDV is about 12.5 kb, and with a virion diameter of 40 – 60 nm, it belongs to the smaller viruses (Collett et al. 1988). Based on visual inspection, BVDV can be grouped into two biotypes, or into two genotypes, depending on the method of molecular characterization (Rümenapf, Thiel 2008). Microscopic observations of BVDV infected tissue cell culture can show the presence of cytopathic effects caused by viral replication and propagation, for example, vacuolization, detachment and cell death by apoptosis. These microscopic changes determine the BVDV biotype as cytopathic (CP). The absence of any such observations in cell culture is used to characterise the BVDV biotype as non-cytopathic (NCP). Initially, BVDV was classified as NCP but evolution of the virus led to the shift of some virus strains to be CP. Nevertheless, over 95 % of all BVDV infections are caused by NCP biotype and, against common belief, both biotypes can produce highly virulent, as well as less aggressive strains. In fact, NCP strains are more often associated with high virulent disease outcomes (Fulton et al. 2000; Fulton et al. 2002).

Apart from different biotypes, BVDV also exists in distinct genotypes (Pellerin et al. 1994; Ridpath et al. 1994). The different genotype classifications initially arouse due to genomic sequence discrepancies recognized in the highly conserved 5’ untranslated region (UTR) (Ridpath, Bolin 1998). Upon this discovery, the two genotypes were first assigned as BVDV type 1 and 2 (Ridpath et al. 2000; ICTV). However, with further advances in molecular biological techniques, such as PCR, nucleic acid sequencing and phylogenetic analysis, it became apparent that an additional subdivision of the genotype 1 into further (at least 16) subgenotypes from BVDV type 1 a to subgenotype 1 p, and an unassigned type, as well as, genotype BVDV type 2 into subgenotype 2 a and 2 b was necessary (Peterhans et al. 2010). Both genotypes, BVDV type 1 and 2 are heterogeneous, can cause severe disease and exist in both biotypes: CP or NCP.

1.2 Bovine Virus Diarrhoea (BVD)/ Mucosal Disease (MD)

BVDV predominantly infects cells of the innate immune system and can lead to pathological effects in various organ systems, which includes the respiratory, hematologic, neurologic, immunologic, and the most common and significant one, the reproductive system (Chase, Christopher C L et al. 2004). The later one is the main system the virus makes use of in order to maintain and spread.

Animals suffering from an infection by BVDV develop Bovine Virus Diarrhoea (BVD), a worldwide distributed viral disease, which causes one of the most significant pathology affecting bovine health (Rümenapf, Thiel 2008). BVDV shows a high tissue tropism for fast replicating cells and thus often targets the foetus when infections occur in pregnant cows. Early intrauterine infections, especially in the first trimester of gestation (day 40 – 125), lead to birth of healthy immunotolerant persistently infected (PI) calves (Lanyon et al. 2013). These animals are the major reservoir of BVDV and have a key status in its epidemiology. Secretions and excretions of PI animals are the source of continuously shed virus and thus enable direct and indirect viral transmission. Only infections with NCP BVDV are known to lead to this persistent infection (Peterhans et al. 2010; Xue et al. 2011).

Infections of pregnant cattle later in gestation can result in abortion, malformations, growth retardation, failure to conceive, mummification, stillbirth, weak or as already mentioned virus-free, seroconverted calves (Beer et al. 2000; Lanyon et al. 2013).

Infections in non-pregnant, naïve cattle are commonly asymptomatic but sometimes can also lead to severe disease outbreaks. The common signs and symptoms are then fever, weight reduction due to appetite loss, mucosal lesions, diarrhoea, thrombocytopenia, immune suppression and infertility (Fray et al. 2000; Lanyon et al. 2013). The fate of infection outcome depends on the immune condition of affected animals, the virulence level as well as the degree of pathogenicity of the infecting virus strain. A severe form of the disease common with NCP BVDV genotype 2 infections, which can lead to a form known as acute haemorrhagic syndrome (HS), is characterized by high virulence, aggressive destruction of erythrocytes, coagulopathy and death (Rümenapf, Thiel 2008; Ridpath et al. 2000).

In the long run, PI animals can develop a deadly condition, which can be acute or chronic and is known as Mucosal Disease (MD) (Grego et al. 2007; Ozer, Duman 2011). In acute or early onset MD the life expectancy is between 2 days to 3 weeks whereas in a chronic or late onset manifestation, the animal might survive up to 18

months only. The cause of development of BVD to MD is mutation or genetic recombination that occurs by superinfection of PI animals with other antigenetically similar viral strains (Ridpath, Bolin 1995; Hilbe et al. 2013). The common signs and symptoms of MD are, amongst others, fever, anorexia, diarrhoea and mucosal erosion, especially in the gastrointestinal tract (GIT), which all consequently result into wasting and finally death.

BVDV can be transmitted by either direct contact, which is the most efficient and effective route or by indirect transmission via vehicles, vectors, infected nose tongs, rectal gloves, semen, artificial insemination, etc. Both acute infected cattle and far more important and significant, PI animals, have the potential to shed the virus and transmit the disease.

BVD and BVDV infections greatly contribute to considerable economic losses in the cattle industry throughout the world (Smith et al. 2013). These are mostly attributed to a reduction in milk production, decreased fertility, reduced weight gains, as well as higher susceptibility and incidences of affected cattle to respiratory tract diseases (Houe 1999; Peterhans et al. 2010).

1.3 Measures against BVDV

BVD is considered to be one of the contributors of Bovine Respiratory Disease Complex (BRDC) (Aiello S.E. and Mays A., 1998). The causative agents, termed under the acronym BRDC, are the primary cause leading to huge economic losses globally. This, furthermore, underlines the importance of designing control and eradication strategies.

Complete control and possible eradication of BVDV infections as well as outcomes of infections can only be accomplished when PI animals are identified early and removed, thereby breaking the cycle of virus transmission and preventing constant shedding of new viruses (Lindberg, Alenius 1999). In some areas, with relative low cattle population such as Norway and Finland, outbreaks are managed without vaccination. In locations with increased cattle population and a higher seroprevalence, vaccination schemes are the primary choice to keep BVDV infections under control (Raue et al. 2011).

Primarily, vaccines against BVDV should ensure protection against viraemia and avoid transplacental infections, and thus the occurrence of immunosuppressed PI calves, and in addition, should prevent severe disease outbreaks.

1.3.1 Vaccination

Although several viral diseases are battled against by vaccination, virus shift and drift as well as emergence of different virulent variants remain to be the main challenges. In the USA alone, there are more than 175 state approved vaccines against BVDV (Ridpath et al. 2000; Newcomer, Givens 2013). Prior to the year 2000, vaccines available in the market included mainly the BVDV- 1 strains, predominantly of the subgenotype 1 a (Xue et al., 2011) but recent findings of different (sub-) genotypes of BVDV led to increased necessity to incorporate other BVDV strains. The existence of antigenic heterogeneity between the various BVDV genotypes and subgenotypes forced manufacturers to produce combination vaccines that include BVDV- 2 strains, as well as other BVDV- 1 subgenotypes so as to guarantee a wider range of protection and efficacy. This high antigenic diversity amongst the different BVDV strains and isolates is also the major reason as to why vaccination programs against the disease are so challenging (Ridpath et al. 2000).

The two main categories, in which vaccines are divided, are live vaccines and inactivated vaccines (Patel, Heldens 2009). In America, most BVDV vaccines are sold as polyvalent cocktails against BRDC, containing live and killed components. In the EU, the majority of vaccines available are as monovalent BVDV abortion vaccines.

1.3.1.1 Modified Live Vaccines (MLV)

Live vaccines are produced by either attenuated strains, recombinant viruses or naturally occurring, non-pathogenic virus strains (Patel, Heldens 2009). The aim and challenge are to ensure that the virus cannot induce the disease but at the same time is capable to elicit suitable immune responses. MLV against BVD/ MD contain live, attenuated BVDV strains. Attenuation is commonly achieved by repeated passaging in bovine or porcine cell lines, chemically altering isolates or by molecular deletion of virulence genes. Immune responses, after successful vaccination with MLV, are comparable to natural BVDV infections and thus often a single dose is sufficient (Newcomer, Givens 2013). This also suggests the higher risk associated with MLV. Pregnant cattle vaccinated with these types of vaccines have shown to have an increased risk to produce PI animals due to potential residual virulence of the vaccine strain, making the vaccine inappropriate for use during gestation (Beer et al. 2000). Live vaccines have also been reported to be a potential source of introduction of new BVDV strains and thus increasing virus epidemiology (Xue et al. 2011; Van et al.

2000). Recent reports also suggest that BVDV vaccinations lead to a state of immunosuppression in vaccinated cattle as a side effect.

1.3.1.2 Inactivated vaccines

Inactivated vaccines are made up of inactivated viral isolates, for example, by chemical means (Patel, Heldens 2009). Multiple doses are frequently necessary in order to achieve primary immunization but still, when compared to MLV, these vaccine types are considered the safer choice. Unlike MLV, which lead to Th1 and Th2 immune answers, inactivated vaccines usually lead to humoral immune responses. These are far less effective and, therefore, adjuvants are commonly used in order to increase the host immune reaction. In the context of BVDV, inactivated vaccines rarely show sufficient foetal protection, which is mainly due to the short immunity period, inefficient cross-type protection and inability of the virus to replicate (Beer et al. 2000; Newcomer, Givens 2013). Therefore, these vaccine types do not actually fulfil the main objective set for BVDV vaccines. Additionally, due to the delay until an optimal protection is ensured, inactivated vaccines are often considered as more costly.

A practical approach to combine the advantages of live-attenuated vaccines with the safety of inactivated vaccines was put forward by a group at the University of Veterinary Medicine, Hannover, led by Prof. Volker Moennig. In the so called two-step regimen the animals are primarily immunized with an inactivated vaccine to elicit a basic immunity against BVDV (Moennig et al. 2005). Subsequently, the dams are boosted with a live attenuated vaccine inducing long-lasting, reliable protection. This two-step regimen was originally developed for a combination of a inactivated and live-attenuated vaccine from one particular manufacturer. Nevertheless, later it was shown, that inactivated vaccines from other manufacturers can also be used.

1.3.1.3 Adjuvants

Adjuvants are additives that are combined with drugs and vaccines in order to increase their function but, at the same time, they should have minimal independent effects (Patel, Heldens 2009). These additives, which can be mineral salts, cytokines, bacterial products, etc., are used to assist by navigating the immune response. The classical examples are aluminium hydroxide, which elicits a strong humoral response, or saponins, which lead to stronger cellular responses. In BVDV vaccines, a high titre of NCP BVDV field strains, in combination with aluminium hydroxide or

Quil A, which is a complex mixture of extracts from a Chilean tree, are commonly used (Beer et al. 2000). They are effective, but the actual potentiating mechanism of most adjuvants is still not clearly understood.

1.3.2 PregSure® BVD

PregSure® BVD by Pfizer Animal Health (now Zoetis) was licensed in the EU as a monovalent inactivated vaccine directed against BVDV type 1 infection (Veterinary Medicines Directorate 2009). In the US, a similar variant, known as CattleMaster® GOLD™ is available, which additionally protects against BVDV type 2, Bovine Herpes Virus Type-1 (BHV-1), Bovine Respiratory Syncytial Virus (BRSV) and Parainfluenza Virus (PI3) – members of the BRDC (Dominowski et al. 2007; United States Patent Application Publication, 2007). According to the manufacturer, the vaccines are produced by the same procedure, the major difference being only that one is a monovalent vaccine, whereas the other is a polyvalent one (Federal Association of Official Veterinarians 2012; Bastian, personal communication).

PregSure® BVD is a unique inactivated vaccine comprising BVDV type 1, which has been combined with a new patented adjuvant system. The virus (BVDV type 1 strain 5960) is disabled by chemical inactivation with binary ethyleneimine (BEI) and adjuvanted with Quil A- Cholesterol- Drakeol 5- Amphigen® (Veterinary Medicines Directorate 2009). This innovative adjuvant composition is collectively known as Procision- A™. It is similar to the PreZent™ adjuvant system in CattleMaster® GOLD™ and provides a unique enhancement and long-term immune answer, primarily based on its capability to enable efficient presentation of BVDV antigens to the host immune system (Pfizer Animal Health 2004; United States Patent Application Publication, 2007).

Although the actual manufacturing procedure is obviously not known to the public, a technical bulletin released by Pfizer Animal Health outlines some of the main points. The vaccine makes use of intact inactivated virus or fragments of BVDV envelope glycoproteins, which are associated with virus neutralizing antibody production, as a nanocomplex with Quil A- cholesterol, attached to amphigen® microdroplets, created by shear force processing (Pfizer Animal Health 2004).

Quil A is a combination of various saponins, i.e. plant secondary metabolites, derived from the bark of *Quillaja saponaria* Molina, a tree found predominantly in Chile (Demana et al. 2004). The unique chemical structure of Quil A, which is composed of characteristic termini of different charge, is the cause for its underlying ability to boost

cell mediated and humoral immune reactions. Depending on the quantity of Quil A added, the substance leads to formation of various liposome structures from micelles to a bilayer. The hydrophobic centre interacts freely with the animal fat cholesterol (Pfizer Animal Health 2004). In right quantity, cholesterol minimizes unwanted side effects of Quil A but at the same time does not alter its immunostimulating characteristics. As shown in figure 1 A, Quil A and cholesterol intercalate to form helices that possess long polymeric-like structures. The hydrophilic immunoactivating polysaccharides are exposed to the surface and thus can freely interact with the immune system, while hydrophobic areas remain buried in the inner regions of the helix (Pfizer Animal Health 2004).

Amphigen®, a further adjuvant system patented by Pfizer, is a de-oiled lecithin mixed with paraffin, which acts as an oil-based adjuvant and thus elicits a long lasting immune reaction (United States Patent Application Publication, 2003). Particles in lecithin are spatially organised similar to cells of the bovine immune system, therefore making it easily accessible (Pfizer Animal Health 2004). The decreased viscosity has, furthermore, the advantage that the mixture becomes easier to draw in a syringe and reduces local side effects at the injection site e.g. lump, swelling or abscesses.

Through using powerful shear forces, the resulting mixture of antigen-Quil A cholesterol-amphigen® is processed into nanocomplexes. The result is that amphigen® is transformed into small homogeneous stable microdroplets of 0.1 to 0.2 µm size (Pfizer Animal Health 2004). Additionally, the applied forces shorten the length of Quil A- cholesterol chain helices to form shorter fragments, which bind optimally to the BVDV envelope antigens, as shown schematically in figure 1 B. This powerful and highly immunogenic combination has not been used in any other vaccine so far (Federal Association of Official Veterinarians 2012).

Procision- A™ is shown to be an improved and powerful adjuvant, which possesses a good capacity to induce strong immunogenic and protective immune responses to BVDV (Raue et al. 2011; Kasonta et al. 2012; Gonzalez et al. 2014). The manufacturer claims that the vaccine provides broad foetal protection against BVDV type 1, when given to BVDV- seronegative cows whereas at lower levels, it also provides protection against BVDV type 2. Pfizer assures that the vaccines are safe for use in “[...] pregnant and nursing cattle and their offspring and meet dairy and beef cow market needs” (United States Patent Application Publication, 2007). The vaccine is thus able to protect against PI caused by BVDV, making it a powerful tool

to battle BVD/ MD. Furthermore, Pfizer's claim that the vaccine induces very high and long term antibody titres, which is incomparable to any other BVDV inactivated vaccine on the market, is indeed confirmed in various publications (Bastian et al. 2011; Federal Association of Official Veterinarians 2012; Raue et al. 2011; Kasonta et al. 2012; Gonzalez et al. 2014).

According to the manufacturer, the vaccine should be administered in two doses, 5 weeks and 2 weeks prior to breeding in order to achieve primary immunization. Overall the new intervention should guarantee protection to breeding age, pregnant and lactating cattle, making it highly attractive in BVDV control programs (Veterinary Medicines Directorate 2009; United States Patent Application Publication, 2007). Despite the manufacturer's instructions however, in some federal states in Germany a two-step vaccination regime is enforced as the method of choice to eradicate BVDV, which is the case in Lower Saxony and Saxony-Anhalt. This regiment implies priming with an inactivated vaccine and boosting with a MLV (Moennig et al. 2005; Kasonta et al. 2012). Although the two-step regimen was initially developed for a combination with an inactivated vaccine from a different manufacturer, in some Federal States PregSure® BVD was also used.

The Paul- Ehrlich- Institute (PEI), the Federal Institute for Vaccines and Biomedicines, issued the market authorisation for PregSure® BVD in the German market, but then raised awareness that the vaccine might be associated with certain adverse side effects and therefore gave recommendations to suspend its authorisation. Consequently, Pfizer Animal Health willingly ceased production and sales in the UK and EU member states (Pfizer Animal Health 2010) and in New Zealand on August 18th 2011 (New Zealand Ministry for Primary Industries 2011). After the company voluntarily removed the vaccine from the European market, the European Medicines Agency (EMA) recommended that the market authorisation for PregSure® BVD should be suspended on July 15th 2010 (EMA 2010).

Observations made were a possible association between PregSure® BVD application and occurrence of Bovine Neonatal Pancytopenia (BNP), also known as blood sweating. As the PEI is the National Competent Authority, it is determined to investigate this in order to prevent similar adverse reactions in future.

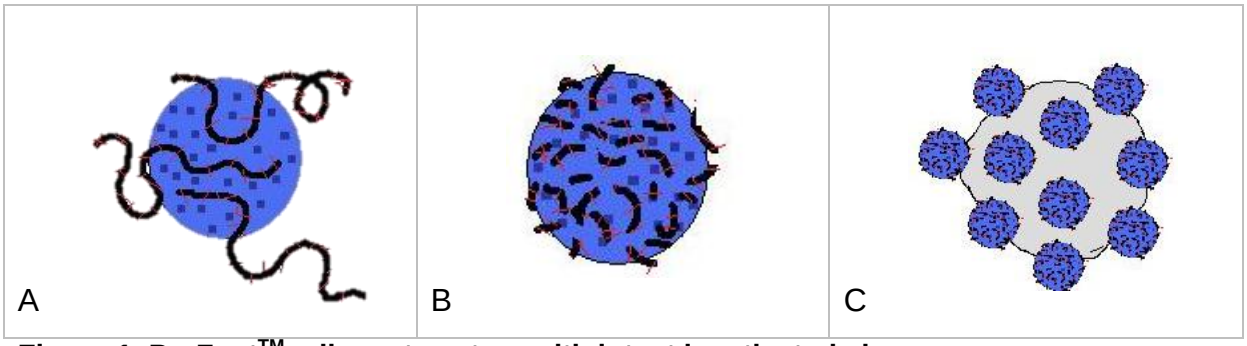


Figure 1: PreZent™ adjuvant system with intact inactivated virus.
(A) BVDV (blue circle) and viral envelop proteins (dark blue dots) are coated with Quil A-cholesterol helices (black). Quil A immunostimulating sugars (red) are uncovered to the surrounding. (B) After high shear force application, the length of helices is shortened enabling optimal binding of more coils. (C) Multiple nanocomplexes interact with amphigen® microdroplets (grey). The result is a potent and highly immunogenic antigen vehicle which can elicit strong and long-lasting immune responses [modified from Pfizer Animal Health, 2004].

1.4 Bovine Neonatal Pancytopenia (BNP)

Starting from 2007, there were incidences reported all over Europe of a new pathological condition in young calves (Friedrich et al. 2011; Friedrich A. et al. 2009; Kappe et al. 2010; Dyer et al. 2010). The observations were characterised by spontaneous bleeding, without obvious prior injuries, high fever (up to 41 °C) and death a short period after first symptoms were observed. Pathohistological analysis, showed bone marrow depletion, pancytopenia and severe thrombocytopenia in affected animals. Based on these signs and symptoms, the novel disorder is now officially known under the terminology “Bovine Neonatal Pancytopenia” (BNP) or “Bleeding Calf Syndrome” (sometimes just referred to as “blood sweating” or in some older literature still as “Idiopathic Haemorrhagic Diathesis” (IDH)) (Ballingall 2011; Bastian et al. 2011).

The first reports originated from Germany, in regions of Bavaria and North Rhine-Westphalia (NRW), followed by an accumulation of reports from various other European countries including the UK, Belgium, France, the Netherlands, Hungary, Italy and Spain (Dyer F. et al. 2010; Federal Association of Official Veterinarians 2012; Defra 2011), affecting cattle of both sexes and of various breeds. Clinical signs are observed in young calves between the ages of one to three weeks. The first signs are that calves, which are apparently healthy and inconspicuous, get a fever spike and turn pale due to haemorrhage from various body parts, and sometimes also

associated signs of petechiae (i.e. tiny red-purple spots on the skin) and melena (dark faeces) (Bell et al. 2010b; Kappe et al. 2010). Histological sections further show that the cause of spontaneous haemorrhage of external and/ or internal organs, is a wide spread bone marrow depletion, similar to the photograph in figure 2.

The bone marrow is the site of residence of pluripotent haematopoietic stem cells, which further differentiate into the common myeloid and lymphoid progenitor cells. Microscopic analysis of blood and bone marrow of calves diagnosed with BNP show total aplasia, non-regenerative anaemia and depletion of all haematopoietic cell types (Bell et al. 2010b; Pardon et al. 2011; Pardon et al. 2010). Due to the different life span of these cells, first, platelets diminishing number (life span of approximately 10 days) is observed, resulting in thrombocytopenia, which is detectable by increased susceptibility to bleeding. The resulting haemorrhagic anaemia is supported and becomes irreversible due to the continuous loss of erythrocyte precursors (existence period of about 60 days). The decrease of neutrophils leads to neutropenia and analysis of the lymph nodes and spleen also reveals diffuse lymphoid destruction, affecting and leading to depletion in both B- and T-lymphocyte compartments (Kappe et al. 2010). This also explains the reason for increased susceptibility of affected calves to secondary infections. Twenty four to forty eight hours after appearance of the first signs, the calves' health increasingly declines followed by death in up to 90 % – 95 % of cases (Bell et al. 2010b; Bell et al. 2010a; Dyer et al. 2010).

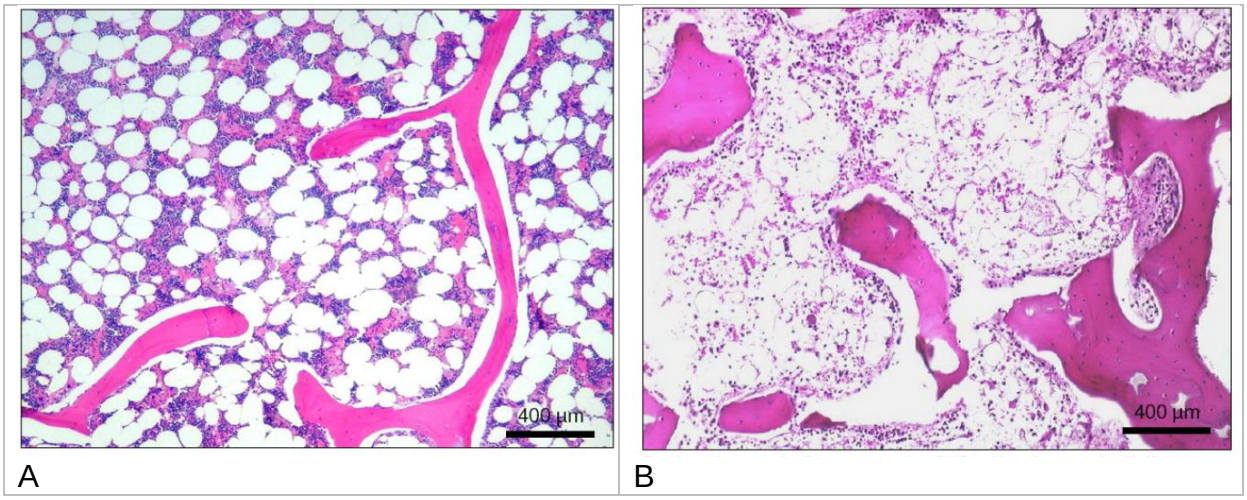


Figure 2: Histopathology of the femoral bone marrow.
(A) Bone marrow section of a healthy calf: high amount of haematopoietic tissue, low in fat (B) Bone marrow section of a BNP calf: severe depletion of haematopoietic cells, high in fibroblast and fat cells. Haematoxylin and eosin stain [Courtesy of: Dr. med. vet. Mark Holsteg; Cattle Health Service NRW].

As described earlier on, BVDV and associated diseases can lead to similar pathological outcomes but research has revealed that there are no associations and that both BVDV type 1 and 2 have been excluded as the causative agents of BNP (Bell et al. 2010b; Friedrich A. et al. 2009). Evidence of other causative virus strains, such as a novel circovirus (Pardon et al. 2011; Willoughby et al. 2010; Pardon et al. 2010), bluetongue virus (Pardon et al. 2010; Pardon et al. 2011), epizootic haemorrhagic disease virus (EHDV) (Bridger et al. 2011) were also not apparent. Other hypothesis, such as plant toxins (e.g. acute bracken fern like toxicity), other toxins, mycotoxins, chemicals, medications, etc. as the causative agent of BNP all revealed to be negative or inconclusive (Friedrich A. et al. 2009; Kappe et al. 2010; Pardon et al. 2011; Pardon et al. 2010).

Friedrich and colleagues, and later on others, carried out studies that showed a clear association between BNP and ingestion of colostrum (i.e. milk-like substance produced during pregnancy) from cows that had previously given birth to BNP calves (Friedrich et al. 2011; Schroter et al. 2011; Bell et al. 2013). They could experimentally reproduce the disease in some young born calves. Unlike in humans, calves do not get their first protective antibodies from their mothers via *in utero* transmission but obtain them by uptake of colostrum. Bovine colostrum is produced in mammary glands prior to delivery; it is low in fat but contains a high amount of proteins, including a substantial amount of protective maternal antibodies (70 – 80 % of IgG isotype) (Renegar K. B 2005).

After further research, it was obvious that in cases reported as BNP, the initial signs and symptoms were apparent only after the first ingestion of colostrum (Friedrich et al. 2011). The resulting new hypothesis, therefore, was that the causative agent must somehow be delivered via the first feeding. Furthermore, calves developed BNP only if fed with colostrum from donors that had previously given birth to calves suffering from BNP (Bell et al. 2013; Schroter et al. 2011; Friedrich et al. 2011). Cell-mediated mechanisms were ruled out because samples were stored at conditions usually not favourable and deleterious for cells (Friedrich et al. 2011). Toxins, as a possible cause, were also improbable based on the fact that most toxins (small molecular weight) normally would cross the placental barrier and no confirmed signs of BNP have been reported in pre-colostral individuals. Ruling out these possibilities, assumptions that the causative agent might be antibodies (alloantibodies) increased (Bridger et al. 2011). Isoantibodies or alloantibodies (Greek: allos means other) are

antibodies that are directed against antigens of another individual but of the same species.

After analysis of BNP cases reported to the various surveillance agencies and evaluation of epidemiological data, a common vaccination history in dams of affected calves was obvious (Dyer F. et al. 2010; Bastian et al. 2011; Friedrich et al. 2011). Also, when considering higher occurrences of BNP in specific regions in Germany only (high incidence in Bavaria and NRW and few cases in Lower Saxony, Saxony-Anhalt and Thuringia), the association between BNP and vaccination strategies against BVDV crystallized (Moennig et al. 2005; Kasonta et al. 2012; Lambton et al. 2012; Jones et al. 2013). In states and countries in which BNP was noted, PregSure® BVD was used as the vaccine of choice against BVDV and in accordance with the manufacturer's instructions. In theory it was hypothesised (Friedrich et al. 2011) and later proven (Bastian et al. 2011), that the vaccine induces the production of alloantibodies, which are then transferred from the vaccinated dam to the calf via colostrum (Bridger et al. 2011; Schroter et al. 2011). These alloantibodies are directed against peripheral blood cells and pluripotent haematopoietic stem cells in the bone marrow. This was also verified by Pardon and co-workers by demonstrating that BNP is caused by alloimmune immunoglobulin G (IgG) that recognize antigen(s) on bovine granulocytes, lymphocytes and monocytes (Pardon et al. 2011; Bridger et al. 2011). Further analysis of BNP colostra revealed the presence of pathogenic IgG1 alloantibodies bound to platelets and leukocytes (Assad et al. 2012). Bell and colleagues (2013) could show that neutrophil, thrombocyte, lymphocyte and monocyte committed cells and very primitive precursor cells of the neutrophil, erythrocyte and eosinophil lineage are affected, in addition to the aforementioned peripheral blood cell depletion. Furthermore, the disease could be circumvented by colostrum substitution (Bell et al. 2010a).

1.4.1 BNP Associated Alloantibodies

Two independent publications soon thereafter revealed the presence of Major Histocompatibility class I (MHC- I) antibodies in colostrum and sera of BNP dams (Deutskens et al., 2011; Foucras et al., 2011). Furthermore, it was also described that bulls that were experimentally vaccinated with PregSure® BVD can develop anti-bovine MHC I alloantibodies (Kasonta et al., 2012).

The bovine analogue of MHC I, termed Bovine Leukocyte Antigen I (BoLA I), is found on all nucleated cells, including thrombocytes. Thrombocytes possess little integral

membrane MHC I (Santoso et al. 1993a) but absorb a considerable amount shed and present in plasma (Lalezari, Driscoll 1982; Kao et al. 1986; Semple et al. 2011). As in humans, BoLA I is highly polymorphic and plays a major role in presenting intracellular proteins to cells of the immune system. Classical BoLA I molecules have a higher polymorphism while non-classical variants have a limited polymorphism and cell expression. In the Immuno Polymorphism Database (IPD) 96 different classical and 18 different non classical BoLA I alleles are listed (Robinson et al. 2005). The encoding gene has been mapped onto the bovine autosome 23. The actual number of loci in cattle is not known but at least six loci have been identified so far (Ellis 2004). Interlocus recombination and the possibility that one individual can express several BoLA I molecules, in various different combinations, increase the diversity in a herd further. Thus, it is unlikely that cattle in a herd carry the exact same BoLA I combination pattern (Babiuk et al. 2007; Ellis 2004). If BNP alloantibodies target a specific set of BoLA I alleles, perhaps even in combination with the expression of another yet unidentified BNP (haematopoietic stem cell) alloantigen, this might explain the rather low incidences (0.016 % per single dose PregSure® BVD) of BNP (EMA 2010).

The proposed BNP model hypothesizes that the cell line expresses a panel of specific BoLA I alleles and since the cell line is present as a bioprocess contaminant, these different variants are found in the vaccine. A vaccinated cow mounts an antibody response against those variants that she does not express herself. If the calf inherits one of these immunogenic variants from the father, the BNP alloantibodies from the dam can bind and can then lead to disease induction in the calf (Deutskens et al., 2011; Foucras et al. 2011).

Another research group examined bone marrow haematopoietic progenitor cells in culture (Laming et al. 2012). Results revealed that the colony forming unit-granulocyte/erythroid//macrophage/megakaryocyte (CFU-GEMM) was markedly decreased when colostrum from BNP dams was added, indicating that these cells are specifically targeted by BNP inducing-alloantibodies. This finding would go hand in hand with histological observations reported in affected calves (compare with figure 2). Varying BoLA I expression patterns, expression levels or expression of a specific variant may be the reason as to why only these cell populations are targeted while others remain unaffected.

When feeding cattle with pooled colostrum from BNP dams, the result is a higher likelihood of all challenged animals to develop BNP (Bell et al. 2013; Laming et al. 2012). The affected animals present a peripheral blood cell depletion, especially of leukocytes and platelets, in addition to haematopoietic stem cell insult, characteristic for BNP. In contrast, challenges with non-pooled colostrum produce a variable number of susceptible calves (Schroter et al. 2011; Friedrich et al. 2011). These findings would support the hypothesis that BNP alloantibodies recognize a highly polymorphic set of antigens, such as BoLA I.

1.4.2 Alloimmune Diseases in Humans

Humans can develop a disease clinically almost identical to BNP known as Foetal or Neonatal Alloimmune Thrombocytopenia (abbreviated FAIT or NAIT). In FAIT/ NAIT, the most common cases are due to a single nucleotide polymorphism in the beta chain of β3 integrin, which is present in the platelet membrane, making the individuals homozygote for the human platelet antigen 1 (HPA-1a). When these individuals become pregnant with a HPA-1a heterozygous foetus, they develop anti-HPA-1a antibodies. The maternal antibodies pass the placental barrier and recognize the paternal-derived antigens on thrombocytes, which eventually leads to thrombocytopenia and foetal bleeding (Bridger et al. 2011; Skogen et al. 2009). In addition to other platelet antigen incompatibilities (e.g. HPA-5b, HPA 4), the role of human MHC class I (i.e. Human Leukocyte Antigen; abbrev. HLA) antibodies have been discussed but it is thought that these alloantibodies only play a weak part in disease induction (Marin et al. 2005; Kaplan 2006). In contrast to BNP, which is an alloimmune disease caused by vaccination, FAIT/ NAIT is an example of natural sensitisation and thus has a different epidemiological profile.

Another transfusion associated alloimmune phenomenon is Transfusion Related Acute Lung Injury (TRALI). HLA I specific antibodies have been detected in TRALI inducing blood products but their specific role still remains under debate (Reil et al. 2008). However, it is evident that a combination of HLA I and human neutrophil antigen 3a (HNA-3a) alloantibodies are causative for most of the TRALI cases (Greinacher et al. 2010; Reil et al. 2011). Although it is assumed that the HLA I antibodies only provide a milder trigger in contrast to HNA-3a alloantibodies (Reil et al. 2008).

1.4.3 Hypothesis

The precise procedure on how Pfizer manufactures PregSure® BVD is a company secret and is not published, but as mentioned before, the major processes are likely to be similar to the production of CattleMaster® GOLD™ and other inactivated viral vaccines (Bastian et al., 2011; Federal Association of Official Veterinarians 2012).

The BVDV antigens are obtained by culturing the virus in a permanent bovine kidney cell line (Bastian et al., 2011). Subsequent purification and removal processes of contaminates are not known, but when taking into account general procedures customary in the veterinary vaccine production industry, the slurry is probably just centrifuged and the supernatant is then checked for potential contaminating bacteria or other bovine virus pathogens (OIE 2012; Bastian, personal communications). This procedure would thus make contaminations with process related impurities and so called Host Cell Proteins (HCP) very likely (Federal Association of Official Veterinarians 2012). Indeed, another scientific group compared the vaccine with a bovine kidney cell line, by SDS-PAGE and mass spectrometry, and could identify several shared cellular components (Euler et al. 2013), thus confirming the presence of HCP in PregSure® BVD.

HCP are bioprocess contaminants, such as biomolecules, derived from the host cell system in which a drug or biopharmaceutical is manufactured (Wang et al. 2009). These residual host or process impurities are specific for a given manufacturing process and can range from cell culture derived contaminants (e.g. medium serum proteins), cell substrate derived contaminants (e.g. immunoglobulins, DNA, endotoxins, viruses, cell wall components) to downstream derived contaminants e.g. Protein A or G affinity ligands (FDA 2010; EMA 2011). Their often foreign/ non-self-characteristics increase the risk of development of an immune response in the consumer, leading, for example, to the production of anti-HCP antibodies (Dagouassat et al. 2001; Hoffman K. 2000). Alternatively, these unintended components can increase, accelerate or prolong immune responses, thus functioning like adjuvants, or they can contribute to various other potential risk scenarios that can affect the actual biopharmaceutical or vaccine functionality.

In order to avoid these drug efficacy interferences as well as adverse reactions and also in order to assure recipient's safety, several purification steps should be undertaken during manufacturing processes so as to reach acceptable safety levels. However, multiple purification steps lead to a decrease in the overall product yield

and a complete removal of contaminants is often virtually impossible. It is common knowledge that this is also the case in most BVDV vaccines and BVDV antigens, like other viral antigens, are challenging to obtain in a 100 % pure form (Federal Association of Official Veterinarians 2012). Nonetheless, in the specific case of PregSure® BVD, this has been shown to lead to a major side effect:

The colostrum-derived alloantibodies, which are shown to cause BNP (Bridger et al., 2011; Schroter et al. 2011) and absorb to peripheral and bone marrow cells (Pardon et al., 2011), have been demonstrated to cross react with antigens on the bovine kidney cell line used for vaccine production (Bastian et al., 2011). The main manufacturing processes of PregSure® BVD, most probably, do not differ greatly from those of other brands. The primary difference, therefore, would be the combination of residual host cell or process contaminants (impurities such as HCP), the antigen and most importantly, the highly potent new adjuvant system (i.e. Procision- A™).

1.4.4 Objectives of the Research

By connecting those facts so far, the research hypothesis, on which the current work is based on, can be formulated:

- First, histopathological analysis show increased destruction and depletion of peripheral blood cells and bone marrow stem cells, which is the cause of the spontaneous bleedings observed.
- Second, the uptake of colostrum, which is the primary source of antibodies, from dams previously vaccinated with PregSure® BVD, can induce BNP in some calves and BNP has only been observed in post-colostral calves. Toxins, viruses and other infectious agents have been ruled out by research and aetiological studies on various reported cases.
- Third, several researchers, including the manufacturers, have demonstrated the capability of PregSure® BVD to induce a potent immune response that lead to a prolonged antibody titre, including a manifold higher titre of antibodies also measurable in colostrum. This is mainly based on the action of a highly potent and new adjuvant system, which is also the major difference between this particular vaccine and other BVDV vaccines.
- The overall hypothesis therefore is that, impurities in vaccine production and the application of the new adjuvant system, somehow lead to increased production of alloantibodies in vaccinated dams (Bastian et al., 2011). These alloantibodies are

then transferred to calves by colostrum and are directed against antigens present on peripheral blood cells and precursor cell lines due to foetal-maternal incompatibility (Bridger et al., 2011; Pardon et al., 2011). Furthermore, the antibodies show a degree of cross reactivity between the calves' host cells and bovine kidney cells, which are closely related to the cell line in which viral vaccine antigens are produced and are present as HCP impurities in the vaccine (Bastian et al., 2011; Euler et al. 2013).

The main aim of this PhD project was to further characterize the novel syndrome BNP and verify that BNP is truly a vaccine-induced alloimmune syndrome. To this end, the objective was also to characterize BNP associated alloantibodies and identify the antigen(s) targeted by BNP inducing alloantibodies. Apart from identifying BNP associated alloantigen(s), a further goal was to define the alloantigen(s) relevant for the induction of BNP by comparing between cattle vaccinated with PregSure® BVD but that did not give birth to calves affected by BNP and dams that gave birth to bleeder calves, the so called BNP dams.

2. Materials and Methods

2.1 Materials

2.1.1 List of Devices and Apparatus

Table 1. 1: List of the devices and apparatus used

Device/ Apparatus	Type	Manufacturer
Blotting device	Semi-Phor™	Hoefer Scientific Instruments
Centrifuge	Centrifuge 5415C	Eppendorf AG
Centrifuge	Centrifuge 5804R	Eppendorf AG
Centrifuge	Avanti J-26 X PI	Beckman Coulter
Centrifuge	Varifuge RF	Heraeus Sepatech
Centrifuge	Cryofuge 8500	Heraeus Sepatech
CO_2-Incubator	BB620	Heraeus Instruments
ELISA reader	Sunrise™	Tecan
Fast Protein Liquid Chromatography (FPLC)	Smartline	Knauer Advanced Scientific Instruments
Flow cytometers	Accuri C6 flow cytometer LSR II	BD Biosciences
Gamma radiation source	Cäsium 137 OB 29/ 932-3	STS Steuerungstechnik u. Strahlenschutz GmbH
Gel chambers	GNA 100 Mini-PROTEAN® Tetra Cell	Pharmacia Biotech Biorad
Image documentation unit	G:Box	Syngene
Incubator	6200	Heraeus Instruments
Inverse fluorescence microscope	Axio Observer.Z1	Carl Zeiss AG
Inverse microscope	Telaval 31	Carl Zeiss AG
Laminar flow cabinet	SterilGard A/B3 Hood - Class 2	The baker company
Multi-channel Pipettes	Research Pro	Eppendorf AG
Nano-Photometer	OD600 DiluPhotometer™	IMPLEN
pH meter	pH 538	WTW
Power supplies	Standard Power Pack P25T DC Power Supply PS3000	Biometra GmbH Hoefer
Single-channel Pipettes	Reference	Eppendorf AG
Thermo cycler	PTC-200 Peltier Thermal Cycler	MJ Research
Thermo mixer	Thermomixer 5437	Eppendorf AG
Ultrasonic bath	Laboson 200	Bender & Hobein
Water bath	1083	GFL

2.1.2 List of Chemicals, Kits and Reagents

Table 1. 2: List of the chemicals and reagents used in different experiments

Name	Manufacturer
100 bp-DNA-Ladder 50 µg	Invitrogen™
2-Propanol, for molecular biology, >99%	Sigma-Aldrich
3,3′,5,5′-Tetramethylbenzidin (TMB)	Sigma-Aldrich
3-Amino-9-Ethylcarbazol (AEC) tablets	Sigma-Aldrich
Acrylamide- Bis solution (37.5: 1) 30 % (w/v)	Serva
Ammonium persulfate (APS) $(NH_4)_2S_2O_8$	Serva
Ampicillin readymade solution (100 mg/mL)	Sigma-Aldrich
Biozym Sieve 3:1 Agarose	Biozym Scientific
Bromphenol Blue (3',3'',5'.5"- Tetrabromophenolsulfonepthalin)	Sigma-Aldrich
Chloroquin (25 mM)	Sigma-Aldrich
Citric acid monohydrate ($C_6H_8O_7.H_20$)	Merck
Coomassie staining (Roti® Blue)	Carl Roth
D (+) Glucose ($C_6H_{12}O_6$)	Sigma-Aldrich
Dulbecco's Modified Eagle Medium (DMEM)	Lonza
EDTA- disodium ($C_{10}H_{14}N_2O_8Na_2.2H_20$)	Serva
Enhanced Chemoluminescence reagents (ECL)	GE Healthcare
Ethanol	Merck
EZ-Link Sulfo-NHS-LC-Biotin	Thermo Scientific
GelRed Nucleic Acid Stain	Biotium, Inc.
Glycerol ($C_3H_8O_3$)	Sigma-Aldrich
Glycine ($C_2H_5NO_2$)	Serva
Hydrogen peroxide (H_2O_2)	Merck
N, N- Dimethylformamide (DMF) (C_3H_7NO)	Sigma-Aldrich
N, N, N', N'- Tetramethyl- ethylene diamine (TEMED) ($C_6H_{16}N_2$)	Serva
PageRuler™ Plus Prestained Protein Ladder	Fermentas
Poly- L- Lysine	Sigma-Aldrich
Polypren (=Hexadimethrine bromide)	Sigma-Aldrich
Potassium chloride (KCl)	Merck
Potassium dihydrogen Phosphate (KH_2PO_4)	Serva
Propidium Iodide (PI)	Sigma-Aldrich
Roswell Park Memorial Institute (RPMI) Medium- 1640	Biowest
Sodium acetate anhydrous (CH_3COONa)	Merck
Sodium chloride (NaCl)	Merck
Sodium Dodecyl Sulfate (SDS)	Carl Roth
Sodium hydrogencarbonate ($NaHCO_3$)	Merck
Tris (Tris hydroxymethyl-aminoethane) ($C_4H_{11}NO_3$)	Carl Roth
Triton- x 100 (2-[4-(2,4,4-trimethylpentan-2yl)phenoxy-ethanol)	Fluka
Trypan blue solution (0.4 %)	Sigma-Aldrich
Tween- 20 (polyoxyethylene sorbitan monolaurate)	Sigma-Aldrich
Urea (CH_4N_2O)	Carl Roth
Vegetable extract No.2	Fluka
ε – aminocaproic acid ($C_6H_{13}NO_2$)	Serva

Distilled water used for buffers was produced via „PURELAB ultra“ from ELGA LabWater. Some buffers were produced by the in-house department at the Paul-Ehrlich- Institute. These include: Alsever's Trypsin-Versen-Solution (ATV), DEPC-Water, EDTA (0.5 M, pH 8.0), EDTA (100 mM), LB-Agar with Ampicillin (0.1 g mL^{-1}), LB-Media, NaCl (100 mM), Phosphate Buffered Saline (PBS) without Ca^{2+} und Mg^{2+}, PBS containing 0.05 % Tween-20, SF-IMDM-Media, SOC-Media, TBE-Buffer (10-fold) and Tris pH 8,0 (100 mM).

2.1.3 List of Buffers

Table 1. 3: List of different buffer names and content

Buffer Name	Ingredients (pH)
10 x Lämmli Buffer	1 % SDS, 0.25 M Tris, 2 M Glycine; pH 8.3
2 x non reducing Loading Buffer	1.7 ml Upper Buffer, 2 ml Glycerol, 4.5 ml 10 % SDS, 0.8 ml Bromphenol blue, 1 ml H20
4 x Lower Buffer	1.5 M Tris- HCl, 0.4 % SDS; pH 8.8
AEC Buffer solution	0.05 M Sodium acetate ; pH 5.0
Anode Solution 1	0.3 M Tris- HCl, 20 % Methanol; pH 10. 4
Anode Solution 2	0.025 M Tris- HCl, 20 % Methanol; pH 10.4
Binding Buffer	20 mM KH2PO4; pH 7.4
Blocking Buffer	0.5 % Vegetable Extract in PBS containing 0.05 %Tween-20
Cathode Solution	0.04 M ε – aminocaproic acid, 20 % Methanol; pH 7.6
Elution Buffer A	0.1 M Citrate, pH 2.5
Elution Buffer B	0.1 M Glycine- HCl; pH 2.7
FACS Buffer	0.5 % FCS in PBS
IP Lysis Buffer	1 % Triton- x 100, 150 mM NaCl, 50 mM Tris, 5mM EDTA
Loading Buffer	6 M Urea, 62.5 mM Tris, 2 % SDS, 10 % Glycerol, 0.025 % Bromophenol Blue
Mild Stripping Buffer	200 mM Glycine, 0.35 % SDS and 0.8 % Tween 20; pH 2.2
Neutralizing Buffer	1 M Tris- HCl; pH 9.0
TMB solution	1 ml sodium acetate (1.1 M, pH 5.5), 9 ml H_20, 167 µl TMB stock solution (6 mg TMB ml^{-1} Ethanol), 2 µl H_2O_2 (30%)
Upper Buffer	0.5 M Tris HCl, 0.4 % SDS; pH 6.8
Wash Buffer	0.9 % NaCl

2.1.4 List of Enzymes, Cells, Vectors, Kits and Antibodies

Table 1. 4: List of cells and cell lines

Name	Manufacturer
Bovine Kidney cell line	Pfizer Deutschland GmbH
Murine pre-B cells (38B9 Cells)	Jörg Kirberg, PEI
One Shot® TOP10 Chemically Competent *E. coli*	Invitrogen™
Platinum-E Retroviral Packaging Cell Line, Ecotropic	Cell Biolabs Inc.

Table 1. 5: List of antibodies

Name	Manufacturer
Goat Anti- Bovine IgG	Dianova
Goat Anti- Mouse IgG$^{PE\text{-}Cy5.5}$	Invitrogen™
Goat Anti- Mouse^{HRP}	Dianova
Mouse Anti- BoLA Class I clone IL- A88	AbD Serotec
Mouse Anti- HLA Class I clone w6/32	Steffen Tenzer, University of Mainz
Mouse Anti- MHC Class I clone H58A	VMRD, Inc.
Rabbit Anti- Bovine- IgG^{HRP}	Dianova
Sheep Anti- Bovine IgG^{Biotin}	AbD Serotec
Sheep Anti- Bovine-IgG^{FITC}	AbD Serotec
Streptavidin^{HRP}	Dianova
Streptavidin$^{Pe\text{-}Cy5.5}$	Invitrogen™

Table 1. 6: List of enzymes and vectors

Name	Manufacturer
AmpliTaq Gold® DNA Polymerase (in Buffer II and $MgCl_2$)	Invitrogen™
*Bam*HI (in purified BSA 100 x and NEBuffer 4)	Biolabs, Inc.
T4 DNA Ligase (in 10 x T4 DNA Ligase Reaction Buffer)	Biolabs, Inc.
*Xho*I (in purified BSA 100 x and NEBuffer 4)	Biolabs, Inc.
pMyc-IRES-eGFP vector	Jörg Kirberg, PEI
pGEM-T easy vector	Promega
BoLA sequence-specific primers (PCR-SSP) Forward: 5' ggatcc**ATG**GGGCCGCGAACC 3' Reverse: 5' ctcgag**TCA**CCCTTTAGGAACCG 3'	Thermo Scientific

Table 1. 7: List of kits used

Kit name	Manufacturer
NucleoBond Xtra Midi-Kit	Machery-Nagel GmbH Co.KG
QIAPrep Spin Miniprep Kit	Qiagen
RNeasy Mini Kit	Qiagen
Wizard SV Gel and PCR Clean-up System	Promega

2.2 Methods

2.2.1 Animals, Serum and Dairy Samples

The majority of BNP cases were identified and kindly provided by Dr. med. vet. Mark Holsteg of the Cattle Health Service (*Tiergesundheitsdienst*) of North Rhine Westphalia. The cattle were vaccinated at least twice with PregSure® BVD and additionally received booster shots, all administered in compliance with manufacturers' recommended instructions. As control, sera samples of cattle not immunized against BVD or treated with alternative BVD vaccines were available. The calves obtained colostrum only from their respective mothers, meaning that colostrum pooling was not performed. Samples were acquired by venipuncture, performed by authorized personnel, and then transported to the institute within a 24 hour period. Additional sera from BNP dams were provided by a veterinary practice in the northern part of Hesse and by the Clinics of Internal Medicine and Surgery of Ruminants of the Veterinary Faculty in Munich.

Definitive diagnosis of BNP in calves was confirmed based on haematology and bone marrow histopathology. The cases have been reported to and reviewed by the national pharmacovigilance system. The complete vaccination history of all animals included was made available to institute employees.

Additionally, serum samples from 12 PregSure® BVD-vaccinated New Zealand BNP dams were obtained. Sera from PregSure® BVD vaccinated animals with no history of BNP in their progeny and from non-vaccinated or alternatively BVDV-vaccinated animals served as controls. In addition, milk and colostrum samples were obtained from 12 and six of the BNP dams, respectively. Raw milk and colostrum samples from individual cows were subjected to continuous-flow High Temperature Short Time pasteurization by: using a peristaltic pump to pass small volumes (600 ml in total) through a miniature plate heat exchanger (PHE), fitted to a hot-water heating supply; then through a holding tube with the peristaltic pump adjusted such that the flow rate provided a residence time of 15 seconds; then past an electronic temperature probe to ensure that the flow of hot water to the initial PHE was adjusted such that the temperature of the liquid within the far end of the holding tube was at least 72.6°C; and finally through a second miniature PHE, fitted to a iced-water cooling supply; and then discarding the first 250 mL portion that emerged, prior to collecting each sample. Commercial lots of New Zealand whole milk powder and two colostrum powders were also obtained. One colostrum powder sample was

manufactured in New Zealand in 2011, prior to the prohibition against PregSure® BVD treatment, while the second sample was manufactured in New Zealand in 2012 from colostrum that had been sourced exclusively from non-PregSure® BVD treated cows. All samples were held at -70 °C for long term storage. The powders were weighed and 0.1 g of each sample was dissolved in 1 ml phosphate buffered saline (PBS), which was freshly prepared before each analysis. Whole colostrum was centrifuged twice at 11,000 x g followed by 25,000 x g to remove cell debris, and then stored at -20 °C.

2.2.2 Continuous Cell Culture

Analysis was carried out using the bovine kidney cell line used to produce PregSure® BVD, which was kindly provided by Pfizer Animal Health. Continuous cell culture was performed in accordance to directions provided. The cells were grown in DMEM, split (usually 1:8) and passaged (never exceeding 20 passages) according to need. Confluency, growth and possible contaminations were monitored by a technical assistant. The cell line was tested to be free of BVDV.

For the different experiments, cell detachment for 175 cm^2 culture flasks was performed as follows: cell media was aspirated and adhering cells were washed by overlaying with PBS. Trypsinization was done with detachment solution containing 0.8 % sodium chloride, 0.04 % potassium chloride, 0.1 % glucose, 0.058 % sodium hydrogencarbonate, 0.02 % EDTA and 0.5 mg ml^{-1} trypsin (ATV solution) for 10 minutes at 37 °C, and it was stopped by addition of 5 ml PBS. The resulting cell suspension was pelleted by centrifugation at 1,500 x g for 10 minutes at 15 °C. The supernatant was decanted and the cells were re-suspended in 10 ml PBS and a viable cell count with trypan blue was performed. The total cell number was calculated by using the Neubauer chamber (W. Schreck, Hofheim) or the Fuchs-Rosenthal-Chamber (Blaubrand®) and the appropriate formulas.

Phytohaemagglutinin (PHA), a T-cell mitogen, was used to produce short-term T cell lines (hereafter referred to as lymphoblasts) as described previously by Bastian and colleagues (Bastian et al. 2011). Shortly, 1 x 10^6 Peripheral Blood Monoclonal Cells (PBMCs) per ml, extracted by Ficoll Paque (1.077 g ml-1, GE Healthcare) from blood of various animals or buffy coats of healthy blood donors (German Red Cross, Frankfurt; Votum 329/ 10; ethics committee; Goethe-University, Frankfurt), were washed twice with PBS containing 1 % FCS and then supplemented with 0.1 µg ml^{-1}

PHA (Oxoid) and a 1:20 dilution of hybridoma supernatant containing human interleukin- 2 (IL- 2) in 2 ml RPMI-1640 media.

2.2.3 Enzyme Linked Immunosorbent Assay (ELISA)

A MaxiSorp™ 96 well plate (Nunc-Immuno) was coated overnight at 4 °C with 2 µg ml^{-1} anti-bovine IgG (Dianova) in PBS, followed by blocking with 200 µl 0.5 % Vegetable Extract in PBS containing 0.05 % Tween-20 (Blocking buffer) for 1 hour at room temperature. After washing three times with PBS containing 0.05 % Tween-20, sample dilutions of serum, milk or colostrum were prepared and left for 2 hours at room temperature. After the time had passed the cavities were again washed thrice and then incubated with biotinylated anti-bovine IgG (AbD Serotec) in a 1:1000 end dilution in PBS containing 0.05 % Tween-20 for 1 hour at room temperature. This was followed by another wash step that was repeated for three times and then incubation with streptavidin coupled to peroxidase (Dianova) in a 1:10000 dilution in PBS containing 0.05 % Tween-20 for half an hour. After another three wash steps the plate was developed with freshly prepared TMB solution, colour development was stopped with 1 M H_2SO_4 (stop solution) and measured at 450 nm/ 620 nm with an ELISA reader (Tecan™ Sunrise). A standard series was made in parallel with bovine IgG with known antibody concentration.

2.2.4 Serum Neutralization Test (SNT)

To detect and quantify the amount of BVDV-antibodies in serum standard SNT was performed according to OIE guidelines (OIE 2012): In summary, serial dilutions of bovine sera were incubated for two hours with 100 Cell Culture Infectious Dose 50 % ($CCID_{50}$) of cytopathogenic BVD virus reference strain, NADL (National Animal Disease Laboratory). Pre-incubated virus was transferred to microtiter plates that had been seeded over night with 4 x 10^4 Madin-Darby bovine kidney (MDBK) cells per well. After three to four days the development of a cytopathic effect (CPE) was assessed by microscopy; alive and intact cells are indicative for a successful neutralization. CombiStats software (distributed by the European Directorate for the Quality of Medicines) was used to calculate individual SN titres.

2.2.5 IgG Affinity Purification Assay

The purpose of the IgG affinity purification assay was to obtain the alloreactive antibodies present in sera of BNP dams. Since it is verified that these antibodies bind to peripheral blood and stem cells of calves (Pardon et al. 2011; Laming et al. 2012;

Bell et al. 2013) and cross react with the bovine kidney cell line (Bastian et al. 2011), the assay was modified and carried out as follows: to 1 x 10^8 bovine kidney cells, re-suspended carefully using a needle, 2.5 ml serum from the respective individual was added. The mixture was transferred into a glass bottle, filled up to 50 ml PBS per 1 x 10^8 cells and left rotating for 1 hour at 4 °C on a rotator (Roller Heraeus Instruments) to allow sufficient time for the alloantibodies to bind to the cell surface. After incubation time had elapsed, the cell suspension was transferred into 250 ml plastic bottles and unbound antibodies were removed by centrifugation at 1,500 x g for 10 minutes at 15 °C (Beckmann coulter Avanti® J-26 x PI). The supernatant was kept for further analysis by flow cytometry and the cells were washed with 50 ml 0.9 % sodium chloride (NaCl; wash buffer) and transferred to a sterile 50 ml falcon tube, followed by centrifugation at the same parameters as above (Heraeus Sepatech Cryofuge 8500). This washing step was repeated four times, after which absorbed antibodies were eluted from the cell surface by incubating cells with 45 ml ice-cold citrate buffer (0.12 M Citrate, pH 2.5; elution buffer A) for 15 minutes on ice.

Detached IgGs were harvested by centrifugation at 7,000 x g for 10 minutes at 15 °C (Heraeus Sepatech Cryofuge 8500) and the supernatant was transferred into a clean 50 ml falcon tube, already containing 7.5 ml neutralizing buffer (1 M Tris- HCl, pH 9.0). To ensure the pH was around 7.4 the value was measured using a pH meter (pH 538, WTW) and adjusted if necessary.

2.2.6 Affinity Chromatography of Eluted Alloantibodies

The reversible antigen-antibody binding characteristic was used in the IgG affinity purification assay. In the procedure alloantibodies, present in sera of BNP dams were absorbed onto intact bovine kidney cell surface, and then eluted with treatment by a low pH buffer (see section 2.2.5). The resulting eluted antibodies were further concentrated via affinity chromatography over a protein G column in a Fast Protein Liquid Chromatography (FPLC) system (Smartline, Knauer Advanced Scientific Instruments).

50 ml of sample containing alloantibodies was injected for 45 minutes at 1 ml min^{-1} flow rate (Knauer Pump 1000). The column was then washed with binding buffer (20 mM potassium phosphate; pH 7.4) for 20 minutes at a flow rate of 2 ml min^{-1} in order to allow sufficient time and optimal conditions for the alloantibodies to interact with the column material. Subsequently, bound antibodies were eluted with elution buffer B (0.1 M glycine- HCl; pH 2.7) for 15 minutes at a flow rate of 1 ml min^{-1}. The FPLC

system continuously measures the absorbance at 280 nm and 214 nm (Knauer UV detector 2550) and collects 1 ml fractions (Knauer fraction collector 3050), when the elution process begins, into sterile Eppendorf tubes containing 120 µl neutralizing buffer (1 M Tris- HCl; pH 9.0).

2.2.7 Flow Cytometry

To demonstrate that sera from the specific cows indeed possess alloimmune antibodies, which bind to bovine kidney cells and lymphoblasts, flow cytometric measurements with sheep-anti-bovine-IgG conjugated with Fluorescein isothiocyanate (sheep-α-bovine-IgG^{FITC}; AbD Serotec) was performed.

Standard Fluorescent Activated Cell Sorting (FACS) protocols were followed. Briefly, 1 x 10^5 bovine kidney cells or lymphoblasts were re-suspended in 100 µl PBS containing 0.5 % foetal calf serum (FCS) and distributed into FACS tubes to which sera, colostrum or milk were added to a final dilution of 1:5 (if not stated otherwise), followed by one hour incubation at 4 °C. Unbound antibodies were washed off with 850 µl PBS containing 0.5 % FCS, followed by centrifugation at 1,500 x g for 5 minutes (Beckmann coulter Avanti® J-26 x PI). The supernatant was discarded and cells were washed again and then re-suspended in 20 µl PBS containing 0.5 % FCS and treated with 1:100 sheep-α-bovine-IgG^{FITC} (AbD Serotec) for 30 minutes at 4 °C. After the incubation time had passed, the previous washing step was repeated and cells were re-suspended in 100 µl PBS with 0.5 % FCS.

In parallel to the aforementioned samples, a negative control, containing only the secondary antibody was prepared. Another FACS staining was performed according to the same procedure described above, but in these experiments, cells were incubated with supernatant from IgG affinity purification assay, affinity elution (section 2.2.5) or the different protein G fractions captured (section 2.2.6).

Alternatively, cells were analysed by flow cytometry to detect presence of MHC-I with the mouse anti-MHC-I monoclonal antibody H58A (VMRD), with a wide species cross reactivity; the mouse anti-HLA (Human Leukocyte Antigen) monoclonal antibody w6/32 (kindly donated by Steffen Tenzer, University of Mainz) or with IL- A88 (AbD Serotec), an anti-bovine MHC class I monoclonal antibody. Primary antibody was detected with 1:100 secondary goat anti-murine PE-Cy5.5 conjugated antibody (invitrogen) in PBS with 0.5 % FCS.

Transduced e-GFP murine pre-B-cells, which express different BoLA I alleles (described in section 2.2.16) were also analysed by flow cytometry. The basic

protocol, as described previously, was adhered to. To eliminate any background binding and examine if there is preferential binding to transduced eGFP positive cells, transduced 38B9 cells and non-transduced 38B9 cells were mixed in a 1:1 ratio and treated with a 1:50 serum dilution. Binding was detected with a biotinylated anti-bovine antibody (AbD Serotec; 1:100) and streptavidin coupled to PE-Cy5.5 (Invitrogen; 1:1000) in PBS with 0.5 % FCS, thus reactive samples are GFP positive as well as PE-Cy5.5 positive. Fluorescence FL3 overlap was corrected by subtracting 0.6 % of FL1.

Median fluorescence intensity (MFI) was determined for each sample using a BD Lsr II Flow Cytometer or a BD Accuri C6 Flow Cytometer. The Accuri device uses a different log scale to display fluorescence data, which results in a baseline shift of two log scales between the two devices. To take this into account, figures using a BD Lsr II data were given an adapted scale (as indicated in the axis title, which modified reads: MFI (x 100)).

2.2.8 Flow Cytometric Detection of Complement- Activating Antibodies

To assess the complement activating activity of opsonising alloantibodies, a flow cytometry based complement-dependent cytotoxicity assay (Diaz et al. 2004) was adopted accordingly. Briefly, 1 x 10^5 human lymphoblasts, re-suspended in PBS containing 0.5% FCS, were incubated with heat inactivated (56 °C, 30 minutes) bovine sera at a final dilution of 1:5 for 45 minutes at 4 °C. Complement activation was assessed by adding 50 µl active or heat-inactivated rabbit complement, i.e. fresh rabbit serum. After 10 minutes at 37 °C, propidium iodide (PI, Sigma-Aldrich) was added, the samples were placed on ice and immediately analysed by flow cytometry. The cytometer settings were limited to run a fixed volume of 20 µl. For each serum sample the number of living, PI-negative cells (N) after incubation with active or heat-inactivated rabbit complement was determined. The percentage of cytotoxicity was calculated according to the following equation:

specific Lysis = $100\% \times (1 - N_{\text{active complement}} / N_{\text{inactive complement}})$.

2.2.9 Fluorescence Microscopy

Fluorescent microscopy was performed to test if alloantibodies involved in BNP pathogenesis bind to surface antigens on bovine kidney cells. Intact bovine kidney cells were detached as described before (section 2.2.2) and incubated with peak fraction of protein G affinity purified alloantibody (section 2.2.6). In the meantime, 50

µl Poly-L-Lysine, a synthetic amino acid chain regularly used as an attachment factor to enhance cell and protein adhesion, for example, in cell culture vessels, was added to a microscopic slide with wells (Diagnostic Slides, Menzel-Gläser). The substance was left to react for 30 minutes in a damp chamber and then sucked off with an aspirator. The pre-treated bovine kidney cells were then added and incubated for 30 minutes in the damp chamber. After this incubation time, 50 µl of the secondary antibody i.e. sheep-α-bovine-IgG^{FITC} (AbD Serotec; 1:100 in PBS containing 0.5 % FCS) was overlaid and left to bind for 1 hour in the damp chamber, in order to prevent samples from drying out and to circumvent photobleaching. Unbound material was then carefully sucked off and the slide was washed four times with 50 µl PBS. For analysis under the fluorescent microscope, 10 µl PBS was added and the cover slide was placed on top.

Alternatively, eGFP positive murine pre-B cells expressing BoLA class I (see section 2.2.16) were incubated with a monoclonal mouse anti-MHC class I antibody for 1 hour, washed twice and then treated with an anti-murine PE-Cy5.5-conjugated secondary antibody for 30 minutes and analysed by fluorescence microscopy.

2.2.10 Immunoprecipitation (IP)

In order to isolate and identify BNP antigens on bovine kidney cells or lymphoblasts, immunoprecipitation (IP) was performed as previously described (Deutskens et al. 2011; Foucras et al. 2011). The cells were labelled with 2 mM EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) for 30 minutes at 4 °C to prevent endocytic intracellular labelling, as described in the instruction leaflet. Subsequently, 100 mM glycine was added to stop the reaction. The cells were washed with PBS, adjusted to 2 x 10^6 bovine kidney cells ml^{-1} in PBS or 2 x 10^7 lymphoblasts ml^{-1} in PBS and treated with 300 µl of the respective bovine serum for 90 minutes at 4 °C on a rotator (NeoLab Rotator). Unbound and nonspecific bound antibodies were removed by four washing steps with PBS. The cells were then solubilized with 1 ml Lysis Buffer (1 % Triton X-100, 150 mM NaCl, 50 mM Tris and 5mM EDTA) and lysed in an ultrasonic bath (Laboson) containing ice for 10 minutes. The solution was clarified by centrifugation at 10,000 x g for 10 minutes and the supernatant was incubated with 35 µl protein G-sepharose beads slurry (GE Healthcare) and rotated for 90 minutes at 4 °C. The resulting precipitate was thoroughly washed four times with 1.5 ml ice-cold Lysis buffer and re- suspended in 40 µl Loading Buffer containing 6 M Urea, 62.5 mM Tris,

2 % SDS, 10 % Glycerol and 0.025 % Bromophenol Blue and subjected to SDS-PAGE under non- reducing conditions.

2.2.11 SDS-Polyacrylamide Gel Electrophoresis Analysis

Standard protocols for SDS-PAGE based on descriptions by Lämmli were followed. Briefly, in order to cast a 0.75 mm mini-gel, a 10 % resolving gel was prepared by mixing 3.1 ml distilled water, 1.85 ml 4 x lower buffer, 2.5 ml 30 % Acrylamide-Bis solution, 150 µl 10 % APS and 20 µl TEMED. In order to improve resolution, a 4 % stacking gel was poured on top, made with 1.5 ml distilled water, 0.626 ml upper buffer, 0.4 ml 30 % Acrylamide-Bis solution, 150 µl 10 % APS and 20 µl TEMED. The samples of interest were pre-incubated with non-reducing loading dye (table 1.3), loaded, and the gels were then run at 125 V for about 1:30 hours (Bio rad Mini Protean N^{TM}). Bands were made visible by Colloidal Coomassie staining (Roti$^{®}$ Blue, Carl Roth) and then subsequently, de-stained with 30 % methanol as instructed by the provider.

2.2.12 Western Blot Analysis

Samples separated by SDS-PAGE were transferred onto a nitrocellulose membrane (0.45 µm, Protran$^{®}$ BA85, Whatman) and blotted at 0.8 mA per cm^2 (Semi-Phor^{TM}, Hoefer Scientific Instruments). The blotted nitrocellulose membrane was blocked (for 2 hours on a shaker or overnight in a refrigerator) with 0.5 % Vegetable Extract in PBS containing 0.05 % Tween-20 (Blocking buffer) in order to reduce unspecific binding. The blot was developed with a mouse monoclonal antibody IL- A88 (1:2000 in blocking buffer), an anti-bovine MHC class I heavy chain antibody, or with w6/32 (1:10 hybridoma supernatant in blocking buffer), an anti-MHC class I monoclonal antibody, for 2 hour on a shaker. The membrane was then washed three times for 10 minutes with the blocking buffer and thereafter probed with a secondary horseradish Peroxidase conjugated anti-mouse antibody (Dianova; 1:3000 in blocking buffer) for 1 hour on a shaker. Unbound antibodies were removed after three washing steps for 10 minutes with blocking buffer. Peroxidase activity was then detected via Enhanced Chemiluminescence reagents (ECL, GE Healthcare) according to manufacturer's instruction.

The same membrane was then washed twice for 10 minutes with PBS containing 0.05 % Tween-20, stripped with mild stripping buffer, containing 200 mM Glycine, 0.35 % SDS and 0.8 % Tween 20 adjusted to a pH of 2.2, twice for 20 minutes,

washed again twice with PBS containing 0.05 % Tween-20 for 20 minutes and re-blocked with blocking buffer on a shaker. Biotinylated proteins were detected with 1:30000 streptavidin coupled to HRP (Dianova) in blocking buffer for 30 minutes on a shaker, followed by five washing steps for 5 minutes each and then visualization with ECL.

2.2.13 Bovine Leukocyte Antigen I (BoLA I) Sequencing

To find out if a specific BoLA I molecule is targeted by BNP alloantibodies, the BoLA I alleles expressed on the cell line, the primary source of alloreactivity, were identified by Reverse Transcription Polymerase Chain Reaction (RT-PCR).

Briefly, via RT-PCR with BoLA-specific primers (Thermo Scientific) the mRNA extracted from the bovine kidney cell line, using an mRNA extraction kit (RNeasy Mini Kit, Qiagen), was amplified and cloned into a pGEM-T easy vector (Promega). The forward primer in cooperates the *Bam*HI cleavage site followed by the start codon and the reverse primer, the *Xho*I cleavage site followed by the stop codon, leaving a sticky end and allowing easy ligation into the vector.

Commercial sequencing was performed with the BLASTn program from the European Molecular Biology Laboratory - The European Bioinformatics Institute (EMBL- EBI). In total, eight productive BoLA I alleles were identified, summed up in table 2.1.

Table 2.1: BoLA alleles sequenced from the PregSure® BVD production cell line

BoLA I Allele	Remarks
BoLA 3*05001	-
BoLA 3*01101	840 bp available, 100% match full productive sequence
BoLA 2*04801	100% match
Undefined (similar to 2*04801)	full productive sequence, 29 mismatches
Undefined (similar to 2*04601)	full productive sequence, 35 mismatches
Undefined (similar to 2*40801)	775 bp available, 6 mismatch; full productive sequence
Undefined (similar to 2*04801)	138 bp missing; full productive sequence
BoLA Nc3*50201	755 bp available, 100% match; full productive Sequence
BoLA Nc3*00101	844/845; full productive Sequence (short cut at the end)

2.2.14 BoLA I Cloning

Recombinant cell lines expressing the identified BoLA I alleles were generated by integrating a eukaryotic vector via retroviruses into murine pre-B cell lines (38B9-cells). The eukaryotic expression vector *pMyc*-IRES-eGFP possesses elements for expression in *E. coli* and eukaryotic cells (see plasmid map S5 in the supplements) and is a well-established system in the institute (Kitamura et al. 2003). The cloning experiments, including transfection and transduction, were performed as a part of the research project to achieve the Bachelor degree by Jacqueline Mauritz.

Briefly, 5 µl of the cloning vector, pGEM-T easy, containing BoLA I, and 5 µl of the expression vector *pMyc*-IRES-eGFP were digested with 1 µl of the restriction enzymes *Bam*HI and *Xho*I (Biolabs, Inc.) in 15 µl DEPC-H_20, 2.5 µl 10 x Buffer 4 and 0.5 µl BSA. The fragments were separated with a 1.5 % Agarose gel in 1 x TBE-Buffer and the respective bands i.e. *pMyc*: approx. 6000 bp and the insert approx. 1200 bp were excised and processed with the *Wizard SV Gel and PCR Clean-up System* kit, purchased from Promega, according to the instructions in the leaflet. 10 µl of the resulting BoLA I insert was then ligated with 7 µl *pMyc*-IRES-eGFP vector via the created sticky ends, by incubating the mixture with 2 µl 10 x Ligase Buffer and 1 µl T4 DNA Ligase (Biolabs).

5 µl of the resulting ligate was then transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen), via incubation for 30 minute on ice followed by heat treatment at 42 °C for 30 seconds and immediate cooling on ice. 1 ml SOC media was then added to the bacteria and left for one hour at 37 °C and 200 rpm. The cells were pelleted and streaked onto LB-selective media containing ampicillin over night at 37 °C.

Colonies were then screened in a screening PCR run using BoLA specific primers (2 mM dNTP Mix). A standard PCR protocol was used: 2 minutes at 95 °C for AmpliTaq Gold® DNA polymerase (invitrogen) activation; 30 cycles of 30 seconds at 95 °C, 30 seconds at 48 °C and 90 seconds at 72 °C; 10 minute at 72 °C.

Positive clones were further isolated and cultured in 200 ml LB-media containing ampicillin over night at 37 °C and 180 rpm and the plasmids retained according to instructions using the *NucleoBond Xtra Midi-Kit* (Marchery-Nagel). After drying, the DNA was stored in 50 µl TE-Buffer. An additional restriction digestion was performed as described previously and the plasmid DNA was then used for further transfection of a virion producing cell line, namely Plat-E cells.

2.2.15 Transfection of Platinum-E Cells

5 x 10^6 Plat-E cells (Cell Biolabs Inc.) were cultured in 12 ml SF-IMDM Media containing 10 % FCS. After 24 hours, the media was aspirated and the cells were treated with 8 ml SF-IMDM containing chloroquin (end concentration 25 µM) for 30 min at 37° C. Chloroquin increases transfection efficiency by inhibiting lysosomal DNAses.

For plasmid preparation: 20 µg isolated plasmid DNA was mixed with 920 µl $CaCl_2$ (0.2717 M) and 80 µl distilled water. 1 ml BES Buffer was added and the solution was mixed by blowing air bubbles with a pipette for 20 seconds.

The pre-treated DNA mixture was then incubated with the Plat-E cells at 37 °C overnight. After approximately 15 hours the media was exchanged with fresh SF-IMDM containing 10 % FCS and after 24 hours the supernatant containing virus particles was harvested.

2.2.16 Transduction of Murine Pre-B Cell Lines (38B9 cells)

Cell debris from the harvested supernatant (see section 2.2.15) was removed by centrifugation at 3,000 x g for 10 minutes at 4 °C, followed by another centrifugation step at 10,000 x g for 1 hour at 4 °C. The resulting virus pellet was re-suspended in 1.5 ml SF-IMDM media containing 2 % FCS and 5 µg ml^{-1} polypren (Sigma-Aldrich) and kept on ice. Polypren increases transduction efficiency by reducing electrostatic repulsion between virions and acid groups on the cell surface.

The so prepared virus was then slowly added to 38B9 cells pre-cultured in 2 ml SF-IMDM media containing 2 % FCS. The cell culture plate was centrifuged at 700 x g for 1 hour at 37 °C followed by a one hour incubation period at 37 °C. After the incubation period had elapsed, cells were treated with fresh cell media containing 2 % FCS.

Transduction efficiency was examined after 24 hours by fluorescence microscopy. Successful uptake of plasmid renders cells eGFP-positive which can be monitored at a wavelength of 488 nm. To obtain a culture of 100 % transduced GFP positive cells, a serial dilution was performed and the cells were co-cultured with a growth-arrested feeder cell population in SF-IMDM media. The feeder cells were generated by exposing non-transduced 38B9 cells to 30 gray radiation (Cäsium 137). Transduced and non-transduced cells were further analysed by flow cytometry as described in section 2.2.7.

2.2.17 Statistical Analysis

The median fluorescence intensity (MFI) i.e. the shift in fluorescence intensity of a specific cell population (in the cases presented, living lymphoblasts' or bovine kidney cells) was determined by the data software FlowJo or BD Accuri C6. To adjust the baseline shift of the two devices, a modified scale (adjusted reads MFI (x 100)) is shown for data obtained with the LSR II cytometer, to make the results comparable to the ones obtained with Accuri C6.

Experiments were performed in multiples of at least three independent repetitions and presented are representative data of these. When applicable, the p-value (significance level $\alpha = 0.05$) was calculated by the Mann-Whitney-Test in GraphPad Prism. Standard deviations are shown by error bars in the graphs. The cut off was determined by taking the geometric mean plus the 3-fold standard deviation. Pearson's test was used to calculate correlation coefficients.

3. Results

3.1 Effect of the vaccination scheme on PregSure® BVD induced alloreactivity and the incidence of Bovine Neonatal Pancytopenia

The first BNP cases observed and reported were in Bavaria and thereafter, there was an accumulation of cases reported in several regions in Germany and later on from other regions in the EU, as well as from Turkey and New Zealand (Lambton et al. 2012; Jones et al. 2013). Different scientific groups presented convincing data that BNP was a PregSure® BVD vaccine-induced neonate-maternal incompatibility phenomenon caused by colostral alloantibodies. The vaccine PregSure® BVD induces alloreactive antibody production. These alloantibodies are directed against cell surface antigens present on bovine cells and are a result of bioprocess related impurities, originating from the cell line used for virus propagation, and present as a contaminant in the vaccine. In EU states, where PregSure® BVD was not marketed, such as Switzerland, Austria and northern European countries, cattle remained unaffected by BNP.

Nonetheless, there was a great regional difference in the prevalence of BNP reported, not only between the different European countries, but also within member states of the EU. What was also puzzling was the three to four year gap noted between the launch of PregSure® BVD and the first reported incidences. To better understand the underlying reason for the regional differences and also confirm the role of PregSure® BVD in BNP induction, field samples from cattle treated with different BVD vaccines, cattle naturally infected with BVDV or cattle not immunized against BVDV were analysed.

Bovine lymphoblasts, produced by stimulating T-lymphocyte cell division of PBMCs extracted by Ficoll Paque from whole blood of healthy animals, were incubated with sera obtained from the field animals and a secondary antibody directed against bovine IgG that was coupled with FITC. This enabled the detection of surface bound IgG by exciting the bound fluorescein in a flow cytometer. The system was gated so that the respective Median Fluorescence Intensity (MFI) of living lymphoblasts could be determined.

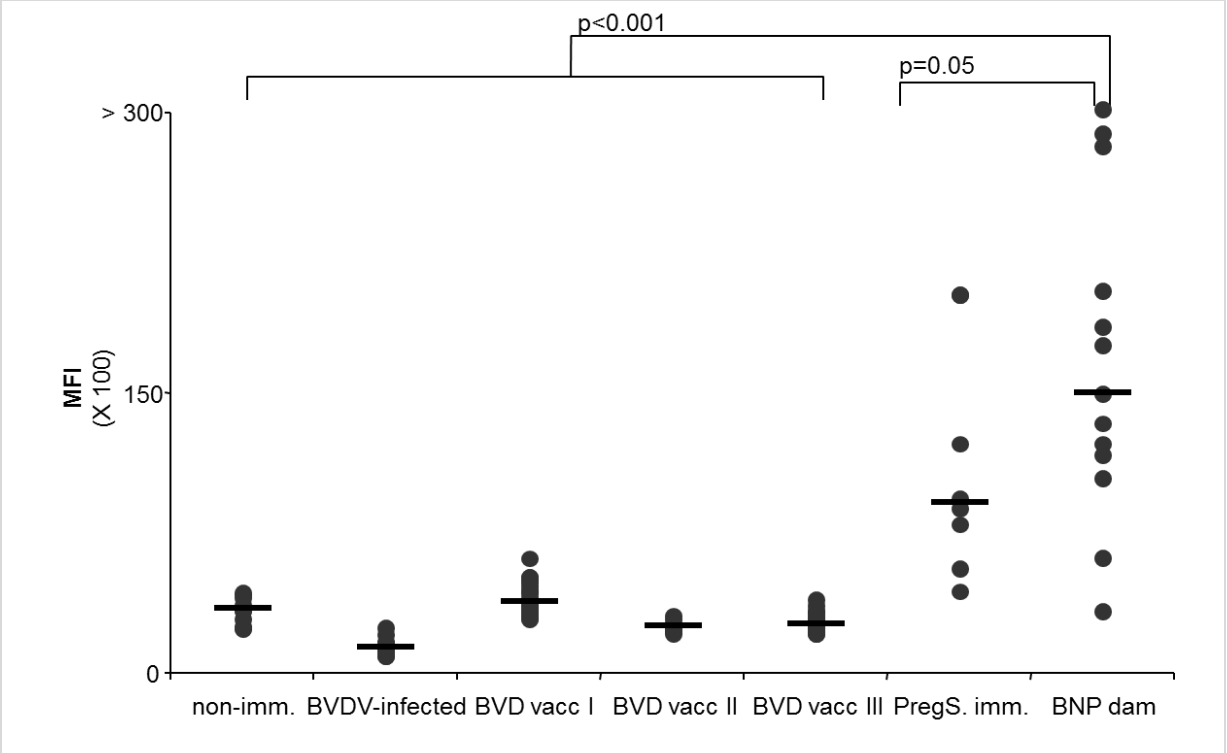


Figure 3.1:PregSure® BVD immunized animals possess high alloantibody titres.
Bovine lymphoblasts were incubated with sera of field animals not immunized against BVDV (non-imm.), BVDV infected animals, animals treated with different BVD vaccines (vac I – III, PregS) or animals that gave birth to a BNP calf (BNP dam). Surface bound antibodies were detected by flow cytometry using a FITC conjugated 2nd antibody and the Mean Fluorescence Intensity (MFI) was determined. Black dots represent the MFI of individual sera; the median for each group is presented as a horizontal black bar.

Only animals that were vaccinated with the incriminated vaccine PregSure® BVD, possessed antibodies that were bound to bovine lymphoblasts of a healthy individual. In contrast, animals vaccinated with alternative BVD vaccines (BVD vac I – III) did not show any or only minor reactivity to the bovine cells; neither did naturally BVDV infected animals nor non-BVDV immunized animals. Confirmed BNP dams, i.e. animals that were vaccinated with PregSure® BVD and previously gave birth to a calf affected by BNP, had a significantly higher alloantibody titre compared to animals that were only vaccinated with PregSure® BVD but did not have BNP calves (PregS imm), as presented in figure 3.1. These data confirmed that the vaccine, PregSure® BVD, induced the production of alloantibodies which bound to surface structures on bovine cells.

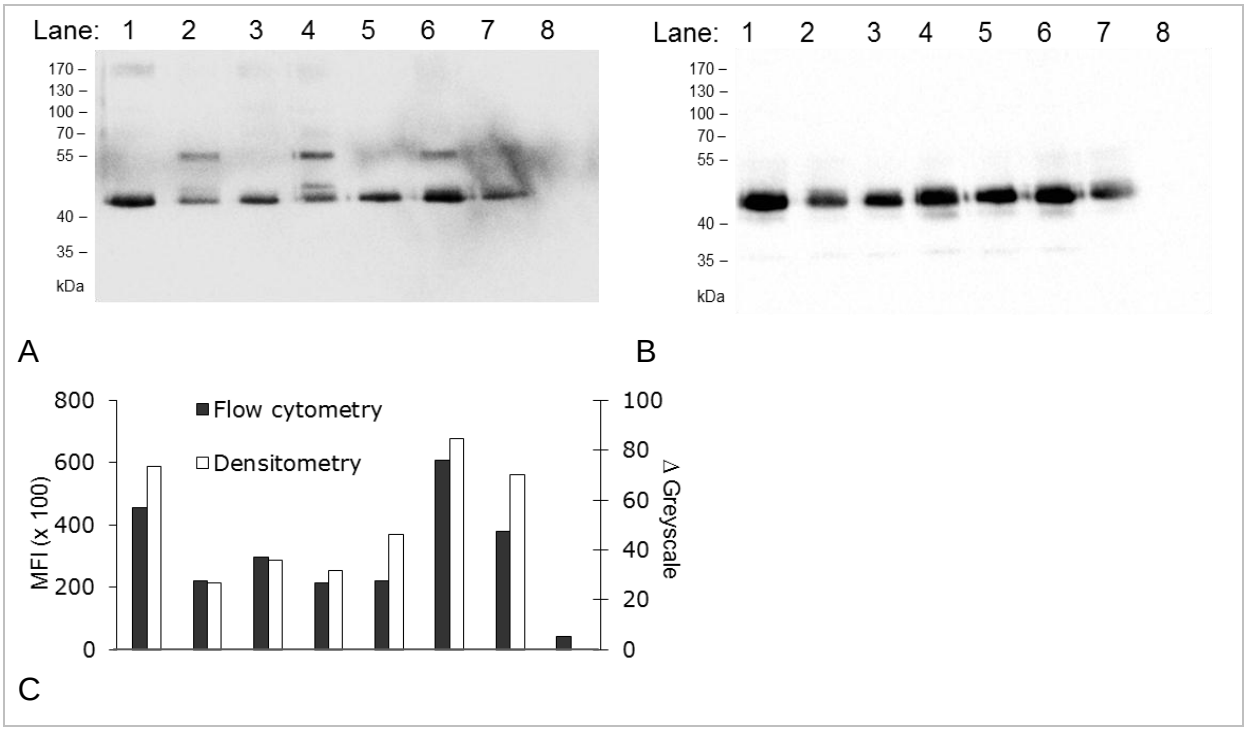


Figure 3.2: Immunoprecipitation of BNP alloantibodies with the production cell line. Intact bovine kidney cells, from the cell line used to produce PregSure® BVD, were extracellularly biotinylated and incubated with serum of vaccinated bulls (lane 1 – 5), confirmed BNP dams (lane 6 – 7) or an unaffected control animal (lane 8) and precipitated with protein G beads. The precipitates were separated by SDS-PAGE and analysed by western blot using (A) HRP conjugated streptavidin and (B) IL- A88, a monoclonal anti-bovine MHC class I antibody. (C) The corresponding MFI determined by flow cytometry (left x-axis, black bars) and the grey scale determined by densitometry (right x-axis, white bars) of the respective sera presented in the western blot.

The bovine kidney cell line used for the viral vaccine strain propagation is present as a bioprocess impurity in the vaccine. Therefore, the cell line is considered to be the primary source of BNP alloreactivity. When the antigen present on the cell line is determined, then the corresponding antigen(s) present on bovine peripheral blood cells and bone marrow stem cells, which possess part cross reactivity, can be identified. In order to identify the target antigen(s) of BNP inducing alloantibodies the bovine kidney cell line – the cell line used for PregSure® BVD production – was therefore the main focus of investigation.

The cell line was extracellularly biotinylated and membrane proteins bound to antibodies present in sera of experimentally PregSure® BVD vaccinated bulls, BNP dams or a control animal, were precipitated with protein G beads.

Animals vaccinated multiply with PregSure® BVD, as well as BNP dams, precipitated a band at ~ 43 kDa (Figure 3.2 A). Densitometry measurements of the greyscale observed with ECL and Streptavidin^{HRP}, compared with the MFI observed in the flow

cytometric experiments revealed a 94 % correlation (Figure 3.2 C). Thus, the surface reactivity observed by flow cytometry was mostly caused by antibodies, which precipitated a membrane protein with a molecular weight of ~ 43 kDa.

By western blot this band was confirmed to be bovine MHC-class I (BoLA I) with the monoclonal antibody IL- A88, as depicted in figure 3.2 B. This was in accordance with previous publications (Deutskens et al. 2011; Foucras et al. 2011) and also with LC-MS-MS analysis using the in-house proteomics platform (data not shown).

In Germany, the majority of BNP cases were reported in Bavaria. In contrast, in Lower Saxony, for example, there were significantly lower numbers of reported BNP cases. Considering the marketing data provided by the vaccine manufacturer, there were theoretically 6 BNP cases per 100,000 doses sold in Lower Saxony, while in Bavaria there were 99 BNP cases per 100,000 doses. The main difference between these two German federal states is the BVD vaccination regimen.

Some federal states use inactivated vaccines and adhere to the instructions provided. On the other hand, other states have a modified BVD vaccination scheme. This program is the so-called two-step vaccination scheme because it consists of priming with an inactivated vaccine and boosting with a live BVD vaccine (Moennig et al. 2005). Bavaria is an example of a federal state that performs the conventional strategy and Lower Saxony is an example of a state that enforced the two-step regimen.

	# 1	# 2	# 3	# 4
T0= day 0	Inactivated BVD vaccine I	Inactivated BVD vaccine II	**PregSure**	**PregSure**
T1= day 21	Inactivated BVD vaccine I	Live BVD vaccine	Live BVD vaccine	**PregSure**
T2= day 42	Inactivated BVD vaccine I	Live BVD vaccine	Live BVD vaccine	**PregSure**
T3= day 63	Blood sampling	Blood sampling	Blood sampling	Blood sampling

Figure 3.3: Experimental vaccination scheme.
Four groups of five bulls each were vaccinated with different commercially available vaccines against BVD using the depicted vaccination scheme. Sera were collected before the first and after each immunization.

To prove that the epidemiological discrepancy observed in BNP was due to differences in the use of PregSure® BVD, we experimentally mimicked the different vaccination strategies. Priming was performed with an inactivated vaccine, including

PregSure® BVD, and two booster shots were given of either an inactivated vaccine (according to the vaccination instructions) or a live vaccine, as it is done according to the two-step vaccination scheme, and summarized in the graphics in figure 3.3 .

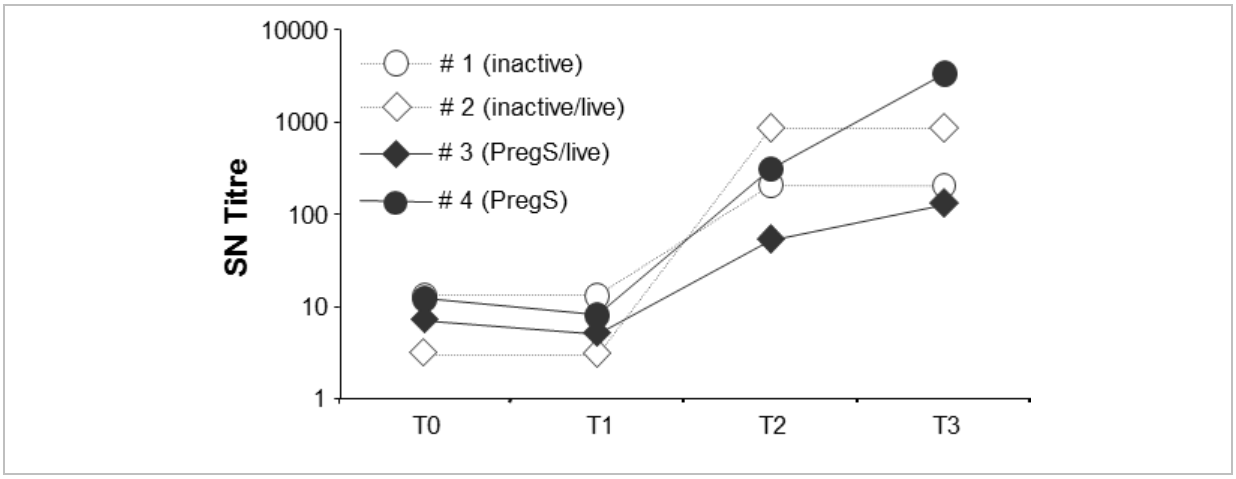


Figure 3.4: Anti-BVDV titres after vaccination using different vaccines.
Serum samples of experimentally immunized cattle were analysed for BVDV-neutralizing antibodies by SNT. The development of the median titres for each immunization group is shown over time. Open circles represent group #1, open diamonds group #2, filled diamonds group #3 and filled circles represent group #4.

Animals vaccinated thrice with PregSure® BVD developed a very high anti-BVDV titre in comparison to the other vaccines used. This could be demonstrated by SNT (Figure 3.4) and confirmed previous publications (Raue et al. 2011). Nonetheless, all four vaccination regimes led to a clear titre increase already after two immunizations.

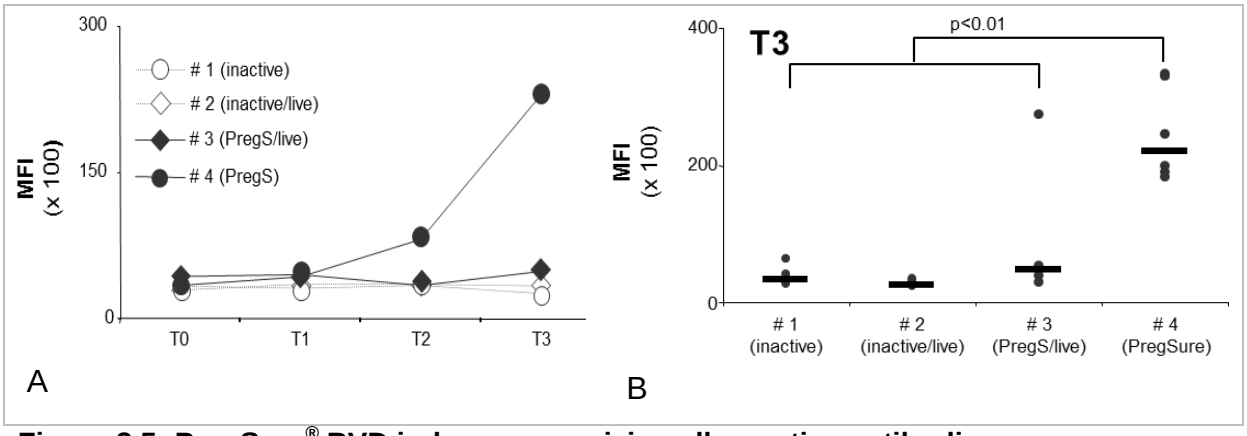


Figure 3.5: PregSure® BVD induces opsonizing alloreactive antibodies.
Bovine lymphoblasts were treated with individual sera and surface bound alloantibodies were detected. (A) Presented is the MFI for each immunization group before the first immunization (T0) and over time after each immunization (T1 – T3). Open circles represent the median for group #1, open diamonds for group #2, filled diamonds for group #3 and filled circles represent group #4. (B) Individual results are shown for the last time point (T3): Black dots represent the MFI of individual sera; horizontal black bars the median for each group.

In order to analyse which experimental group produced alloantibodies, that recognize surface structures on bovine lymphocytes and are known to be associated with BNP, sera of these differently treated animals were further examined by flow cytometry. Similar as before, pre-treated bovine lymphoblasts were examined for the presence of surface bound opsonizing alloantibodies.

Only the group of animals which received three PregSure® BVD vaccinations, developed alloantibodies, especially after being vaccinated several times (Figure 3.5 A). Three immunizations seem to be a requirement for a full BNP alloantibody titre to develop. This finding explains the lag period observed between PregSure® BVD market authorization and the first reported BNP cases. The basic immunization consists of two vaccinations; a booster immunization is given one year later, as in accordance with the instructions by the manufacturer.

Figure 3.5 B portrays individual MFI values at time point three i.e. after the third vaccination. At the group level there was a highly significant difference between group # 1 - 3 and group # 4. It was clearly observable that three PregSure® BVD vaccinations induced significantly higher alloantibody titres but nonetheless, one individual only primed once with PregSure® BVD (group # 3) also developed BNP alloantibodies comparable to group # 4 (figure 3.5 B).

This was also confirmed with the IP data performed with the hyperimmune sera obtained after the third vaccination. As shown in figure 3.6 A, this individual (animal # 1) precipitated the band at 43 kDa. All animals in Group IV i.e. the animals that were vaccinated thrice with PregSure® BVD, precipitated the same band, whereas only this one animal in group # 3 did so. Thus, the IP results could again be correlated to the previously determined flow cytometric signal. The band at about 58 kDa was observed with several pre-immune sera and respective negative controls (not shown), and was not associated with flow cytometric results, thus it was considered to be irrelevant in the context of BNP and obtained results.

The band at 43 kDa was confirmed to be BoLA class I with the monoclonal anti-BoLA I antibody IL- A88, as presented in the figure 3.6 B.

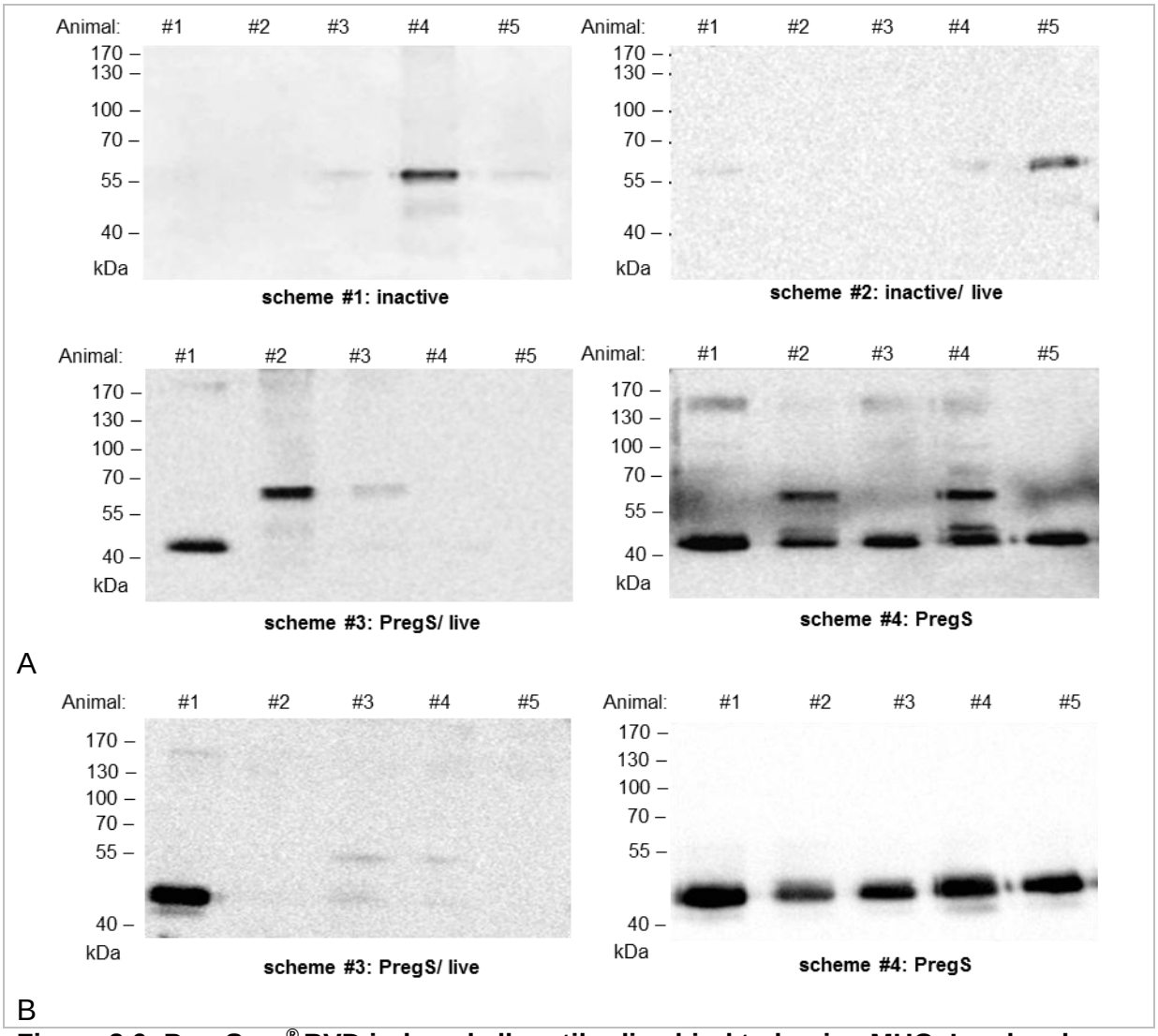


Figure 3.6: PregSure® BVD induced alloantibodies bind to bovine MHC- I molecules.
(A) After three immunizations serum samples of experimentally immunized cattle were analysed for their specificity by immunoprecipitation. Cells of the production cell line of PregSure® BVD were extracellularly biotinylated and incubated with the respective sera. The immunoprecipitate was analysed with a streptavidin-HRP conjugate. The four blots show individual immunoprecipitates for each group. (B) The blots of group #3 and #4 were also developed with IL- A88, an anti-BoLA I antibody. BoLA class I heavy chain has a molecular weight of approximately 43 kDa.

The observed IP and flow cytometric reactivity was directed only against the bovine kidney cell line used for PregSure® BVD production, as shown in figure 3.7 A. There was minimal cross reactivity to the other cell lines used to produce the alternative vaccines (figure 3.7 B and C), although also these cell lines expressed BoLA class I to a similar (and even higher) extent, when analysed with the monoclonal antibody H58A, an anti-MHC-I monoclonal antibody, as seen in figure 3.7 D.

A dam that already gave birth to a calf affected by BNP, had a significant stronger reactivity to surface structures expressed on the bovine kidney cell line used to

produce PregSure® BVD (figure 3.7 E). Whist minimal to no binding was observed with the cell lines used to produce the alternative vaccines.

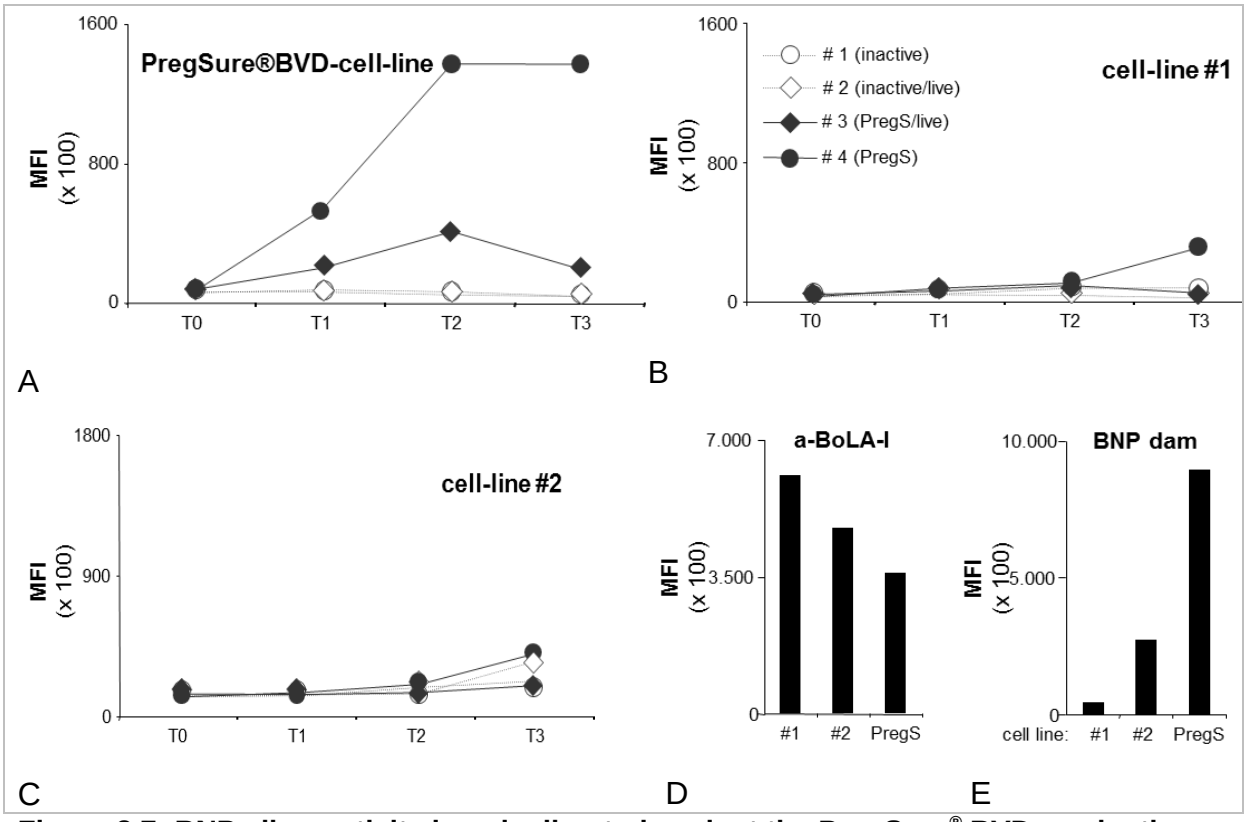


Figure 3.7: BNP alloreactivity is only directed against the PregSure® BVD production cell line.

Flow cytometric reactivity of the groups treated with the different BVD vaccination schemes against (A) the PregSure® BVD production cell line and (B, C) the cell lines used to produce the other vaccines (cell line #1 and #2). (D) BoLA I expression levels in the different cell lines determined by an anti-MHC- I monoclonal antibody. (E) A confirmed BNP dam reactivity to the different cell lines.

3.2 BoLA I Antibodies and the induction of Bovine Neonatal Pancytopenia – A twin calves study

The first indications that MHC-I is one possible BNP alloantigen were published in 2011 (Foucras et al. 2011; Deutskens et al. 2011). Our results further clearly demonstrate that PregSure® BVD vaccination induce the production of BNP anti-bovine MHC- class I (BoLA I) alloantibodies. However, the broad expression of BoLA I molecules on all nucleated cells, as well as thrombocytes, seems to be incompatible with the observation that only circulating blood cells and haematopoietic progenitor cells are specifically targeted in BNP.

Table 2.2: Anamnestic Information of twin calves born on a farm in Germany.

	Calf # 1	**Calf # 2**
16.10.2011	normal birth	normal birth
26.10.2011	**†** – clinical symptoms of **BNP** – *post mortem:* - haemorrhages - complete destruction of the red bone marrow	healthy
20.11.2011	–	**†** – **No** symptoms of BNP – *post mortem:* - purulent pneumonia - fibrinous perikarditis - **normal appearance of the bone marrow**

Fortunately, we obtained samples from a highly interesting twin calf case observed on a farm in Germany. The twin calves were born healthy in October, 2011 and obtained approximately 3 litres of fresh maternal colostrum shortly after birth. Their dam had received three vaccinations with PregSure® BVD in July and August, 2008 and a booster immunization in June, 2009. Shortly after birth one calf (calf # 1) died after showing clinical signs of BNP. Post mortem diagnostics revealed haemorrhage and extensive destruction of the haematopoietic tissue, similar to findings observed in confirmed BNP cases. The sibling remained healthy without any observable abnormal signs but five weeks after birth it died. The cause of death was due to infection of several organs but the bone marrow, a clear indicator for BNP, appeared normal at the time of the necropsy (Table 2.2).

Figure 3.8: The healthy calf ingested opsonizing alloreactive BNP antibodies.
(A) Cells of the bovine kidney cell line used for PregSure® BVD production were incubated with serum of calf #2 (bold line) or a reference serum of a confirmed BNP dam (dotted line). Mean fluorescence intensity (MFI) was determined by flow cytometry. Secondary antibody only (grey line) served as a control. (B) Immunoprecipitation of the bovine kidney cell line with serum from calf #2 and the reference BNP dam (ref.) revealed a specific band at 43 kDa, which is absent with the unvaccinated control (ctrl).

Flow cytometric analyses revealed that the healthy twin calf did receive colostrum, as the alloreactivity observed with the calves' serum was very similar to the binding pattern of serum from a BNP dam that was used as a positive control (Figure 3.8 A). Furthermore, as with sera from BNP dams, we could confirm that the calves' serum precipitated BoLA class I molecules, as depicted in the western blot in Figure 3.8 B.

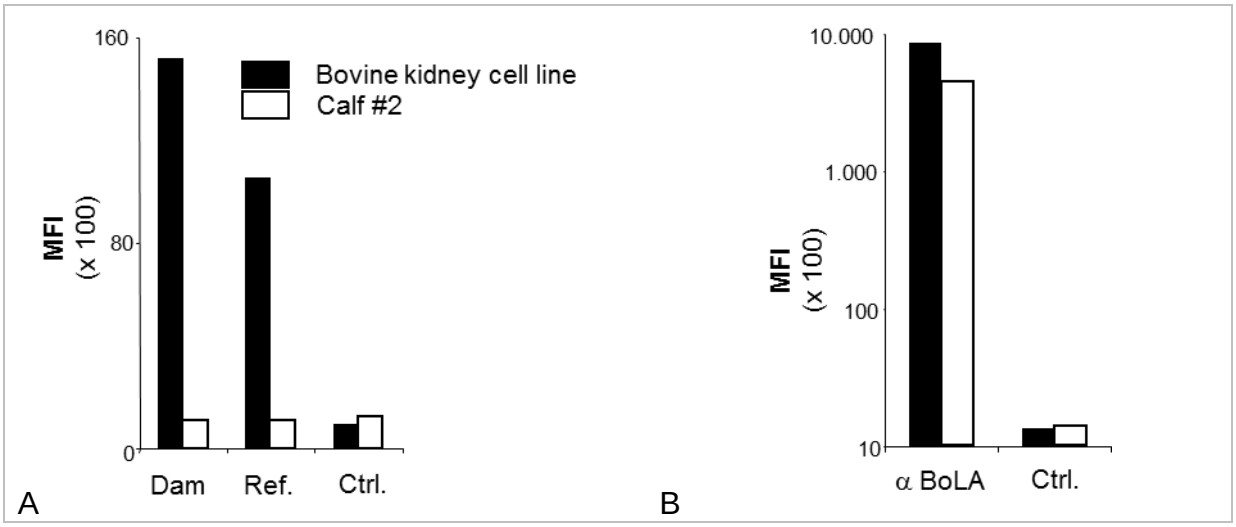


Figure 3.9: Lymphoblasts of the healthy calf are not recognized despite high level BoLA I expression.
(A) Sera of the dam of calf #2 (dam) and the reference BNP dam (ref), were incubated with lymphoblasts of calf #2 (white bars) or the bovine kidney cell line (black bars). Alloantibody binding was determined by flow cytometry. (B) BoLA I expression level was determined using a BoLA I specific, monoclonal antibody. Bars represent individual MFI-values for the lymphoblast's of calf #2 (white bars) and for the bovine kidney cell line (black bars).

Although the calf received sufficient alloantibodies, the alloantibodies of the dam – even though highly reactive to the bovine kidney cell line – did not interact with the cells of the calf (Figure 3.9 A). This was, however, not due to differences in the expression level of BoLA I molecules because both, the lymphoblasts of the calf and the bovine kidney cell line, expressed similar levels as confirmed by flow cytometry using H58A, a MHC class I monoclonal antibody, as shown in figure 3.9 B. Unfortunately, any additional experiments could not be performed due to limitations in sample amount and poor growth of the calves' lymphoblasts.

From these observations we conclude that the dam of the twin calves had a sufficient alloantibody titre to induce BNP in her progeny because one twin succumbed to the syndrome. This was also in accordance with the dam's vaccination history of repeated PregSure® BVD injections and confirms the previous hypothesis that three vaccinations (i.e. basic immunization with one booster) are sufficient to induce a long-lasting alloimmune response. The fraternal twin also ingested sufficient amounts of colostrum to develop a high BoLA I specific alloantibody titre. However, in this calf no pathology occurred because the maternal alloantibodies were not specific for the set of BoLA I alleles the calf expressed.

3.3 BNP associated alloantibodies recognize individual BoLA I alleles

As we observed that BNP associated anti-BoLA I antibodies are obviously variant specific, we sought to define the fine specificity of these antibodies. Up to date, six gene loci for BoLA I have been mapped on chromosome 23, which means an individual can carry up to 12 different BoLA class I alleles (Robinson et al. 2005; Birch et al. 2008). To identify if there is a specific BoLA I molecule targeted by BNP associated alloantibodies, we isolated, amplified and sequenced the BoLA I genes of the production cell line by RT-PCR using BoLA I specific primers.

The resulting eight productive BoLA I alleles (listed in table 2.1) were cloned into the eukaryotic expression vector *pMyc*-IRES-eGFP and used to transform chemically competent *E. coli.* After successful transformation of competent *E. coli* with the various *pMyc*-BoLA plasmids, restriction digest, screening PCR and plasmid midi preparation, the nucleic acid yield was determined by measuring the absorption (OD600 DiluPhotometer™, IMPLEM). The concentration of the different plasmids containing the different BoLA I alleles was between 0.7 µg μl^{-1} to 3.0 µg μl^{-1} (summarized in Table S1, supplements) i.e. enough for transfection of the Plat-E retroviral packaging cell line. The packaging cell line produces a high-titre of retroviruses that contain the produced plasmids encoding the individual BoLA I genes. These viral particles were further used to transduce the murine pre-B cell line, 38B9.

The cloning experiments, including transfection and transduction, were part of a BSc degree project performed by Jacqueline Mauritz entitled “Eukaryotic expression of bovine leucocyte antigen for the determination of the specificity of BNP-alloantibodies” (BSc in Molecular Biology; Johannes Gutenberg-University- Mainz).

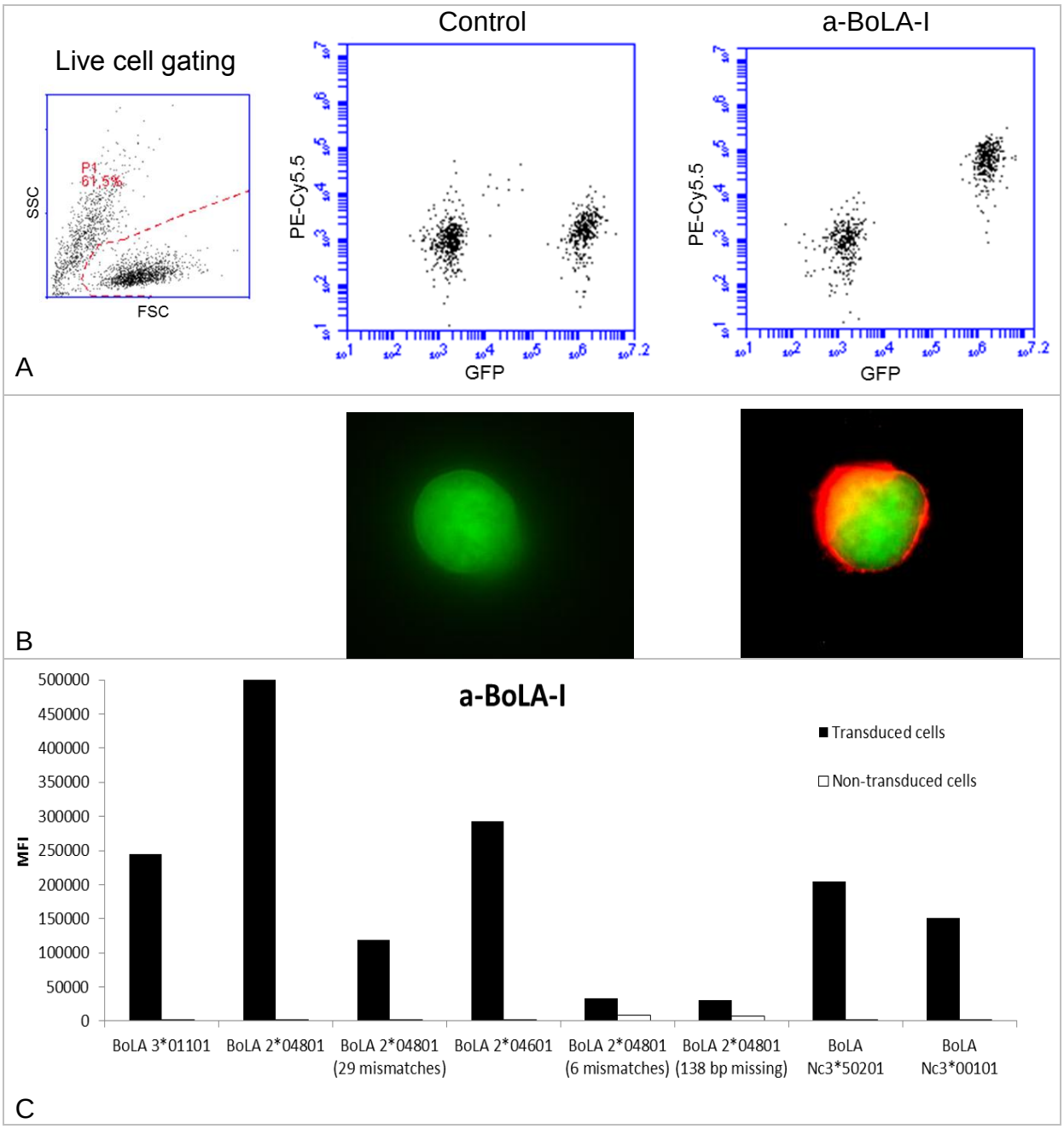


Figure 3.10: Recombinantly produced murine cell lines express BoLA class I.
BoLA I genes were cloned and stably expressed in a murine pre-B cell line using a MoMLV-based transduction system, using the *pMyc*-IRES-GFP vector. The cells were treated with a monoclonal anti-BoLA antibody and an anti-murine PE-Cy5.5 conjugated secondary antibody and analysed by (B) fluorescence microscopy and (A) flow cytometry: Live cell gating; transduced cells and non-transduced cells were mixed in a 1:1 ratio to determine preferential binding to BoLA I. Non-transduced cells are GFP and PE-Cy5.5 negative (bottom left corner), transduced cells are GFP positive (bottom right corner) and additionally PE-Cy5.5 positive if antibodies have bound (upper right corner). (C) MFI of 38B9 cells treated with an anti-MHC I mAb.

The transduction efficiency of 38B9 cells with virus particles produced by Plat-E cells was monitored by fluorescence microscopy, as the BoLA I gene was incorporated in front of the IRES, followed by eGFP (see plasmid map S5 in supplements). Therefore, green fluorescence was indicative for a successful transduction. To further prove that the cells expressed the different BoLA I alleles on their surface, the cells were treated with a monoclonal anti-BoLA antibody and an anti-murine PE-Cy5.5 conjugated secondary antibody and then analysed by fluorescence microscopy (figure 3.10 B) and flow cytometry (figure 3.10 A and C). The expression of all BoLA I alleles, except for BoLA 2*40801 (6 mismatches) and BoLA 2*40801 (138 bp missing), could be confirmed, as observed by the high MFI with the anti-BoLA monoclonal antibody. In case of the two exceptions, binding was considerably weaker.

After having established the transduced cell lines, we went ahead to analyse the fine specificity of BoLA-reactive, BNP-associated alloantibodies. To this end, we tested the recombinant cells in a flow-cytometric assay against a well-defined panel of cattle sera from a heavily BNP affected herd in North Rhine Westphalia. The herd was observed for three years, mainly by Dr. med. vet. Mark Holsteg of the Cattle Health Service NRW.

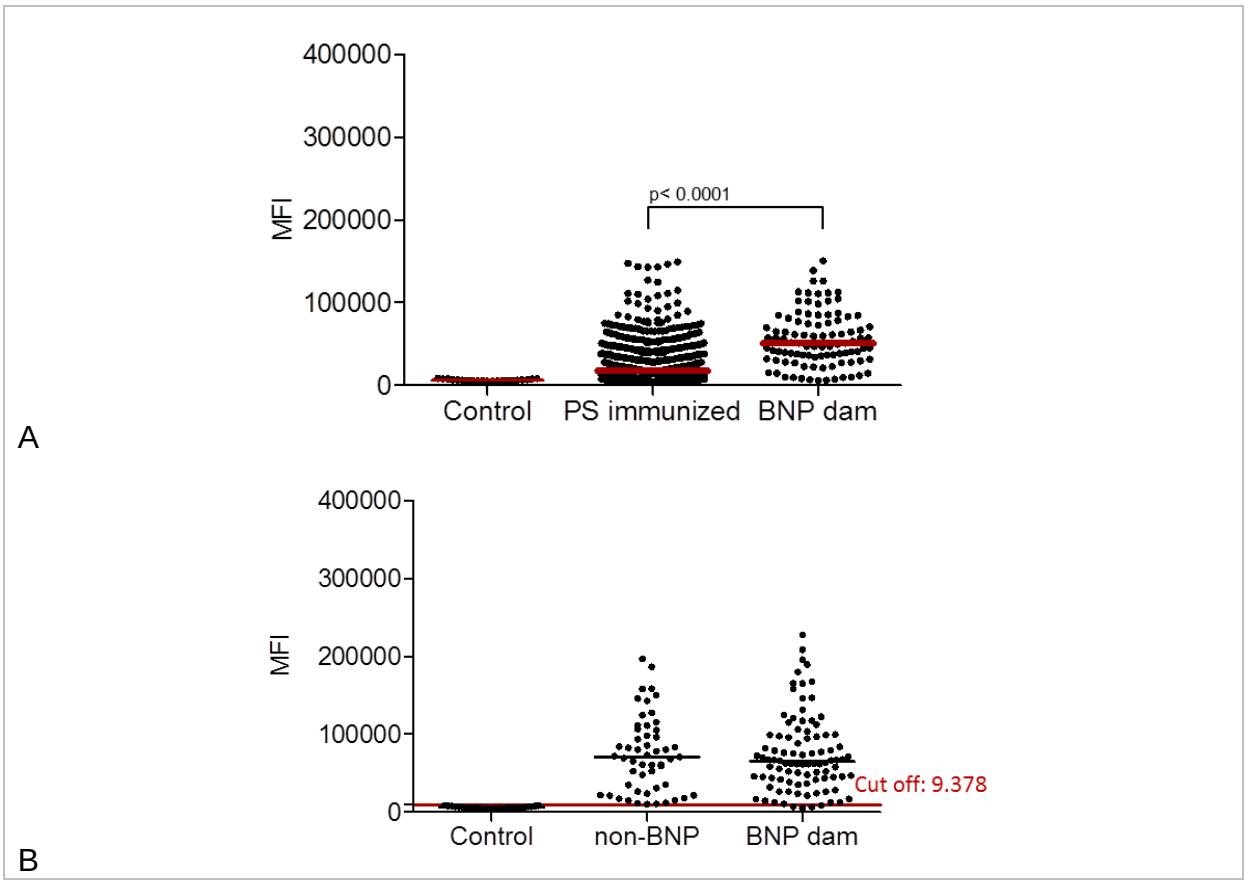


Figure 3.11: Flow cytometric reactivity to the production cell line.
Depicted is the MFI of animals not vaccinated with PregSure® BVD (control), animals vaccinated (PS immunized) and animals that gave birth to bleeder calves (BNP dam). Black dots represent the MFI of individual sera (A) BNP dams have a significantly higher BNP alloantibody content. The red bar represents the median value for each group (B) The groups were modified into control (n= 21), highly reacting PregSure® BVD vaccinated animals that did not give birth to BNP calves (non-BNP, n= 94) and BNP dams (n= 80). The median of each group is presented as a horizontal bar. The cut off (red line) was determined using the geometric mean of the control group plus 3 times the standard deviation.

The cattle herd consisted of 969 animals, out of which 21 animals were not immunized against BVDV and these served as the control group. The rest were vaccinated with PregSure® BVD, whereby 851 did not give birth to calves affected by BNP (non-BNP) and 97 dams gave birth to bleeder calves i.e. BNP dams. Presented in figure 3.11 A is the MFI of these groups to the production cell line. Confirming previous findings, BNP dams had a significantly higher BNP alloantibody titre.

From these sera, 94 BNP dams and 80 non-BNP dams were selected to test whether high reacting non-BNP dams targeted different BoLA I alleles as compared to BNP dams. Using these modified groups, the difference between the MFI of sera from high reacting non-BNP dams and BNP dams to the production cell line, was not significant anymore (figure 3.11 B).

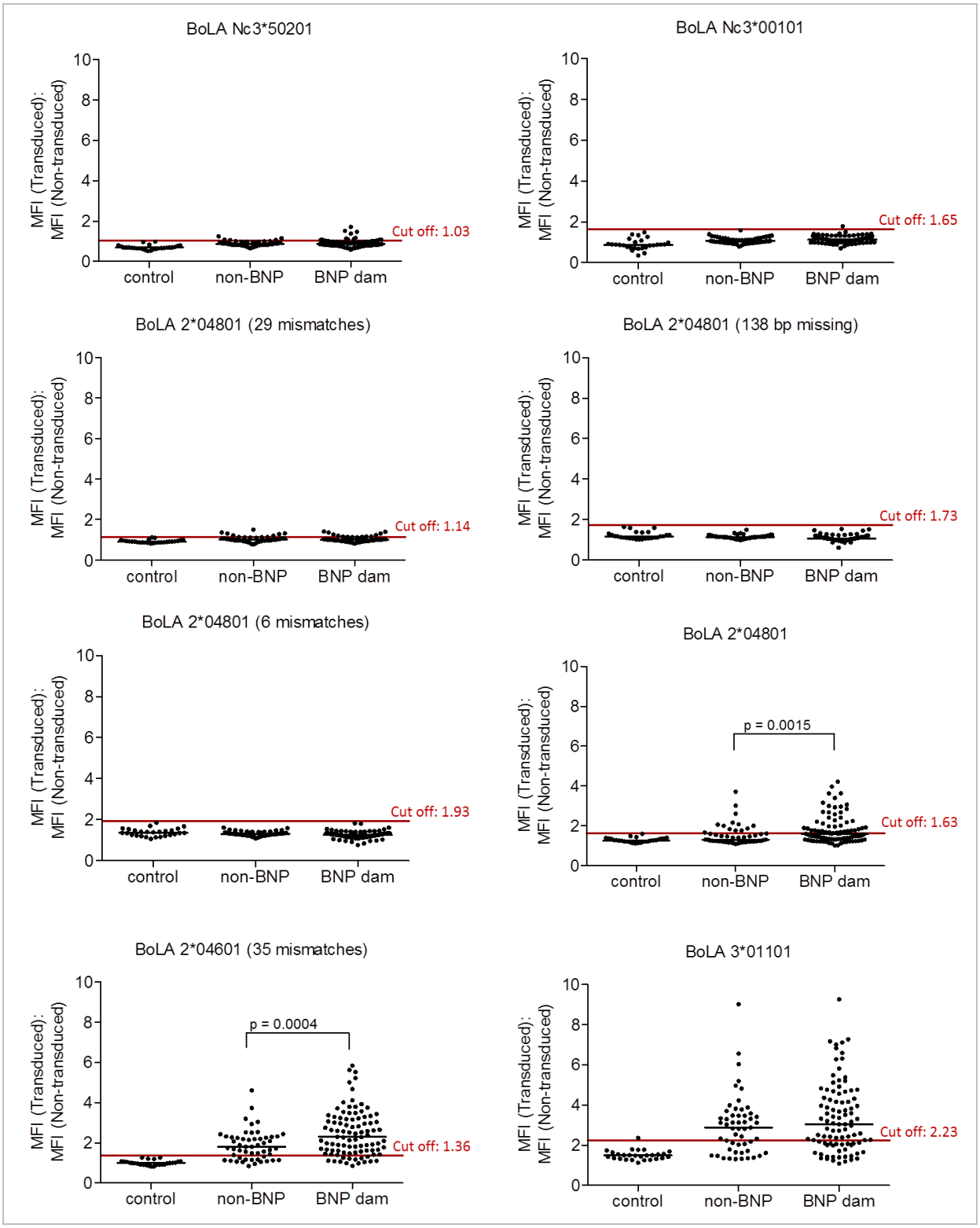


Figure 3.12: Flow cytometric analysis of recombinant BoLA I expressing cell lines. Presented is the quotient of the MFI (of transduced 38B9 cells)/ MFI (non-transduced 38B9 cells). The cut off (red line) is calculated by taking the geometric mean of the control group plus 3 times the standard deviation. Symbols represent the MFI for individual serum samples and the median of each group is presented as a horizontal black bar. Three BoLA I alleles are recognized by BNP dams and high reacting PregSure® BVD immunized non-BNP dams only.

Of special interest to us was to see if animals that react strongly to the bovine kidney cell line but did not give birth to BNP calves (non-BNP) recognized different BoLA I alleles in contrast to dams that already gave birth to calves affected by BNP (BNP dam). To this end, a flow-cytometric assay with the recombinant BoLA I expressing cell lines was established.

With individual immune sera, we observed considerable background staining also with non-transduced cells and in order to take this into account, we chose to add non-transduced and transduced cells simultaneously into one well. Therefore, the results are presented as the quotient of the MFI (of transduced 38B9 cells)/ MFI (non-transduced 38B9 cells). Thus an increased or preferential binding of antibodies to transduced cells, in contrast to non-transduced cells, can be directly determined.

Using a control group consisting of animals that were not vaccinated, a cut off was determined. This was calculated by taking the geometric mean of the group plus the 3-fold standard deviation. The cut off is presented as a red line in the graphs 3.12.

From the eight transduced cell lines, we identified three cell lines that were frequently recognized. Recombinant cells expressing the BoLA alleles 2*04801, BoLA 2*04601 (35 mismatches) and BoLA 3*01101 were often recognized only by non-BNP dams and BNP dams but not by the control group.

No principle difference that could explain the differential clinical observations in the offspring could be defined. However, it was noted that the overall number of alleles recognized was higher in BNP dams compared to non-BNP dams i.e. 55% of BNP dams recognized 2 or more alleles, whereas only 31% of non-BNP dams targeted 2 or more alleles, as summarized by the percentage of cross reactivity in table 2.3.

Table 2.3: Percentage of number of BoLA I alleles recognized

Number of alleles	0	1	2	3	4
Non-BNP dams	27 %	42 %	17 %	12 %	2 %
BNP dam	14 %	31 %	37 %	15 %	3 %

Allele 2*04801 was recognized by 25 % of all non-BNP dams and 45 % of all BNP dams. BoLA 2*04601 (35 mismatches) was recognized by 73 % of all non-BNP dams and 87 % of all BNP dams, whereas BoLA 3*01101 was recognized by 65 % and 68 % of all non-BNP dams and BNP dams, respectively.

Overall, there were more sera that recognized BoLA 3*01101 and BoLA 2*04601 (35 mismatches), compared to BoLA 2*04801. The sequence similarity between these

different alleles is over 90 %, therefore we did a sequence alignment to compare differences and possibly deduce which epitope might be recognized by PregSure® BVD associated alloantibodies. Depicted in figure 3.13 is the alignment of these three BoLA alleles. Highlighted are the signal peptide, the alpha-1, the alpha-2 and the alpha-3 domain, as well as the transmembrane region.

Since BoLA 2*04801 was recognized by far fewer animals (figure 3.12), we focused on amino acid differences noted between this allele and the two other BoLA alleles, BoLA 3*01101 and BoLA 2*04601 (35 mismatches).

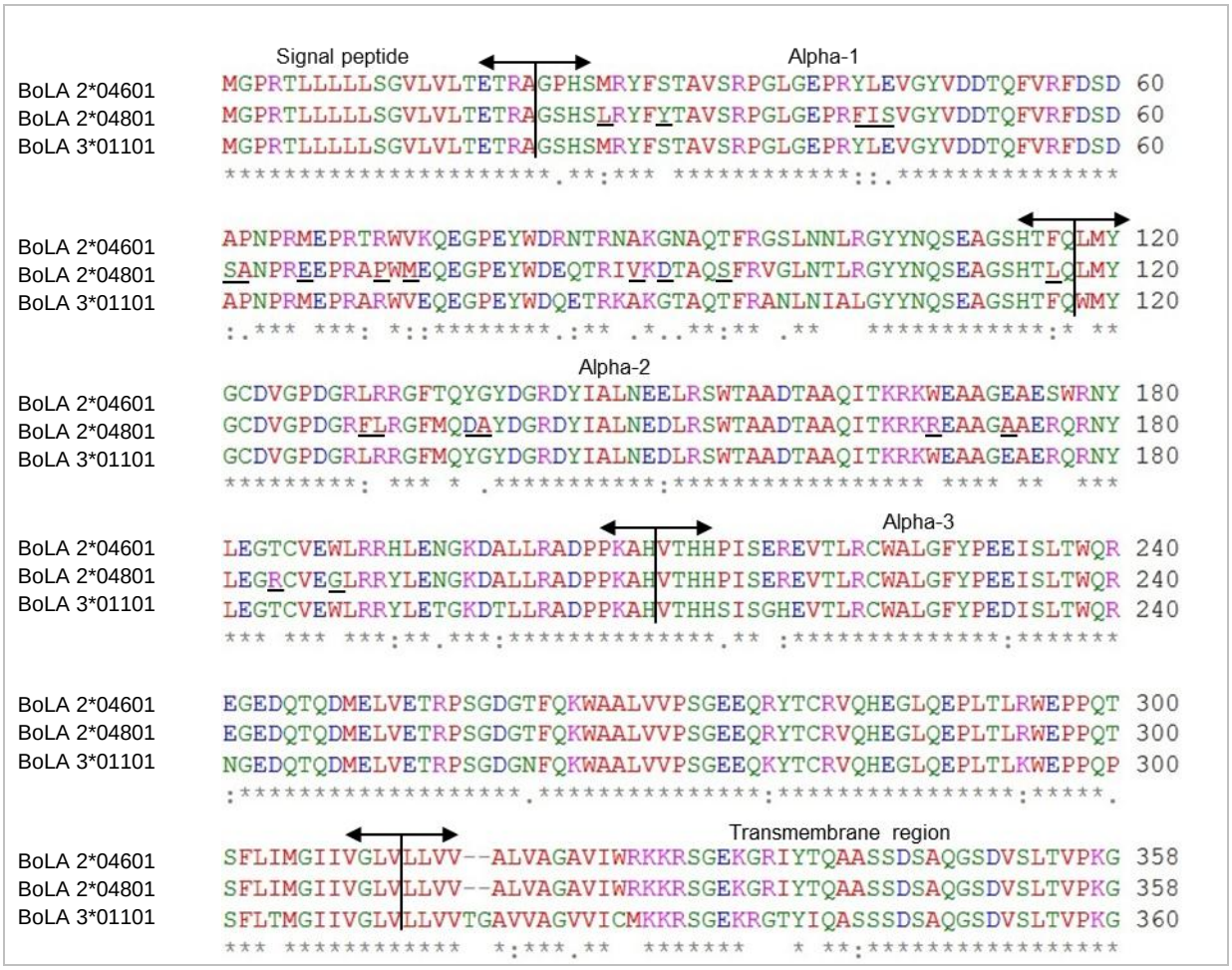


Figure 3.13: Multiple sequence alignment of three BoLA alleles.
The sequences for BoLA 2*04601 (35 mismatches), BoLA 2*04801 and BoLA 3*01101 were aligned with the program clustal W 2.1 multiple sequence alignment. The signal peptide, the alpha-1, alpha-2, alpha-3 and the transmembrane region are indicated according to information available on the UniProt database. An asterisk (*) denotes that residues at that position are exactly the same in all sequences. A colon (**:**) indicates a conserved substitution i.e. the residues share the same properties. A dot (**.**) indicates semi-conserved substitutions and a blank denotes that there is no common property. The numbers to the far right signify the position number of the nucleotide strand. Underlined are the amino acids that differ between BoLA 2*04801 and the two other variants, which were recognized more often by BNP dams and non-BNP dams.

The majority of differences between BoLA 2*04801, the allele recognized by fewer PregSure® BVD antibodies, and BoLA 2*04601 (35 mismatches) and BoLA 3*01101, the alleles where stronger reactivity was observed, were noted in the alpha-1 and alpha-2 domain. There were three amino acid pairs and sixteen single amino acid substitutions (i.e. in total 22 substitutions) in these two domains in BoLA 2*04801, compared to BoLA 2*04601 (35 mismatches) and BoLA 3*01101 (underlined in figure 3.13). Differences in the alpha-3 domain and the transmembrane region were predominantly present in BoLA 3*01101.

In view of our previous observations with the twin calves and since we could not observe a principle difference between high reacting non-BNP dams and BNP dams, we rationalized that the calves of non-BNP dams developed no symptoms because they did not express the BoLA I alleles their dams reacted to. To test this notion, we identified offspring's from a selection of BNP and non-BNP dams and hypothesised that cells of calves free of BNP symptoms are not recognized by the PregSure® BVD induced alloantibodies of their respective dams.

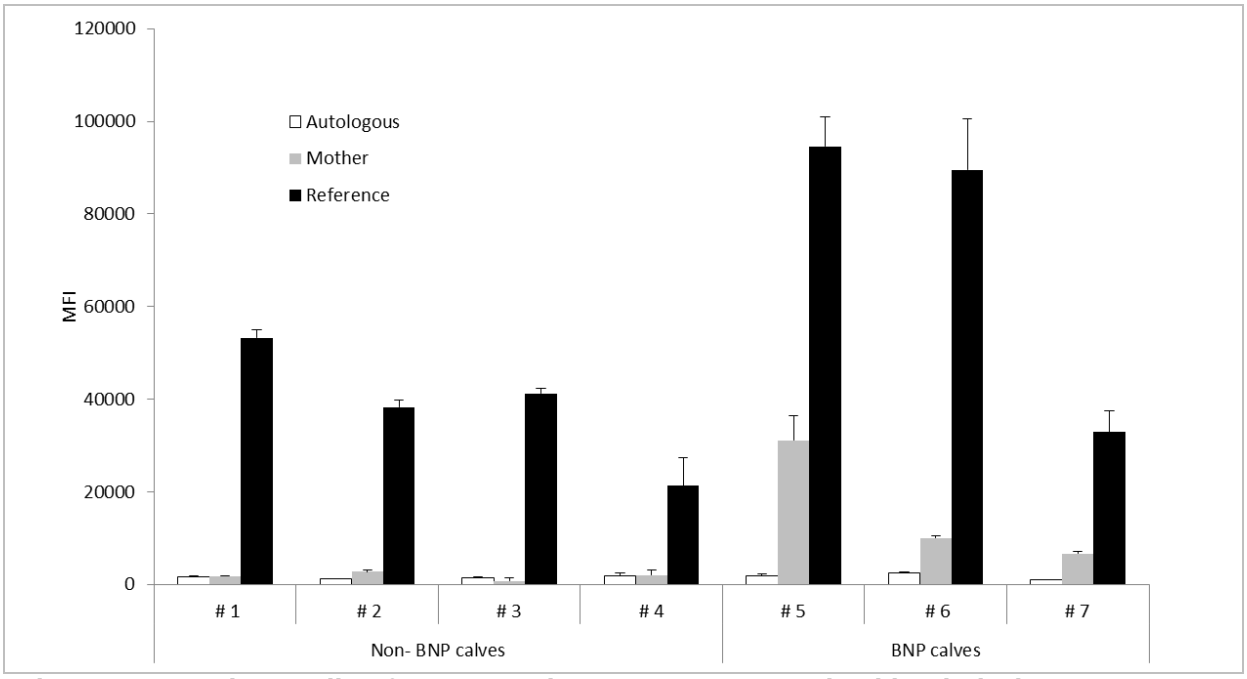


Figure 3.14: Calves cells of non-BNP dams are not recognized by their dams.
The surface binding reactivity of serum from respective dams (grey bars), another reference BNP dam (black bars) and autologous sera (white bars) was examined on calf's cells. Presented is the MFI. The three calves #5, #6 and #7 were BNP calves.

To prove the postulated hypothesis, we obtained serum and whole blood for generation of lymphoblasts, from seven different calves and their respective dams. Out of the seven dams, three had given birth to calves affected by BNP in the previous years. Serum of the respective dams, another reference BNP dam and of the calves themselves was examined for binding reactivity to the calves' cells via flow cytometry.

Indeed, non-BNP dams did not recognize cells of their offspring, although the reference serum of another confirmed BNP dam, bound well to the calves cells, as shown in figure 3.14 (and the calves' cells expressed BoLA I to a high extent; depicted in the supplements S8).

Dams of the calf #5, #6 and #7, had previously given birth to BNP calves. These dams did react to their respective calves cells. These calves showed symptoms of BNP, such as thrombocytopenia (for example calf #6 had a thrombocyte count of 46 G/l which was life threatening low as the normal range is 100 – 600 G/l), but fortunately treatment was administered timely and the animals survived.

Interesting was also the case of calf #2. Calf #2 was born in 2008. We obtained serum from its dam from 2010, 2011, 2012 and 2014. In the figure 3.14 reactivity of sera from the year 2010 is presented. The dam probably received a BVD primary immunization with PregSure® BVD in 2006 or 2007, as this was generally the case in the examined herd.

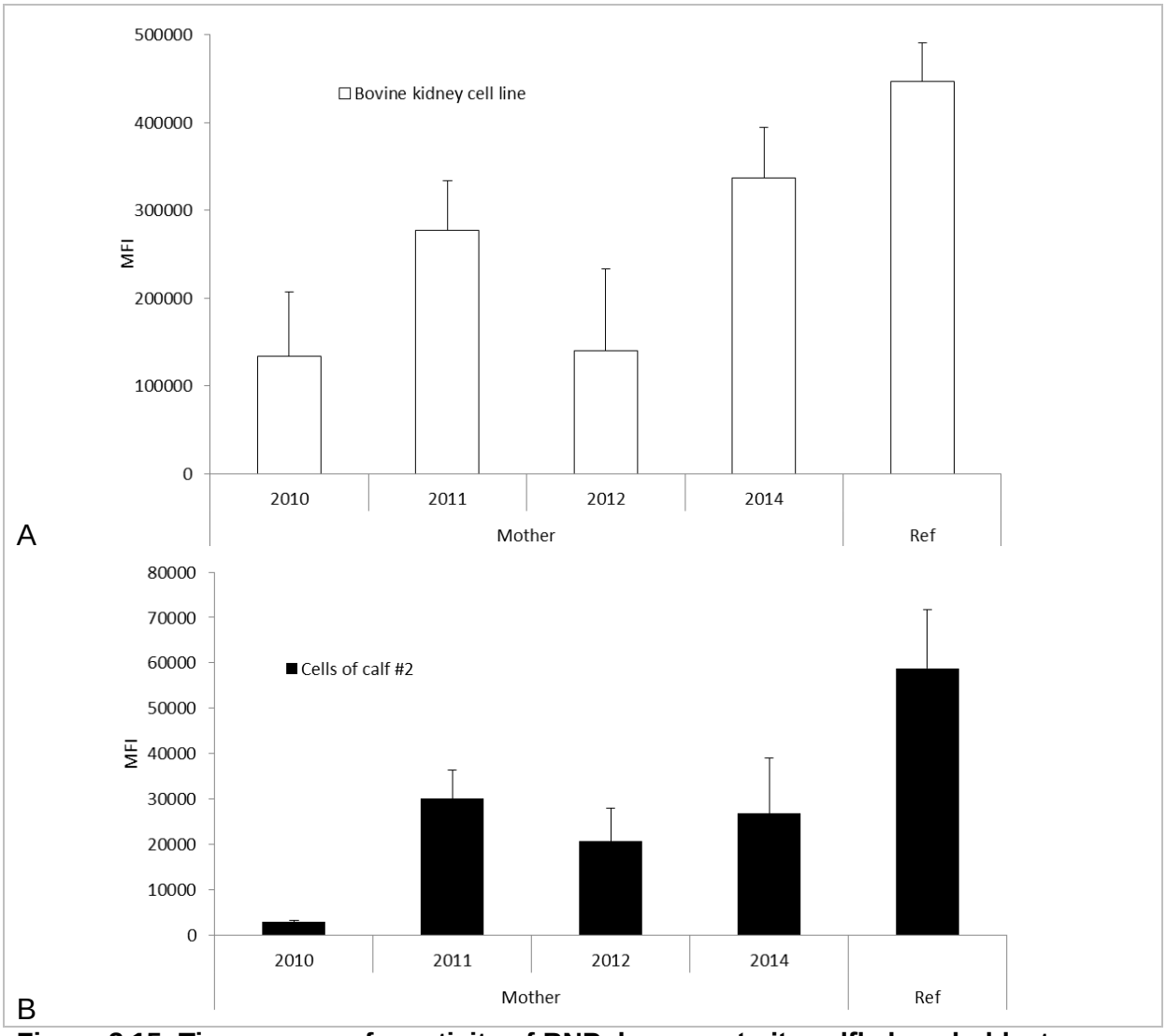


Figure 3.15: Time course of reactivity of BNP dam sera to its calf's lymphoblasts.
The dam of calf #2 was vaccinated with PregSure® BVD in 2006/7. Sera were obtained at four different time points in four years. The serum was analysed for its binding reactivity to (A) the bovine kidney cell line and (B) calf#2 lymphoblasts. Another BNP dam (ref) was used as a positive control.

In the year 2010, after the first PregSure® BVD vaccinations, serum of the dam did possess BNP alloantibodies as seen by the reactivity to the permanent cell line (figure 3.15 A) but these antibodies did not recognize the calf's cells (figure 3.15 B). After another booster with PregSure® BVD in 2010, the dam produced a higher BNP alloantibody titre, as seen by the higher reactivity to the bovine kidney cell line. From 2011 onwards, the serum alloantibodies also recognize cells of the calf. Therefore, it is tempting to speculate that if the calf had been born after 2010 and received colostrum from its mother, it might have developed BNP.

Concluding, the calves that remained free of BNP symptoms, are hypothesized not to express the BoLA I alleles that are recognized by PregSure® BVD induced alloantibodies of their dam. Unfortunately, the exact BoLA I repertoire of the calves cells could not be analysed due to time constrains but our assumption is that the calves do not express the three alleles identified as of interest.

3.4 Colostrum from cows immunized with a vaccine associated with BNP contains alloantibodies that cross-react with human MHC-I molecules

In 2011, three years after the market launch of PregSure® BVD in New Zealand the first BNP cases were reported (Bryan M 2012; New Zealand Ministry for Primary Industries 2011). This was consistent to observations in Europe, where there was also a three years period between the vaccine availability and BNP occurrence (Euler et al. 2013). Fonterrra™, a New Zealand based international dairy manufacturer, produces colostrum based dietary supplements for human consumption. They initiated a collaborative study with the PEI to examine the cases reported and analyse whether there might be any risk for human consumers of colostrum products that are manufactured from colostrum of BNP dams.

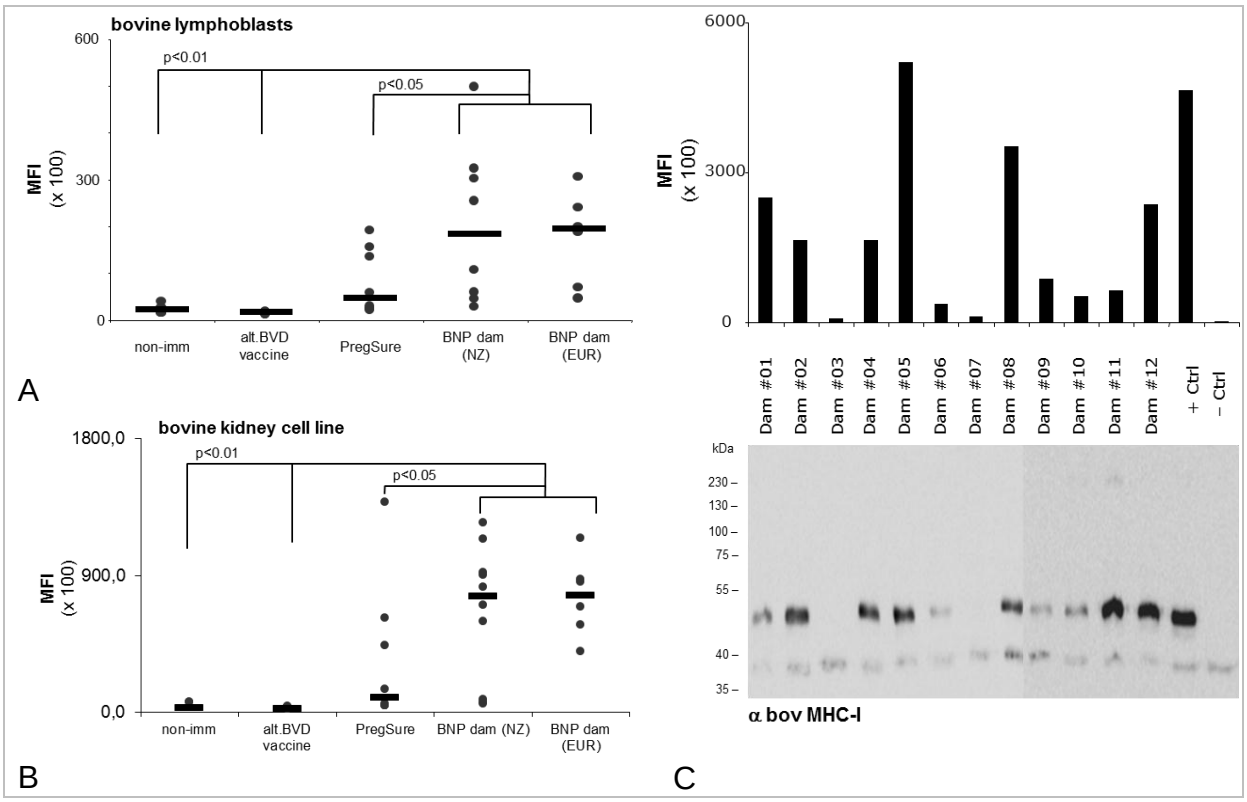


Figure 3.16: Sera of NZ-BNP dams show identical reactivity compared to EU dams. Sera were obtained from NZ dairy cows that had either not been immunized against BVD (non-BVD-imm.), vaccinated with an alternative BVD vaccine (alt. BVD vaccine), vaccinated with PregSure® BVD and birthed healthy calves (PregSure) or cows that had been vaccinated and gave birth to bleeder BNP calves (BNP dams (NZ)). By flow cytometry eight sera per group were tested for alloreactive binding to (A) bovine lymphoblasts and (B) the bovine kidney cell line. As a control we included six serum samples of European BNP dams (BNP dam (EUR)). Symbols represent the MFI for individual serum samples; black bars indicate the median value for each group. The cell line was tested in parallel by (C) flow cytometry and immunoprecipitation with serum samples of twelve NZ BNP dams. The upper panel shows the MFI for the individual serum samples, the lower panel shows the corresponding immunoprecipitate as revealed by IL- A88. Serum from a European BNP dam served as a positive control, foetal calf serum was used as a negative control.

As depicted in figure 3.16 A and B, the reactivity pattern of New Zealand BNP dams was almost identical to European BNP dams. This was also reflected in the results obtained by IP. Reactivity to the cell line was notably stronger compared to that detected to bovine lymphoblasts (figure 3.16 B and A, respectively).

Figure 3.16 C presents data obtained from individual serum samples of the obtained New Zealand BNP dams. The individuals that reacted strongly in the flow cytometric analysis precipitated bovine MHC-class I on the bovine kidney cell line, as demonstrated with the monoclonal antibody IL- A88 (figure 3.16 C). However, in two out of the 12 BNP dam sera, no alloreactivity was detectable. Whether this was due to serum degradation or that these calves just suffered from BNP-like symptoms not related to PregSure® BVD vaccination, was not clear.

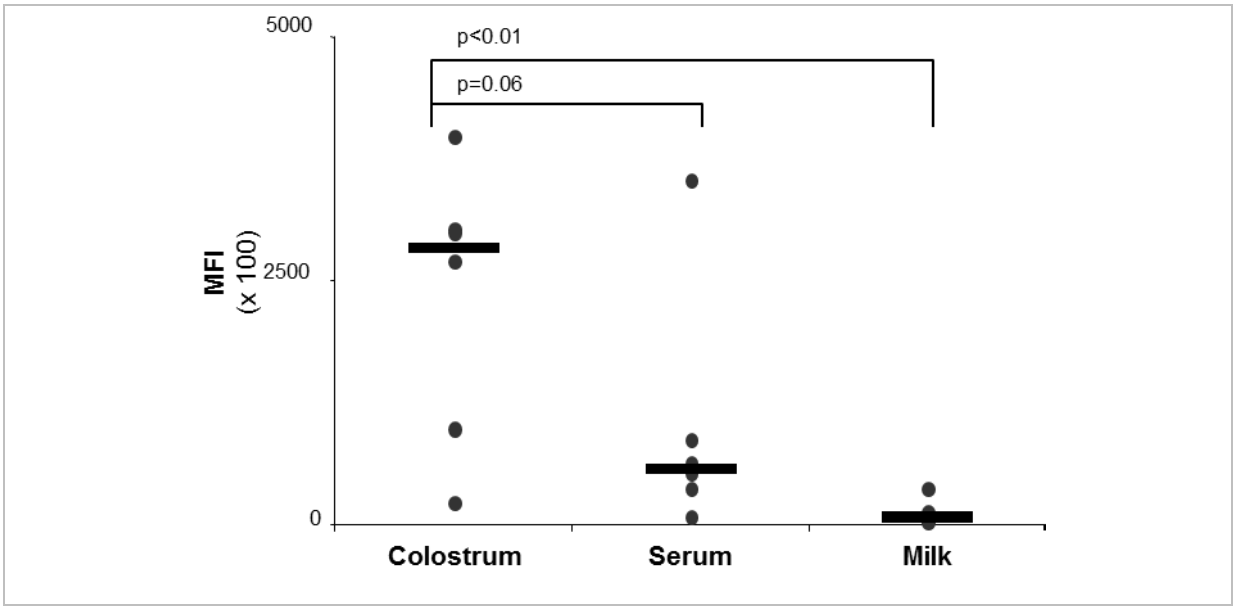


Figure 3.17: BNP-associated alloantibody content in dairy products.
BNP dam colostrum, serum and milk were collected from six NZ BNP dams. The samples were diluted 1:500. Using the cell line the samples were tested by flow cytometry for the presence of BNP associated alloantibodies. Symbols represent the MFI for individual samples as determined by flow cytometry, black bars indicate the median value for each group.

We obtained colostrum, serum and milk from six New Zealand BNP dams. These samples were further investigated for their BNP alloantibody content by flow cytometry. The amount of alloantibodies was considerably higher in colostrum than in serum and was negligible in milk (independent of whether pasteurized or unpasteurized, not shown), as seen in figure 3.17. Nonetheless, serum was easier to work with in contrast to the creamy, fatty composition of colostrum and therefore, we concentrated on serum.

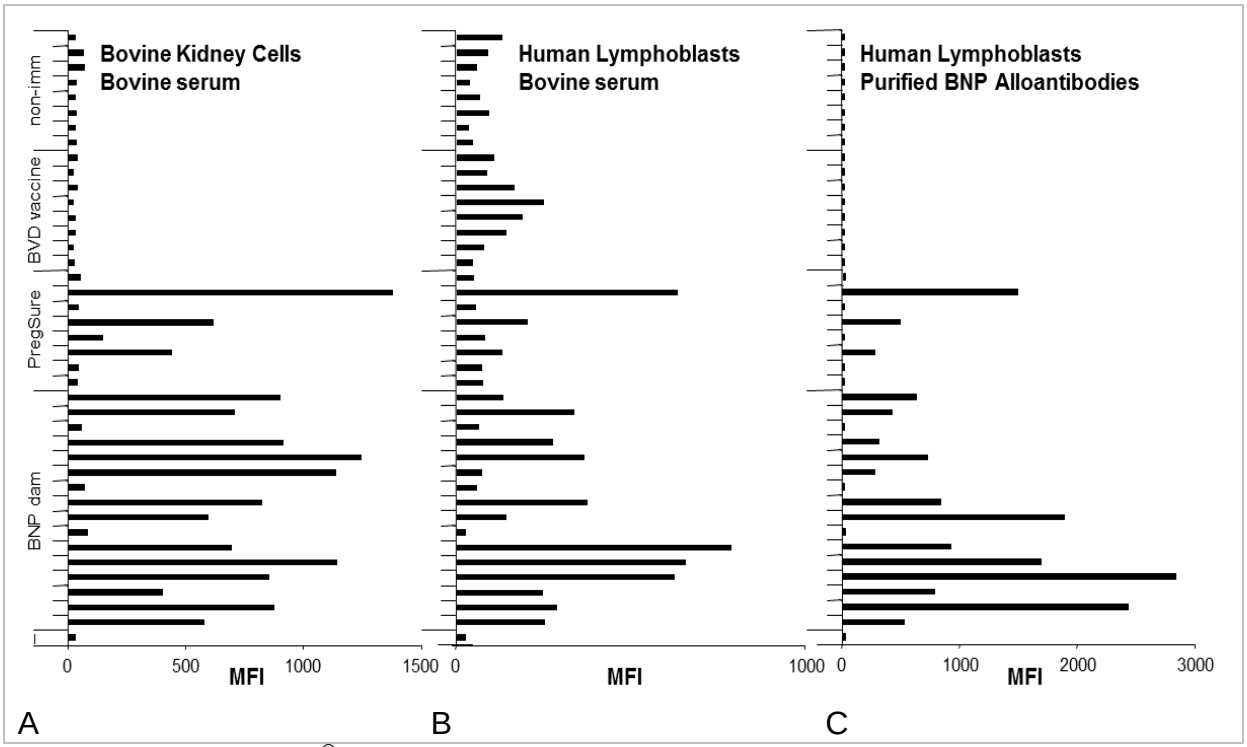


Figure 3.18: PregSure® BVD induced BNP alloantibodies cross-react with human lymphoblasts.
(A) A panel of sixteen BNP dam sera was tested by flow cytometry for the presence of alloreactive antibodies that bind to the cell line. As controls sera from non-immunized (non-imm), alternatively vaccinated (BVD vaccine) or PregSure® BVD-immunized non-BNP dams were included. Secondary antibody only (-) (B) The same serum panel was tested for cross-reactive binding to human lymphoblasts. (C) From the entire serum panel BNP-associated alloantibodies were purified by affinity purification. The affinity purified alloantibodies were again tested for cross-reactive binding to human lymphoblasts. Black bars indicate the MFI obtained with individual sera or the corresponding affinity-purified alloantibodies.

Serum was also examined for the presence of opsonising alloimmune antibodies which may cross-react with human cells. By using flow cytometry, we can again confirm that alloreactivity to the bovine kidney cell line was only detectable in the PregSure® BVD and BNP dam group (figure 3.18 A). Reactivity of these groups to lymphoblasts of healthy human donors was highest but nevertheless some reactivity was observed in the alternatively vaccinated and non-immunized group (figure 3.18 B). There was a significant correlation (R^2 =0.71) between the reactivity to the cell line and to human lymphoblasts. However, when we analysed affinity purified alloantibodies, the antibodies that are according to our hypothesis of importance in the context of BNP; as well as BNP induction, only antibodies in the PregSure® BVD and BNP dam group detected the surface of human lymphoblasts (figure 3.18 C). The previously observed reactivity of alternatively vaccinated and non-immunized animals to human lymphoblasts was completely abolished.

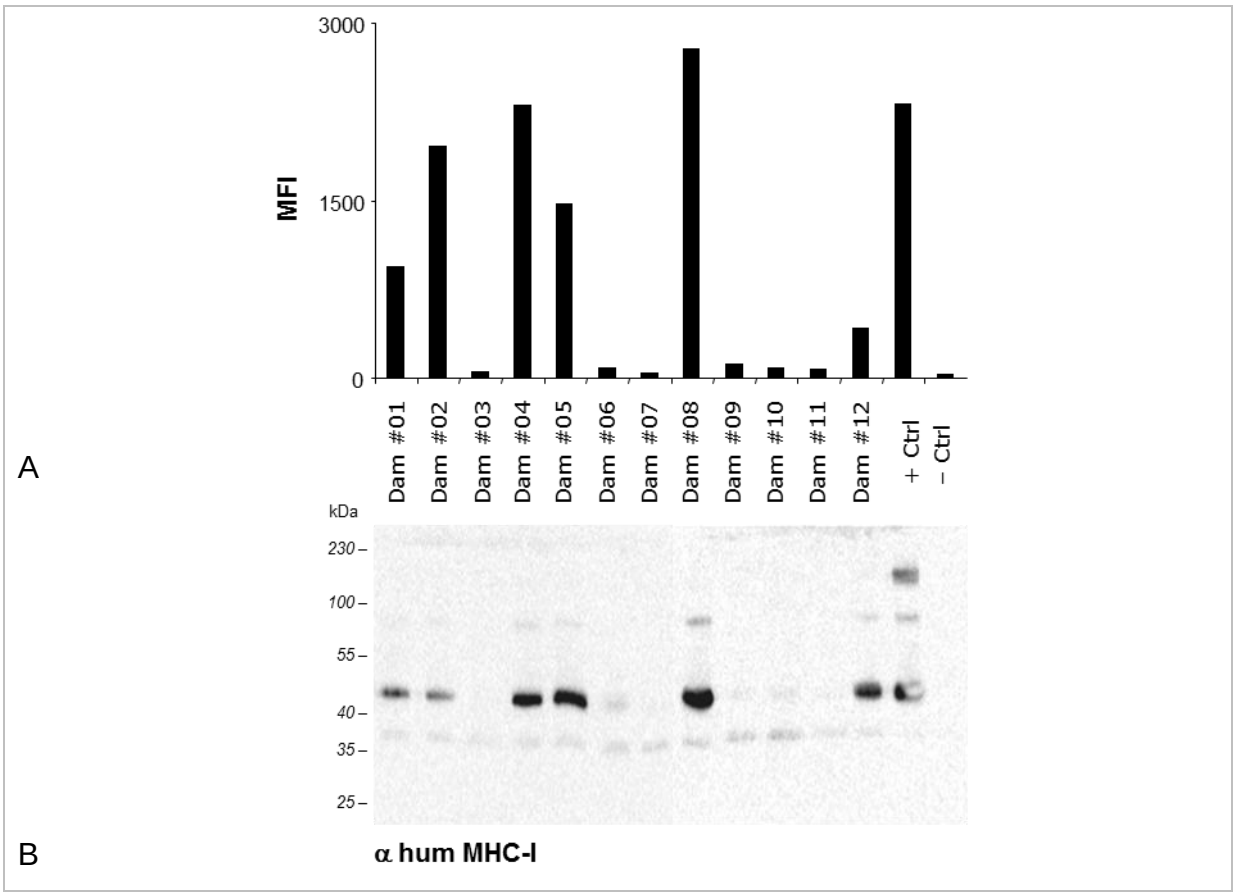


Figure 3.19: BNP-associated alloantibodies bind human MHC- I molecules.
The panel of BNP dam sera was tested in parallel by (A) flow cytometry and by (B) immunoprecipitation using human lymphoblasts. The black bars, in the upper panel, show the MFI as determined by flow cytometric analysis. The lower panel shows the corresponding immunoprecipitates as revealed by w6/32, a monoclonal antibody specific for human MHC-I molecules.

Figure 3.19 A represents the flow cytometric reactivity of individual New Zealand BNP dams to human lymphoblasts. These cross-reactive BNP antibodies precipitate the human variant MHC-class I, i.e. HLA class- I, as seen after IP and development of the western blot with the monoclonal antibody w6/32 (figure 3.19 B).

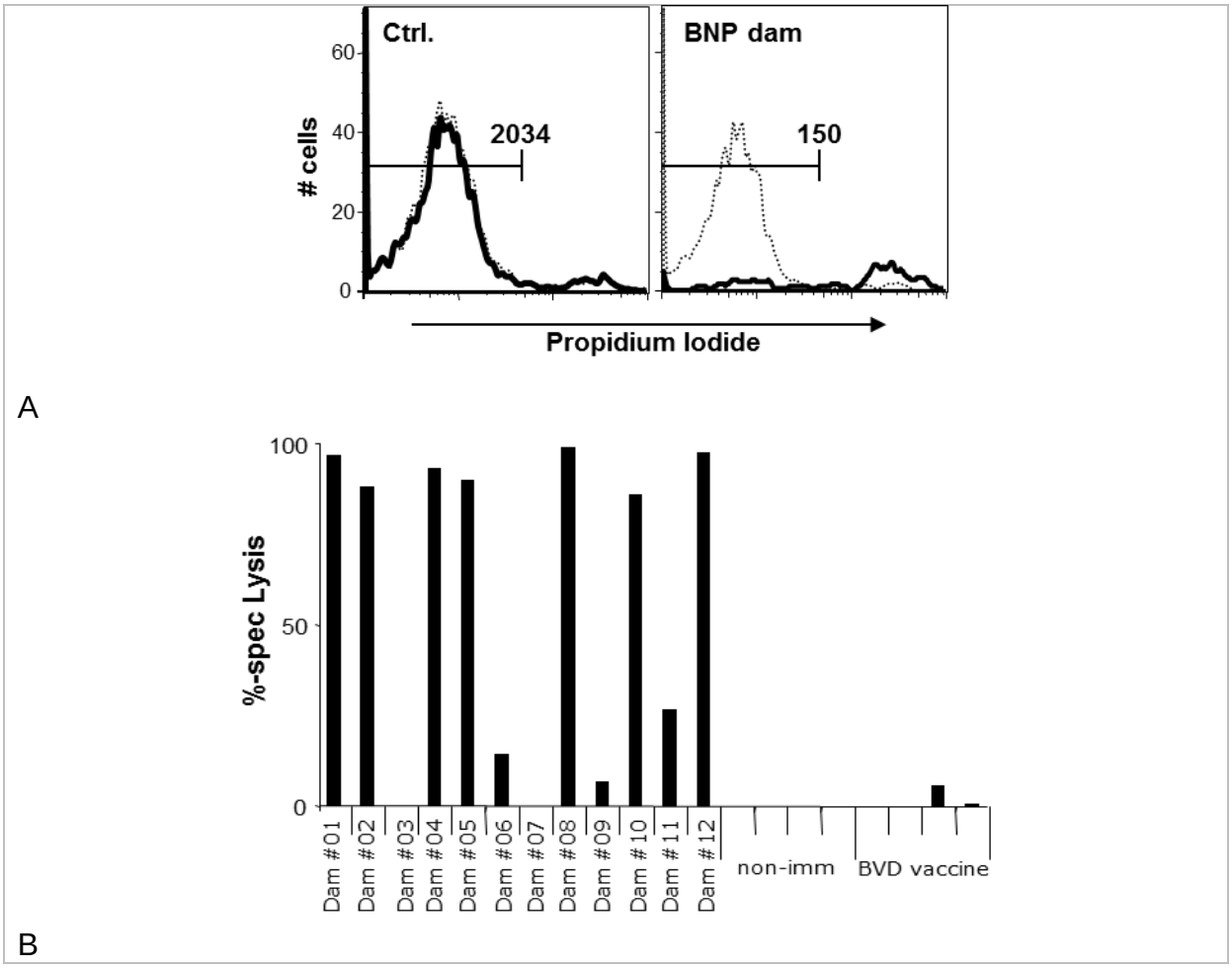


Figure 3.20: BNP-associated alloantibodies opsonise and sensitize human lymphoblasts for complement-mediated cell lysis.
Human lymphoblasts were co-incubated with heat-inactivated bovine serum and rabbit complement. Cell viability was measured by flow cytometry counting live, propidium iodide (PI) negative cells. (A) The histograms show human lymphoblasts incubated with serum from a non-PregSure® BVD immunized control dam (left) or BNP dam serum (right) before adding active complement (bold line) and after heat inactivated rabbit complement was added (dotted line). Numerical figures indicate the absolute number of living, PI negative cells (B) The serum panel was tested for its complement sensitizing activity on human lymphoblasts. Sera from four non-immunized and four alternatively vaccinated animals served as a control. Black bars represent the specific lysis for the individual sera.

One pathomechanism of BNP alloantibodies that has been discussed so far, is complement-mediated lysis of opsonised cells (Friedrich et al. 2011; Foucras et al. 2011). To assess, whether BNP-associated alloantibodies could theoretically destroy human lymphocytes through such a mechanism, we modified a flow-cytometric approach to determine complement activity *in vitro*. All ten BNP sera that contained alloreactive antibodies induced a specific complement-lysis of human lymphoblasts, whereas the corresponding control sera showed no effect, as depicted in figure 3.20 B.

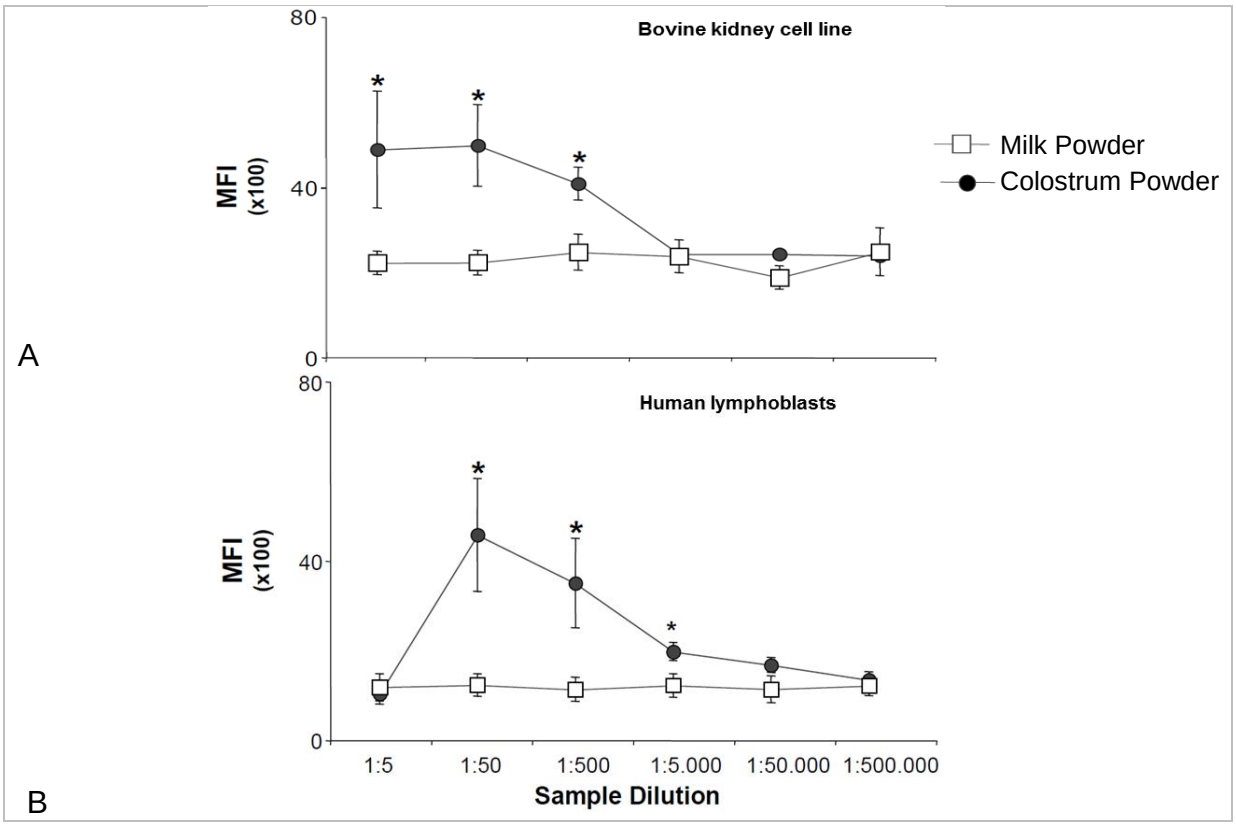


Figure 3.21: A commercial lot of colostrum powder contains cross-reactive BNP-associated alloantibodies.
Commercial lots of BNP colostrum powder and whole milk powder were tested by flow cytometry for the presence of alloreactive antibodies. Filled circles represent the MFI for colostrum powder, open quadrates for whole milk powder. Depicted is the reactivity to (A) the cell line and (B) human lymphoblasts. Symbols represent the median over three independent flow cytometric analyses; error bars the corresponding standard deviation. Asterisks indicate a significant difference between re-suspended colostrum and whole milk powder at the indicated dilution.

Apart from the several sera samples, we also obtained commercially available dairy products. Of particular interest was to determine whether there was a difference in reactivity of dairy products that had been produced during and after the 2011 calving season i.e. after omission of any PregSure® BVD treated cows from colostrum collection. As seen in figure 3.21, we can show for the first time that colostrum powder, although produced by pooling colostrum from several herds, does show significant reactivity to the cell line as well as to human lymphoblasts. Nonetheless, these production-inherent dilution procedures led to a lower reactivity compared to the reactivity of individual BNP dam colostra (as shown in the figure 3.17) but the low reactivity observed with milk can be confirmed and replicated with milk powder. The new colostrum powder batch (from the year 2012), produced by omitting animals that had been vaccinated with PregSure® BVD, displayed neither surface reactivity to the bovine kidney cell line nor to human lymphoblasts (figure 3.22 A and B, respectively).

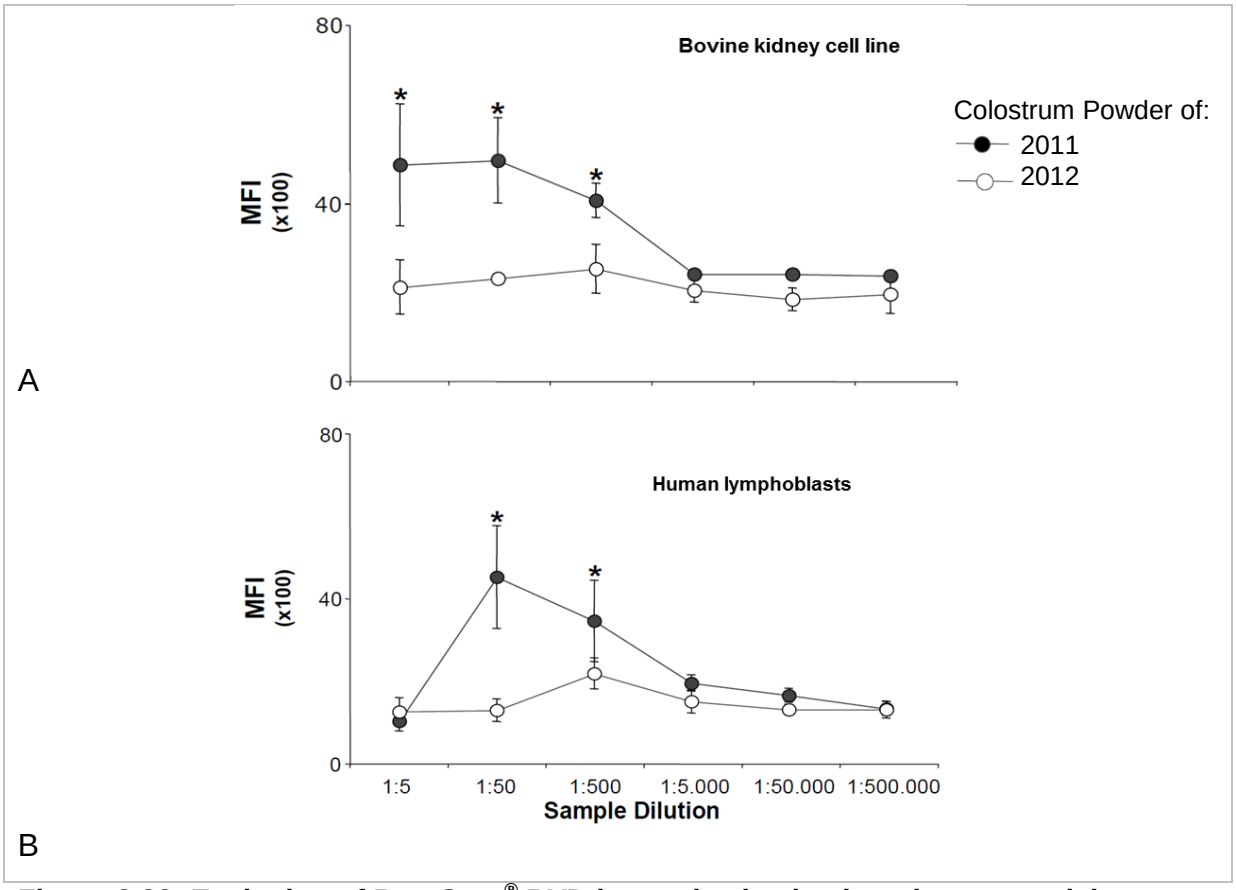


Figure 3.22: Exclusion of PregSure® BVD immunized animals reduces reactivity.
Two commercial lots of BNP colostrum powder were compared by flow cytometry for the presence of BNP-associated alloantibodies, one produced during the calving season of 2011, the other produced in 2012 according to a new harvesting policy which excludes herds that had been vaccinated with PregSure® BVD. Filled circles represent the MFI for colostrum powder produced in 2011, open circles for 2012 colostrum. Depicted is the reactivity for (A) the cell line and (B) human lymphoblasts. Symbols represent the median over three independent flow cytometric analyses; error bars the corresponding standard deviation. Asterisks indicate a significant difference between the two colostrum batches at the indicated dilution.

In conclusion, the obtained data clearly demonstrate that the cases reported in New Zealand are BNP cases. Further results show that BNP alloantibodies cross-react with human lymphoblasts. In accordance with previous findings, these alloantibodies are specific for MHC-I molecules, and sensitize opsonised human cells for *in vitro* complement lysis. Cross-reactive antibodies were observed in serum and colostrum of individual BNP dams, and can also be traced back to commercial colostrum powder lots. Although, alloreactive MHC-I specific antibodies are considered non-hazardous to humans, these results suggest that manufacturers of bovine colostral products for human consumption, should consider to use colostrum only from animals that have not been exposed to the vaccine associated with BNP.

4. Discussion

4.1 BNP: a Vaccine-Induced Alloimmune Disease

It is virtually impossible to completely remove bioprocess impurities, such as Host Cell Proteins (HCP), even after multiple purification steps (Wang et al. 2009). Thus it is not surprising that virus antigens are difficult to obtain in a homogeneous and pure composition (Federal Association of Official Veterinarians 2012). Purity tests, such as the ones described in the European Pharmacopoeia and in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2012), require demonstrations that ingredients are free from potentially harmful contaminations such as certain bacteria, extraneous viruses, mycoplasm, fungi, etc. Additional certification on the absence of other foreign or non-self-impurities are usually not required if Good Manufacturing Practice (GMP) compliances are met and safety as well as efficacy of the product are guaranteed (EMA 2011; FDA 2010). However, in the particular case of the inactivated vaccine PregSure® BVD the presence of process-related impurities results in a major side effect because apart from BVDV antigens, the vaccine contains cellular components originating from the bovine kidney cell line, used for virus cultivation (Bastian et al. 2011; Kasonta et al. 2012; Euler et al. 2013).

Serum from animals vaccinated with different BVD vaccines, were incubated with intact bovine kidney cells from the cell line that is used by the manufacturer to produce the vaccine or bovine lymphoblasts from a healthy individual. There is a significant increased surface binding noted with cells pre-treated with sera of PregSure® BVD immunized individuals. Furthermore, serum from PregSure® BVD immunized BNP dams i.e. dams that already gave birth to so called bleeder calves, resulted in significantly higher surface reactivity. This confirms further the hypothesis that PregSure® BVD vaccination is a common factor associated with an increased production of antibodies, which bind to the bovine kidney cell line (Bastian et al. 2011).

As cattle repeatedly immunized with PregSure® BVD develop alloantibodies that are cross-reactive with the production cell line as well as with peripheral blood cells and haematopoietic progenitor cells. These alloantibodies do not recognize own antigens but are directed against cells or tissues of foreign origin (Bridger et al. 2011; Bastian et al. 2011). The problem arises when these alloantibodies are transferred from the vaccinated dams to their calves, via colostrum, and destroy targeted cells due to foetal-maternal incompatibility (Schroter et al. 2011; Bell et al. 2013). The result is an

induction of BNP in the calf, a novel condition, which is characterized by sudden and spontaneous haemorrhage, without prior visible injuries; thus the primary terminology blood sweating (*Blutschwitzen*), extensive bone marrow depletion and high mortality rates (Bell et al. 2010b; Pardon et al. 2011; Friedrich et al. 2011).

Similar results obtained with the bovine kidney cell line are noted when examining bovine leukocytes, as also published by Bastian et al. 2011, Pardon et al. 2011 and Assad et al. 2012, validating that sera of BNP dams possess alloantibodies which recognize antigens on peripheral and bone marrow calf leukocytes. These alloantibodies are primarily directed against antigens on the production cell line, as observable by the higher MFI, but a degree of similarity and cross reactivity is present with structures on leukocytes or haematopoietic stem cells (Pardon et al. 2011; Bastian et al. 2011). This notion further strengthens assumptions that BNP-associated antibodies result from bioprocess impurities such as residual HCP i.e. in this case, of the manufacturing cell line present in the vaccine. Euler and colleagues (2013) performed a proteomic characterization and could show that there are several serum and cellular derived molecules that are shared between PregSure® BVD and a bovine kidney cell line. Additional evidence for a causal role of cell line associated bioprocess impurities in the induction of alloantibodies comes also from the fact that affinity purified alloantibodies, i.e. BNP antibodies that were desorbed from the cell surface of bovine leukocytes, strongly reacted to the cell line used for vaccine production. Furthermore, it has been shown that BNP associated alloantibodies show higher affinity to the cell line used for vaccine production compared to other cell lines, including alternative bovine cells (Bastian et al. 2011). Together these observations provide compelling evidence that the alloreactivity leading to the clinical syndrome of BNP are due to a vaccination with PregSure® BVD and specifically caused by immunogenic antigens derived from the cell line.

In order to identify BNP-associated alloantigens, we therefore focus on the analysis of the cell line used for PregSure® BVD production. Once individual antigens have been identified, it has to be confirmed whether these antigens are additionally expressed on peripheral blood cells and bone marrow progenitor cells.

It has been postulated that only alloantibodies recognizing cell surface antigens are clinically relevant because the pathomechanisms – that have been discussed so far – leading to cell depletion and as a consequence, to the observed clinical symptoms, depend on surface opsonisation of target cells (Bastian et al. 2011; Pardon et al.

2011). The immune-mechanism of the alloantibodies is thought to include Fc-receptor mediated phagocytosis and/ or complement activation. These mechanisms would additionally explain the increased presence of activated macrophages and haemophagocytosis in histological bone marrow sections (Bell et al. 2010b; Kappe et al. 2010; Pardon et al. 2010) as well as the onset of haematological changes, such as the decline of thrombocytes, directly after colostrum intake (Bridger et al. 2011; Bell et al. 2013; Schroter et al. 2011). Thus, surface antigens, in contrast to hidden epitopes, for example, of cytoplasmic origin, are likely the main target of alloantibody recognition and resulting BNP pathogenesis (Bastian et al. 2011).

One alloantigen identified by two independent publications is BoLA class I (Deutskens et al. 2011; Foucras et al. 2011). This could also be confirmed by our immunoprecipitation data using a monoclonal anti-BoLA class I antibody as well as LC-MS-MS. The flow cytometric cell surface reactivity measured using the described FACS protocol correlates by over 90 % with the grey scale measured by densitometry after IP of BNP sera with biotinylated bovine kidney cells. Together these observations prove that the BNP associated alloreactivity is directed to the most part to BoLA I molecules.

Astonishingly, was that there was a regional difference in BNP induction not correlated to the amount of PregSure® BVD doses sold. One clear example observed in Germany, is the high incidence of BNP reported in Bavaria in contrast to Lower Saxony. To prove that this epidemiological discrepancy was due to differences in the use of PregSure® BVD according to the two-step vaccination regimen as outlined in the Introduction section (page 13 following), we experimentally mimicked the different vaccination strategies. Four groups of five bulls each were primed with an inactivated vaccine, including PregSure® BVD, followed by two booster shoots of either an inactivated vaccine or a live vaccine, as it is done according to the two-step vaccination scheme. Although the vaccine PregSure® BVD results in high and long-lasting anti-BVD antibody titres, anti-BoLA class I BNP-associated alloantibodies are also induced. In one individual we could observe BNP alloantibody production already after only one PregSure® BVD shot (priming). Furthermore, we could again confirm a clear relationship between the flow cytometric reactivity and IP results. Overall, we could experimentally reflect the varying regional vaccination strategies and show a clear correlation between BNP alloantibody production and multiple PregSure® BVD vaccinations.

The main target and primary source of alloreactivity is indeed directed against surface antigens expressed on the bovine kidney cell line used to produce PregSure® BVD, as there is minimal to no cross reactivity of antibodies to surface structures of the production cell lines of the alternative vaccines. In fact, a dam that already gave birth to a BNP affected calf reacts significantly stronger to the cell line used to produce PregSure® BVD and not to the cell lines used to produce the other alternative BVD vaccines.

Since, BoLA class I is so far the sole BNP alloantigen described, one assumption was that the other cell lines simply do not express BoLA I to the same extent or express a different BoLA I variant. The former hypothesis could be refuted because in comparison, the PregSure® BVD production cell line seems to actually even express a lower degree of BoLA I.

BoLA class I is highly polymorphic and expressed on all nucleated cells— essentially, all cells with the exception of red blood cells. One individual can express several alleles, in different combinations and due to other phenomena such as interlocus recombination, there is an extremely low chance that two individuals in a herd carry the exact same BoLA I combination pattern (Babiuk et al. 2007; Ellis 2004). However, selective breeding in order to increase production traits and the tightly controlled use of a small number of breeding bulls, which is common practice in commercial cattle herds, might limit the BoLA I diversity in a commercial herd (Babiuk et al. 2007; Ellis 2004). This would explain why there were sometimes accumulations of BNP cases, hinting to a common familial background (Krappmann et al. 2011; Demasius et al. 2014) whereas, some calves seem to have a lower degree of susceptibility likely due to their genetic make-up (Deutskens et al. 2011).

Fortunately, we had the chance to obtain samples from a twin, whose fraternal sibling succumbed to BNP. Our analysis show that the BNP unaffected calf did ingest BoLA I BNP associated alloantibodies, presumably, to an extent that was potentially lethal, as indicative by the death of the affected twin. Nevertheless, the maternal alloantibodies (as well as another confirmed BNP dam) did not recognize the cells of the calf. The lack of recognition was not because of differences in the expression level of BoLA I molecules, since the calves' cells and the PregSure® BVD production cell line expressed identical levels as confirmed by flow cytometry using a BoLA I specific monoclonal antibody. Similar cases, where only one calf succumbed to BNP

are not unique but we just had this one chance to obtain samples. Unfortunately, there was no possibility to acquire whole blood from the affected calf prior to wasting.
Nonetheless, this presented twin calf study strongly suggests that anti-BoLA I BNP associated alloantibodies seem to be linked to induction of BNP. However, due to the ubiquitous expression, it was questionable how BoLA I targeting BNP associated alloantibodies can induce the specific insult observed on peripheral blood cells and the red bone marrow. A diverse BoLA I expression pattern, varying expression levels or expression of a particular variant might explain why only a specific cell population is targeted.
Laming and colleagues (2012) demonstrated that BNP alloantibodies specifically target an *in vitro* culture of bone marrow biopsies consisting of granulocyte/erythroid/macrophage/megakaryocyte (CFU-GEMM) i.e. pluripotent progenitor cells. Bell and her fellow researchers (2013) meticulously analysed the effect of BNP pooled colostrum on peripheral blood cells and on haematopoietic stem cells at different maturation stages. The first cells that disappear after colostrum ingestion are circulating lymphocytes, neutrophils and monocytes, followed by thrombocytes. The quick and initial destruction was observed as soon as 4 hours and 8 hours after antibody uptake, for leucocytes and thrombocytes respectively, which supports the hypothesis of complement lysis and/ or enhanced phagocytosis susceptibility. Laming and partners (2012) tried to elucidate subset-specific diminution of peripheral PBMCs but could only show that there was an increased drop in $CD25^{+ve}$, γδT cell and B cells in challenged animals only.
The extensive and complete depletion of the red bone marrow has been narrowed down to affect only very early and immature neutrophils, erythrocytes and eosinophil cells but immature precursors as well as already committed thrombocyte, lymphocyte and monocyte cells (Bell et al. 2013; Laming et al. 2012). Furthermore, mature erythrocytes are not affected, thus the alloantigen targeted by BNP alloantibodies is expressed at sufficient levels on only the affected lineages at the particular maturation stages.
Several possibilities are conceivable to explain why only these specific cell populations are affected: a) a particular BoLA I variant is solely expressed on these cells; b) a particular pathomechanism is exclusively functional on blood cells and in the bone marrow and might account for the highly specific insult; c) an additional stem cell alloantigen is involved in the induction of BNP etc. Such a specific antigen

has for example been identified to play a role in human alloimmune phenomena, such as TRALI or NAIT. In both cases there has been a long ongoing discussion to what extent anti-HLA-I (the human homologue of BoLA I antigens) play a role in disease induction. TRALI is an alloimmune graft rejection phenomenon, where in addition to human MHC class I alloantibodies, Human Neutrophil Antigen 3a (HNA-3a) alloantibodies together induce the majority of cases (Greinacher et al. 2010; Reil et al. 2008; Reil et al. 2011). The exclusive presence of MHC class I alloantibodies only to induce TRALI is still questionable and debated. Similarly, in NAIT/ FAIT anti-Human Platelet Antigen 1a (HPA-1a) alloantibodies are identified as the major pathogenic antibodies beside anti-MHC I alloantibodies, which seem to only provide a mild trigger (Marin et al. 2005; Kaplan 2006).

MHC I molecules can be subdivided in classical and non-classical variants. In contrast to classical MHC I molecules, non-classical variants are less polymorphic, have a restricted cellular expression, a limited range of antigens presented and can be encoded within or outside the MHC loci (Birch et al. 2008). Preferential expression of a specific MHC I allele on a particular cell population was one possible hypothesis why only specific cells are targeted by BoLA class I BNP associated alloantibodies.

Therefore, through using RT-PCR, we sequenced in total 8 different BoLA I alleles, including 2 non classical variants, from the PregSure® BVD production cell line. These genes were cloned into *pMyc*-GFP expression vectors, used for transfection of Platinum-E cells and subsequent transduction of a mouse pre-B cell line (38B9). This moloney Murine Leukaemia Virus (MoMLV) based transduction system is well established at the institute and provides a stable, efficient and safe expression in the murine pre-B cell line (Kitamura et al. 2003; Morita et al. 2000).

Positive expression of BoLA I was monitored with monoclonal MHC class I antibodies and by fluorescence microscopy. Successful expression of the two variants, BoLA 2*40801 (6 mismatches) and BoLA 2*40801 (138bp missing), cannot be completely confirmed. The recombinant cells had a bright green appearance but the monoclonal anti-MHC class I antibodies showed relative week reactivity. Whether this reflects a lack of expression or whether the antibodies simply do not recognize these alleles is not entirely clear at the moment.

Another possible explanation could be due to the fact that only the heavy chain of BoLA class I is introduced into the murine cells. The smaller soluble light chain is encoded by the cell and is of murine origin. It is possible that the bovine heavy chain

of BoLA 2*40801 (6 mismatches) and BoLA 2*40801 (138bp missing) is incompatible with the murine light chain and thus that the BoLA I allele cannot be stabilised and expressed efficiently on the cell surface.

To discriminate reactivity of sera to murine MHC I, transduced GFP positive cells and non-transduced GFP negative cells were simultaneously analysed. Using the established flow cytometric protocol, we were particularly interested on whether dams that gave birth to BNP calves (BNP dams) recognize a different BoLA I variant in comparison to dams that were vaccinated with PregSure® BVD but did not produce BNP calves. Prior experiments have already led to the observation that there are dams that react strongly to the cell line but delivered healthy calves.

Results revealed that three BoLA I alleles, namely BoLA 2*04801, BoLA 2*04601 (with 35 mismatches) and BoLA 3*01101, are frequently recognized by BNP dams and PregSure® BVD vaccinated non-BNP dams only, as the reactivity was above the cut-off point which was calculated by taking the geometric mean plus the 3-fold standard deviation of the control group. There is a statistical significant difference between these groups but this would not explain the differential clinical outcome. Therefore, the subsequent hypothesis is that unaffected calves of high responding non-BNP dams most likely lack the respective BoLA I alleles. Furthermore, the results tend to suggest that BNP dams have a higher likelihood to recognize more than one of the BoLA I alleles, thus the expression of more than one allele might be a prerequisite for BNP induction.

The majority of PregSure® BVD induced alloantibodies was directed to the two alleles BoLA 2*04601 (35 mismatches) and BoLA 3*01101, while another allele, BoLA 2*04801, was recognized only by a few BNP and non-BNP dams. Therefore, we aligned and compared differences between these sequences. In view of this reactivity pattern, we considered those polymorphisms relevant for BNP alloreactivity that varied between BoLA 2*04801 and the two stronger reacting alleles, BoLA 2*04601 (35 mismatches) and BoLA 3*01101. Such differences were mainly observed in the alpha-1 and alpha-2 domain. Differences in the alpha-3 domain and the transmembrane region were mostly noted between the two immunogenic alleles. From this observation we conclude that PregSure® BVD associated alloantibodies mainly target epitopes in the alpha-1 and alpha-2 domain. This is in accordance with the fact, that the first two domains form the epitope binding groove and represent the

most polymorphic part of the molecule, while the alpha-3 domain interacts with the beta2-microglobulin and is less accessible to antibodies.

In NAIT/ FAIT or post transfusion purpura single amino acid substitutions in the respective target proteins have been identified as epitopes of pathogenic alloantibodies (Santoso et al. 1993b; Santoso et al. 1994 and Wang et al. 1993). In analogy to these human alloimmune phenomena, it is well conceivable that also in BNP alloantibodies target epitopes consisting of single amino acid polymorphisms. Although we conclude from our observations that the epitope for BoLA I reactive BNP alloantibodies lies in the alpha-1 and -2 domain, we have not conclusively identified the exact amino acid polymorphism that is recognized. Deep sequencing of BoLA I alleles expressed by bleeder calves, non-bleeder calves and the production cell line would potentially help to pin point which exact BoLA variant(s) is recognized by BNP associated alloantibodies.

Nevertheless, a comparison between BNP dams and non-BNP dams revealed that the two identified non-classical BoLA I alleles do not seem to play any major role in BNP induction. Since non-classical variants have a limited polymorphism, this result is not as astonishing, because the BNP incidence would otherwise be much higher.

In spite of these results, the consequential step is to investigate the BoLA expression pattern of unaffected calves from high responding non-BNP dams. Based on the results obtained in the twin calf study, the assumption is that the mother does not recognize the respective cells of the calf, thus the calf remains healthy and does not develop BNP. The underlying reason, being that the calves do not express the BoLA I alleles targeted by the PregSure® BVD associated alloantibodies of their dams.

So far, six BoLA I loci have been mapped onto the chromosome 23, that means one individual can carry up to 12 different alleles (Babiuk et al. 2007). Thus, although we were able to sequence a total of 8 different alleles from the cell line, there is still a certain chance that not all alleles were isolated. A down regulation or complete shutdown of mRNA of other additional alleles by the cell line, at a particular time of isolation, could account for missing hits.

However, out of the 7 calves, only the non-bleeder calves were not recognized by their respective mothers, despite expressing BoLA I and being recognized by another BNP reference dam. In contrast, the cells of calves that showed BNP symptoms were recognized by their respective mothers and even to a much higher extent by the reference BNP dam.

The gradual production of BNP associated alloantibodies that recognize its calves cells can also be observed in one particular dam that received PregSure® BVD primary immunization in 2006/7. Prior to the production of sufficient alloantibodies, the alloantibodies of the dam did not interact with its calves lymphoblasts. After another PregSure® BVD booster vaccination, the dam produced a higher BNP alloantibody titre, which eventually recognizes the calf's cells. One might hypothesize, that if the calf had been born after 2010, and had ingested colostrum from its dam, it would have developed BNP.

The final step, after flow cytometric experiments, would be to sequence the exact BoLA I allele expressed by bleeder calf cells, so to pin point which variants (possibly out of the three already identified as highly interesting) are the alleles targeted by BNP associated anti-BoLA I alloantibodies. In addition to a preferential expression of these BoLA I molecule(s) on haematopoietic stem cells of the affected lineages at the susceptible maturation stages (as described by Bell et al. 2013 and Laming et al. 2012), the outcome would explain the observed panmyelophthisis, which is pathognomonic for BNP. Nonetheless, sequencing the BoLA I alleles expressed by individual bleeder calf cells would have been too time consuming and could not be achieved during the PhD project. Thus the exact BoLA I repertoire of bleeder calves in comparison to non-bleeder calves could unfortunately not be analysed.

4.2 BNP Alloantibodies React Across the Species Barrier

There was a three to four year time lag between the launch of PregSure® BVD and the first BNP observations. This was the case in Germany and more recently in New Zealand (New Zealand Ministry for Primary Industries 2011; Lambton et al. 2012). One plausible explanation, as also presented by data shown here, is that repetitive vaccinations (up to three), in particular, are required to induce alloantibody titres high enough to provoke clinical symptoms (Kasonta et al. 2012).

Our findings confirm that the conditions reported in bovine neonates in New Zealand are indeed BNP cases. The reactivity pattern of New Zealand BNP dams is identical to confirmed European BNP cases. In addition to an anti-BoLA I reactivity in serum directed against bovine lymphoblasts and the production cell line, we observed an even higher BNP alloantibody reactivity in colostrum of BNP dams. This is in accordance with the general perception that colostrum contains extremely high antibody concentrations (Stelwagen et al. 2009) and is in line with the current

concept of the pathoaetiology of BNP (Bridger et al. 2011; Bell et al. 2013; Schroter et al. 2011). On the other hand, we did not detect any alloreactivity in normal milk, thus excluding milk as a potential source of BNP alloantibodies.

In this context, previous observations already indicated that BNP colostrum might be able to induce BNP in other species, such as lambs (Winter 2011) and supplementary experiments with cell lines from non-bovine origin show that BNP alloantibodies can react across the specie barrier.

In Germany the general perception was that such a cross reactivity does not pose a potential risk to human consumers of dairy products, because according to § 18 of the German milk regulations (*Milchverordnung*; complete title: *Verordnung über Hygiene- und Qualitätsanforderungen an Milch und Erzeugnisse auf Milchbasis*) it was prohibited to sell colostrum as milk, a milk-based product or mixture with other dairy substances. However, it is legal to market special dietary supplements or sports food on colostrum basis and in other countries colostrum based products – even infant formula – are very popular. Since colostrum products represent an important economic sector to the New Zealand dairy company Fonterra Ltd., the company was particularly interested if dairy products from BNP dams contained alloantibodies potentially capable of cross-reacting with human cells.

In accordance to previous findings, we observed, through flow cytometry and IP, that BNP-associated antibodies do indeed cross react across the species barrier and even recognize human MHC class I, i.e. HLA class I molecules. Flow cytometry reactivity to human lymphoblasts is only detectable with serum from PregSure® BVD immunized animals. This is particularly the case when BNP associated, affinity purified alloantibodies are analysed (i.e. antibodies that have been eluted from the surface of the bovine kidney cell line used for PregSure® BVD production). *In-vitro*, BNP alloantibodies bound to human lymphoblasts can induce complement-lysis. This indicates that BNP-associated alloantibodies could be clinically relevant as they have the capacity to exert a cytotoxic effect on human lymphoblasts.

Despite a production-inherent dilution resulting from pooling colostrum from PregSure® BVD vaccinated and non-vaccinated herds the alloreactivity to human cells could also be traced in commercial colostrum powder. In view of these observations Fonterra Ltd. changed their sampling policy: As from 2012 on, colostrum is only collected from animals that had never been vaccinated with PregSure® BVD. This new colostrum powder displayed no cross-reactivity, neither to

the cell line nor to human lymphoblasts. This result confirms that the previously observed reactivity was indeed due to BNP associated alloantibodies.

Taking into consideration that so far, only BoLA I has been identified as a BNP alloantigen these results are of significance. Nonetheless, despite these findings, it is debatable whether these observations suggest that colostral products from BNP dams are dangerous for human consumers. Considering the assumption that MHC- I specific antibodies cause BNP pathology, one hypothesis is that a large amount of MHC- I alloantibodies are a prerequisite in order to saturate peripheral MHC- I prior to induction of a tissue specific insult. It is conceivable that such a sudden and immediate uptake of several grams of maternal alloantibodies is possible in calves during nursing. However, it is unlikely that such a massive influx of antibodies can happen in humans, also due to the fact that, in contrast to bovine neonates, bovine dietary antibodies are not effectively transported across the human gut mucosa (Ober 2001).

The neonatal Fc-receptor, FcR_n, is a specific transport system, which transports antibodies across the gut-blood barrier and is expressed throughout life (Shah et al. 2003; Israel et al. 1997). In humans, the foetus is provided with maternal antibodies via the placenta during gestation (Pentsuk, van der Laan, Jan Willem 2009; Butler, J., and M. Kehrli. 2005), whereas ruminants actively absorb intact macromolecules after birth due to the impermeable placental architecture. Absorption of immunoglobulins occurs by non-selective pinocytosis and is only possible for approximately 24 – 48 hours after the calf is born (Fetcher et al. 1983; Bush, Staley 1980; Staley, Bush 1985; Jochims et al. 1994). The affinity of human FcR_n for bovine IgG is up to four-fold lower than that of human IgG (Ober 2001) and since the concentration of HLA cross-reactive alloantibodies in commercial colostrum products is relatively low, the uptake of alloantibodies would be inefficient. As a consequence only an insignificant amount of HLA cross-reactive BNP antibodies would reach the blood circulation of human consumers.

In conclusion, the risk of colostrum-containing dietary supplements, from cows vaccinated with PregSure® BVD or BNP dams is probably low for human consumers, but since the reactivity of BNP alloantibodies to human lymphoblasts is principally indistinguishable from the reactivity to bovine cells, a residual, theoretical risk cannot be absolutely excluded. Based on this conclusion, Fonterra Ltd. also decided to withhold the entire colostrum production of 2011. Despite the recall of the vaccine in

New Zealand in 2011 (New Zealand Ministry for Primary Industries 2011), from 2012 onwards, any PregSure® BVD treated cows were also eliminated from colostrum collection. This approach proved to be effective and reasonable as no alloreactivity to bovine or human cells was detectable in colostrum powder manufactured according to the new sampling policy.

4.3 A Closer Look at PregSure® BVD

It has to be emphasized that non-product related proteins and impurities are not uncommon in biopharmaceuticals and are regulated as well as monitored to ensure safe levels (Dagouassat et al. 2001; Wang et al. 2009). This condition is, therefore, not limited to the vaccine PregSure® BVD, but a unique factor in this case was the combination of a potent and highly immunogenic novel adjuvant system, the viral antigen (Pfizer Animal Health 2004) and the presence of host cell contaminants (Euler et al. 2013), which together play a major role in alloantibody production (Bastian et al. 2011; Federal Association of Official Veterinarians 2012). Bell et al (2013) postulated that apart from HCP directed alloantibodies, the adjuvant in PregSure® BVD might boost normal pregnancy-induced maternal foetal MHC antibodies (Hines, Newman 1981; Newman, Hines 1980). Although being considered generally non-hazardous and produced at a very low titre, it has been shown that, especially multiparous cows possess foetally stimulated antibodies that are directed against paternally-derived foetal MHC molecules. Bell and colleagues postulate that the production of these alloantibodies might be exponentiated due to the potent function of Procision- A™, leading to an increased titre, which might then be pathogenic. However, unpublished experiments (Bastian *et al*.) in which animals were immunized only with the adjuvant Procision- A™ revealed no alloantibody production, thus this scenario seems to be very unlikely according to our state of knowledge.

Nonetheless, special attention should be directed to the novel oil-in-water adjuvant system. The unique architecture of the nanocomplexes, composed of Quil A- Cholesterol- Drakeol 5- Amphigen® and BVDV antigens complexed microdroplets, could be a plausible explanation as to why bioprocess related membrane impurities might be optimally presented to the immune system only in the case of PregSure® BVD. Surface antigens, such as BoLA class I, might be stabilized and efficiently presented in the microdroplets, thus making them easier accessible to the immune

system. The additional immunestimulating components might further enhance immunogenicity. In contrast, in other BVDV vaccines adjuvanted, for example, with aluminium hydroxide, HCP might be hidden in the crystalline structures and not accessible to the immune system. However, this assumption would question how on the other hand BVDV antigens are efficiently presented, as seen by a sufficient (although lower) anti-BVDV titre after vaccination.

However, with PregSure® BVD, Pfizer (now Zoetis) has been able to achieve a major breakthrough in vaccine production and adjuvant manufacturing. The novel adjuvant Procision- A™ shows incredible enhancement mechanisms, which is advantageous and fulfils adjuvant purpose. In theory, PregSure® BVD and Procision- A™ could actually be an excellent example of a good-response optimized vaccine. Nevertheless, the vaccines association with BNP underlines the significance of extensive risk assessments necessary in vaccine production. It is a good example of how complex and difficult it is to find the right balance between efficient vaccine production to combat infections and risks from new strategies and innovations. Therefore, BNP could be used as a model for a vaccine induced alloimmune syndrome in order to define the premises for a safe and efficient use of highly immunogenic adjuvants in livestock vaccines.

5. Conclusion and Proposed Future Work

In view of the massive increase of novel idiopathic haemorrhagic diathesis (HD) cases in cattle since 2007, those cases were given a new entity known as *Bovine Neonatal Pancytopenia* (BNP). In the current work, it has been demonstrated that cattle multiply vaccinated with PregSure® BVD and more significantly, BNP dams, possess alloantibodies, which bind to surface antigens on calf lymphoblasts and are cross reactive to the permanent bovine kidney cell line that is used for PregSure® BVD production. These BNP associated alloantibodies target highly polymorphic Bovine Leucocyte Antigen class I (BoLA I) molecules. Three BoLA I alleles have been identified, which seem to be responsible for the majority of PregSure® BVD induced BoLA I reactivity. Additionally, BNP alloantibodies react across the specie barrier and recognize human MHC class I and can even be found in commercial colostral products.

Further work should include the investigation of the precise pathomechanism leading to BNP and on ongoing analysis in which exact BoLA I allele and possibly even combination out of the three identified; perhaps even in addition to a yet unknown alloantigen, is relevant to induce BNP in a calf. Alloreactivity is usually due to single amino acid polymorphisms thus the affinity of alloreactive antibodies is often low and conformational dependent. This could also be observed in our laboratory through, simple western blotting after protein denaturation, for instance, did not result in any bands. Therefore, one suggestion was to use guinea pigs as a heterologous system. The research group has already published that vaccinated guinea pigs also produce BNP alloantibodies (Bastian et al. 2011). The animals do not have an autotolerance and all immunogenic BNP antigens are recognized as truly foreign. Therefore, the reactivity to surface antigens is stronger and likely not conformational dependent, making the animals a good system to identify further BNP alloantigens. Preliminary experiments have already been performed and further confirmation of identified hits as possible alloantigens in cattle should be the aim in future BNP research.

Together these efforts will provide deeper insight into vaccine-induced alloimmune diseases, which is necessary to prevent occurrence of similar problems in the future. The results presented here strengthen the importance of and necessity to optimize purification technologies of vaccines and to analyse effects that might be caused by residual host cell proteins and process contaminants. Future work could enable the establishment of contamination thresholds that should be considered in veterinary vaccines as the occurrence of BNP strongly emphasizes.

6. Supplementary Figures

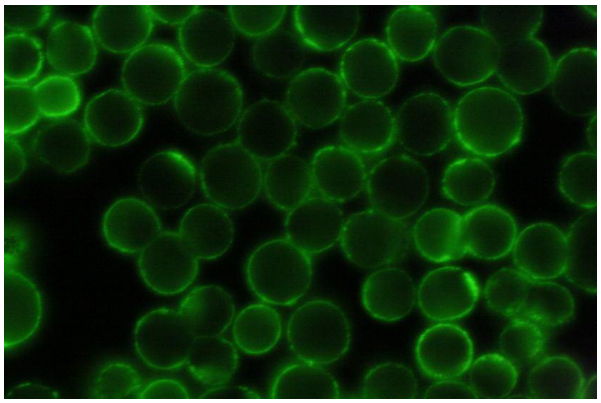


Figure S1: Fluorescence microscopy of intact bovine kidney cells. Cells were incubated with affinity purified alloantibodies of a BNP dam and analysed with a secondary sheep- α- bovine- IgG^{FITC} antibody.

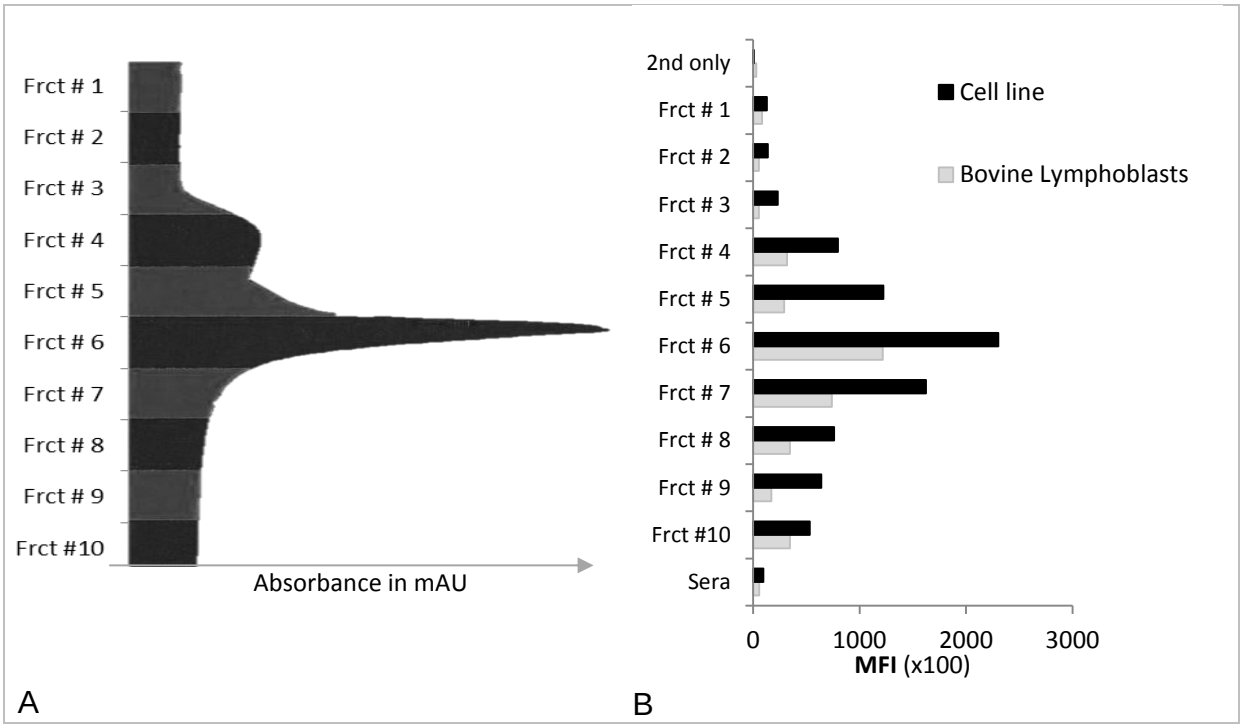


Figure S2: Affinity chromatography of eluted alloantibodies. (A) Antibodies in sera of a BNP dam were absorbed on intact bovine kidney cell surface, eluted at a low pH and concentrated by protein G coupled affinity chromatography. Ten fractions were collected with the beginning of elution (different coloured columns). The x- axis shows the absorbance at 280 nm (B) MFI of affinity purified antibodies determined by flow cytometry. Primary antibodies bound to bovine kidney cells or bovine lymphoblasts were analysed by sheep- α-bovine- IgG^{FITC}. The cells were incubated with sera of a BNP dam (sera), the different fractions collected by affinity chromatography (Frct #) or PBS and secondary antibody only (2nd only).

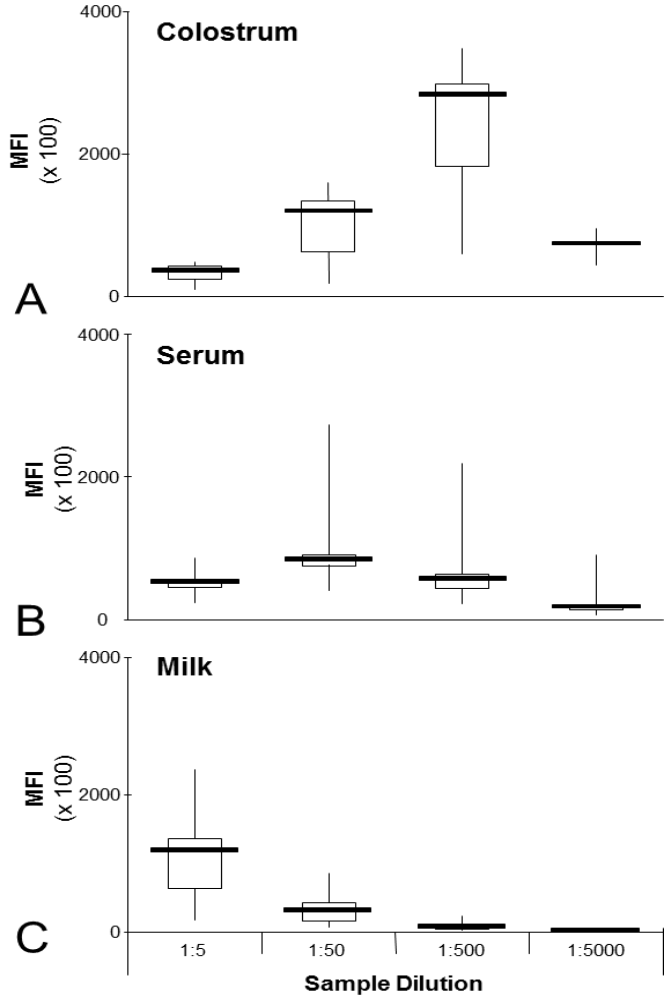


Figure S3: MFI of colostrum, serum and milk from BNP dams. The bovine kidney cell line was treated with different dilutions of (A) colostrum, (B) serum or (C) milk of BNP dams. The 1:500 dilution proved to be the most reliable one. Too high concentrated milk reveals unspecific binding. Bars represent the range of MFI from 6 different dams, the black bar the median value for each group with the standard deviation.

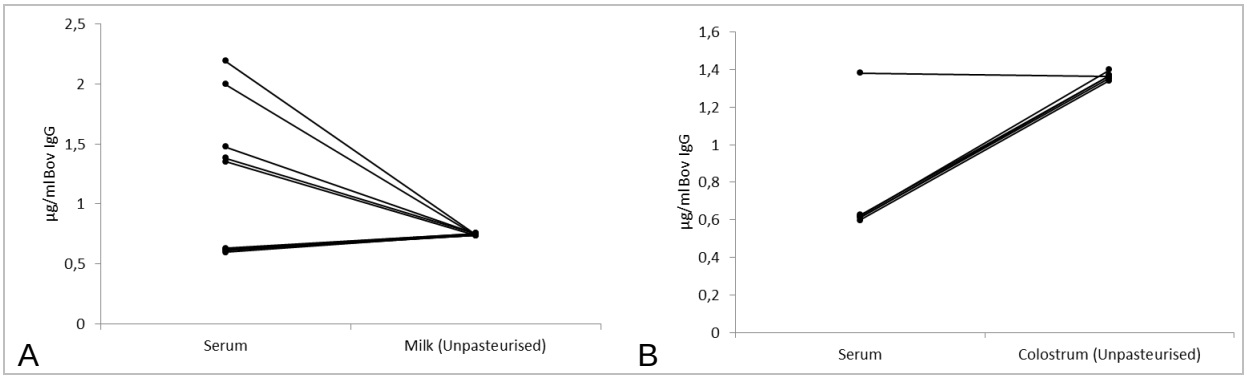


Figure S4: Antibody content of colostrum, serum and milk from BNP dams. The antibody content of bovine IgG in colostrum, serum and milk of BNP dams was determined by ELISA. (A) Serum and corresponding milk of 12 dams; as well as (B) serum and corresponding colostrum of 6 dams was available. The antibody content in milk is lowest, followed by serum and the highest concentration is found in colostrum.

Table S1: Nucleic acid yield from BoLA I transformed *E. coli* determined by photometry

BoLA I Allele	Nucleic acid- concentration
BoLA Nc3*00101	1.6 µg/µL
BoLA Nc3*50201	0.7 µg/µL
BoLA 2*40801 (6 mismatches)	3.0 µg/µL
BoLA 2*40801 (138 bp missing)	0.9 µg/µL
BoLA 2*04801 (29 mismatches)	1.9 µg/µL
BoLA 2*04801	2.9 µg/µL
BoLA 2*04601 (35 mismatches)	1.2 µg/µL
BoLA 3*01101	1.8 µg/µL

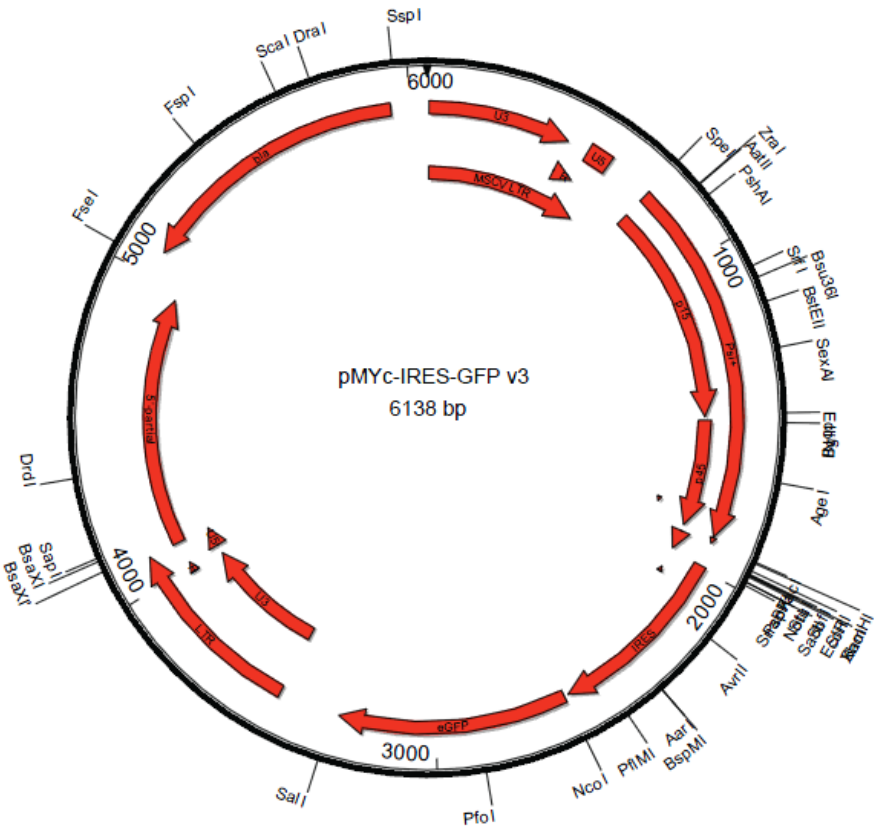


Figure S5: Plasmid map of the eukaryotic *pMyc* vector. The vector possesses the *E. coli* origin of replication (ori) and information on ampicillin resistance as a possible selection marker (Amp^R). In addition to elements for expression in eukaryotic cells i.e., the *Long Terminal Repeat* sequence (LTR) to integrate DNA into the eukaryotic genome, eGFP to select successful transduced cells and a *Multiple Cloning Site* (MCS) containing restriction sites for different enzymes including *Bam*HI and *Xho*I.

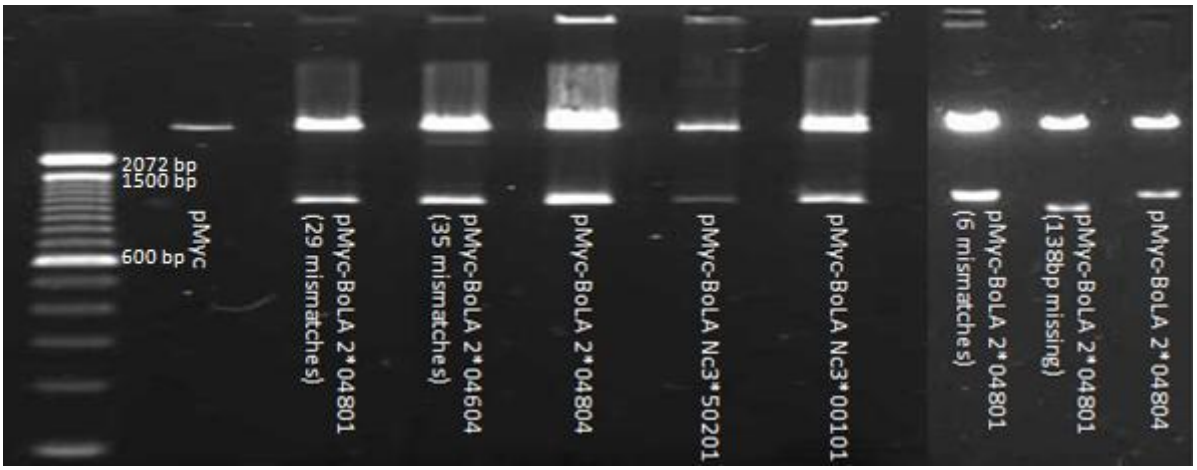
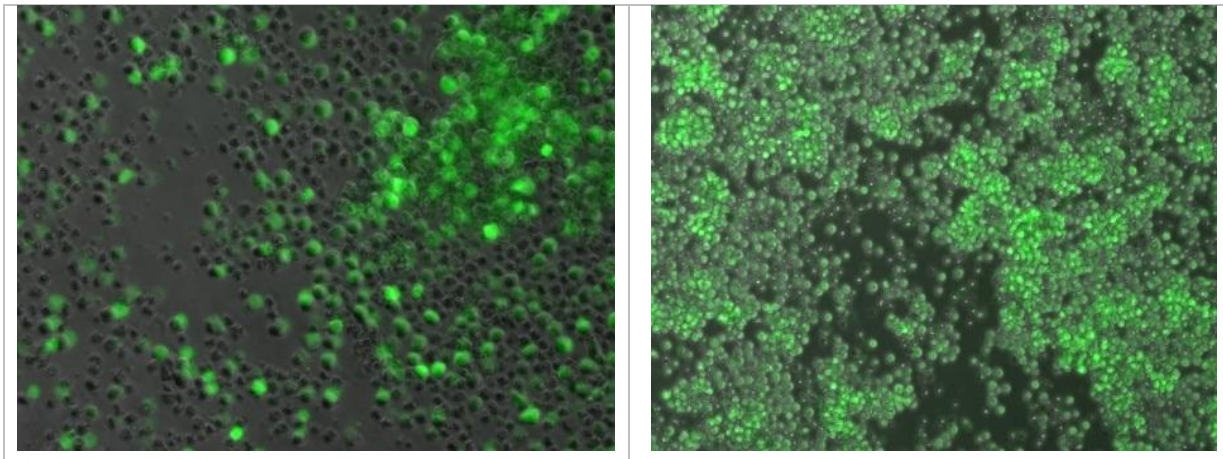


Figure S6: Ligation success monitored by restriction digest. After ligation and restriction digestion the *pMyc*-eGFP-BoLA plasmid fragments were analysed by gel electrophoresis. On the left is the 100 bp-DNA-Ladder, followed by the indicated digested BoLA Plasmid constructs. The *pMyc* vector has a size of about 6000 bp and the BoLA I alleles approximately 1200 bp.



A B

Figure S7: Fluorescence microscopy of transduced murine pre-B cells. (A) The percentage of transduced 38B9 cells after 24 h with virus particles is low (shown as an example of *pMyc*-BoLA Nc3*00101 at 200 x magnification). (B) Transduction efficiency was increased by co-culturing cells with a gamma irradiated growth-arrested feeder cell population. BoLA I is incorporated before the IRES followed by eGFP, thus a successful transduction results in green fluorescence.

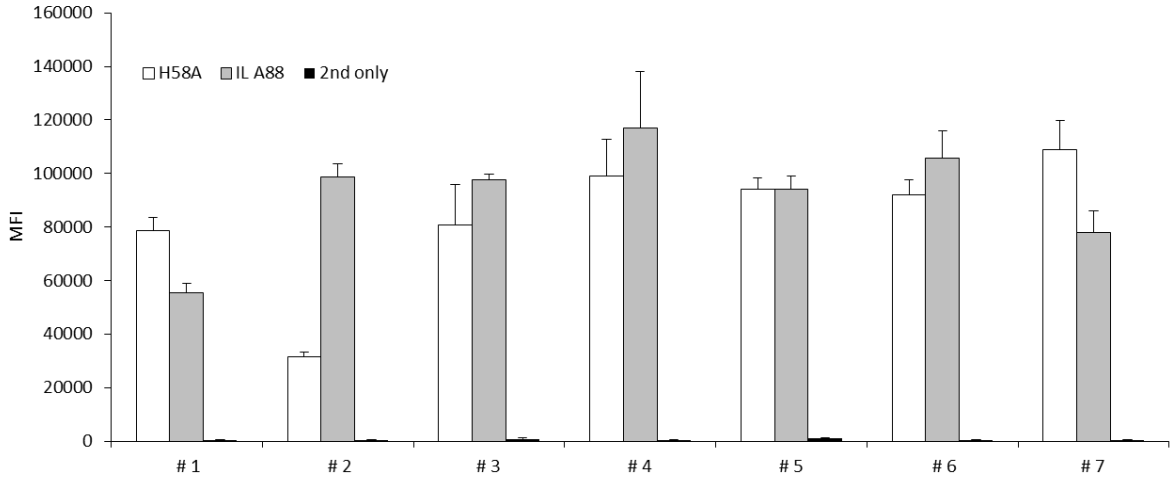


Figure S8: BoLA I expression of calves cells. The BoLA I expression of different calves cells was analysed using the mouse anti-MHC-I mAb H58A (white bars), or with IL- A88 an anti-BoLA-I mAb (grey bars). A control with PBS and secondary antibody only (black bars) was done in parallel.

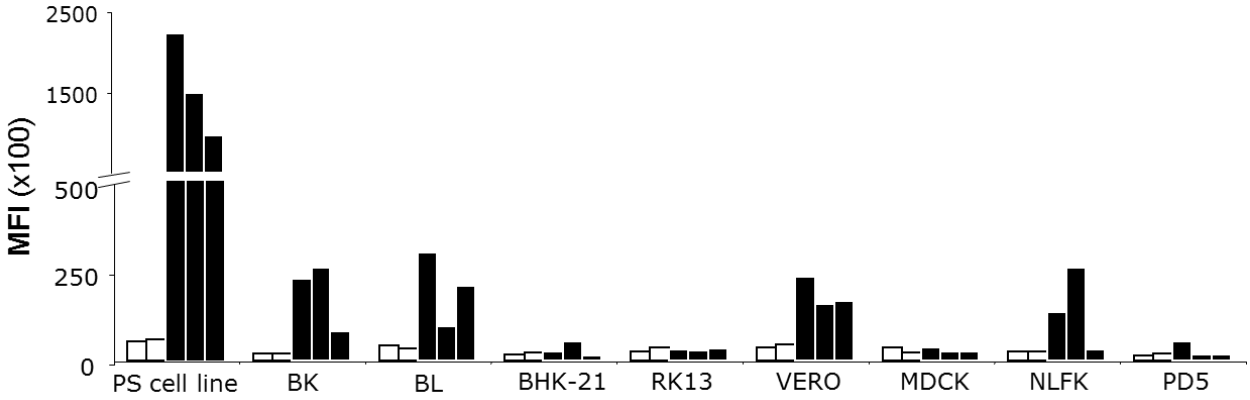


Figure S 9: Flow cytometric reactivity to cell lines of different species. Depicted is the MFI of animals not vaccinated with PregSure® BVD (white bars) and three confirmed BNP dams (black bars). The first three cell lines are of bovine origin and used by manufacturers for veterinary vaccine production (the first being the PregSure production cell line, the second is another bovine kidney cell line (BK), the third a bovine lung cell line (BL)). Followed by the cell lines: Baby Hamster Kidney (BHK-21), Normal Rabbit Kidney Epithelial Cells (RK13), Normal African Green Monkey Kidney Epithelial Cells (VERO), Madin Darby Canine Kidney (MDCK), Norden Laboratory Feline Kidney (NLFK) and Porcine Kidney (PD5). BNP dams do not only recognize surface structures of bovine cells but also on cells from monkey and feline species.

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8. Annex

Publication 1:

Kasonta, R.; Sauter-Louis, C.; Holsteg, M.; Duchow, K.; Cussler, K.; Bastian, M. (2012): Effect of the vaccination scheme on PregSure® BVD induced alloreactivity and the incidence of Bovine Neonatal Pancytopenia. In Vaccine 30 (47), pp. 6649–6655. DOI: 10.1016/j.vaccine.2012.08.069.

Publication 2:

Kasonta, R.; Sauter-Louis, C.; Holsteg, M.; Cussler, K.; Bastian, M. (2014): BoLA-1 antibodies and the induction of bovine neonatal pancytopenia: a twin calves study. In Veterinary Record Case Reports, 2, DOI:10.1136/vetreccr-2014-000082.

Publication 3:

Kasonta, R.; Holsteg, M.; Duchow, K.; Dekker, J.; Bendall, J.; Bastian, M. (2014): Colostrum from cows immunized with a vaccine associated with Bovine Neonatal Pancytopenia contains allo-antibodies that cross-react with human MHC-I molecules. In PLoS One, DOI:10.1371/journal.pone.0109239

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Effect of the vaccination scheme on PregSure®BVD induced alloreactivity and the incidence of *Bovine Neonatal Pancytopenia*

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ABSTRACT

Bovine Neonatal Pancytopenia (BNP) is a new neonate-maternal incompatibility phenomenon caused by vaccine-induced, maternal alloantibodies. The syndrome affects newborn calves at the approximate age of ten days and is characterized by spontaneous bleeding, severe anemia with an almost complete destruction of the red bone marrow. During the past two years the causal role of bioprocess impurities in PregSure®BVD, a strongly adjuvanted, inactivated vaccine against Bovine Virus Diarrhoea (BVD), in the induction of BNP causing alloantibodies has clearly been established. Despite intensive research efforts that have elucidated the basic principles of the BNP immunopathology still a number of questions remain open.

In the current manuscript we address the puzzling observation that BNP incidences vary widely between different regions: as an example we compare the BNP incidences in the German Federal States of Bavaria and Lower Saxony. In Bavaria the BNP-incidence reaches 100 cases per 100,000 doses PregSure®BVD, while in Lower Saxony the incidence is as low as 6 cases per 100,000 doses. In Bavaria the vaccine has always been used according to the instructions for use. By contrast, in Lower Saxony BVD-immunization was performed according to a two-step vaccination protocol including a first immunization with an inactivated BVD-vaccine followed by booster immunizations with a live-attenuated BVD-vaccine. As a consequence, those cattle that received PregSure®BVD received in general more than two doses in Bavaria, while in Lower Saxony cows received at maximum one dose. By experimental immunization we can show that the two-step regimen including PregSure®BVD as a priming vaccine results in significantly lower alloantibody titers as compared to repetitive immunizations with the inactivated vaccine. The lower alloantibody titer after two-step vaccination explains the lower BNP-incidence in Lower Saxony and – generally speaking – indicates that variations in the vaccination regimen have a great influence on the induction of adverse reactions through bioprocess impurities.

1. Introduction

Bovine Neonatal Pancytopenia (BNP) is a very severe and often lethal hemorrhagic syndrome in bovine neonates that is characterized by spontaneous bleeding and an almost complete destruction of the red bone marrow (panmyelophthisis) [1]. BNP was first described in Bavaria and thereafter frequently observed in several regions within Germany. However, very few cases were observed in other parts of Germany, for example in Lower Saxony. On the European level an increase in hemorrhagic disease cases was also noted in some neighboring countries first in Belgium and The Netherlands, followed by France, the UK and several other Member States. Most recently, the occurrence of BNP was described in New Zealand.

During the past two years convincing evidence accumulated that BNP is a neonate-maternal incompatibility phenomenon that is caused by vaccine-induced, maternal alloantibodies. Indeed, the disease only occurred where PregSure®BVD, a potently adjuvanted vaccine against *Bovine Viral Diarrhoea* (BVD), was marketed. European countries where the vaccine was not marketed such as Switzerland, Austria and the Scandinavian countries stayed free from BNP. Only in very few countries outside Europe PregSure®BVD has been launched including New Zealand and Turkey. In a previous publication we were able to demonstrate that PregSure®BVD induces alloreactive antibodies against cell surface molecules on bovine cells due to bioprocess related impurities originating from the cell line used for virus propagation [2]. This was later confirmed

Abbreviations: BNP, *Bovine Neonatal Pancytopenia*; BVD, *Bovine Viral Diarrhoea*; MFI, median fluorescence intensity; PEI, Paul-Ehrlich-Institut; SNT, Seroneutralisation test.

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by two independent publications that identified cell line derived MHC-I molecules in the vaccine as a potential source of alloantigens [3,4]. The vaccine-induced alloantibodies are transferred to the neonate via colostrum. If surface antigens of neonatal cells match with the specificity of the maternal antibodies the neonatal blood cells become opsonised [5] and are subsequently cleared from circulation by the reticulo-endothelial-system, as discussed previously [2]. The resulting thrombocytopenia and leukopenia explains the clinical presentation, namely the severe hemorrhages [6].

Although the basic principles leading to BNP are now largely understood several puzzling observations still seek for an explanation and scientific prove: how can the great regional differences in the prevalence of BNP that have been reported not only between different European countries, but also within member states [7] be explained? And why is it, that PregSure®BVD had been launched in Germany in 2004, while BNP was only recognized three years later as a new disease? The current report addresses these epidemiological questions. By the comparison of the two German federal states, Lower Saxony and Bavaria, we identify differences in the usage of BVDV-vaccines as a key element for the variability of BNP incidences.

2. Materials and methods

2.1. Epidemiological data

Epidemiological data on the occurrence of BNP was collected via suspected or confirmed cases reported to the Clinic for Ruminants, LMU Munich. Calls to support the study were published twice in the German Veterinary Journal (Deutsches Tierärzteblatt, 5/2009 and 6/2011) to guarantee an even distribution of the information all over Germany. In addition, pharmacovigilance (PhV) data collected within the German PhV database VigilanceVET (PharmUP e solutions) system for Immunological Veterinary Medicinal Products run at the Paul-Ehrlich-Institut (PEI) were used. Due to the very complex epidemiological situation of BNP the PEI, in cooperation with the Pharmacovigilance Working Party of the CVMP, had created a specific reporting form for BNP. This reporting form was also published via the German Veterinary Journal (Deutsches Tierärzteblatt, 4/2009).

During data collection and especially when the two data sets were filtered and combined case definitions were harmonized between LMU and PEI. In all BNP cases included in the present study the calves were younger than 4 weeks and presented clinical signs of hemorrhagic diathesis. BNP was confirmed by histology of bone marrow and/or by examination of blood parameters (thrombocytopenia [<200 G/L] [8] and leucocytopenia [<4 G/L] [9]). The reports provided by LMU and PEI were cross-checked for duplicates. However, due to the confidentiality of the PhV data and the similarities of the case reports it cannot be excluded that a few cases were not identified as duplicates. However, it can be ruled out that these few cases have an influence on the overall BNP incidences as they were calculated for Bavaria and Lower Saxony. Information on the sales figures for PregSure BVD in Bavaria and Lower Saxony were kindly provided by Pfizer Animal Health (Pfizer GmbH, Berlin). For graphical display the cases were plotted according to their postal zip-code. Due to reasons of pseudonymisation the postal zip code was not fully available in many cases. For these reports only the first three of the five numbers of the zip code were available. Thus, the zip code for graphical display was chosen randomly within the possible region.

Table 1
Vaccination scheme: four groups of five bulls were vaccinated with commercially available vaccines against BVD using the depicted vaccination scheme.

	vaccination scheme			
	# 1	# 2	# 3	# 4
T_0 = day 0	inactivated BVD vaccine I	inactivated BVD vaccine II	**PregSure**	**PregSure**
T_1 = day 21	inactivated BVD vaccine I	live BVD vaccine	live BVD vaccine	**PregSure**
T_2 = day 42	inactivated BVD vaccine I	live BVD vaccine	live BVD vaccine	**PregSure**
T_3 = day 63	*blood sampling*	*blood sampling*	*blood sampling*	*blood sampling*

2.2. Immunization of animals and clinical material

Groups of five Holstein-Frisian bull calves at the age of four to six months were immunized with four different BVDV vaccines licensed in Germany including PregSure®BVD.[1] Bull calves were chosen, because due to animal welfare considerations the induction of BNP in potential progeny was to be avoided. Group #1 received three consecutive immunizations with an inactivated BVD vaccine (BVD vaccine 1) at an interval of three weeks. This inactivated BVD vaccine represents one of three inactivated BVD vaccines that are currently available on the German market. Group #2 received a first immunization with another inactivated BVD vaccine (BVD vaccine 2) followed by two booster immunizations with a live attenuated BVD-vaccine (live BVD-vaccine). This immunization scheme represents the so-called "two-step" vaccination. It was initially developed for the MERIAL vaccines Mucobovin® and Vacoviron® (see also Ref. [10]). Group #3 received the first immunization with a dose of Pregsure®BVD (L84680), followed by two booster immunizations with the live attenuated BVD vaccine. This vaccination scheme represents the so-called "modified two-step" combination. Group #4 received three consecutive immunizations with PregSure®BVD and is therefore in accordance with the instructions for use of PregSure®BVD (see Table 1). All vaccines were administered subcutaneously at the right shoulder. Before and three weeks after each immunization serum samples were obtained by venipuncture. For leukocyte preparations whole blood samples were taken from a healthy calf at the age of 10 weeks. The immunization study was notified to the responsible authority. Since all vaccines were legally approved at that time, it was decided that no permission according to animal welfare legislation was required.

2.3. Cell preparation and cell culture

Peripheral blood mononuclear cells (PBMCs) were prepared from whole blood of a healthy calf by ammonium-chloride lysis and Ficoll gradient centrifugation, as described elsewhere [2]. Short-term T cell lines were obtained by phytohaemagglutinin (PHA, Oxoid) stimulation. To this end PBMCs were re-suspended in complete medium (i.e. RPMI 1640 (Gibco) supplemented with L-glutamine, penicillin–streptomycin and 10% FCS and stimulated at 1×10^6 cells/ml with 0.1 μg/ml purified PHA (Oxoid, Germany) and a 1:20 dilution of a hybridoma supernatant containing human

[1] On 30th August 2011 Pfizer Animal Health has voluntarily withdrawn the license for PregSure®BVD in Germany and consecutively in all other countries.

Interleukin-2. The resulting polyclonal T cell lines are referred to as PHA blasts. The bovine kidney cell line used for PregSure®BVD manufacturing was kindly provided by Pfizer Animal Health and cultured according to manufacturers instructions.

2.4. Seroneutralisation test (SNT)

SNT was performed according to OIE guidelines [11]: briefly, serial dilutions of bovine sera were incubated for two hours with 100 $CCID_{50}$ of cytopathogenic BVD virus strain, NADL. Preincubated virus was transferred to microtiter plates that had been seeded over night with 4×10^4 MDBK (*Madin Darby Bovine Kidney*) cells per well. After three to four days the development of a cytopathic effect (CPE) was assessed by microscopy. CombiStats software (European Directorate for the Quality of Medicines) was used to calculate individual SN titers.

2.5. Flow cytometry

To test for the presence of alloreactive antibodies up to 1×10^5 bovine PHA blasts were distributed in 1 ml tubes and re-suspended in PBS containing 0.5% FCS. Sera were added to a final dilution of 1:5. After one hour incubation at 4 °C, cells were washed twice with PBS containing 0.5% FCS. Bound bovine IgG was subsequently detected using a FITC conjugated polyclonal sheep-α-bovine IgG antibody (AbD serotec) and analyzed using a flow cytometer (BD LSR II). In general, the median fluorescence intensity (MFI) of the FITC signal was determined for the respective cell population and used for further analysis. All sera were tested in five independent experiments using the bovine kidney cell line or PHA blasts derived from two different animals.

2.6. Immunoprecipitation (IP)

To test for the specificity of alloreactive antibodies we adopted the IP-protocol described by Deutskens et al. [3]. Briefly, membrane proteins of the bovine kidney cell line used for the production of PregSure®BVD were labeled with 2 mM EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific). The cells were then incubated with the respective bovine serum, washed and solubilized with Lysis Buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris and 5 mM EDTA). The solution was clarified and bovine IgG precipitated with protein G-sepharose beads (GE Healthcare). The precipitate was washed and analyzed by non-reducing SDS–PAGE. The gels were blotted on nitrocellulose membranes (Whatman). Bovine MHC-I molecules were detected with a mouse monoclonal antibody against bovine MHC-I molecules (IL-A88), and a secondary Horseradish-Peroxidase (HRP) conjugated anti-murine IgG antibody (Dianova). Bound antibodies were visualized by enhanced chemoluminescence reagents (GE Healthcare). The membrane was stripped and biotinylated proteins were detected with a streptavidin–HRP conjugate (Dianova) and again visualized by enhanced chemoluminescence reagents.

2.7. Statistical analysis

Unless otherwise stated the data are presented as median. Simple Student's *T* test was used to determine statistical significance. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Regional differences in vaccination regimens influence the incidence of BNP

For Germany more than 3500 BNP cases were registered within the PhV-system as suspected adverse events related to PregSure®BVD. However, due to the strict case definition only 959 cases were eligible for the current study, mainly because the majority of case reports within the PhV-system was not substantiated by post-mortem or a haemogram. Most of the cases ($n = 558$) occurred in Bavaria, whereas only 17 cases of BNP were reported in Lower Saxony, see Fig. 1. According to marketing data provided by the company, about 259,000 doses of PregSure®BVD were sold in Lower Saxony, and around 564,000 doses in Bavaria. This leads to an incidence of 6 BNP cases per 100,000 doses sold in Lower Saxony (95% confidence interval: 4–10 cases per 100,000 doses sold) and 99 in Bavaria (95% confidence interval: 97–100 cases per 100,000 doses sold), a highly significant difference ($p < 0.001$).

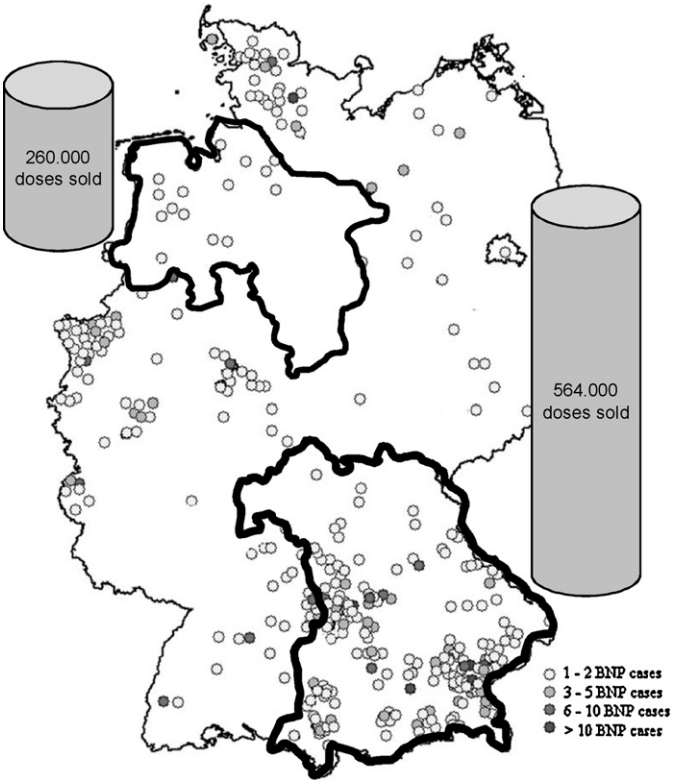

Fig. 1. BNP incidence varies greatly between federal states: individual BNP cases were identified and are depicted as dots on the map of Germany. White dots represent 1–2 cases, light gray dots represent 3–5 cases, dark gray dots represent 6–10 and black dots represent more than 10 BNP-cases within one postal zip-code area. The borders of two federal states, Lower Saxony and Bavaria are outlined. The gray columns indicate the number of PregSure®BVD doses sold in the two federal states.

3.2. Experimental BVD vaccination using different vaccination regimens

To proof the hypothesis that different vaccination schemes account for the varying incidence of BNP in German federal states we immunized young bulls using four different vaccination schemes as listed in Table 1. Sera collected before the first and after each immunization were analyzed for BVDV-neutralizing activity (Fig. 2A). All four vaccination regimens led to a clear titer increase already after two immunizations. Due to the small group sizes the differences are not statistically significant, but there are obvious tendencies: the fastest titer increase in this vaccination experiment was observed with the prime-boost regimen used for group #2. The highest titer after three immunizations was observed with repetitive doses of PregSure®BVD. The lowest response was observed with a two-step vaccination regimen including PregSure®BVD as a priming vaccine (group #3). Supplemenatry Fig. S1 shows SNT values for individual sera over all timepoints.

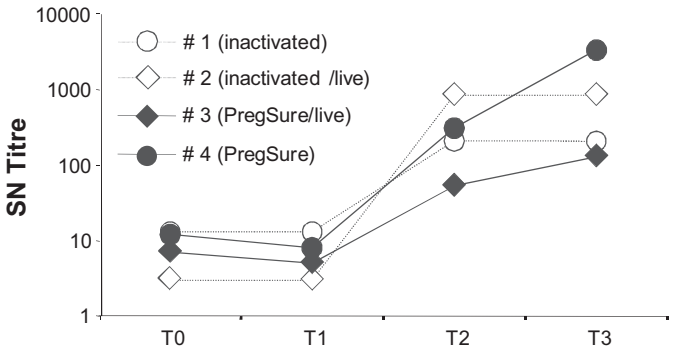

Fig. 2. Repetitive immunization with PregSure®BVD induces the highest anti-BVDV titers: (A) serum samples of experimentally immunized cattle were analyzed for BVDV-neutralizing antibodies by SNT. The development of the median titers for each immunization group is shown over time. Open circles represent group #1, open diamonds group #2, filled diamonds group #3 and filled circles represent group #4.

3.3. The two-step regimen mitigates PregSure®BVD induced alloreactivity

Sera of experimentally immunized cattle were analyzed by flow cytometry for the presence of alloreactive antibodies. PHA blasts of a healthy calf were incubated with the respective sera and surface bound alloantibodies were detected using a FITC conjugated secondary antibody. Fig. 3A shows the development of alloantibody titers: only group #4 samples – receiving three doses of PregSure®BVD – demonstrate a clear induction of alloreactive antibodies. In Fig. 3B results are shown for individual sera obtained after the third immunization. When the results are compared groupwise by Student's *T* test there is a highly significant difference between group #1–3 and group #4. Only one animal in group #3 showed an alloantibody titer comparable to individuals immunized according to vaccination regimen #4. The corresponding results for individual sera over timepoint T0–T2 are shown in Supplemental Fig. S2. Supplemental Fig. S3 shows the development of alloreactive binding for two additional cell lines.

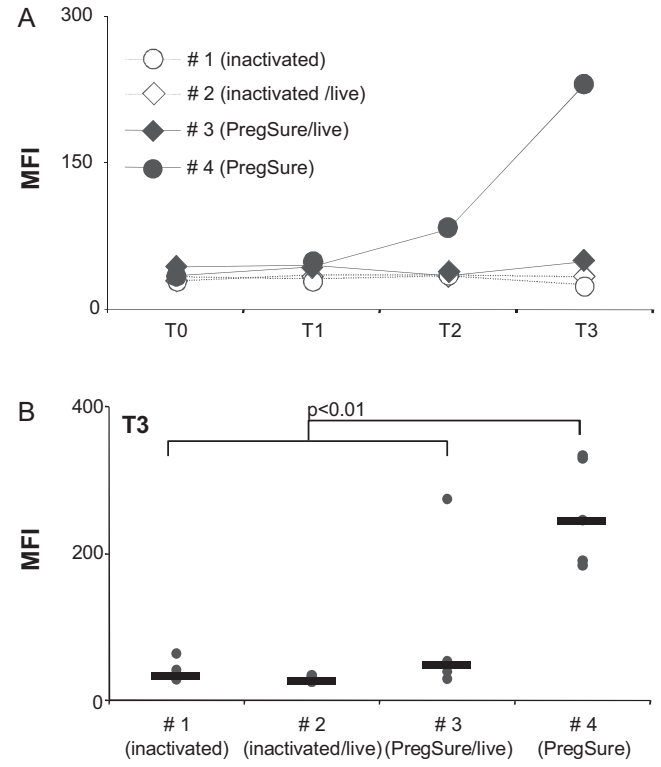

Fig. 3. Alloreactivity is induced by repetitive doses of PregSure®BVD: sera of experimentally immunized cattle were investigated for the presence of alloreactive antibodies. Bovine PHA blasts were incubated with individual sera and surface bound antibodies were detected by FITC-conjugated anti-bovine IgG. (A) The development of the MFI for each immunization group is shown over time. Open circles represent the median for group #1, open diamonds for group #2, filled diamonds for group #3 and filled circles represent group #4. (B) Individual results are shown for the last time point (T3): black dots represent the MFI of individual sera; black bars the median for each group. Results were group wise compared to group #4 and *p* values were calculated by simple Student's *T* test.

3.4. PregSure®BVD induced alloantibodies recognize bovine MHC-I antigens

It has been described that BNP-associated alloantibodies are specific for bovine MHC-I molecules [3,4]. To investigate whether alloantibodies induced by experimental PregSure®BVD vaccination recognize bovine MHC-I molecules we tested the hyperimmune sera after the third vaccination by immunoprecipitation. The protocol was designed to identify only antibody bound surface molecules. As shown in Fig. 4 with all sera of group #4 a reactive band at about 43 kDa size was identified. With one serum of group #3 a specific band in the same size was observed. It was this serum that showed a signal when tested by flow cytometry (see Fig. 3C for comparison). The reactivity at about 58 kDa did not correlate with the flow cytometric signal and was identically observed with several pre-immune sera and the respective negative controls (see Supplementary Fig. S4). To confirm that the 43 kDa reactivity corresponds to bovine MHC-I molecules, we probed the same immunoblots with an anti-bovine MHC-I specific antibody. The reactivity pattern shown in Fig. 4B reveals that the 43 kDa band corresponds to bovine MHC-I.

4. Discussion

BNP is a severe hemorrhagic disorder in bovine neonates. It is now well established that the syndrome is caused by maternal alloantibodies induced by a dam vaccination with the inactivated BVD vaccine, PregSure®BVD [2–4,6,12,13]. However, the elucidation of the causative role of PregSure®BVD has long been hampered by the fact that BNP emerged only three and more years after the product launch in the different countries. Furthermore large regional differences in the incidence of BNP were noted within Germany but also between different European member states and regions [7].

BVD is a notifyable disease in Germany since 2004 and measures to combat the disease, including vaccination, are within the responsibility of the federal states. Detailed investigations concerning the use of BVD vaccines in the different federal states and discussions with the authorities, cattle health services and cattle practitioners revealed that vaccination practices differed largely between regions. In contrast to the majority of federal states which used inactivated vaccines according to the instructions for use, it turned out that the BVD eradication programme of some federal states, including Lower Saxony, propagated a modified vaccination programme. This scheme consists of a primary vaccination with an inactivated BVD vaccine followed by a booster immunization with a live BVD vaccine. This so-called two-step vaccination scheme has been extensively used in Germany for many years [10,14]. It was originally developed for the MERIAL vaccines MUCOBOVIN and VACOVIRON [10]. Later on it was opened for other inactivated vaccines to avoid competitive disadvantages. To this end studies were performed that demonstrated the equivalence of the other inactivated products in the two-step scheme [15,16]. Our observation that the two-step regimen including PregSure®BVD resulted

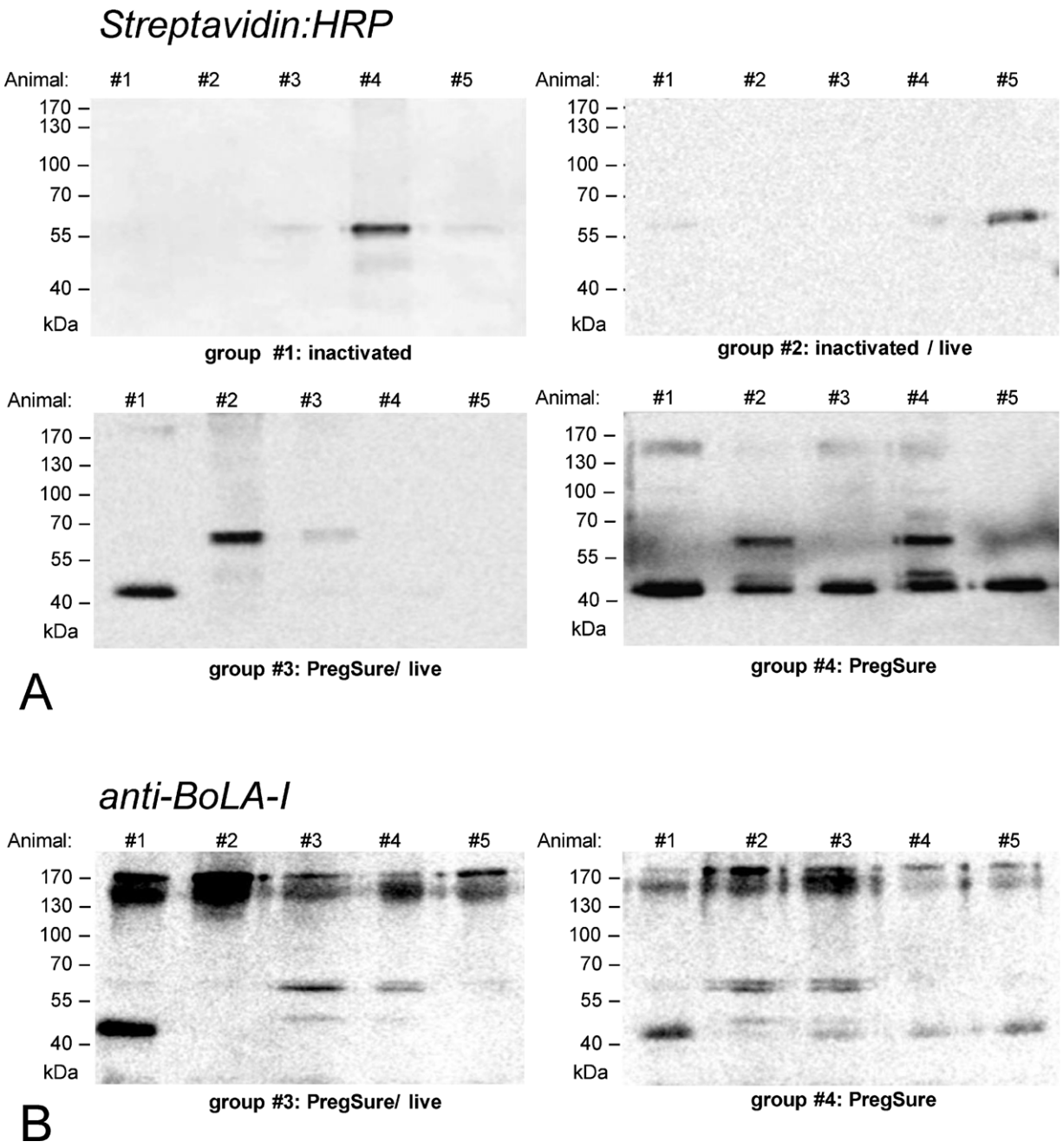

Fig. 4. PregSure®BVD induced alloantibodies bind to bovine MHC-I molecules: (A) after three immunizations serum samples of experimentally immunized cattle were analyzed for their specificity by immunoprecipitation. Cells of the production cell line of PregSure®BVD were extracellularly biotinylated and incubated with the respective sera. Bound antibodies were immunoprecipitated. Precipitates were separated by SDS page, blotted and developed with a streptavidin–HRP conjugate. The four blots show individual immunoprecipitates for each group. (B) The same blots were probed with an anti-bovine MHC-I specific monoclonal antibody.

in a six-fold lower neutralizing BVD titer compared to the original two-step combination (see Fig. 2) is in contrast to these previous studies [15], but may be due to the fact that we chose a short boosting interval of three weeks as it is recommended for the use of PregSure®BVD. The animal disease fund (Tierseuchenkasse) in Lower Saxony only compensated the cost for vaccination if the two-step protocol was used. Due to this compensation strategy most cattle in Lower Saxony that were vaccinated with PregSure®BVD received only a single dose of PregSure®BVD. By contrast, in Bavaria and most other federal states veterinarians in general used only one inactivated BVD vaccine according to the instructions for use and therefore most cattle immunized with PregSure®BVD received two and more doses.

In this context the vast differences in the incidence of BNP between Lower Saxony and for example Bavaria are highly remarkable. In both federal states high numbers of PregSure®BVD doses were marketed. By contrast, we identified a huge difference in the incidence of BNP between the two regions. To this end we collected and evaluated all information that was available within the German PhV system and the epidemiological studies performed by the University of Munich. Although there may be a slight bias in the notification discipline between the two regions and although we can not entirely rule out the possibility that individual duplicate reports have gone unnoticed, we are convinced that the data set presented in the current manuscript represents the most comprehensive epidemiological analysis of the occurrence of BNP that is

available for Germany. We used the rather conservative approach to calculate the incidence as cases per doses sold. Using this approach the incidence reached almost 100 cases per 100,000 PregSure®BVD doses in Bavaria. In Lower Saxony only very few cases were identified equalling an incidence of 6 cases per 100,000 PregSure®BVD doses. Since we know that the vaccination practice differed largely between the two federal states it would also be justified to calculate the incidence as cases per animals vaccinated. In this case the incidence would be at least twice as high in Bavaria because there all cows that were vaccinated with PregSure®BVD received at least the basic vaccination schedule with two doses.

To assess whether this differences in the vaccination regimen may account for the different BNP-incidence rates we experimentally immunized young cattle. Bulls were vaccinated either according to the two-step regimen or with repetitive doses of PregSure®BVD or another inactivated BVD-vaccine, respectively. Subsequently, we investigated the sera in detail for the presence of alloreactive antibodies. The data presented in the current study prove that the induction of significant alloreactivity requires in general repetitive immunizations with PregSure®BVD. This provides a plausible explanation for the time lag between launch of the product and the first recognized BNP cases. According to the recommendations for use the primary immunization comprises two doses, one year later the animals are boosted. So, if three immunizations are required it takes three years before the first calf may develop BNP. This is exactly what has been observed in Germany: PregSure®BVD was licensed in 2004 and the first cases of BNP occurred in 2007. Similar observations were made in other EU member states and in New Zealand. There, the product was marketed since 2008 and the first BNP cases were reported in August 2011 [17].

Repetitive doses of PregSure®BVD are required for the induction of alloreactivity. Accordingly, we find that animals vaccinated with the modified two-step vaccination, as it has been used in Lower Saxony, developed significantly lower alloantibody titers compared to animals receiving repetitive doses of PregSure®BVD. However, the two-step regimen does not completely prevent the induction of alloreactivity by PregSure®BVD: One animal of group #3 developed alloantibodies to a similar degree as the animals of group #4 receiving three doses of PregSure®BVD. Obviously, for some animals one immunization with PregSure®BVD is sufficient to induce an alloreactive antibody response. This fits well to the rare cases of BNP in Lower Saxony and is in accordance with field reports of BNP dams that delivered bleeder calves although they received only one dose of PregSure®BVD (C.S.-L., personal communication).

Two recent publications [3,4] propose that bovine MHC-I molecules act as target for BNP causing alloantibodies. In accordance with these publications we observed a prominent reactivity at 43 kDa with sera of animals that received repetitive doses of PregSure®BVD and with the one animal of group #3 that showed binding by flow cytometry. The band at 58 kDa was considered an unspecific artifact, because it did not correlate with the flow cytometry and was also observed by IP using pre-immune sera or the respective negative controls (see Supplemental Fig. S4). Together with the immunoblot shown in Fig. 4B these observations prove that at least a prominent part of the PregSure®BVD induced alloreactivity targets bovine MHC-I molecules. How a mere MHC-I reactivity is able to cause the severe bone marrow damage, which is considered to be pathognomonic for BNP, is currently controversially discussed (Greinacher; personal communication).[2] Some additional effects are probably at work, because it is hard to conceive that MHC-I specific antibodies should pass all MHC-I expressing tissues without causing any tissue damage and specifically target haematopoietic stem cells in the bone marrow. In addition, it is not yet understood why BNP is a relatively rare event while the alloreactive sera of PregSure®BVD immunized cows bind to lymphocytes of virtually all individuals tested (see [2] for comparison). On-going efforts are aiming to resolve this enigma.

In summary, it can be concluded that PregSure®BVD is unique in its ability to induce opsonising, anti-bovine alloantibodies. This is presumably due to the combination of the homologous cell line derived bioprocess impurities and the highly potent adjuvant system. Our data show that the type of vaccination regimen for PregSure®BVD has great influence on the occurrence and height of vaccine induced antibody responses and as a consequence on the incidence of BNP. This adds to our growing understanding of the complex etiology and epidemiology of this vaccine induced alloimmune phenomenon. On the other hand, there are still a number of open questions that need to be addressed and that will hopefully be answered to avoid similar adverse effects in the future.

[2] Professor Dr. Andreas Greinacher is director of the Institut für Immunologie und Transfusionsmedizin, Ernst Moritz-Arndt-Universität, Greifswald. He was invited speaker at a small workshop on BNP that took place on 22nd September 2011 at the Paul-Ehrlich-Institute.

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Conflict of interest: The authors have no financial conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.08.069.

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Figure S1

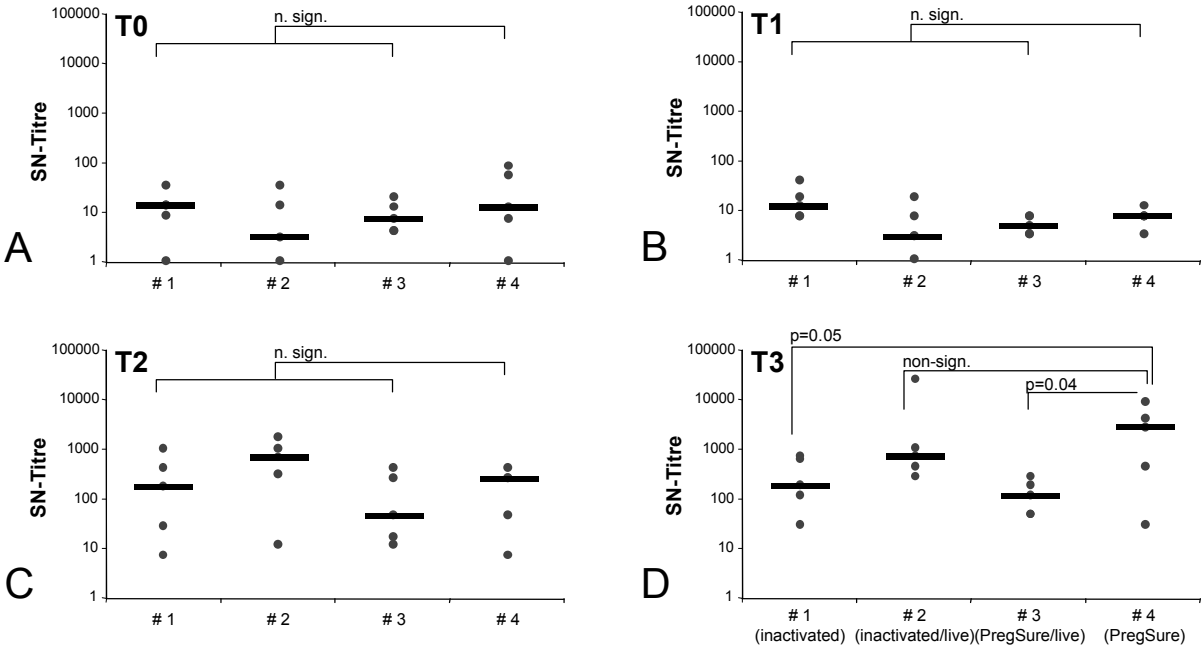

Fig S1 Repetitive immunization with PregSure®BVD induces high anti-BVDV titres: Serum samples of experimentally immunized cattle were analyzed for BVDV-neutralizing antibodies by SNT. Grey dots represent SN-titres for individual serum samples, black bars the median over each immunization group. Results are shown before (A) and three weeks after the respective immunization (B-D). Results were group wise compared to group #4 and P values were calculated by simple Student`s T Test.

Figure S2

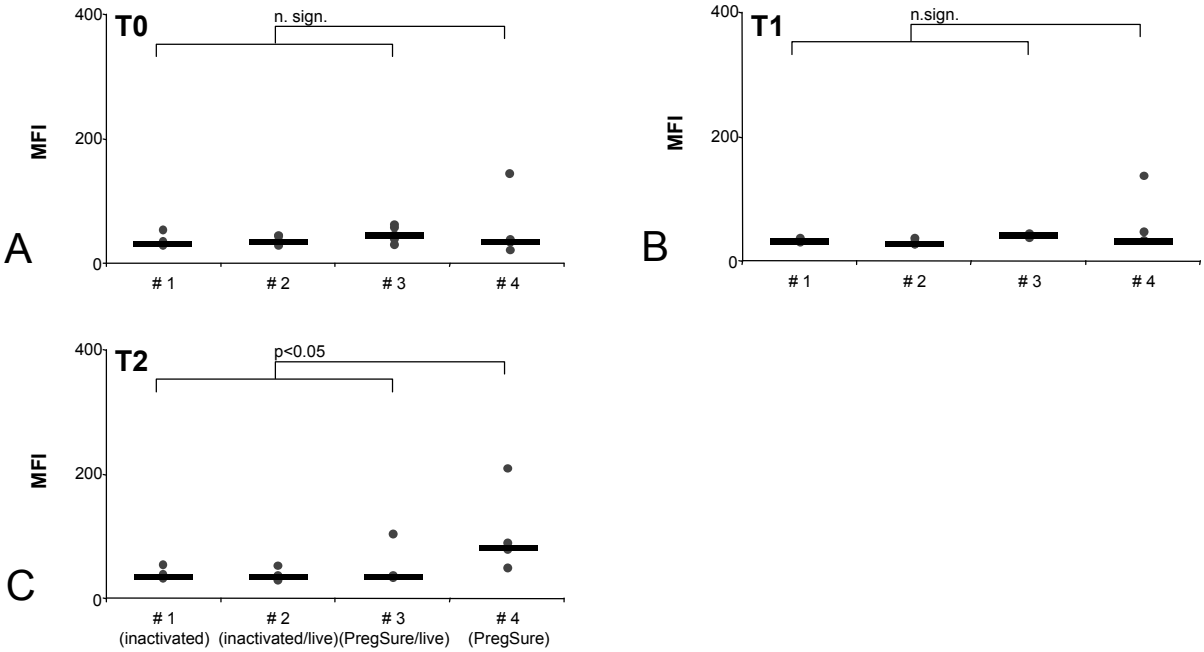

Fig S2 Alloreactivity is induced by repetitive doses of PregSure®BVD: Sera of experimentally immunized cattle were investigated for the presence of alloreactive antibodies. Bovine PHA blasts were incubated with individual sera and surface bound antibodies were detected by FITC-conjugated anti-bovine IgG. Grey dots represent mean fluorescence intensitiy values (MFI) for individual serum samples, black bars the median over each immunization group. Results are shown before (A) and three weeks after the first and the second immunization (B-C). Results were group wise compared to group #4 and P values were calculated by simple Student`s T Test. These results correspond to the data shown in Fig 3.

Figure S3

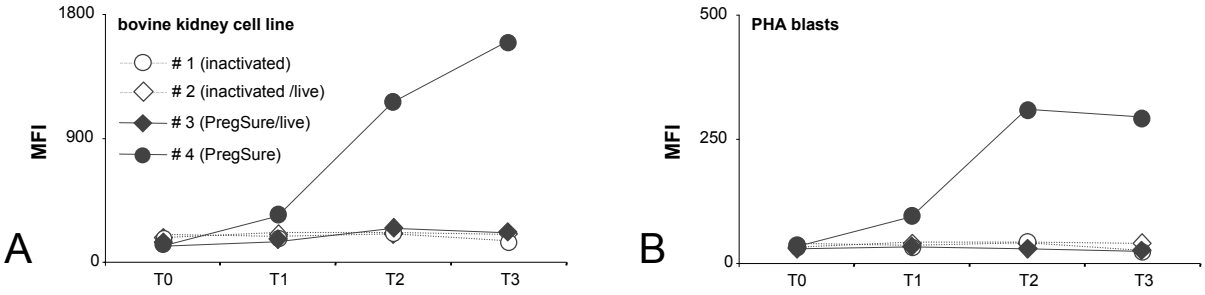

Fig S3 Alloreactivity is induced by repetitive doses of PregSure®BVD: Sera of experimentally immunized cattle were investigated for alloreactive binding to the bovine kidney cell line used for PregSure®BVD production (A) or to PHA blasts derived from a different animal as in Fig 3 and Fig S2 (B). Cells were incubated with individual sera and surface bound antibodies were detected by FITC-conjugated anti-bovine IgG. The development of the mean fluorescence intensity (MFI) for each immunization group is shown over time. Open circles represent the median for group #1, open diamonds for group #2, filled diamonds for group #3 and filled circles represent group #4.

Figure S4

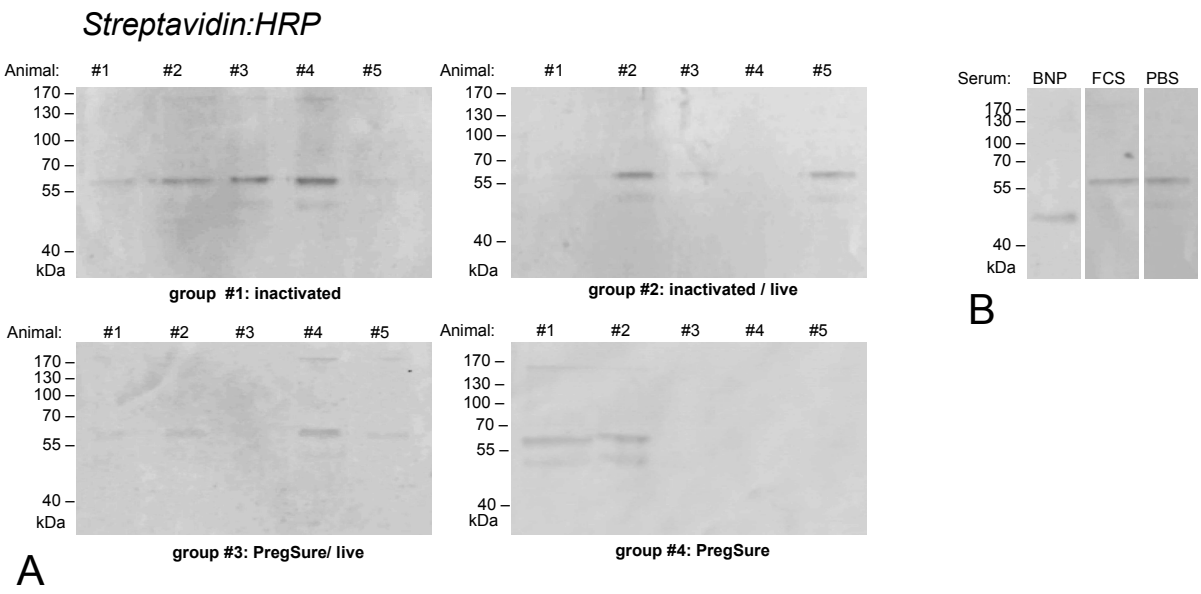

Fig S4 MHC-I reactivity is not detectable with pre-immune sera: (A) Pre-immune sera were analyzed for alloreactivity by immunoprecipitation. Cells of the production cell line of PregSure®BVD were extracellularly biotinylated and incubated with the respective sera. Bound antibodies were immunoprecipitated. Precipitates were separated by SDS page, blotted and developed with a streptavidin-HRP conjugate. The four blots show individual immunoprecipitates for each group. (B) As a control the same was done with serum from a BNP-dam (BNP), with fetal calf serum (FCS) or with the saline control (PBS).

Only with the BNP-dam serum the typical band at about 43 kDa that has been described as bovine MHC-I is detectable. Unspecific bands at about 58 and 50 kDa are observed with many pre-immune sera across all immunization groups and with the fetal calf serum and the saline control.

FOOD/FARMED ANIMALS

BoLA-1 antibodies and the induction of bovine neonatal pancytopenia: a twin calves study

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SUMMARY

Bovine neonatal pancytopenia (BNP) is a vaccine-induced alloimmune disease. The syndrome affects young calves and is characterised by haemorrhages, pancytopenia and severe destruction of the haematopoietic tissue. Recent evidence indicates that vaccine-induced alloantibodies target bovine major histocompatibility complex class I (MHC-I) antigens (bovine leucocyte antigen 1 (BoLA-1)). However, whether BoLA-1-specific antibodies suffice to induce BNP is still a matter of debate. In the current case study, we investigate samples from twin calves where only one calf succumbed to BNP. The fraternal twin also ingested high amounts of BoLA-1-specific alloantibodies but it did not develop BNP because the maternal antibodies did not recognise its BoLA-1 alleles. This association between lack of BoLA-1 binding and the absence of disease provides another important piece of evidence that BoLA-1-specific alloantibodies play a role in the induction of BNP.

BACKGROUND

Bovine neonatal pancytopenia (BNP) is a novel haemorrhagic disease in bovine neonates characterised by spontaneous bleeding within four weeks after birth, destruction of the haematopoietic tissue, and a high fatality rate (Friedrich and others 2009, Pardon and others 2011). The disease is induced by alloreactive, maternal antibodies that are present in the colostrum and serum of cows that were previously vaccinated with PregSure Bovine Viral Diarrhoea (BVD), a highly adjuvanted vaccine against BVD. It could be shown that bioprocess impurities in the vaccine, originating from the cell line used for manufacturing, induce these alloreactive antibodies in vaccinated cattle (Bastian and others 2011). Recently, BNP-associated alloantibodies have been reported to target highly polymorphic major histocompatibility complex class I (MHC-I) molecules, also known as bovine leucocyte antigen 1 (BoLA-1) (Deutskens and others 2011, Foucras and others 2011). This report investigates an interesting case of BNP in one of a pair of twin calves observed on a farm in Germany.

CASE PRESENTATION

The female twin calves were born healthy in October 2011, and obtained fresh maternal colostrum (approximately 3 l) within three hours after birth. The dam had received three vaccinations with PregSure BVD in July and August 2008, and a booster shot in June 2009. Ten days after birth, one calf died after showing clinical signs of BNP. Postmortem diagnostics revealed haemorrhage and extensive destruction of the haematopoietic tissue, similar to findings observed in confirmed BNP cases (Pardon and others 2010).

The sibling remained healthy and did not show any abnormal signs within the first month of life. This second calf (calf 2) died five weeks after birth. The postmortem examination revealed that the death was due to infection of several organs. The bone marrow appeared normal at the time of the postmortem examination (see Table 1). The case was reported to the national authority within the pharmacovigilance scheme in a timely manner, to allow further investigations and laboratory tests.

INVESTIGATIONS

Whole blood and serum of the healthy calf (calf 2), as well as serum of the dam, were collected four weeks after calving by the local veterinarian. Peripheral blood mononuclear cells were isolated and short-term T-cell lines (hereafter referred to as phytohemagglutinin (PHA) blasts) were generated as described previously (Bastian and others 2011).

Opsonising alloreactive antibodies were determined as described (ibid), briefly, PHA blasts from the calf, or cells of the bovine kidney cell line used for PregSure BVD production, were incubated with serum of the calf, the dam or another confirmed BNP dam. Surface-bound antibodies were detected by flow cytometry (BD LSR II) using a secondary fluorescein isothiocyanate-conjugated polyclonal sheep-α-bovine IgG antibody (AbD serotec). Alternatively, the cells were analysed with an anti-MHC class I antibody (clone H58A, VMRD) and an antimurine Pe-cy 5.5-conjugated antibody (Invitrogen).

Identification of the alloantigen was performed as described by Deutskens and others (2011). In summary, the cell line was extracellularly biotinylated, incubated with the respective serum from the calf, a confirmed BNP dam, or a non-vaccinated calf, washed and disrupted in an ultrasonic bath. The solubilised immune complexes were precipitated with protein-G. The resulting precipitate was separated by non-reducing SDS-PAGE and western blotting, with subsequent detection of biotinylated membrane proteins by horseradish-peroxidase (HRP)-conjugated streptavidin (Dianova). Alternatively, the blot was developed with an anti-BoLA-1 antibody (IL-A88, AbD Serotec) and HRP conjugated antimouse IgG antibody (Dianova). Unfortunately, any additional or further experiments could not be performed due to limitations in sample amount and poor growth of the PHA blasts.

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TABLE 1: Anamnestic information

	Calf 1	Calf 2
October 16, 2011	Normal birth	Normal birth
October 26, 2011	† ▶ clinical symptoms of BNP ▶ postmortem: – haemorrhages – complete destruction of the red bone marrow	Healthy
November 20, 2011		† ▶ **No** symptoms of BNP ▶ postmortem: – purulent pneumonia – fibrinous pericarditis – normal appearance of bone marrow

†Deceased.
BNP, bovine neonatal pancytopenia

OUTCOME AND FOLLOW-UP

The presence of alloreactive antibodies in the serum of calf 2 was assessed by comparing the calf serum to a reference serum of a confirmed BNP dam. By flow cytometry, it was shown that the calf ingested alloreactive colostrum—because the alloreactive staining pattern was almost identical when the respective sera were tested for alloreactive binding to the bovine kidney cell line used for PregSure BVD production (Fig 1A). Immunoprecipitation experiments with the bovine kidney cell line revealed that the calf serum did possess anti-MHC class I antibodies as they precipitated a band at 43 kDa that has previously been identified as BoLA-1 (Deutskens and others 2011, Foucras and others 2011). The BNP reference serum precipitated a band at the same size (Fig 1B). BoLa-1 was also additionally confirmed with the IL-A88 antibody and a secondary HRP-conjugated antimouse IgG antibody.

Despite the abundant presence of BoLA-1-specific alloantibodies, neither the reference serum nor serum from the dam (nor from the calf itself (not shown)) did interact with PHA blasts of calf 2 (Fig 2A, white bars). This was in marked contrast with the kidney cell line, which was recognised by both sera (Fig 2A, black bars). This discrepancy was not due to differences in the expression level of BoLA-1 molecules, because both cell lines expressed similar levels as confirmed by flow cytometry using a BoLA-1 specific monoclonal antibody (Fig 2B).

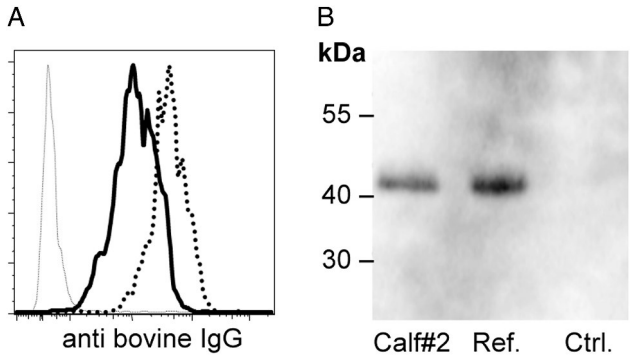

FIG 1: Calf 2 possesses high titres of maternally derived opsonising alloantibodies specific for bovine leucocyte antigen 1 (BoLA-1): (A) Cells of the bovine kidney cell line used for PregSure bovine viral diarrhoea production were incubated with serum of calf 2 (bold line) or a reference serum of a confirmed bovine neonatal pancytopenia (BNP) dam (dotted line). Mean fluorescence intensity was determined by flow cytometry. Secondary antibody only (grey line) served as a control. (B) Immunoprecipiation of the bovine kidney cell line with serum from calf 2 and the reference BNP dam, reveals a specific band at 43 kDa, which is absent with the unvaccinated control

DISCUSSION

Our data show that the dam of the twin calves had a sufficient alloantibody titre to induce BNP in her progeny because calf 1 succumbed to the syndrome. This is in accordance with the dam's vaccination history of repeated PregSure BVD injections, and confirms our hypothesis that three vaccinations with PregSure BVD (basic immunisation with one booster) are sufficient to induce a long-lasting alloimmune response (Kasonta and others 2012). This also explains why BNP still occurs after the withdrawal of PregSure BVD.

The fraternal twin (calf 2) also ingested sufficient amounts of colostrum to develop a high BoLA-1-specific alloantibody titre. However, this calf did not develop BNP because the maternal alloantibodies did not recognise its set of BoLA-1 antigens. Regrettably, this second calf (calf 2) died on November 20, 2011, but no signs of BNP were observed. Autopsy revealed that the cause of death was unrelated to BNP (i.e. pneumonia and pericarditis). The bone marrow was free from any insult and had a normal appearance (see Table 1). The incapability of the maternal BoLA-1 alloantibodies to bind to the calf's BoLA-1 alleles, therefore, is in absolute agreement with the absence of the immune-mediated disease.

The influence of the calf's sex on the development of BNP has been initially discussed (Friedrich and others 2009, Pardon and others 2011). For the presented case, an influence of the sex can be excluded, as both calves were females.

In the Immuno Polymorphism Database (IPD) 112 different BoLA-I alleles are listed (Robinson and others 2005). The encoding gene has been mapped onto the bovine autosome 23, and so far, at least six loci have been identified (Ellis 2004). The

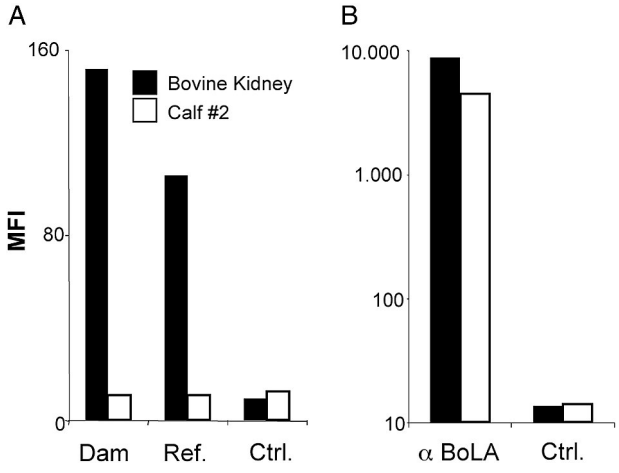

FIG 2: Phytohemagglutinin (PHA) blasts of calf 2 are not recognised despite high-level bovine leucocyte antigen 1 (BoLA-1) expression: (A) Sera of the dam of calf 2 (dam) and the reference bovine neonatal pancytopenia dam (ref) were incubated with PHA blasts of calf 2 (white) or the bovine kidney cell line (black). Alloantibody binding was determined by flow cytometry. (B) BoLA-1 expression level was determined using a BoLA-1 specific, monoclonal antibody. Bars represent individual mean fluorescence intensity-values for the PHA blasts of calf 2 (white), and for the bovine kidney cell line (black)

diversity is further increased by the fact that these alleles can be expressed in different combinations and due to interlocus recombination, there is an extremely low chance that two individuals in a herd carry the exact same BoLA-I combination pattern (Ellis 2004, Babiuk and others 2007). Therefore, the current notion suggests that only particular BoLA-I alleles are targeted by BNP alloantibodies. Our results indicate that in the healthy calf no pathology occurred because the maternal alloantibodies were not specific for the set of BoLA-I alleles the calf expressed. Due to the leukopoenic condition, we did not obtain cells from the affected calf precluding any attempt to directly compare the expression pattern of BoLA alleles between the two twin calves. Nonetheless, currently ongoing research in the group aims at identifying the BoLA alleles targeted by BNP-associated alloantibodies.

Why BoLA-1-targeting alloantibodies induce only insults on blood cells and the bone marrow is still a matter of discussion. In this line we, and others, have previously hypothesised that there might be an additional bone marrow-specific alloantigen(s) involved. However, the data provided in this case study strongly suggest that BoLA-1-specific alloantibodies indeed induce the syndrome. Whether the preferential expression of particular BoLA-1 molecules on haematopoietic stem cells or a particular pathomechanism that is exclusively functional on blood cells and in the bone marrow account for the pancytopenia and the panmyelophtisis that is pathognomonic for BNP, still needs to be clarified.

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Contributors RK and MB carried out all experiments described. CSL, MH and KC have been involved in drafting the manuscript and revising it critically for important intellectual content. CSL, MH and KC contributed in the epidemiological analysis. All authors read and approved the final manuscript.

Competing interests None.

Patient consent Obtained.

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Colostrum from cows immunized with a vaccine associated with Bovine Neonatal Pancytopenia contains allo-antibodies that cross-react with human MHC-I molecules

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Running Title: BNP-Colostrum binds human MHC-I

Abstract

In 2006, a new haemorrhagic syndrome affecting newborn calves, *Bovine Neonatal Pancytopenia* (BNP), was reported in southern Germany. It is characterized by severe bleeding, destruction of the red bone marrow, and a high case fatality rate. The syndrome is caused by alloreactive, maternal antibodies that are ingested by the calf with colostrum and result from a dam vaccination with one particular vaccine against Bovine-Viral-Diarrhoea-Virus. Because bovine colostrum is increasingly gaining interest as a dietary supplement for human consumption, the current study was initiated to elucidate whether BNP alloantibodies from BNP dams (i.e. animals that gave birth to a BNP-affected calf) cross-react with human cells, which could pose a health hazard for human consumers of colostral products. The present study clearly demonstrates that BNP alloantibodies cross-react with human lymphocytes *in vitro*. In agreement with previous reports on BNP, the cross-reactive antibodies are specific for MHC-I molecules, and sensitize opsonised human cells for *in vitro* complement lysis. Cross-reactive antibodies are present in serum and colostrum of individual BNP dams. They can be traced in commercial colostrum powder manufactured from cows immunized with the vaccine associated with BNP, but are absent from commercial powder manufactured from colostrum excluding such vaccinated cows. In humans alloreactive, MHC-I specific antibodies are generally not believed to cause severe symptoms. However, to minimize any theoretical risk for human consumers, manufacturers of bovine colostrum for human consumption should consider using only colostrum from animals that have not been exposed to the vaccine associated with BNP.

Keywords

bovine viral diarrhoea; veterinary virology; veterinary vaccines; zoonoses; bovine neonatal pancytopenia; alloantibodies; bovine colostrum; dietary supplements

I. Introduction

During the past two decades, bovine colostrum has gained increasing interest as a dietary supplement for human consumption. Several studies have proposed beneficial effects for colostral, antimicrobial antibodies (1-3), while other studies have postulated that small molecules such as peptidic growth factors may have an advantageous influence on gastrointestinal disorders (4). Consequently, international food and pharmaceutical companies have developed an array of different colostrum based products ranging from sports food (5) to dietary supplements aiming to ameliorate unspecified diarrhea in AIDS patients (6). Annual sales of dry colostrum ingredients reached a volume of 2,600 tonnes with a value of US$80 million in 2007 (7).

However, in 2006 a new hemorrhagic syndrome of bovine neonates, *Bovine Neonatal Pancytopenia* (BNP) was first observed in Central European countries. The syndrome affects newborn calves and is characterized by a complete destruction of the red bone marrow, pancytopenia, severe bleeding and high lethality. The syndrome is triggered by ingestion of colostrum from dams that have previously been vaccinated with PregSure®BVD, a strongly adjuvanted, inactivated vaccine against bovine viral diarrhea virus (BVDV) (8, 9). Due to its particular composition that combines substantial amounts of bioprocess impurities with a very efficient adjuvant system, this vaccine induces high titres of bovine MHC-I-specific alloantibodies (10-12). Transfer of alloreactive, maternal antibodies via colostrum to a newborn calf carrying the corresponding alloantigens leads to severe pancytopenia (13). The pathoetiology of BNP is therefore similar to human alloimmune diseases such as the Rhesus-Incompatibility Syndrome or Neonatal Alloimmune Thrombocytopenia (NAIT), except that such diseases involve transfer of alloimmune antibodies to the fetus *in utero* rather than via breast milk to neonates.

Due to the compelling evidence for the causal role of PregSure® BVD vaccine in the induction of BNP, the European Medicines Agency (EMA) suspended the marketing authorisation for PregSure® in 2010 (14). Shortly after the problem had been identified in Europe, a number of BNP cases were observed in New Zealand leading to a withdrawal of the vaccine from the New Zealand market (15).

This animal disease, amongst calves, raises questions about the safety of such colostral products for human consumption. Therefore a joint study was initiated between the Fonterra Research and Development Centre and the Paul-Ehrlich-Institute (PEI) to clarify whether the New Zealand haemorrhagic cases amongst calves were due to BNP and to assess whether the colostrum of the respective dams contained antibodies capable of cross-reacting with human cells.

II. Material and Methods

II.1 Serum and dairy samples

Two sets of serum samples from 8 and 4 PregSure®BVD-vaccinated New Zealand BNP dams were obtained. During the calving season 2011 these cows gave birth to calves, which developed BNP within the first three weeks of life. Sera from PregSure®BVD vaccinated animals with no history of BNP in their progeny (n=79) and from non-vaccinated (n=20) or alternatively BVDV-vaccinated animals (n=20) served as controls. The vast majority of cows was of Holstein-Frisian breed. The collection of blood samples from NZ dairy cows was being undertaken by veterinarians as part of routine disease investigations. In addition, milk and colostrum samples were obtained from 12 and six, respectively, of the BNP dams. Raw milk and colostrum samples from individual cows were subjected to continuous-flow High Temperature Short Time pasteurization by: using a peristaltic pump to pass small volumes (600 ml in total) through a miniature plate heat exchanger (PHE), fitted to a hot-water heating supply; then through a holding tube with the peristaltic pump adjusted such that the flow rate provided a residence time of 15 seconds; then past an electronic temperature probe to ensure that the flow of hot water to the initial PHE was adjusted such that the temperature of the liquid within the far end of the holding tube was at least 72.6°C; and finally through a second miniature PHE, fitted to a iced-water cooling supply; and then discarding the first 250 mL portion that emerged, prior to collecting each sample. Commercial lots of New Zealand whole milk powder and two colostrum powders were also obtained. Milk and colostrum powder is batchwise produced from a total amount of about 100.000 ℓ of raw milk or colostrum sourced from different herds. The milk or colostrum is pasteurized and subsequently dried *James, help!*. One colostrum powder sample was manufactured in New Zealand in 2011, prior to the prohibition against PregSure®BVD treatment, while the second sample was manufactured in New Zealand in 2012 from colostrum that had

been sourced exclusively from non-PregSure®-treated cows. All samples were held at -70°C for long term storage. The powders were weighed and 0.1 g each was dissolved in 1 ml phosphate buffered saline (PBS, PEI), prepared fresh before each analysis. Whole colostrum was centrifuged twice at 11,000 × g followed by 25,000 × g to remove cell debris, and stored at -20°C. For comparison, serum samples of six European BNP dams form North-Rhine-Westphalia were included in the study. According to the new German animal welfare legislation, the study was announced to and approved by the competent authority (State Office for Nature, Environment and Consumer Protection of North-Rhine-Westfalia, LANUV Recklinghausen, Germany; ref. 84-02.05.40.14.032).

II.2 Cell preparation and cell culture

Bovine leukocytes were prepared from whole blood of healthy heifers by ammonium chloride lysis and Ficoll gradient centrifugation as described elsewhere (10). Short-term T cell lines were obtained from peripheral blood mononuclear cells (PBMCs) by phytohaemagglutinin (PHA) stimulation as previously described (10). The resulting polyclonal T cell lines are hereafter referred to as lymphoblasts. Human lymphoblasts were prepared accordingly from buffy coats of healthy blood donors. The buffy coats were purchased from the German Red Cross Bloodbank, Frankfurt. All donors have given informed consent to the medicinal, scientific or pharmaceutical use of their blood or preparations thereof. Before distribution the material is anonymized. To the customer no information is disclosed about individual donors. This procedure has been approved by the local ethics committee (Votum 329/ 10; ethics committee; Goethe-University, Frankfurt).

The bovine kidney cell line used for the production of PregSure®BVD, hereafter referred to as BK cell line, was kindly provided by Pfizer Animal Health (10). The cell

line was tested to be free of BVDV and was maintained according to manufacturer's instructions as previously described (10).

II.3 Flow cytometry

To examine samples for the presence of opsonising alloimmune antibodies, flow cytometry analyses were carried out as previously described (10). Briefly, up to 1×10^5 BK cells or lymphoblasts were re-suspended in PBS containing 0.5% fetal calf serum (FCS, Gibco). Sera, colostrum or milk and also individually pasteurized colostrum samples and reconstituted commercial milk and colostrum powders were added to a final dilution of 1:5 (if not stated otherwise) followed by one hour incubation at 4°C. Cells were then washed twice with PBS containing 0.5% FCS and cell surface bound bovine IgG detected using a FITC-conjugated polyclonal sheep-α-bovine IgG antibody (Invitrogen). Median fluorescence intensity (MFI) of living cells as defined by Forward-Scatter (FSC)/ Sideward-Scatter (SSC)-gating was determined for each sample using a BD Lsr II Flow Cytometer (Figs 1–4) or a BD Accuri C6 Flow Cytometer (Figs 6). The Accuri device uses a different log scale to display fluorescence data, which results in a baseline shift of two log scales between the two devices. To take this into account, figures using Accuri data were given an adapted scale (as indicated in the axis title, which now reads: MFI ($\times$ 100)).

II.4 Immunoprecipitation

To isolate and identify BNP associated alloantigens on BK cells or lymphoblasts immunoprecipitation (IP) was performed as previously described (16). In summary, cells were surface labeled with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific) and incubated with bovine serum samples. Cells were then solubilized with Lysis Buffer (1 % Triton X-100, 150 mM NaCl, 50 mM Tris and 5 mM EDTA) in an ultrasonic bath. Solutions were clarified by centrifugation (10,000 $\times$ g, 10 min) and the immune

complexes precipitated with protein G-sepharose beads (GE Healthcare), eluted with Loading Buffer containing 6 M urea, 62.5 mM Tris, 2 % SDS, 10 % glycerol and 0.025 % bromophenol blue, and subjected to SDS-PAGE under non-reducing conditions.

Proteins were blotted onto nitrocellulose membranes (Whatman). Biotinylated cell membrane proteins were detected using a Streptavidin-HRP conjugate (Dianova) and visualized using enhanced chemoluminescence (ECL; GE Healthcare) reagents. MHC-I molecules were visualized by developing the membranes with an anti-bovine MHC I antibody (IL-A88; AbD Serotec) or an anti-HLA I antibody (w6/32, kindly provided by Steffen Tenzer, University of Mainz) followed by a secondary anti-murine-IgG^{HRP} conjugate (Dianova).

II.5 Affinity purification of BNP alloantibodies

BNP alloreactive antibodies were affinity purified as previously described (10). Sera of PregSure®BVD vaccinated dams, alternatively vaccinated animals, or non-immunized controls were diluted 1:10 in PBS and incubated with BK cells. The cells were repeatedly washed with 0.9% sodium chloride. Cell surface-bound antibodies were detached by treatment with ice-cold citrate buffer (0.12 M sodium citrate, pH 2.5) for 15 minutes. After centrifugation (10,000 $\times$ g, 5 min) supernatants were harvested and immediately treated with neutralization buffer (1 M Tris HCl, pH 9.0).

II.6 Flow cytometric detection of complement-activating antibodies

To assess the complement activating activity of opsonising alloantibodies, we adopted a flow cytometry based, complement-dependent cytotoxicity assay (17). Briefly, 1×10^5 human lymphoblasts, re-suspended in PBS containing 0.5% FCS, were incubated with heat inactivated (56°C, 30 minutes) bovine sera at a final dilution of 1:5 and incubated for 45 minutes at 4°C. Complement activation was assessed by

adding active or heat-inactivated rabbit complement, *i.e*. fresh rabbit serum. After 10 minutes at 37°C, propidium iodide (PI, Sigma-Aldrich) was added, the samples were placed on ice and immediately analysed by flow cytometry. The cytometer settings were limited to run a fixed volume of 20 µl. For each serum sample the number of living, PI-negative cells (N) after incubation with active or heat-inactivated rabbit complement was determined. The percentage of cytotoxicity was calculated according to the following equation: specific Lysis = 100% x (1 – $N_{\text{active complement}}$/ $N_{\text{inactive complement}}$).

II.7 Statistical analysis

Single-tailed, paired or unpaired, homoskedastic Student's t test was performed to test for significant differences between groups, indicated by the p-value in the respective graphs (significance level α= 0.05). Pearson's test was used to calculate correlation coefficients.

III. Results

III.1 New Zealand dams with BNP calves have high alloreactive antibody titres

During the 2011 calving season, several New Zealand farms experienced haemorrhagic disorders in bovine neonates. To determine whether BNP, the vaccine-induced alloimmune syndrome that had previously been described only in Central Europe, was the underlying cause, serum samples from affected dairy herds and unaffected controls were collected and investigated for the presence of alloreactive antibodies by flow cytometric assay. Bovine lymphoblasts obtained from an adult Holstein-Frisian cow were incubated with respective sera and cell surface-bound alloantibodies quantified by flow cytometry. Living cells were identified according to their FSC/ SSC characteristics and median fluorescent intensity (MFI) was determined for the living cell population (suppl. Fig S1). The MFI served as a

correlate for the amount of alloantibodies present in each serum sample. MFI obtained from New Zealand BNP dam sera was similar to the confirmed BNP cases from German dams (Fig 1A). In accordance with previous observations made in Central Europe (10), we found that BNP dams had significantly higher levels of alloantibodies than PregSure®BVD vaccinated animals giving birth to calves that did not develop BNP, or animals that received an alternative BVDV vaccine, or non-vaccinated controls (Fig 1A).

To confirm that bovine MHC-I molecules were targeted by the vaccine induced alloantibodies, as previously described (11, 12, 16), BNP dam sera were tested by immune precipitation. BK cells (i.e. the cell line used in the production of PregSure®BVD) were biotinylated and incubated with each test sera. Cell surface molecules complexed to the alloantibodies were precipitated, separated by SDS-PAGE, blotted and visualized by peroxidase-conjugated streptavidin (data not shown) or MHC-I-specific staining. In parallel, the same samples were tested against BK cells by flow cytometry. The level of alloantibody binding as measured by flow cytometry correlates significantly with the amount of bovine MHC-I detected, as revealed by the presence of a Western blot band of approximately 43 kDa (Fig 1B and suppl. Fig S2A and S3). However, in two out of the 12 BNP dam sera, no alloantibody binding was detectable. Whether this is due to serum degradation, or whether these calves suffered from BNP-like symptoms not related to PregSure®BVD induced MHC-I alloantibodies, is not clear.

III.2 BNP associated alloantibodies are concentrated in colostrum

Bovine colostrum represents an antibody concentrate that supplies the neonate with maternal antibodies, and it has been shown that BNP-associated alloantibodies are present in the colostrum of BNP dams (13). To assess the amount of BNP-associated alloantibodies in colostrum and milk, matched samples of: colostrum,

serum and milk from six individual New Zealand BNP dams were tested. We found that the reactivity to BK cell surface molecules was highest in colostrum, followed by serum, while only low to negligible amounts of BNP-associated alloantibodies were present in milk. Presumably, due to one outlier the difference between colostrum and serum is not significant (Fig 2). To test whether pasteurization potentially reduces the activity of colostrum contained alloantibodies, individually pasteurized colostrum samples were compared to unpasteurized colostrum samples. However, no significant change in binding capacity of the alloantibodies could be detected (data not shown). Colostrum is only produced during the first days after birth, whereas the first clinical signs of BNP occur ten days after birth making it challenging to obtain colostrum from BNP dams. Since the alloreactivity was sufficiently detectable in both colostrum and serum, we performed the majority of the subsequent experiments on serum samples.

III.3 BNP-associated alloantibodies cross-react with human lymphoblasts

To investigate whether BNP-associated alloantibodies cross-react with human cells, we compared the surface-binding reactivity of our serum panel to the BK cell line with human lymphoblasts. There was a significant correlation (R^2 =0.71) of the reactivity to BK cells and to human lymphoblasts (Figs 3A and B). To confirm that this reactivity was due to PregSure®BVD vaccination, the experiment was repeated using affinity-purified, vaccine-induced alloantibodies. While affinity-purified alloantibodies from PregSure®BVD-vaccinated animals exhibited the same cross-reactivity pattern to human lymphoblasts as the sera, the minor reactivity seen with sera from animals not vaccinated with PregSure®BVD disappeared indicating that those signals were non-specific. To identify the antigen BNP-associated alloantibodies recognized on human lymphoblasts, we performed immunoprecipitation experiments using human lymphoblasts. Again, for BNP dam sera the flow cytometric reactivity correlated

significantly with the precipitation of human MHC I (Fig 4 and supplementary Fig S2B), while sera from non-immunized or alternatively vaccinated animals displayed no MHC I-specific reactivity (data not shown).

It has been speculated that complement-mediated lysis of alloantibody-opsonised cells contributes to the pathogenesis of BNP (10, 18). To assess whether BNP-associated alloantibodies could potentially destroy human lymphocytes through such a mechanism, we adapted a flow-cytometric approach to measure complement activity *in vitro* (17). All ten BNP sera that contained alloreactive antibodies induced a specific complement-lysis, while the corresponding control sera showed no effect (Fig 5). This indicates that BNP-associated alloantibodies not only recognize human MHC-I molecules, but also have the capacity to exert a cytotoxic effect on human lymphoblasts.

III.4 BNP Alloantibodies are present in commercially available dairy products

To test whether BNP-associated alloantibodies are present in commercial lots of colostrum powder, i.e. the material used by down-stream industries to produce colostrum based dietary supplements for human consumption, we investigated milk and colostrum powders that had been produced during and after the 2011 calving season. Flow cytometry showed significant reactivity against BK cells and human lymphoblasts in the 2011 colostrum powder lot (Fig 6), although due to a production-inherent dilution effect the binding is much lower compared to the reactivity of individual BNP dam colostra (see Fig 2 for comparison). In view of this observation, Fonterra withheld all 2011 colostrum from sale, and in 2012 changed the colostrum harvesting policy to exclude all animals that had been vaccinated with PregSure®BVD. Colostrum powder manufactured during the 2012 calving season displayed no cross-reactivity to BK cells or human lymphoblasts.

IV. Discussion

The aim of the current study was to clarify whether cases of bovine neonate haemorrhages in New Zealand were caused by the vaccine induced feto-maternal incompatibility syndrome, BNP. Furthermore, the question was addressed whether the haemorrhage causing alloantibodies in the colostrum of PregSure®BVD immunized cows could cross-react with human cells and might therefore pose a theoretical risk to human consumers of products manufactured from such colostrum.

The data presented in the current study are consistent with the contention that the cases of bovine neonate haemorrhage observed in NZ were caused by BNP. The alloreactivity in sera of NZ dairy cows showed the same reactivity pattern as that previously observed in Europe (10, 13). Only sera from PregSure®BVD immunized animals showed alloreactive binding to bovine lymphocytes, and this alloreactivity was directed against MHC-I molecules of BK cells. Although this alloreactivity was present in the serum, it was clearly higher in the colostrum of BNP dams. This is in concordance with observations in Europe and was expected because, in ruminants, colostrum is the only source of maternal antibodies and contains high amounts of immunoglobulins (3). Compared to serum or colostrum, the level of alloantibodies in milk was negligible. From this observation there is no evidence that the consumption of milk from BNP dams is hazardous for human consumers.

By contrast, for colostrum or serum our data clearly prove that BNP-associated alloantibodies cross-react with human MHC-I molecules. Whether this could potentially be hazardous for human consumers, cannot be conclusively answered in this study: In humans, alloimmune syndromes with severe clinical signs are generally attributed to specific alloantigens that are expressed on particular target cell types only. For example, NAIT is induced in most cases by alloantibodies targeting a dimorphic epitope in the beta chain of Human Platelet Antigen 1 (HPA-1a), which is only present on megakaryocytes and thrombocytes (19). The particular expression

pattern of the alloantigen explains the specific insult of NAIT antibodies on the haemostatic system (20). Although MHC-I specific antibodies have originally been implicated in the induction of NAIT, it was later shown that they rather play an inferior role (20). In the case of BNP no specific target antigen other than MHC-I has yet been identified. Instead, there is evidence for a causal role of MHC-I specific alloantibodies. Several studies have identified MHC-I specificity as the only common denominator in the serum of BNP dams (11, 12, 16, 21). Furthermore, Bridger *et al*. demonstrated in an elegant study that the surface opsonising activity of orally administered BNP dam antibodies — which has been shown to correlate with MHC-I reactivity — is directly associated with the severity of clinical symptoms in challenged calves (13). Finally, we observed in two cases that fraternal twins of BNP-affected calves, expressing MHC-I molecules that were not recognized by the alloantibodies of the dam, survived the ingestion of toxic colostrum and exhibited a completely normal appearance of their red bone marrow (22). So, one important current hypothesis is that MHC-I specific alloantibodies cause BNP in bovines.

However, despite the complement activating effect on human lymphoblasts observed *in vitro*, it is questionable whether the bovine alloantibodies could cause any effect in humans. The main difficulty with a pathoetiological role of MHC-I-specific antibodies is that MHC-I proteins are ubiquitously expressed on the vast majority of cells. Even though the actual pathomechanism leading to cell destruction after alloantibody intake is not elucidated, it can be assumed that large amounts of MHC-I alloantibodies would be required to saturate peripheral MHC-I before such antibodies could reach a tissue and induce a specific insult. It is conceivable that with the sudden uptake of several grams of maternal antibodies in calves, such levels of alloantibodies are reached. However, it is improbable for such a massive influx to occur in humans. Firstly, in contrast to bovine neonates, bovine dietary antibodies are not effectively transported across the human gut mucosa. The neonatal uptake of maternal antibodies via the gut mucosa is mediated by the neonatal Fc-receptor,

FcR_n, a dedicated transport system that shuttles maternal antibodies across the gut-blood barrier. It is known that the mucosal barrier closes 24–48 hrs after birth and at least in rodents it has been shown that FcR_n is downregulated directly after birth (23). In humans, the fetus is provided with maternal antibodies via the placenta during gestation (24, 25). While the neonatal Fc-receptor is expressed mainly in the fetal placenta, it is also found in the gut mucosa (26) where it is expressed throughout life (27, 28). *In vitro* studies have shown that whilst the expressed receptor is functional and transports antibodies across polarized cell layers (29), the main function of human FcR_n-expression in the gut is to shuttle antibodies from the submucosal side into the gut lumen (30). The direction of the net transport is determined by the IgG concentration gradient (26). Since the affinity of the human FcR_n for human IgG is four-fold higher than that of bovine IgG (31), and since the concentration of cross-reactive alloantibodies in commercial colostrum products is relatively low, the uptake of cross-reactive alloantibodies is inefficient, such that only insignificant amounts of MHC-I-specific BNP antibodies would reach the circulation.

Secondly, there are cases where human breast milk can contain alloreactive antibodies. For instance, for NAIT, platelet-specific antibodies have been identified in the breast milk of affected mothers, but this breast milk is still considered safe to consume by the infant (32). Similarly for other maternal autoimmune diseases, such as lupus or Hashimoto's thyroiditis, disease manifestation in the infant is due to maternal alloantibodies transferred *in utero* rather than via lactation, such that disease exacerbation is not associated with breast feeding (33, 34). This suggests that alloantibodies present in the infant diet are unlikely to result in detrimental health effects.

Taking all these observations and considerations together, we conclude that the risk of colostrum-containing dietary supplements, from cows treated with the vaccine associated with BNP, is low for human consumers. However, since the reactivity of BNP alloantibodies to human lymphoblasts is principally indistinguishable from the

reactivity to bovine cells, a residual risk cannot be absolutely excluded. In what may have been an overly cautious approach, Fonterra decided to withhold the entire colostrum production of 2011, and from 2012 onwards excluded colostrum collection from PregSure®-treated cows. This approach was effective, as no alloreactivity to bovine or human cells was detectable in colostrum powder manufactured according to the new supply policy.

Acknowledgements

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Abbreviations

BK cell, a bovine kidney cell line used for vaccine production; BNP, *Bovine Neonatal Pancytopenia*; BVD, *Bovine Viral Diarrhoea*; MFI, Median Fluorescence Intensity; MHC-I, Major Histocompatibility Complex Class I Antigen; NAIT, Neonatal Alloimmune Thrombocytopenia; PEI, Paul-Ehrlich-Institute.

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Captions

Fig 1 Sera of NZ-BNP dams show identical alloreactivity compared to European BNP dams (A) Sera were obtained from NZ dairy cows that had either not been immunized against BVDV (non-BVD-imm.), vaccinated with an alternative BVD vaccine (BVD-vaccine), vaccinated with PregSure®BVD and birthed healthy calves (PregSure®) or cows that had been vaccinated with PregSure®BVD and gave birth to BNP calves (BNP dams (NZ)). By flow cytometry a first set of eight sera per group was tested for alloreactive binding to bovine lymphoblasts. As a control we included six serum samples of European BNP dams (BNP dam (EUR)). Symbols represent the median fluorescence intensity (MFI) for individual serum samples, black bars indicate the median value for each group. (B) Using BK cells, *i.e*. the cell line used for the production of PregSure®BVD, the full panel of serum samples from twelve NZ BNP dams were tested in parallel by flow cytometry and immunoprecipitation for the presence of BNP associated alloantibodies. The upper panel shows the MFI for the individual serum samples, the lower panel shows the corresponding immunoprecipitate as revealed by a monoclonal antibody specific for bovine MHC-I molecules. Serum from a European BNP dam served as a positive control, fetal calf serum was used as a negative control.

Fig 2 Comparison of BNP-associated alloantibody content in BNP dam colostrum, serum and milk Colostrum, serum and milk samples were collected from six NZ BNP dams. The samples were diluted 1:500. Using BK cells the samples were tested by flow cytometry for the presence of BNP associated alloantibodies. Symbols represent the median fluorescence intensity (MFI) for individual samples as determined by flow cytometry, black bars indicate the median value for each group.

Fig 3 PregSure®BVD induced BNP alloantibodies cross-react with human lymphoblasts (A) A panel of sixteen BNP dam sera was tested by flow-cytometry for the presence of alloreactive antibodies that bind to the BK cell line. As controls sera from non-immunized, alternatively vaccinated or PregSure®BVD-immunized non-BNP dams were included. (B) The same serum panel was tested for cross-reactive binding to human lymphoblasts. (C) From the entire serum panel BNP-associated alloantibodies were purified by affinity purification. The affinity purified alloantibodies were again tested for cross-reactive binding to human lymphoblasts. Black bars indicate the MFI obtained with individual sera or the corresponding affinity-purified alloantibodies.

Fig 4 BNP-associated alloantibodies bind human MHC-I molecules The panel of BNP dam sera was tested in parallel by flow-cytometry and by immunoprecipitation using human lymphoblasts. The black bars in the upper panel show the MFI as determined by flow cytometric analysis. The lower panel shows the corresponding immunoprecipitates as revealed by a monoclonal antibody specific for human MHC-I molecules.

Fig 5 BNP-associated alloantibodies opsonise and sensitize human lymphoblasts for complement-mediated cell lysis: Human lymphoblasts were coincubated with heat-inactivated bovine serum and rabbit complement was added. Cell viability was measured by flow cytometry counting live, propidium-iodide negative cells. (A) The histograms show human lymphoblasts incubated with serum from a non-PregSure®BVD immunized control dam (left) or BNP dam serum (right) after adding active complement (bold line). As a control, heat inactivated rabbit complement was added (dotted line). Numerical figures indicate the absolute number of living, propidium-iodide negative cells. (B) The serum panel was tested for its complement sensitizing activity on human lymphoblasts. Sera from four non-immunized and four alternatively vaccinated animals served as a control. One representative of three experiments is shown. Black bars represent the specific lysis for the individual sera.

Fig 6 Exclusion of PregSure®BVD immunized animals reduces the amount of cross-reactive antibodies in commercial colostrum powder Two commercial lots of colostrum powder were compared by flow cytometry to whole milk powder for the presence of BNP-associated alloantibodies, one lot was produced during the calving season of 2011, the other was produced in 2012 according to a new harvesting policy excluding herds that had been vaccinated with PregSurer®BVD. Filled circles represent the MFI for colostrum powder produced in 2011, open circles for 2012 colostrum, open quadrates for whole milk powder. Panel (A) depicts the reactivity for BK cells, panel (B) the reactivity to human lymphoblasts. Symbols represent the median over three independent flow cytometric analyses, error bars the corresponding standard deviation. Asterisks indicate a significant difference between the two colostrum batches at the indicated dilution. For the analyses an Accuri C6 flow cytometer was used.

Figure 1

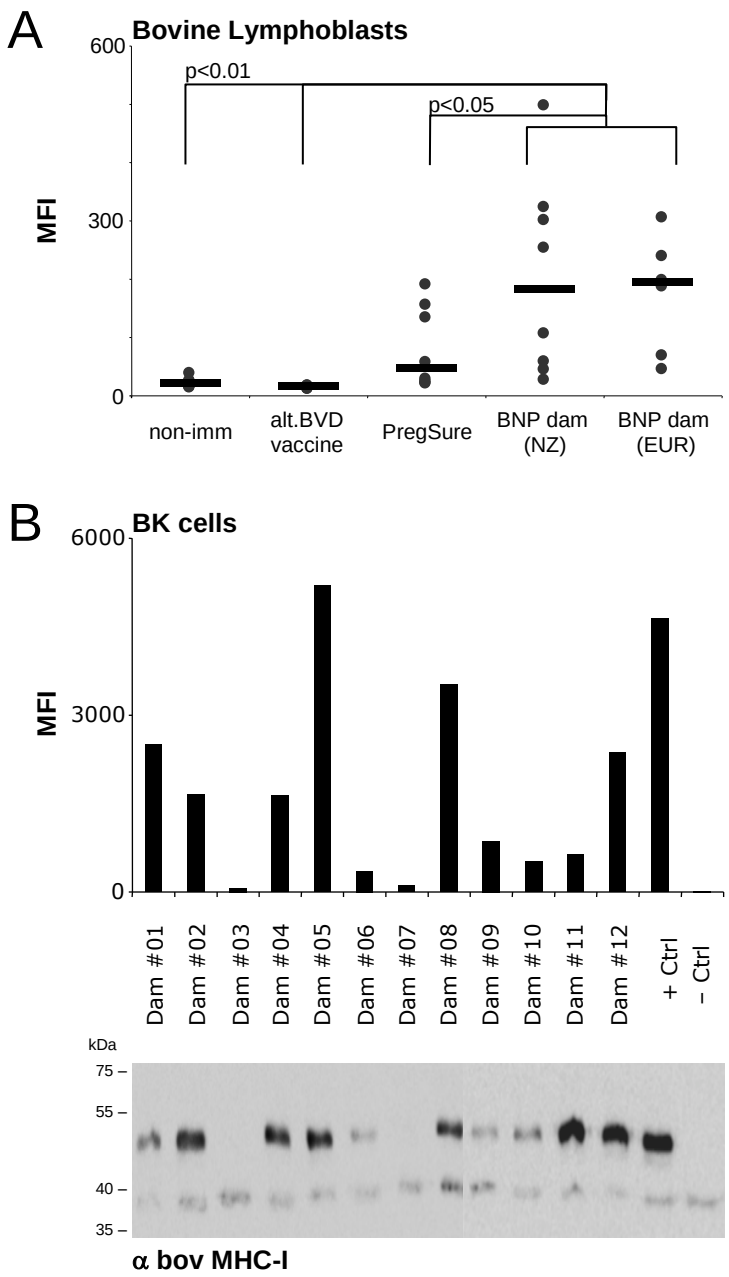
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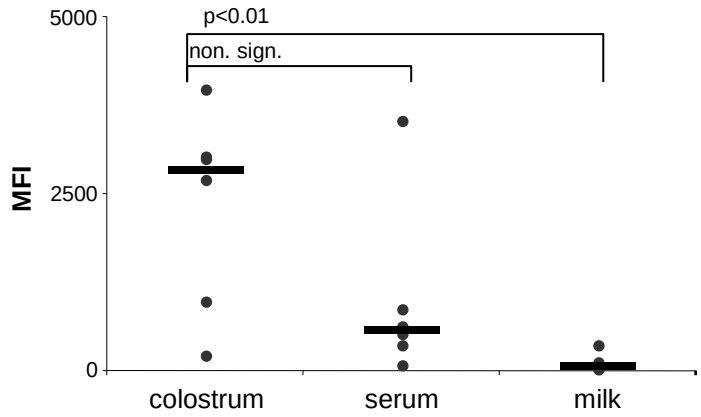
Figure 2



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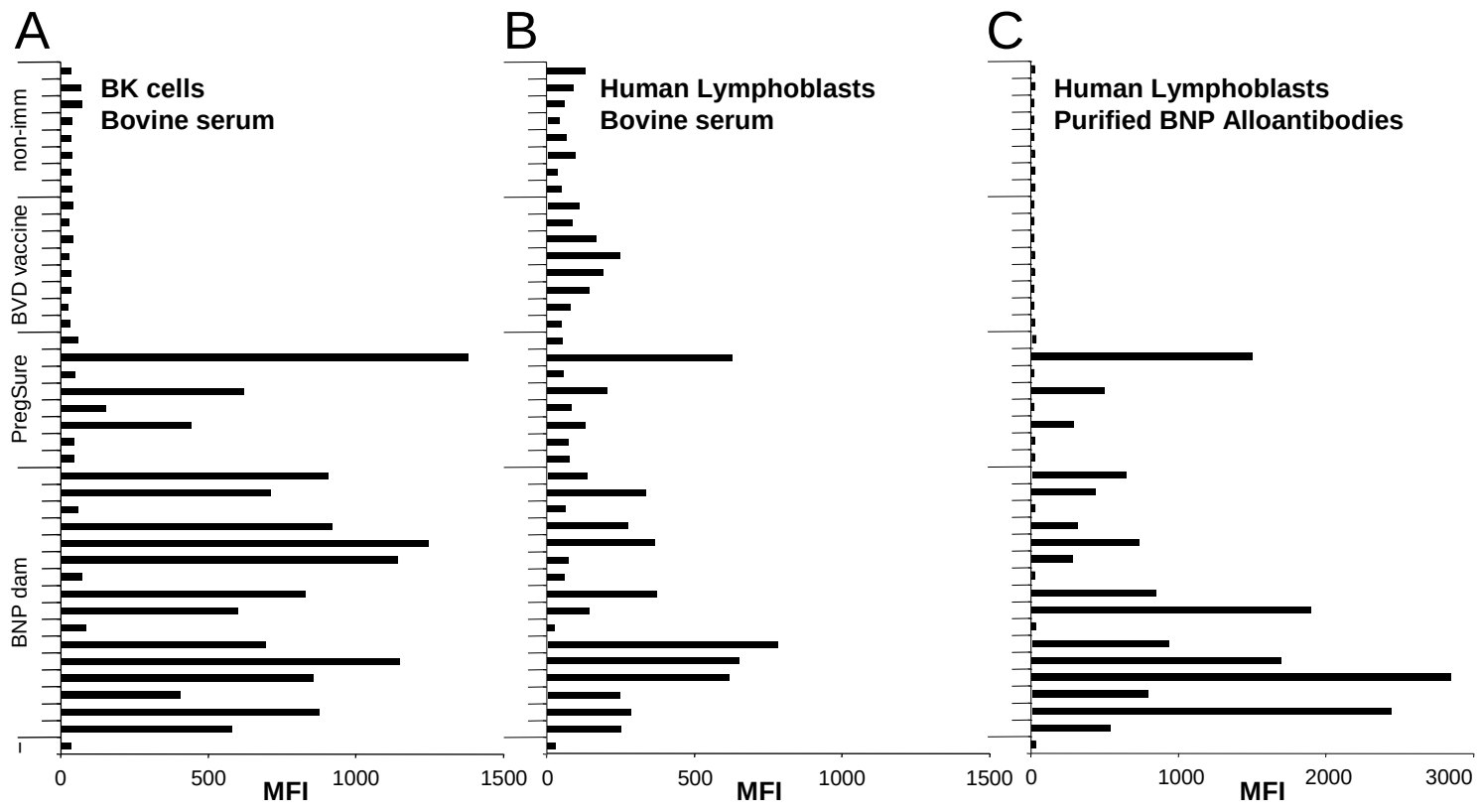
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Figure 4

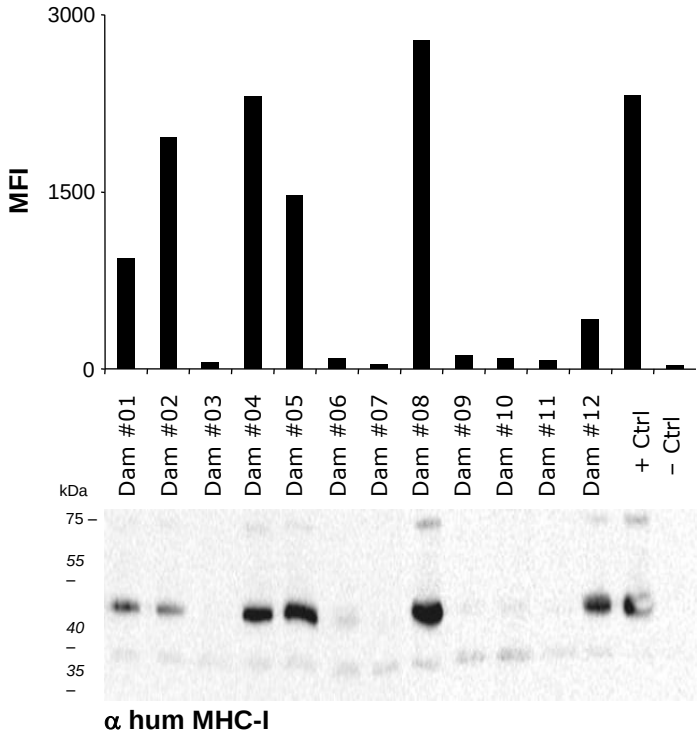
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Figure 5

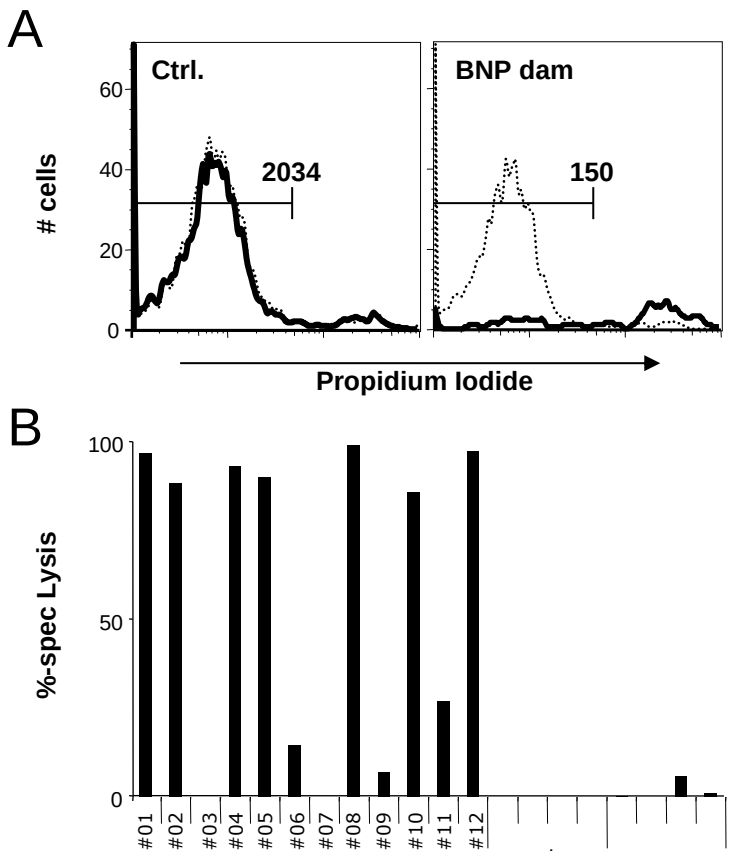
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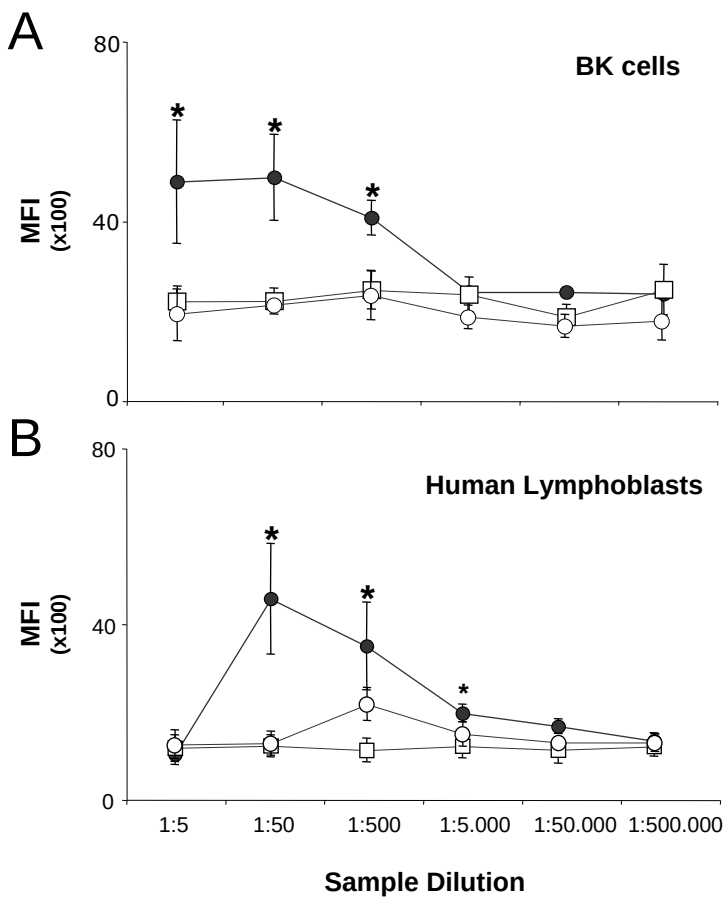
Figure 6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Sample Dilution
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Milk Powder:

Figure S1

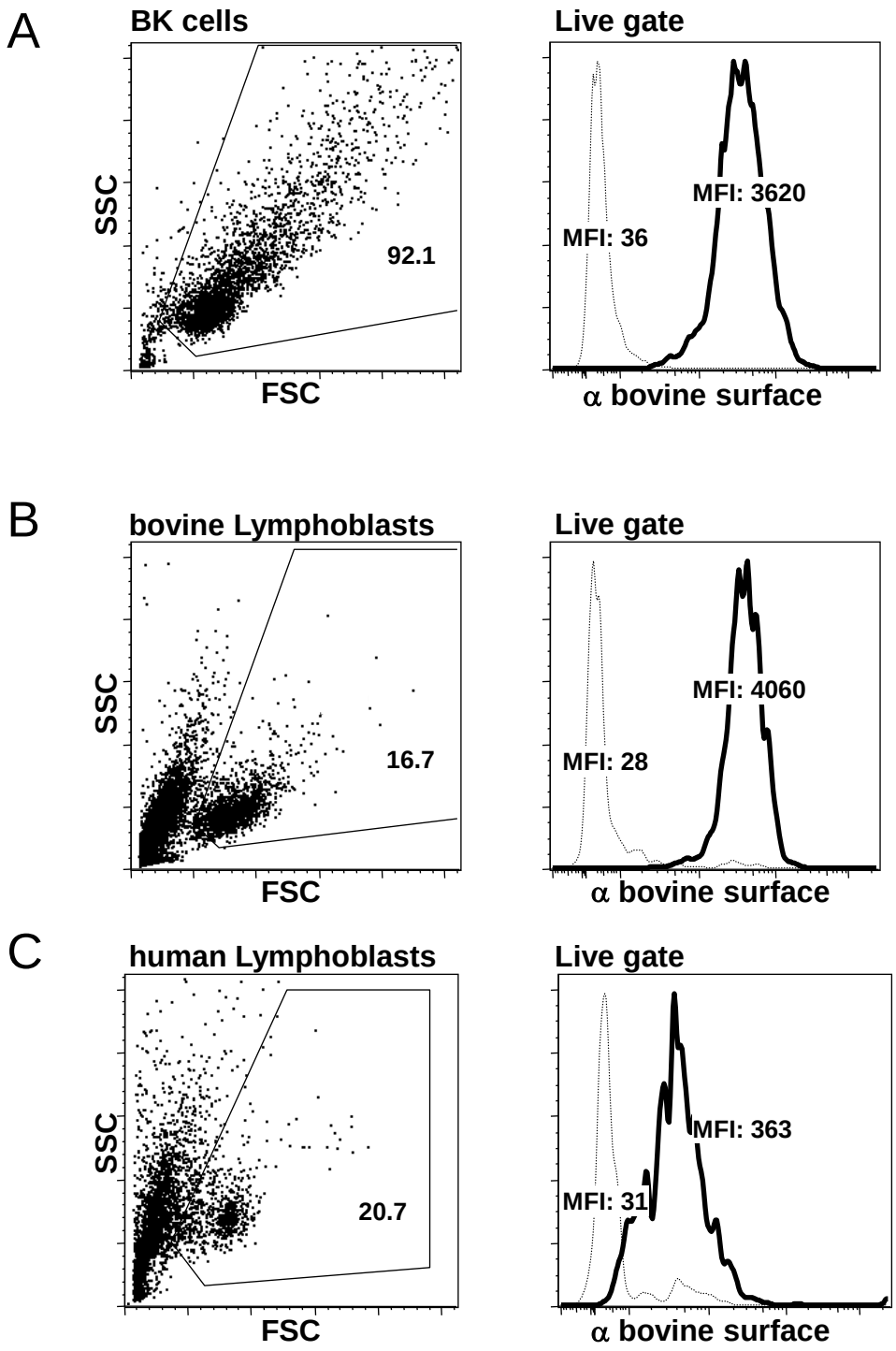


Fig S1 The gating strategy of the flow-cytometric analysis: BK cells (A) and bovine (B) or human lymphoblasts (C) were incubated with serum from a non.-immunized cow (punctuate line) or a BNP dam (bold line). Cells were washed twice and incubated with a FITC-conjugated secondary antibody. Subsequently, cells were analyzed by flow-cytometry. Live cells were identified and gated according to their FSC/ SSC characteristics (black lined area in the left panels). The median fluorescence intensity was analyzed for all events within the live cell gate (right panels).

Figure S2

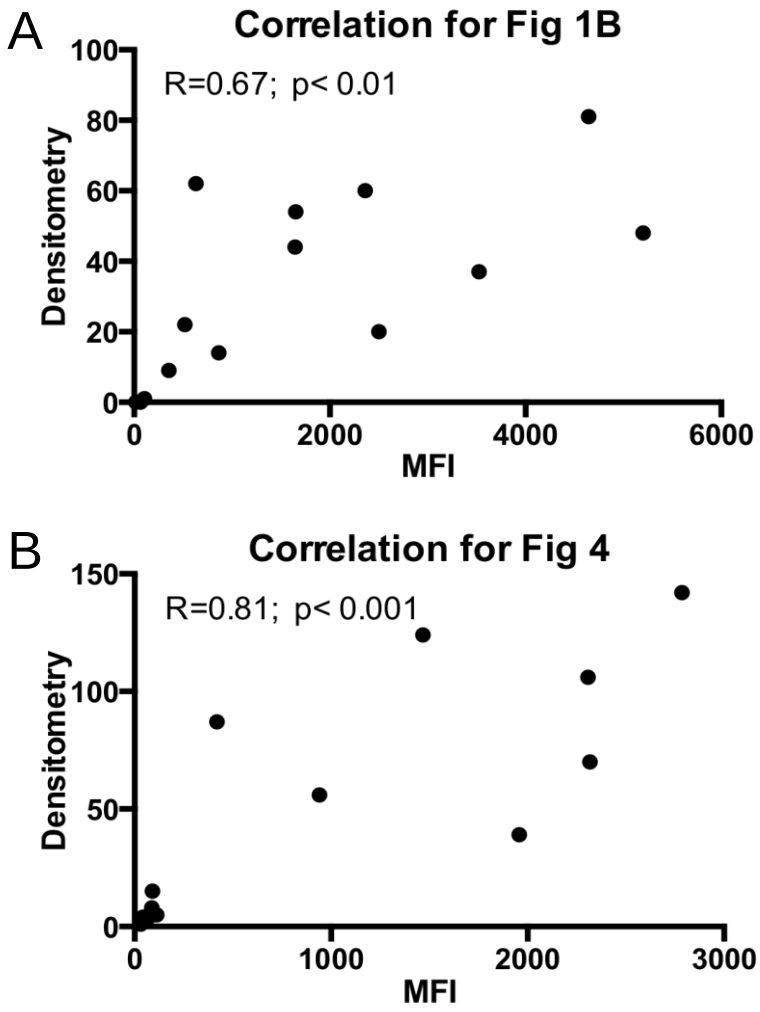


Fig S2 Alloantibody reactivity measured by FACS correlates with MHC-I immunoprecipitation: The intensity of immunoprecipitated MHC-I bands shown in Fig 1B and Fig 4 was analyzed by densitometry. The arbitrary grey value of the densitometry was plotted against the corresponding alloantibody binding as determined by flow-cytometry (MFI). The correlation is shown for BK cells (A) and for human lymphoblasts (B). Pearson correlation coefficients and p-values are indicated.

Figure S3

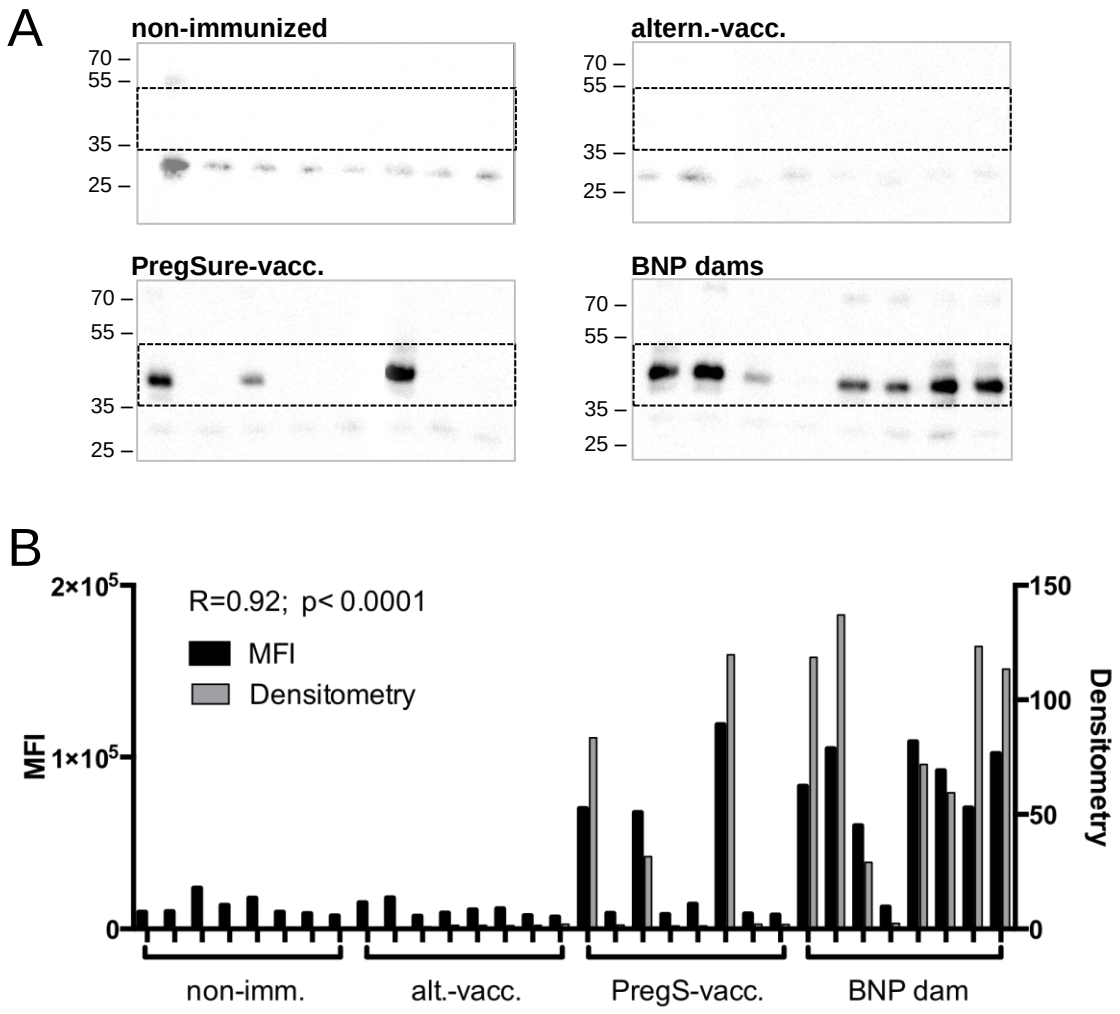
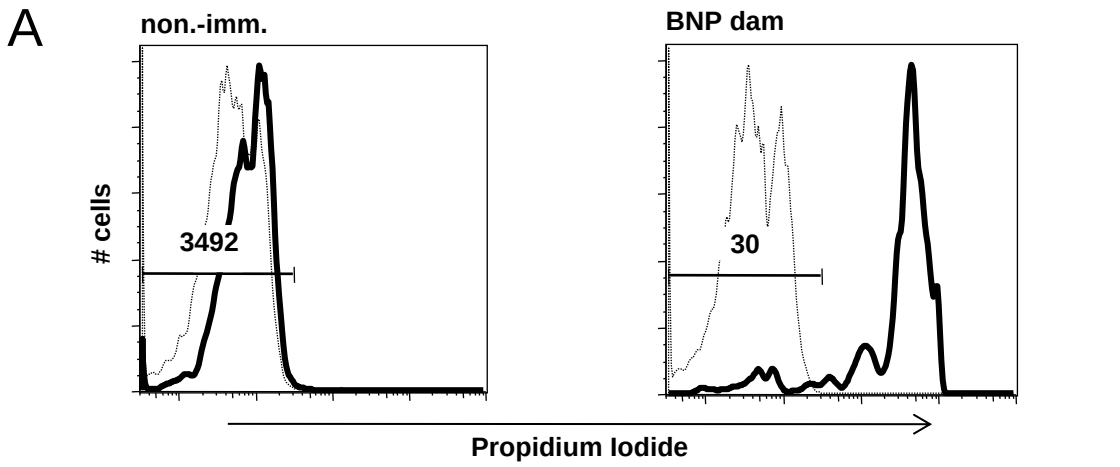


Fig S3 Alloantibody reactivity measured by FACS correlates with MHC-I immunoprecipitation: (A) BK cells were incubated with eight sera of non-immunized or alternatively BVD-vaccinated cows or of PregSure®BVD-vaccinated non-BNP dams and PregSure®BVD vaccinated BNP dams. Surface molecules were immunoprecipitated as described. The precipitates were analyzed by SDS-PAGE followed by anti BoLA-I westernblot using monoclonal antibody IL A88. Specific MHC-I bands at about 40 kDa (lined area) were analyzed by densitometry. (B) The same panel of sera was tested in parallel by flow-cytometry for alloantibody binding. MFI values (black bars) are plotted side by side with the corresponding grey values as determined by densitometry (grey bars). The correlation was calculated, the respective Pearson coefficient and the p-value are indicated.

Figure S4



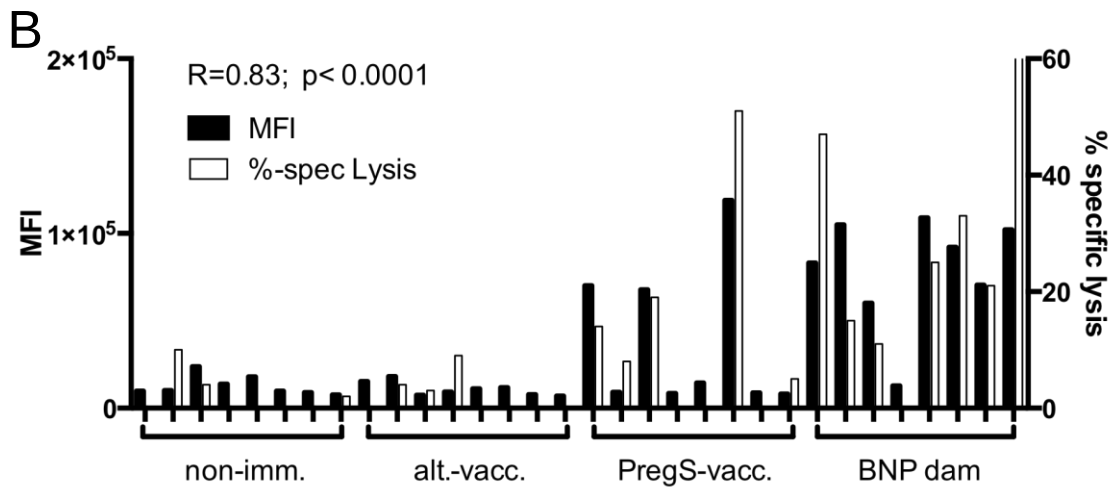


Fig S4 Alloantibody mediated complement lysis correlates with FACS reactivity: (A) BK cells were incubated with serum from a non-immunized cow (left panel) or a BNP dam (right panel). Heat-inactivated (dotted line) or active rabbit complement (bold line) was added and samples were incubated at 37° C. To identify dead cells red fluorescent propidium iodide was added and samples were analyzed by FACS. The absolute number of living, propidium iodide negative cells in a defined sample volume of 20 µl was determined. Numerical figures in the graphs represent the respective number of living cells after adding active complement. (B) Specific cell lysis was determined for the same serum panel as in suppl. Fig S3 and tested in parallel by flow-cytometry for alloantibody binding. MFI values (black bars) are plotted side by side with the corresponding %-specific cell lysis (open bars). The correlation was calculated, the respective Pearson coefficient and the p-value are indicated.