CHARACTERISATION OF T CELL RESPONSES TO BOVINE VIRAL DIARRHOEA VIRUS PROTEINS AND ITS APPLICATION TOWARDS THE DEVELOPMENT OF IMPROVED VACCINES

by

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DEDICATION

To the women in my life: Rose Nyakwea Riitho, Lynne Njeri Riitho, Winifred Wanjiku Riitho and Rose Nyakwea Njeri.

In the words of Gill-Scott Heron,

My life has been guided by women
~~But~~ And because of them – I am a man.
God bless you mama – and thank you.

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ABSTRACT

Bovine viral diarrhoea virus (BVDV) is an important pathogen that causes infectious disease of cattle worldwide and results in significant economic losses. Vaccination has long been used as a tool for control of BVDV but inadequacies of existing vaccines have hampered eradication efforts. Attempts to develop sub-unit vaccines have focused on the structural envelope protein E2, which is a dominant target of neutralising antibodies and as well as CD4 T cell responses. This study aimed to rationally address the development of more efficacious vaccines by characterising the kinetics and specificity of T cell responses to a BVDV type 1 peptide library in calves rendered immune to BVDV following recovery from experimental infection. Upon identification of E2 and NS3 as the dominant targets of CD4 T cell responses, we assessed whether T cells induced by one virus genotype were capable of responding to a heterologous virus genotype and to identified E2 and NS3 as targets of genotype-specific and genotype transcending responses, respectively. This finding strengthened the argument for inclusion of both antigens in a subunit vaccine formulation. A nanoparticulate formulation of E2 and NS3 adjuvanted with poly(I:C) was shown to induce protective responses comparable to a commercial available BVDV vaccine in a vaccination and challenge experiment. It is hoped that the data generated will have implications for the design of improved vaccines against BVD.

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ABBREVIATIONS

ADCC	Antibody-Dependent Cell-mediated Cytotoxic
ADCVI	Antibody-Dependent Cell mediated Virus Inhibition
ANOVA	Analysis of Variance
APC	Antigen Presenting Cell
APHA	Animal and Plant Health AgencyBorder Disease Virus
BDV	Border Disease Virus
BHV	Bovine Herpes Virus
BNP	Bovine Neonatal Pancytopenia
BRDC	Bovine Respiratory Disease Complex
BRSV	Bovine Respiratory Syncytial Virus
BSA	Bovine Serum Albumin
BT	Bovine Turbinate Cells
BVDV	Bovine Viral Diarrhoea Virus
C	Caspid
CCD	Charge-coupled Device
CCP	Complement Control Protein
CCR7	C-C chemokine Receptor type 7
CD	Cluster of Differentiation
cDNA	Complementary DNA
COD	Corrected OD
cp	Cytopathic
CpG	Cytosine-phosphate-guanine
cRPMI	Complete RPMI
CSFV	Classical swine fever virus
CTL	Cytotoxic T Lymphocytes
CXCL9	Chemokine (C-X-C motif) Ligand 9
DC	Dendritic cell
DIVA	Differentiation between Infected versus Vaccinated Animals
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleid Acid
DNA-PKcs	DNA-dependent Protein Kinase catalytic subunits

dNTP	Deoxynucleotide Triphosphates
dpc/dpi/dpv	days post challenge/infection/vaccination
E	Envelope
E-MEM	Eagle's Minimum Essential Medium
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
FACS	Fluorescence Activated Cell Sorting
FBS/FCS	Foetal Bovine/Calf Serum
FBT	Foetal Bovine Turbinate Cells
FITC	Fluoroescein Isothiocynate
FMDV	Foot and Mouth Disease Virus
FSC	Forward Scatter
HA	Hemagglutinin
HBSS	Hank's Balanced Salt Solution
HCV	Hepatitis C virus
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic Acid
Ho916	Horton 916
HRP	Horseradish peroxidase
IAH	Institute of Animal Health
IAV	Inactivated Vaccine
IC	Internal Control
IFN-α/β/γ	Interferon Alpha/Beta/Gamma
Ig	Immunoglobulin
IL	Interleukin
IRES	Internal Ribosome Entry Site
IRF	Interferon Regulatory Factor
ISG	IFN stimulated genes
LIN^-	Lineage Negative
LPS	Lipopolysaccaride
kb	kilobases
kDa	kiloDalton
mAb	Monoclonal antibody

MACS	Magnetic Cell Sorting
MAD	Mucosal Atomization Device
MD	Mucosal Disease
MDA-5	Melanoma Differentiation-Associated gene 5
MEM	Minimum Essential Medium
MHC-I/II	Major Histocompatibility Complex class I/II
MIG	Monokine Induced by Gamma
MIP	Macrophage Inflammatory Protein
MLV	Modified Live Vaccine
MOI	Multiplicity of Infection
mRNA	Messenger RNA
MSD	Meso Scale Discovery
MVA	Modified Virus Ankara
N/NS	Non-structural
NA	Not applicable
NC	Negative Control
ncp	non-cytopathic
ND	Not determined
NF-kB	Nuclear transcription factor kappa B
NK	Natural Killer
NO	Nitric Oxide
NP	Nanoparticle
OD	Optical Density
OIE	Organisation for Animal Health
ORF	Open Reading Frame
P	Percentage
p7	Protein-providing Nucleocapsid 7
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
PC	Positive Control
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid DC

PE	Phycoerythrin
pH	Potential Hydrogen
PI	Persistently Infected
PI3V	Parainfluenza Type 3 Virus
PLGA	Poly(D,L-lactic-co-glycolic) acid
Poly(I:C)	Polyinosinic-polycytidylic Acid
PPD	Purified Protein Derivative
pro	Protease
PWM	Pokeweed Mitogen
qRT-PCR	Quantitative Reverse Transcriptase PCR
RIG-I	Retinoic acid Inducible Gene I
RLU	Relative Light Units
R-PE	Red Phycoerythrin
RNA	Ribonucleic Acid
rns	RNAse
RPMI	Roswell Parks Memorial Institute
RT	Room Temperature
RT-PCR	Reverse Transcriptase PCR
S	Sample
SA	Streptavidin
SD	Standard Deviation
SEM	Standard Error of Means
S/P	Sample to Positive Ratio
SSC	Side Scatter
ssRNA	Single Stranded Ribonucleic Acid
$TCID_{50}$	Tissue Culture Infectious Dose 50
TGF	Transforming Growth Factor
Th1/2	T-helper 1/2
TKK	Th/04_KhonKaen
TLR	Toll-like receptor
TMB	3, 3', 5, 5'-tetramethylbenzidine
TNF	Tumour Necrosis Factor
TSV	Tunisian Sheep Virus

UTR	Untranslated Region
VD	Viral Diarrhoea
VI	Virus Isolation
VNT	Virus Neutralization Titre
α	Alpha
β	Beta
γ	Gamma
δ	Delta

1 GENERAL INTRODUCTION

1.1 BOVINE VIRAL DIARRHOEA VIRUS

Bovine viral diarrhoea virus (BVDV) represents a group of enveloped, single-stranded, positive-sense RNA viruses of the genus *Pestivirus* in the family *Flaviviridae*, which are closely related to classical swine fever virus (CSFV) and ovine border disease virus (BDV) species (Simmonds et al., 2011). BVDV is divided into two antigenically and genetically distinct species, BVDV-1 and BVDV-2 (Ridpath et al., 1994). Two biotypes exist in both species, noncytopathic (ncp) and cytopathic (cp), based on the effect of the virus on cells in culture (Zhang et al., 1996). BVDV-1 is further divided into at least 12 subgenotypes (BVDV-1a to l) (Vilcek et al., 2001) while BVDV-2 is divided into two subgenotypes (BVDV-2a and b) (Flores et al., 2002). More recently, atypical bovine pestiviruses have been discovered that have a sister phylogenetic relationship to BVDV-1 and BVDV-2 and have been proposed as a third species, BVDV-3 (Liu et al., 2009c).

The BVDV virion consists of a 40-60nm-diameter particle made up of a central icosahedral capsid, enclosing genomic RNA, surrounded by a lipid bilayer envelope. The genome is approximately 12.3kb in length with 5' and 3' untranslated regions (UTR) bracketing a single open reading frame (ORF). The uncapped 5'UTR is highly conserved and consists of tertiary structures that make up an internal ribosomal entry site (IRES) required for initiation of translation (Pestova and Hellen, 1999) while the 3' UTR, like in other pestivirus genomes, terminates in a short polycytosine (C) tract. The 3' UTR is known to contain conserved structural elements thought to be important for minus-strand initiation and replication (Yu et al., 1999). The ORF encodes a polyprotein of about 4000 amino acids which is co- and post-translationally processed by host and viral proteases into 11 or 12 viral

proteins, including four structural proteins (C, E^{rns}, E1 and E2) and seven or eight non-structural (NS) proteins (N^{pro}, p7, NS2-3 (further cleaved to NS2 and NS3 in cytopathic virus), NS4A, NS4B, NS5A, and NS5B) (Meyers and Thiel, 1996) as shown in **Figure 1.1**.

The conserved capsid, C, protein forms the virion nucleocapsid, while the viral envelope is made up of E^{rns}, E1 and E2 glycoproteins. p7 is a small protein of unknown function that is vital for production of viable virus but is neither associated with the virion structure nor necessary for RNA replication (Harada et al., 2000). Cleavage between E2 and p7, like other structural proteins, is by host cellular proteases (signal peptide peptidase and signal peptidases) and has been reported to be incomplete, resulting in p7, E2 and E2-p7 proteins. Unlike p7 and E2, E2-p7 is not essential for the generation of infectious virus. E^{rns} is an envelope glycoprotein with intrinsic RNase activity (Schneider et al., 1993) and is unique to pestiviruses. It has the ability to translocate across the plasma membrane, which suggests that it may have a role in virus entry into target cells (Fetzer et al., 2005).

N^{pro}, the N-terminal self-cleaving cysteine protease (Rumenapf et al., 1998), like E^{rns}, is unique to pestiviruses. NS2/3 protein is a highly conserved protein with multiple enzymatic functions (N-terminal serine protease (Bazan and Fletterick, 1989), C-terminal RNA helicase (Gorbalenya et al., 1989) and NTPase (Gu et al., 2000)) and is known to cleave both itself and downstream NS proteins from the viral polyprotein employing NS4A protein as a cofactor (Tautz et al., 2000). NS5B is an RNA-dependent RNA polymerase that together with NS4B, the zinc-binding phosphorylated NS5A and unknown host factors, make up the viral replicase complex in association with intracytoplasmic membranes (Zhong et al., 1998).

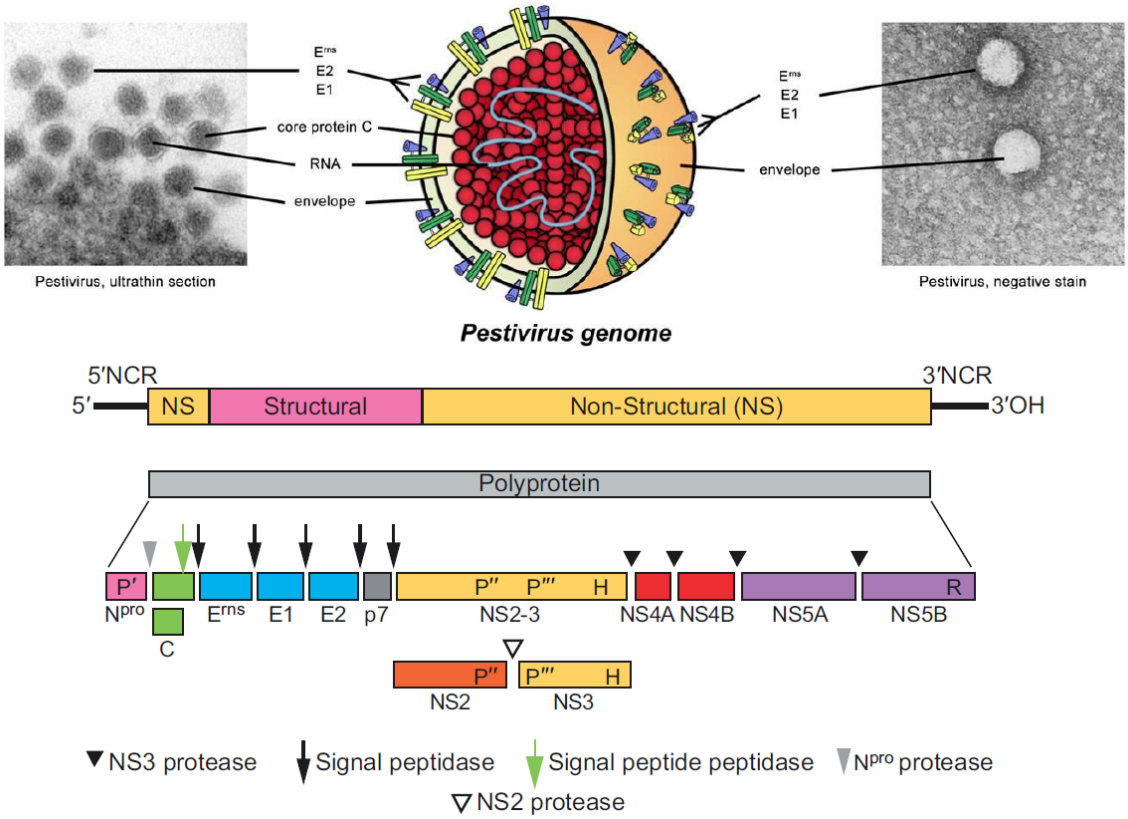

Figure 1.1. The virion morphology and genome organisation of BVDV and other pestiviruses. Electron micrographs show pestiviruses as spherical enveloped viruses with genomic RNA in an enveloped capsid core. The genome encodes a polyprotein which is cleaved by host and viral proteases to produce 11-12 proteins. The locations of structural and non-structural proteins are indicated. In ncp viruses, the NS2-3 protein is further processed to an NS2 and NS3 protein (H. Granzow, Friedrich-Loeffler-Institut, Insel Riems, Germany, with permission. © 2010)

Insertion of host cellular sequences, genome deletion, duplication and other rearrangements are commonly observed in cp viruses (Baroth et al., 2000, Becher et al., 1999, Becher et al., 1998, Meyers et al., 1998, Qi et al., 1998). Cp strains are reported to have an in-frame insertion of ubiquitin and ubiquitin-like genes (UBL) (Randow and Lehner, 2009) upstream of the viral NS3 gene providing a cleavage site allowing for the release of NS3 by the NS2 autoprotease (Becher et al., 2002). J-domain protein interacting with viral protein, jiv, a cellular chaperone protein, has been shown to interact with and activate autoprotease activity in NS2 (Lackner et al., 2006). The cytopathic effect of BVDV has been associated with the induction of apoptosis by the protease activity of secreted NS3 (Gamlen et al., 2010). A single point mutation in NS4B (Y2441C) has also been implicated in changing the virus from cp to ncp, suggesting that NS4B may play a role in BVDV pathogenesis (Qu et al., 2001).

The binding and entry of BVDV into cells involves the interaction of viral glycoproteins with cell surface receptors. BVDV E2 binds to the complement regulatory protein CD46 (Maurer et al., 2004), whilst it is believed that E^{rns} interacts with heparin sulphate (Iqbal and McCauley, 2002), after which, entry has been shown to be mediated by receptor-mediated, clathrin-dependent endocytosis in a pH dependent manner (Lecot et al., 2005, Krey et al., 2005). The viral membrane then fuses with the endocytic vesicle membrane resulting in the release of the viral genome into the cytoplasm. Replication then takes place at the surface of the endoplasmic reticulum (ER), where the positive sense genome is translated into the polyprotein, while a negative sense complimentary ssRNA is synthesized and serves as a template for new genomic material. Virus assembly occurs at the ER, from

which the virion is transported to the Golgi apparatus and buds from the cell membrane. E2 is a major determinant of both cell and host tropism (Liang et al., 2003), and through interaction with the ubiquitous CD46 which is present on all nucleated cells, BVDV appears to infect most bovine cells. E2 binding has been mapped to two domains on the complement control protein 1 (CCP1) of CD46 (Krey et al., 2006) and genetic variation in these regions has been shown to affect permissivity and host tropism to BVDV (Zezafoun et al., 2011). The host range for BVDV includes most even-toed ungulates (order *Artiodactyla*), and although many species of animals such as goats, sheep, pigs, deer, reindeer, bison llamas and alpacas are susceptible to infection with BVDV, domestic cattle are the primary hosts (Ames, 2008).

1.2 BOVINE VIRAL DIARRHOEA: DISEASE, EPIDEMIOLOGY AND IMPACT

BVD was first described in North America in the 1940s as a transmissible disease of unknown origin affecting cattle (Childs, 1946, Olafson et al., 1946). The disease was characterized by leucopoenia, fever, depression, diarrhoea, dehydration, anorexia, salivation, nasal discharge, gastrointestinal erosions and tissue haemorrhages. The causative agent was isolated from separate cases of mucosal disease (MD) and viral diarrhoea (VD) in cattle and was found to be cytopathic and noncytopathic BVDV respectively (Lee and Gillespie, 1957, Underdahl et al., 1957). A cytopathic virus, Oregon C24V was isolated in 1960 from a case of VD and attenuated in cell culture for use as a vaccine against BVD (Gillespie et al., 1960, Coggins et al., 1961).

BVD clinical presentation is dependent on a number of factors such as virus strain, immune, reproductive and age status of the host, as well as the presence of co-infections (Ridpath, 2010, Baker, 1995). The majority of BVDV isolates are well adapted to cattle and cause transient acute infection in healthy animals that is cleared within 10-14 days. Transient immunosuppression, thought to be a consequence of immune cell death within lymph nodes and gut-associated lymphoid tissue (GALT) and reduced numbers of circulating leukocytes, may increase susceptibility to secondary infection resulting in respiratory and enteric disease (Bolin, 2002).

BVDV infection has a major impact on the reproductive success of the host (Fray et al., 2000). Infection of bulls, in which virus has been shown to be able to persist in the testes, can lead to a poor semen quality, while infection in cows can result in poor conception rates and reproductive failure. In pregnant animals, depending on the time of infection, BVDV may cause abortions, stillbirths or teratogenic effects (congenital defects) (Roeder et al., 1986). Foetal infection with ncp virus during the first trimester (40 to 120 days) of pregnancy, prior to the development of immunocompetence, can result in the birth of persistently infected (PI) calves. Only ncp BVDV are able to establish persistent infections. PI calves are chronically infected and, in the absence of an adaptive immune response, continuously shed virus. Superinfection of PI animals with a related cp virus or by mutation of the persistent ncp virus into a cp virus may result in MD, a fatal condition characterised by severe lesions of the oral and intestinal mucosa (Ramsey and Chivers, 1957, Brownlie et al., 1984). While there has been no direct association between virus biotype and severity of disease *in vivo*, with both cp and ncp viruses exhibiting a spectrum of virulence, ncp viruses, which make up the majority of

viruses in the field, are more commonly associated with high virulence (Ridpath, 2005).

Like BVDV-1, the majority of BVDV-2 infections are asymptomatic. However, BVDV-2 infection associated with severe thrombocytopenia and haemorrhagic syndrome in cattle was observed in North America in the late 1980s (Rebhun et al., 1989) and subsequently in Europe in the early 1990s (Thiel, 1993). BVDV-1 viruses makes up more than 90% of isolates in Europe (Vilcek et al., 2005), with BVDV-2 being more prevalent in North America (50%) (Ridpath, 2005). Despite BVDV being recognized as a global problem, there is only sparse information on the prevalence and genotypes of BVDV outside Europe and America. Recent studies have attempted to characterize the disease in Africa (Kabongo and Van Vuuren, 2004, Handel et al., 2011), Asia (Kampa et al., 2004), South America (Stahl et al., 2002) and Australasia (Heuer et al., 2007, Ridpath et al., 2010). Atypical bovine pestiviruses which had previously been detected in commercial foetal calf serum batches (Peletto et al., 2010, Schirrmeier et al., 2004, Stalder et al., 2005, Xia et al., 2011), have also been reported in natural field infections (Ståhl et al., 2007). These viruses have been detected in aborted bovine foetuses in Brazil (Cortez et al., 2006) and in association with cases of abortion and respiratory disease in Italy (Decaro et al., 2011b, Decaro et al., 2011a) suggesting that they may have similar clinical implications as classical BVDV species.

BVD has a complex epidemiology, central to which is the role played by PI calves that constantly disseminate virus in herds. BVDV transmission in herds is either horizontal by contact with PI or acutely infected animals and infection by virus in aerosol through the oropharynx and respiratory tracts, or vertically in pregnant

animals to the foetus. It is estimated that between 1-2% of cattle worldwide are persistently infected with BVDV, with about 50% of herds having PI animals and 60-80% of cattle becoming exposed in their lifetime (Houe, 1999). BVDV infection causes significant economic losses to the cattle industry (Houe, 2003). Economic costs of BVDV in Europe have been estimated at between €10 to 40 per calving (Houe, 2003) while recent estimates in the United States have put average economic costs at approximately $88 per animal (Hessman et al., 2009). Losses are associated with reproductive failure, the birth of PI animal, co-infections or highly virulent strains resulting in severe disease and death, reduced feed conversion efficiency for meat and milk production and costs associated with BVDV control.

1.3 CONTROL AND ERADICATION EFFORTS

As a result of its economic impact, significant efforts are made to prevent and control BVD. BVD control programmes have been classified as either systematic, involving a monitored, goal-oriented reduction in incidence and prevalence, or non-systematic where measures are implemented on a herd to herd basis without systematic global monitoring (Lindberg and Houe, 2005). Three key elements for the systematic control of BVDV have been described: increased biosecurity to prevent the introduction of the infection in BVDV-free herds, the identification and elimination of PI animals and increased surveillance to monitor the progress of intervention and detect new infections (Lindberg et al., 2006).

Successful BVD eradication programmes have been implemented in Scandinavia (Hult and Lindberg, 2005, Valle et al., 2005) and Austria (Rossmanith et al., 2010), while Switzerland is in advanced stages.(Presi et al., 2011). Eradication

efforts are also underway in Finland (Rikula et al., 2005), Scotland (Logue, 2010) and Germany (Moennig et al., 2005), while the Republic of Ireland has recently initiated a voluntary eradication scheme which is planned to be mandatory in 2013 (Barrett et al., 2011). A pilot eradication scheme has also been launched in England (Booth and Brownlie, 2012).

While virus isolation (VI) is considered the gold standard for virus detection in samples, antigen detection by immunohistochemistry (IHC), antigen capture ELISA (ACE) and nucleic acid detection by reverse transcriptase-polymerase chain reaction (RT-PCR) are more broadly applied (Saliki and Dubovi, 2004, Sandvik, 2005). VI is the only way of detecting viable virus; although these alternative tests have proven robust, reproducible and have good correlation with VI. These tests are, however, dependent on monoclonal antibody (mAb) binding to antigen or detection of viral RNA that is likely to vary between different diverse virus genotypes. In addition to the selection of conserved targets to prevent detection failure, these tests are regularly adapted to ensure detection of novel viruses as well as distinguish different pestivirus species (Decaro et al., 2012). Detection of BVDV specific antibody in milk or blood samples by ELISA or by virus neutralisation test (VNT) in exposed or vaccinated animals are also useful for diagnosis. Antibody and antigen tests, when used together, help distinguish acute from persistently infected animals in which the virus persists in the absence of immune responses.

Vaccination against BVDV is viewed as an additional biosecurity measure aimed primarily at the protection of postnatal calves against disease after maternal antibody wanes and of heifers to prevent foetal infections which may result in reproductive failure, foetal losses and birth of PI calves (Fulton et al., 2003a).

Adoption of BVDV vaccination in Europe is voluntary with usage from 20% to 75% (Moennig and Brownlie, 2006). BVDV eradication without vaccination has been successfully carried out by large-scale eradication schemes in Scandinavian countries where 90-99% of herds are considered free of BVDV (Bitsch and Ronsholt, 1995). These schemes are however expensive and intensive and take a long time to implement. Vaccination remains the most cost-effective measure of disease control in animal health (van Oirschot et al., 1999). Furthermore, in regions with high cattle densities and BVDV prevalence, and therefore an increased probability of virus reintroduction into fully susceptible BVDV free herds, vaccination can be incorporated into systematic control strategies as is the case in Germany (Moennig et al., 2005).

1.4 BVDV VACCINES

According to an online database of livestock veterinary vaccines, (www.vetvac.org), there are around 135 registered BVDV vaccine products currently in use around the world, mostly in North and South America. These are conventional modified live virus (MLV) or inactivated/killed virus vaccines, formulated as either monovalent BVDV preparations or multivalent vaccines including other pathogens implicated in the bovine respiratory disease complex (BRDC) such as members of the *Pasteurellaceae* family (including *Mannheimia haemolytica*, *Pasturella multicoda* and *Haemophilus somnus*), bovine herpesvirus (BHV), parainfluenza type 3 virus (PI3V) and bovine respiratory syncytial virus (BRSV) (Allen et al., 1992). Whilst good cross protection is observed against BVDV-1 subgenotypes, the failure of BVDV-1 based vaccines to protect against some emerging BVDV-2 viruses, in

spite of a degree of antigenic cross-reactivity, has resulted in inclusion of the latter in new vaccine preparations (Ridpath, 2005). The need to adapt vaccines to prevalent BVDV viruses has also been considered in the development of new vaccines (Fulton, 2008).

MLV vaccines are generally thought to be more efficacious since they induce high titres of virus neutralising antibodies and provide a longer duration of protection from clinical disease than inactivated vaccines that often require booster immunizations to achieve improved protection. However, there is a risk that MLVs can revert to a virulent form or recombine with field viruses and cause disease and vaccinated animals have been reported to develop transient viraemia and shed vaccine virus (Fulton et al., 2003b, Grooms et al., 1998). In pregnant animals, live vaccines pose the risk of vertical transmission of vaccine virus that can result in foetal complications or persistent infection (Ficken et al., 2006). A number of MLV vaccines have however been shown to confer foetal protection after BVDV-1 and, in a few instances, BVDV-2 challenge (Xue et al., 2009, Fairbanks et al., 2004, Kovacs et al., 2003). Live vaccination has also been implicated in post-vaccination mucosal disease (pvMD) (Becher et al., 2001). As a result of these safety concerns, live vaccines are not licensed in a number of countries such as the United Kingdom, Ireland, The Netherlands and Slovenia.

Whilst inactivated vaccines are thought to be much safer and are therefore preferred for vaccination of breeding cattle, bovine neonatal pancytopenia (BNP), a syndrome affecting newborn calves characterised by pancytopenia, severe bleeding and high lethality, has recently been described in Europe and associated with alloreactive antibodies against bovine MHC class I linked to the use of a particular

inactivated BVDV vaccine (Bastian et al., 2011, Foucras et al., 2011). This highlights a safety concern with regards to the use of crude inactivated virus preparations that contain residual host cell derived material. Neither MLV nor inactivated vaccines allow for differentiation between infected versus vaccinated animals (DIVA) (van Oirschot, 1999), which makes them less suitable for use in BVDV eradication efforts. Inactivated vaccines were previously thought to allow DIVA because of a lack of production of non-structural proteins such as NS3 and hence diminished responses to these targets (Makoschey et al., 2007). This has however proved not be the case in a number of studies (Alvarez et al., 2011, Raue et al., 2010), due to the presence of non-structural proteins in the crude virus preparation prior to inactivation.

Recent BVDV vaccine developments aim at addressing the shortcomings of existing vaccines. An ideal vaccine should: prevent disease, vertical and horizontal virus transmission, be safe in pregnant animals, unable to revert to virulence (in the case of a live virus vaccine), have broad efficacy to account for virus diversity and permit DIVA (Fulton, 2008). Various approaches towards the development of the next generation of BVDV vaccines have been evaluated in cattle such as DNA vaccination (Nobiron et al., 2003, Young et al., 2005, Liang et al., 2005, Harpin et al., 1999), the use of baculovirus or mammalian cell expressed subunit proteins (Thomas et al., 2009, Bolin and Ridpath, 1996, Chimeno Zoth et al., 2007), or combined as a DNA prime-protein boost regime (Liang et al., 2008, Liang et al., 2006), all with mixed but encouraging results. Others, such as replication defective viral vectored replicons (Elahi et al., 1999b, Mehdy Elahi et al., 1999, Toth et al., 1999, Baxi et al., 2000, Elahi et al., 1999a), synthetic attenuated infectious cDNA

clones (Meyer et al., 2002, Meyers et al., 1996), RNA vaccination (Vassilev et al., 2001), as well as chimeric pestivirus marker vaccination (Luo et al., 2012) that allow DIVA are being assessed for use in vaccines against BVDV and other pestiviruses (Tautz and Meyers, 2011). Recent and on-going BVDV vaccine development efforts have been the subject of recent reviews (Fulton, 2008, Chase et al., 2004). Development of safer, efficacious vaccines will require a better understanding of immune responses to BVDV.

1.5 IMMUNOLOGY OF BVDV

1.5.1 BVDV and Innate Immunity

BVDV has a variety of effects on both innate and adaptive immune responses as summarized in **Figure 1.2** (Chase et al., 2004). BVDV has been shown to infect myeloid cells of the innate immune system and affect their function. The microbicidal, chemotactic and antibody-dependent cell-mediated cytotoxic (ADCC) functions of neutrophils are impaired (Potgieter, 1995). BVDV infects monocytes, producing progeny virus, and in the case of cp BVDV, inducing apoptosis in infected monocytes as well as uninfected 'bystander' monocytes and lymphocytes via soluble factors derived from infected cells (Glew et al., 2003, Lambot et al., 1998). Infection with both biotypes of BVDV has been shown to affect alveolar macrophages by decreasing phagocytosis, Fc and complement receptor expression, microbicidal activity and chemotactic factor production (Peterhans et al., 2003). Infection of macrophages with cp or ncp BVDV has also been shown to decrease LPS-induced tumour necrosis factor alpha (TNF-α) and superoxide anion production while ncp BVDV induces enhanced nitric oxide (NO) synthesis, which may contribute to the

immunosuppressive effects associated with BVDV infection (Adler et al., 1997, Adler et al., 1996). Other immunosuppressive effects have also been described such prostaglandin E2 production (Van Reeth and Adair, 1997), the release of IL-1 inhibitors (Jensen and Schultz, 1991) and depression of cytokine induced chemotaxis (Ketelsen et al., 1979). Additionally, BVDV has been shown to down-regulate MHC class II expression in monocytes and macrophages but no effect was observed in antigen presentation ability of dendritic cells (Glew and Howard, 2001).

Cp BVDV induces interferon-alpha (IFN-α) production while ncp virus has been shown to inhibit double-stranded RNA (poly(I:C)) induced apoptosis and type I IFN (α, β) synthesis *in vitro* (Peterhans et al., 2003). Ncp virus has also been shown to fail to induce type I IFN in persistently infected (PI) foetuses following experimental infection of pregnant cows with ncp virus (Smirnova et al., 2008, Charleston et al., 2001). Type I IFN plays a role in the elimination of virally infected cells through apoptosis and it has been proposed that this response may be responsible for preventing foetal infection by cp viruses, whereas ncp BVDV, which can inhibit this response *in vitro*, is able to establish persistent infections. Interestingly, while blocking type I IFN to itself, BVDV does not appear to disrupt these responses to other unrelated viruses (Schweizer et al., 2006). However, both cp and ncp viruses have also been shown to induce type I IFN production *in vivo* (Charleston et al., 2002) and from lineage negative (LIN^-) $CD4^+$ plasmacytoid dendritic cells (pDCs) which are present at low frequency in peripheral blood (Gibson et al., 2011, Brackenbury et al., 2005). Furthermore, a mutant virus with a codon deletion affecting the RNase activity of E^{rns} and deletion of part of the N-terminal protease, N^{pro}, both of which have been shown to inhibit type I IFN, has

been shown to prevent the establishment of persistent foetal infection (Meyers et al., 2007). N^{pro} and E^{rns} have been described to suppress host immune responses by blocking the activity of interferon regulatory factor 3 (IRF3) and preventing type I IFN production, and by preventing IFN-β production by binding and degrading double-stranded RNA, respectively (Hilton et al., 2006, Iqbal et al., 2004).

Another feature in the establishment of PI is the evasion of adaptive immune responses because of the presence of viral antigen during selection of T lymphocytes that subsequently recognize the virus as self (Hansen et al., 2010). Interestingly, superinfection of PI animals with an antigenically homologous virus may result in fatal MD, suggesting that the immune system in these animals is able to recognize and mount an active unregulated response against a related virus from the PI virus (Collen et al., 2000). The role of natural killer (NK) cells in BVDV immunity has not been reported but NS5A has been reported to impair NK responses following infection with the related hepatitis C virus (HCV) in humans (Sene et al., 2010).

1.5.2 BVDV and Adaptive Immunity

1.5.2.1 Antibody Responses

The effect of BVDV on circulating B cells varies by study; from an observed decrease (Ellis et al., 1988), to showing no effect (Archambault et al., 2000), to a transient increase (Brodersen and Kelling, 1999). Antibody responses to BVDV are detectable 2-3 weeks after infection and may plateau after 10-12 weeks (Howard, 1990). Serum antibodies may be acquired following an active immune response due to infection or from maternal colostrum. E^{rns}, E1 and E2 glycoproteins induce neutralizing antibodies, with E2 being immunodominant and antibodies to structural proteins NS2-3 or NS3 have also been detected (Bolin, 1993, Mignon et al., 1992).

Whilst neutralizing antibodies have been shown to offset disease following homologous viral challenge, this does not necessarily result in viral clearance (Nobiron et al., 2003). Ncp BVDV infection is associated with higher levels of neutralising antibody which develop more quickly than cp BVDV, which showed evidence of a stronger cell mediated response and suggesting predominantly Th_2 and Th_1 regulated mechanisms respectively (Lambot et al., 1997).

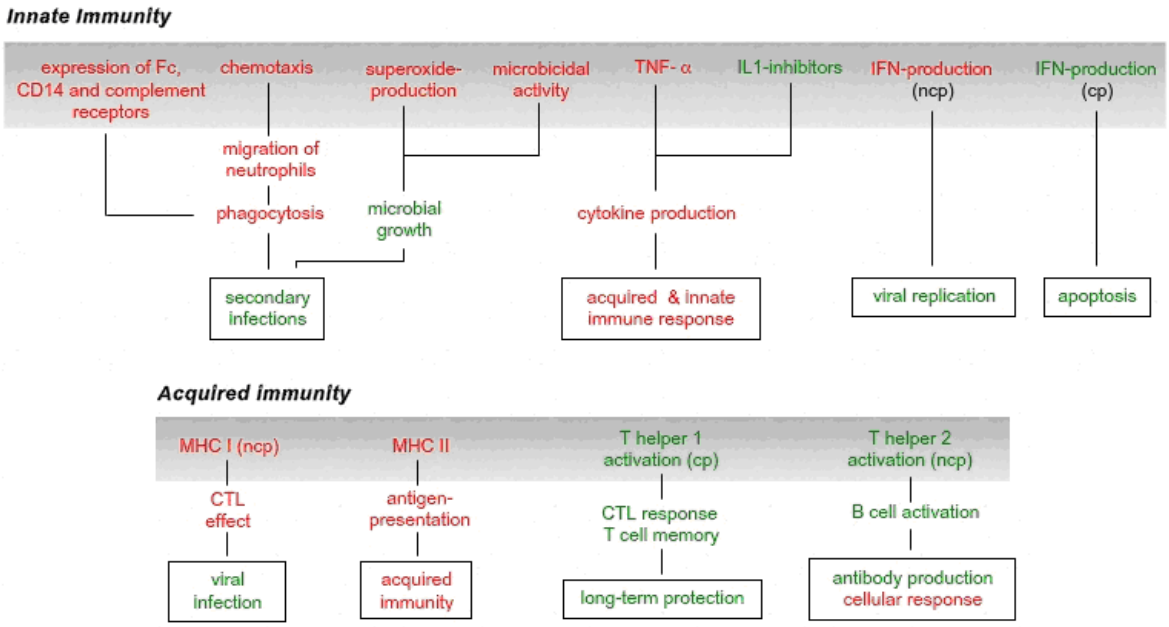

Figure 1.2. Interaction of BVDV with innate and adaptive immunity. BVDV has various effects on both innate and adaptive immunity with some functions, depending on biotype, decreased (in red) or increased (in green). (Institute of Veterinary Virology, University of Bern, Bern, Switzerland © 2006 based on Chase et al. (2004). Used with permission.

1.5.2.2 T cell responses

The effect of BVDV infection on circulating T cells varies from mild (10-20%) to severe (50-60%) lymphopenia depending on strain virulence (Brodersen and Kelling, 1999). $CD8^+$ cytotoxic T-lymphocytes (CTL) exhibit the greatest decrease in numbers followed by $CD4^+$ helper T cells but there is seemingly little effect on γδ T cells (Chase et al., 2004). T cell responses to BVDV have been demonstrated following acute infection, as well as after vaccination with killed, MLV and subunit E2 vaccines (Beer et al., 1997, Collen et al., 2000, Liang et al., 2005, Rhodes et al., 1999). Monoclonal antibody mediated cell depletion studies have shown that CD4 T cell depletion increased the duration of virus shedding, while CD8 and γδ T cell depletion had little effect, suggesting the importance of CD4 T helper cells in mediating immunity to BVDV (Howard, 1990). The possible role of depletion of other $CD4^+$ population such as pDCs was however not addressed.

Characterisation of T cell responses to BVDV has shown that both CD4 and CD8 T cell responses are evoked, with the former producing IL-4 and the latter IFN-γ, suggestive that the major role for CD4 T cells is to mediate help for the induction of B cell antibody responses (Rhodes et al., 1999). However, MHC restricted CTL specific for BVDV infected targets have also been reported although their phenotype was not discerned (Beer et al., 1997). Assessment of the specificity of the T cell response has been limited and focused on CD4 T cells. A preliminary study revealed that of a number of viral proteins analysed, C, E^{rns}, E2 and NS2-3, were all recognised by T cells (Collen and Morrison, 2000). An extension of this study mapped an antigenic region on the NS3 protein and showed that amino acid substitutions in this area altered T cell recognition (Collen et al., 2000).

1.6 AIMS AND OBJECTIVES

Whilst published data provides good evidence for the role of neutralising antibody responses in immunity against BVD, the assessment of T cell responses to the various BVDV proteins has been limited. Most studies have assessed T cell responses in the context of the whole virus conducted outside the context of the identification and selection of vaccine candidate antigens. This study aimed to rationally address the development of effective BVDV vaccines by generating and using information on T cell responses, which may play a role in the development of protection. We hypothesised that T cell responses to certain key BVDV antigens play a critical role in immunity to BVD and their inclusion in a subunit vaccine formulation will enhance protection against BVDV challenge. The project therefore focused on the characterisation of T cell responses following experimental infection with BVD viruses, including the identification of viral proteins that are the targets of T cell responses. The project then evaluated a novel vaccine formulation of BVDV antigens for its ability to stimulate T cell responses and protect calves against challenge infection.

The specific objectives were:

1. Characterisation of T cell responses to BVDV proteins following experimental infection of calves with cytopathic BVDV-1a virus. (Chapter 3)
2. Comparative analysis of immune responses following experimental infection of calves with BVDV-1a and an Asiatic atypical bovine pestivirus (Chapter 4)
3. Evaluation of the immunogenicity and efficacy of biodegradable nanoparticle vectored viral proteins as a vaccine against BVDV-1 (Chapter 5)

2 MATERIALS AND METHODS

2.1 Bovine Viral Diarrhoea Viruses

Cytopathic BVDV-1a Oregon C24V (Coggins et al., 1961) was propagated *in vitro* by inoculation of sub-confluent foetal bovine turbinate (BT) cell monolayers. BT cells were maintained in Minimum Essential Medium (MEM) with 7.5% sodium carbonate, 5% lactalbumen hydrosate, 1mM sodium pyruvate, 2mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 20µg/ml nystatin (all Invitrogen, Life Technologies, Paisley, UK) and 10% BVDV-free foetal bovine serum (FBS; Autogen Bioclear, Calne, UK) (BT medium). After 4 days, the supernatant was collected and pooled with a freeze-thawed cell lysate. The resultant pool was clarified by centrifugation at 524 x g for 10 minutes, aliquoted and stored at -80°C. Virus titres were determined as the median (50%) tissue culture infectious dose ($TCID_{50}$) using the Spearman-Kärber method (Finney, 1978) following immunoperoxidase staining (Meyling, 1984). Mock virus supernatant was prepared in an identical manner from uninfected BT cells. BVDV-1 Oregon C24V was select as it is a prototype BVDV-1 strain which has been attenuated for vaccination purposes and for which full genome sequence data was available for synthesis of a proteome-wide peptide library. Two noncytopathic BVDV isolates were used in the second study: BVDV-1a strain Horton-916 (Ho916), isolated from a clinically severe fatal outbreak of BVD in the United Kingdom (Hibberd et al., 1993) and an atypical bovine pestivirus strain, Th/04_KhonKaen (TKK) which was recovered from a dairy calf in Thailand (Liu et al., 2009a). BVDV-1 Horton-916 was selected as a strain known to cause disease in cattle in the UK and TKK virus was included as a genetically distant atypical bovine pestivirus. BVDV-1 Horton-916 was also used as the challenge virus in the final vaccination study. All experimental calves received an

intranasal (*i.n.*) inoculation on 41 dpv with $5x10^6$ $TCID_{50}$ in 2ml, which was confirmed by back titration. The virus dose was selected as it had been previously used at this dose to induce clinical disease in an experimental infection (Dr. Rebecca Strong, personal communication).

2.2 Ethical Consideration

All animal work was approved by the Animal and Plant Health Agency Ethics Committee and all procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act 1986 under UK Home Office Project Licence permit number 70/6836.

2.3 Animals, experimental infection and vaccination

Six 3-4 month old Holstein-Friesian calves were recruited; four were inoculated intransally (*i.n.*) with a 'low' dose (10^4 $TCID_{50}$) of BVDV Oregon C24V (2 ml divided equally between each nostril and administered using a mucosal atomization device MAD-300, Wolfe Tory Medical, USA) and then similarly inoculated with a 'high' dose (10^7 $TCID_{50}$) after a period of four weeks. BVDV Oregon C24V was selected as a reference cytopathic BVDV-1a strain attenuated for vaccine use. The 'low' dose was expected to prime immune responses and the the higher dose to boost them. Two calves received two doses of control mock virus supernatant and were housed separately. Animals were monitored for clinical signs of infection and blood samples longitudinally collected to assess viraemia, antibody and T cell responses.

For the second experiment, twenty 3–5 month old, BVDV-free Holstein calves were divided into 4 groups of 5 calves each and inoculated with 10^5 $TCID_{50}$ in 10ml (5ml intranasal and 5ml intramuscular) of Ho916, TKK or both viruses (Ho916/TKK). Control animals (Controls) were inoculated with 10ml of Eagle's Minimal Essential Medium (E-MEM) (Invitrogen). The viruses used represented a typical field virus, Ho916, and newly described genetically divergent atypical pestivirus, TKK, for comparative purposes. The doses used were considered adequate to induce clinical disease. Assessment of clinical scores including rectal temperature and sampling of blood and nasal secretions was conducted up to 45 days post-infection (45 dpi). The details of the procedures and outcome of the experimental infection are described by Larska et al. (2012) (Appendix A).

For the final experiment, eighteen 6-8 month old male Holstein-Friesian calves were used. Published data from an experimental E2 subunit vaccine study (Thomas et al., 2009) was used to calculate a sample size sufficient to measure differences in immune responses between vaccinated and control animals with significant statistical power. A group size of six was found to enable measurement of virus neutralising antibody responses with a power of 0.93 based on mean variances (range of 0.68-1, based upon maximum and minimum variances) and total virus-specific antibody response with a power of 0.81 (range, 0.54-1). The animals were tested for the absence of BVDV antibody and antigen and randomly assigned into three experimental groups prior to vaccination.

2.4 Vaccine preparations and immunization

A formulation of PLGA nanoparticles encapsulating *Escherichia coli* expressed helicase domain of BVDV-1 CP7 NS3 protein (a kind gift from Prof. Till

Rümenapf, Institute of Virology, Giessen, Germany) and poly(I:C) (Sigma-Aldrich, Poole, UK) and coated with BVDV-1 NCP7 and NADL baculovirus derived E2 protein ectodomain (a kind gift from Dr. Thomas Krey, Pasteur Institute, Paris, France) (Vaccine NP; approximately 150μg NS3, 50μg E2 and 80μg poly(I:C) per dose) was prepared and quality controlled at the APHA (Walters et al., unpublished data). PLGA nanoparticles similarly formulated with poly(I:C) and ovalbumin protein (Sigma-Aldrich) were used as a negative control (Control NP). The quantity of antigen and TLR ligand coating or encapsulated in the nanoparticles was determined empirically during formulation and dosage was determined by quantities that were considered adequate in published literature. (Bruschke et al., 1997, Thomas et al., 2009). A licensed inactivated vaccine (IAV), Bovidec® (Novartis Animal Health, Camberley, United Kingdom), was used as a reference standard for both immunogenicity and efficacy. According to the manufacturer's specifications, Bovidec consists of an inactivated BVDV-1a strain, KY1203nc (Clarke et al., 1987) in Minimum Essential Medium (5×10^6 $TCID_{50}$ per 4ml dose), which is adjuvanted with Quil A (1 mg per dose) and stabilised with thiomersal. Calves (six per group) received a primary subcutaneous (*s.c.*) inoculation of Vaccine NP or Control NP in 2ml or IAV in 4ml. A similar booster inoculation was given at 21 days post-vaccination (dpv).

2.5 Clinical and haematological monitoring

Clinical scores and rectal temperatures were monitored prior to viral challenge and on a daily basis subsequently. For clinical scoring, seven parameters were assessed: depression, ocular discharge, nasal discharge, dyspnoea, cough, inappetance and diarrhoea. These were individually scored as normal (0), slightly

altered (1), a distinct clinical sign (2) or a severe symptom (3). Weights were also monitored weekly prior to and after viral challenge.

Platelet and leukocytes were enumerated by volumetric flow cytometry prior to viral challenge and every 2-3 days thereafter. Briefly, for platelets, 500µl EDTA blood was centrifuged at 200g for one minute in a microcentrifuge and 5µl of platelet-rich plasma was transferred into a FACS tube containing 2ml of CellFix solution (BD Biosciences, Oxford, UK), mixed and incubated for 10 minutes at room temperature (RT). 25µl was then acquired on a volumetric flow cytometer (MACSQuant Analyzer; Miltenyi Biotec, Bisley, UK) and counts obtained by gating on platelets based on their forward and side scatter characteristics on logarithmic scales. Platelet gating was validated by staining with mouse anti-bovine CD61 (platelet glycoprotein IIIa, AbD Serotec, Oxford, UK). For total leukocyte counts, 50µl EDTA blood was stained with 5µl of anti-ovine CD45-FITC mAb (AbD Serotec) diluted 1 in 5 in PBS with 1% FBS and 0.1% sodium azide (FACS buffer) and incubated for 15 minutes at RT in the dark. 0.945ml of FACS Lysing solution (BD Biosciences) was added for 10 minutes at RT to lyse erythrocytes and fix leukocytes. 25µl was acquired and cell counts were obtained by gating FITC positive events. Granulocytes (eosinophils and neutrophils), monocytes and lymphocytes were distinguished by plotting intracellular complexity (side scatter) against CD45 expression (Pelan-Mattocks et al., 2001). Both platelet and leukocyte counts per ml were obtained by multiplying the count by dilution factor and acquired volume conversion to 1ml.

2.6 Assessment of viraemia

2.6.1.1 Detection of viral antigen

The BVDV envelope RNase glycoprotein, E^{rns}, was detected in heparinised blood samples using the HerdChek® BVDV Antigen/Serum Plus ELISA kit according to the manufacturer's instructions (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland). Briefly, 50µl of sample, including positive and negative controls, were added to microtitre plate wells coated with immobilized anti-BVDV E^{rns} mAb and incubated for two hours at 37°C. The captured antigen was then detected using a secondary E^{rns} specific mAb and horseradish peroxidase (HRP) conjugate. Unbound conjugate was then washed off and a chromogenic substrate solution was added. The reaction was stopped by the addition of 100µl of sulphuric acid to each well and absorbance at 450nm measured on a spectrophotometer. The presence of BVDV antigen was determined as corrected optical density (COD) by subtracting the mean negative OD from the mean sample OD. COD values which were greater than 0.3 were considered to be positive.

2.6.1.2 Viral nucleic acid detection

BVDV RNA was extracted from EDTA blood samples using the QIAamp Viral RNA extraction kit (Qiagen, Crawley, UK) as described by the manufacturer. The RNA quality (OD_{260}/OD_{280} ratio) and quantity (OD_{260}) was assessed on a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the material stored at −80°C. BVDV RNA extracted from EDTA blood was detected using a single tube nested quantitative RT-PCR (qRT-PCR) TaqMan® assay (McGoldrick et al., 1999). cDNA synthesis from RNA was first performed by

reverse transcription using the V326 (5' TCA ACT CCA TGT GCC ATG TAC 3') primer (Vilcek et al., 1994). First round PCR (10 cycles) using the pestivirus primers V326 and V324 (5' ATG CCC W(A/T)TA GTA GGA CTA GCA 3') produced a 300bp amplicon, after which a second round nested PCR (40 cycles) was used to copy the first round amplicon using primers A11 (5' AGT ACA GGG TAG TCG TCA GTG GTT CG 3') and A14 (5' CAA CTC CAT GTG CCA TGT ACA GCA G 3') to produce a 220bp amplicon (Belak and Ballagi-Pordany, 1993). A pan-pestivirus TaqMan® probe (FAM 5' CTG ATA GGG TGC TGC AGA GGC CCA CT 3' TAMRA) was detected during the extension phase in each PCR cycle on a Stratagene MX3000P Real-Time PCR machine (Agilent Technologies, La Jolla, CA, USA) and data analysed on the MX Pro 4.1.0c Software (Agilent Technologies). Relative quantities of viral RNA in samples were quantified by interpolation against a 10 fold dilution of RNA extracted from an equivalent volume of virus (BVDV-1a Oregon C24V) of known titre (standard range from $10^{7.55}$ to $10^{0.55}$ $TCID_{50}$/ml) (La Rocca and Sandvik, 2009).

2.7 BVDV sequence analysis

E2 and NS3 coding sequences from Ho916 were provided by Dr. Rebecca Strong (APHA, Addlestone, United Kingdom). Predicted amino acid sequences were aligned against E2 and NS3 sequences from the published genome of reference strain BVDV-1 Oregon C24V (C24V) (AF091605.1) (Coggins et al., 1961) and Th/04_KhonKaen (FJ040215.1) using the *clustalw* algorithm on MegAlign (DNAStar Lasergene 9 Core Suite, Madison, WI, USA). Sequence distances were calculated as *divergence* based on comparison of sequence pairs in relation to a

phylogeny reconstruction by MegAlign and as *percent identity*, which compares both sequences directly without accounting for phylogenetic relationships.

2.8 Detection of virus and antigen specific antibody responses

2.8.1 BVDV specific antibody

Total BVDV-specific antibody was detected in serum samples using a commercial indirect ELISA (HerdChek® BVDV Antibody ELISA, IDEXX Laboratories, Wetherby, UK). In brief, 25µl of serum samples, negative and positive controls were each diluted to 100µl and added to a 96 well plate coated with immobilized BVDV antigen, mixed thoroughly and incubated for 90 minutes at room temperature. After, the wells were washed five times and 100µl of anti-bovine HRP conjugate added to each well and incubated for a further 30 minutes at RT. Following another wash step and 10 minutes incubation with 100µl per well of 3, 3', 5, 5'-tetramethylbenzidine (TMB) substrate at RT in the dark, 100µl of stop solution was added and absorbance measured at 450nm. Results were calculated as sample to positive control ratios (S/P) using the following formula:

$$S/P = \left[\frac{OD_S - OD_{Nc}}{OD_{Pc} - OD_{Nc}}\right]$$

Where, OD – Optical density, S – Sample, N_C – Negative control, P_C – Positive control. Samples with an S/P value greater than or equal to 0.30 were considered positive, those less than 0.2, negative, and anything between 0.2 and 0.3 were inconclusive.

In Experiment two (Chapter four), BVDV specific antibody was detected in serum samples using an indirect BVDV antibody ELISA (Svanovir®, Svanova Biotech AB, Uppsala, Sweden). In brief, 10µl of samples, negative and positive controls in 90µl of diluents were added to a 96 well plate with immobilized BVDV or control antigen and incubated for one hour at 37°C. After, the wells were washed five times and 100µl of anti-bovine HRP conjugate was added to each well and incubated for a further hour at 37°C. Following another wash step and 10 minutes incubation with 100µl per well of TMB substrate at RT in the dark, 50µl of stop solution was added and absorbance measured at 450nm. Corrected OD values were obtained by subtracting the OD values of the control antigen well from the corresponding BVDV antigen well. Antibody values were calculated as follows:

$$PP = \left[\frac{cOD_S \text{ or } cOD_{Nc}}{cOD_{Pc}}\right] \times 100$$

Where, PP – percent positive value, cOD – corrected optical density, S – sample, N_C – negative control, P_C – positive control. Samples with a PP value greater than or equal to 10 were considered positive in accordance with the kit instructions.

2.8.2 E2 and NS3 specific antibodies

Commercial blocking ELISA kits were used for the detection of antibodies against NS3 (SVANOVIR® BVDV p80, Svanova Biotech, or Pourquier® ELISA BVD p80, Institut Pourquier, Montpellier, France) and E2 (VDPro® BVDV, Jeno Biotech Inc., Chuncheon, Republic of Korea) according to the manufacturer's instructions. Briefly, 10µl of serum diluted in 90µl of diluent (NS3 ELISA) or 50µl serum in 50µl of diluent buffer (E2 ELISA) was plated in duplicate in recombinant antigen coated plates and incubated for one hour. A positive and negative control

was provided for each assay. The plates were then washed three times in PBS with 0.05% Tween-20 (PBS-Tween) wash buffer, 100µl of conjugate (anti-NS3 or E2-horseradish peroxidise) added to each well and incubated for another hour. The sample and conjugate incubations for the NS3 ELISA were done at 37°C, while the E2 assay incubations were performed at room temperature (RT). After a second wash step, 50 or 100µl of TMB substrate was added and incubated for 10-20 minutes at RT. Sulphuric acid was used to stop the reaction and the optical density measured at 450nm. For both assays, the percentage inhibition was calculated for each sample as follows:

$$PI = \left| \frac{OD_{Nc} - OD_S}{OD_{Nc}} \right| x\ 100$$

Where, PI – Percentage inhibition, OD – Optical Density, Nc – Negative Control, S – Sample. Samples with a PI value greater than 30% were considered positive.

2.8.3 Serum virus neutralization titres

To determine virus neutralising antibody titres (VNT), test sera was heat inactivated for 30 minutes at 56°C after which 50µl of a two-fold dilution in BT medium from 1:5 to 1:640 was added into duplicate wells of a flat-bottomed 96-well microtitre plate (Nunc). Positive and negative control sera were also tested. An equal volume containing 100 50% tissue culture infective dose ($TCID_{50}$) of BVDV-1a Oregon C24V was added and incubated for 1 hour at 37°C. A back titration of virus was performed to check the potency of the virus (working range 30-421 $TCID_{50}$). BT cells were then added (1.5×10^4 in 50µl) and incubated at 37°C for 5 days in a humidified 5% CO_2 atmosphere. After, the plates were washed in PBS and then fixed with 20% acetone (v/v) for 10 minutes, excess acetone removed by tapping on a

paper towel and dried under a lamp for 3-4 hours. The plates were then stored at -20°C for at least 30 minutes after which immunoperoxidase staining for virus was conducted as described previously (Meyling, 1984). Serum VNTs were calculated as reciprocal of the highest serum dilution that inhibited virus growth by 50% using the Spearman–Kärber method and expressed on a $\log_2$ scale.

In Experiment 2 (Chapter 4), VNTs were determined as described previously on serum samples from the different inoculation groups at 42 dpi. A two-fold dilution series (1:10 to 1:1280) of heat inactivated (56°C for 30 minutes) serum, in duplicate with Ho916, TKK, BVDV-1a C24V (Coggins et al., 1961), BVDV-2 50263 (Wakeley et al., 2004) or Border Disease Virus 1 (BDV-1) S137/4 (Vantsis et al., 1976). The extent of cross reactivity (serological relatedness) was calculated as a percentage, P, of heterologous virus neutralisation (VN) compared to homologous VN values as follows (Ridpath et al., 2010):

$$P_{B>A} = 100 \times \left(\frac{BA}{AA}\right)$$

Where BA is the VNT of antiserum A against virus B, and AA is the homologous VNT against virus A.

For Experiment 3 (Chapter 5), virus neutralising antibodies titres were determined by incubation of two-fold dilution of heat-inactivated serum (1:5 to 1:81920) with the challenge virus, BVDV-1a Horton 916 (100 $TCID_{50}$ per well).

2.9 BVDV T cell Responses

2.9.1 BVDV Peptide Library

A library of 972 overlapping 16-mer peptides offset by 4 amino acids (Pepscan Presto BV, Lelystad, The Netherlands) representing the BVDV-1a Oregon C24V polypeptide sequence (GenBank Accession No. AF091605) were dissolved in sterile acetonitrile (Sigma-Aldrich, Poole, UK) to make 5mg/ml stocks and stored at -80°C. The peptide stocks were diluted in RPMI-1640 medium (Invitrogen) supplemented with 2mM L-glutamine, 20mM HEPES, 100U penicillin and 100µg/mL streptomycin with 10% sterile FBS (cRPMI) and used at 2µg/ml for each peptide. The peptides were pooled to represent different viral proteins with minimum overlap between proteins and the longer proteins represented by two pools. Bovine peripheral blood mononuclear cells (PBMC) were cultured in the presence of peptide pools (1µg/ml final) for 24 hours and then viability assessed by incubating cells for a further 10 minutes with a cell viability dye (7AAD, BD Biosciences) and analysed on a flow cytometer.

For Experiment 2 (Chapter 4), overlapping 15-mer peptides offset by 4 amino acids (JPT Peptide Technologies GmbH, Berlin, Germany) were synthesised covering the E2 protein from Ho916 and TKK, and the NS3 protein of C24V based on the divergence of the E2 and conservation of NS3 between the three viruses. Lyophilised peptides were dissolved in sterile 0.1M HEPES, pH 7.4-buffered 40% acetonitrile and combined to make the respective E2 and NS3 protein pools. The peptide stocks were diluted in cRPMI.

2.9.2 Isolation and ex *vivo* stimulation of peripheral blood mononuclear cells

Heparinised blood was collected from calves on a weekly basis and peripheral blood mononuclear cells (PBMC) isolated by density centrifugation. Blood was diluted 1:1 in HBSS (Invitrogen) and layered over an equal volume of Biocoll Separation Solution (density of 1.077g/ml; Autogen Bioclear) and centrifuged at 1000g for 30 minutes at room temperature (RT) with the brakes off. PBMC were harvested from the biocoll interface and washed thrice in HBSS by centrifugation at RT. PBMC were resuspended in cRPMI. Cell counts were obtained by flow cytometry as described above, with events gated on typical linear SSC and FSC characteristics for PBMC. The PBMC density was adjusted to 5 x 10^6 cells/ml and 100µl transferred to wells of a 96 well round bottom microtitre plate. In Experiment 1 (Chapter 3), cells were stimulated in duplicate wells with 100µl of peptide pools representing the individual BVDV proteins at 2µg/ml or BVDV-1a Oregon C24V at a multiplicity of infection (MOI) of 5. Live virus was used for the *ex vivo* stimulation to ensure the production and presentation of both structural and non-structural BVDV proteins in antigen presenting cells. cRPMI or mock-infected BT supernatant and 10µg/ml pokeweed mitogen (PWM; Sigma) were used as negative and positive controls respectively. The cells were incubated at 37°C for 24 or 48 hours for flow cytometry or IFN-γ ELISA analysis respectively. In Experiment 3 (Chapter 4), cells were stimulated in duplicate wells with 100µl of peptide pools representing the E2 and NS3 proteins at 1µg/ml or viruses at a multiplicity of infection (MOI) of 1. Medium or mock-infected BT supernatant and PWM were again used as negative and positive controls respectively. The cells were incubated

at 37°C for 48 hours after which cell-free supernatants were harvested after centrifugation and stored at -80°C until analysis for detection of cytokines.

2.9.3 Detection of bovine IFN-γ and MIG/CXCL9

Forty-eight hours post-stimulation, PBMC were resuspended and then centrifuged at 300g for 2 minutes and the cell-free supernatants collected for cytokine detection or stored at -80°C until use. Interferon-gamma (IFN-γ) in culture supernatants was measured using a bovine IFN-γ ELISA (Bovigam, Prionics AG, Schlieren-Zurich, Switzerland) according to the manufacturer's instructions or an in-house ELISA protocol. Briefly, 96 well NUNC MaxiSorp microplates (Thermo Fisher Scientific, Loughborough, UK) were coated with 100µl/well of mouse anti-bovine IFN-γ mAb (CC330, AbD Serotec) at 5µg/ml in 0.05M carbonate/bicarbonate coating buffer (pH 9.6) (Sigma) and incubated overnight at RT. The plates were then washed with PBS with 0.05% Tween-20 (wash buffer) and incubated for an hour at RT with 300µl/well PBS supplemented with 1% bovine serum albumin (BSA) and 0.05% Tween-20 (blocking buffer). The blocking buffer was aspirated and 50µl of test supernatant added to duplicate wells. A two-fold serial dilution of recombinant bovine IFN-γ (AbD Serotec) in cRPMI, starting at 50ng/ml, and medium alone was added as standard/positive and negative controls respectively. After one-hour incubation at RT, the plates were washed five times with wash buffer and 100µl/well biotin-conjugated mouse anti-bovine IFN-γ mAb at 5µg/ml (CC302-biotin, AbD Serotec) added. The plate was incubated and washed as above and 100µl/well streptavidin conjugated horseradish peroxidise (SA-HRP; GE Healthcare, Little Chalfont, UK) diluted 1/1000 in blocking buffer and incubated for 45 minutes at RT.

After a final wash, 100µl/well of TMB substrate (Sigma) was added and incubated for 15 minutes at RT, before stopping the reaction with 100µl/well 1.8N sulphuric acid and absorbance at 450nm measured with a background correction (reference filter 630nm) within 5 minutes on a FLUOstar Optima Plate Reader (BMG Labtech, Aylesbury, UK). In Experiment 3 (Chapter 5), the in-house IFN-γ ELISA was modified to use black opaque 96 well flat-bottom plates (View-plate-96, PerkinElmer, Seer Green, USA) and after the final wash, 100ul of Supersignal® ELISA Femto Substrate (equal parts luminol/enhancer and stable peroxidise solution; Thermo Scientific) was added per well and mixed for one minute using a microplate mixer and the relative light units (RLU) measured on a luminometer (≈425nm) (Victor™ X4 Multiplate Plate Reader; Perkin Elmer). IFN-γ was measured as it is considered the prototype marker of anti-viral type 1 T cell responses (Wheelock, 1965).

Levels of bovine monokine induced by gamma (MIG/CXCL9) were assessed using a commercial ELISA kit (Bovine CXCL9 (MIG) VetSet™ ELISA Development Kit (Kingfisher Biotech Inc., St Paul, MN, USA). Briefly, 100µl of sample per well in duplicate was added to CXCL9 antibody coated plate and incubated for an hour at RT. A two-fold dilution series of standard from 30 to 0.469ng/ml was also added. The plates were then washed four times in wash buffer after which 100µl of detection antibody was added and incubated for another hour at RT. Following a similar wash step, 100µl of Streptavidin-HRP was added and incubated for 30 minutes at RT. TMB substrate solution (100µl per well) was added after a final wash and incubated in the dark for 30 minutes. The reaction was then stopped by adding 100µl of 0.18M sulphuric acid and absorbance measured at

450nm on a spectrophotometer (Victor™ X4). MIG/CXCL9 was selected as it has been reported to be a monokine induced by IFN-γ and serves as an amplified and functional measure of IFN-γ responses (Berthoud et al., 2009).

2.9.4 Multiparametric Flow Cytometry

To accumulate intracellular cytokine, brefeldin A (GolgiPlug, BD Biosciences) was added (0.2µl/well) to PBMC cultures and for the final 4 hours of the 24 hour incubation period before performing flow cytometric analysis. PBMC were washed twice in PBS by centrifugation at 300g for 2 minutes, resuspended in 50µl PBS and incubated with 0.2µL/well Near Infra-Red Live/Dead Viability Dye (Invitrogen) for 30 minutes at 4°C protected from light. Cells were washed with FACS buffer and stained with mouse monoclonal antibodies: CD4-FITC, CD8β-PE or CD335-Alexa Flour 488 (all AbD Serotec) and γδ-TCR (GB21A; VMRD, Pullman, WA, USA) for 15 minutes at room temperature. Since the γδ-TCR antibody was unconjugated, cells were subsequently stained with anti-mouse $IgG_{2a,b}$-PE secondary antibody (BD Biosciences) for an additional 15 minutes at room temperature. The cells were then washed twice with FACS buffer and then fixed and permeabilised using CytoFix/CytoPerm Solution (BD Bioscience) for 20 minutes at 4°C. After two washes in BD Perm/Wash Buffer (BD Biosciences), the cells were resuspended in a 50µL volume and stained with mouse anti-bovine IFN-γ antibody or an irrelevant isotype control antibody both conjugated to Alexa Flour-647 (AbD Serotec), at 4°C for 30 minutes in the dark. The cells were given two final washes in BD Perm/Wash buffer and resuspended in FACS buffer prior to flow cytometric analysis. Cells were analysed by gating on viable cells (Live/Dead Fixable Dead Cell

dye negative) in the lymphocyte population, and defined lymphocyte subpopulations were then gated upon and their expression of IFN-γ assessed.

2.9.5 Measurement of cytokines by ELISA and MSD multiplex assay

A number of cytokines associated with a cell-mediated response were assessed following *ex vivo* stimulation of PBMC with viruses or peptide pools. IFN-γ in culture supernatants was measured using a bovine IFN-γ ELISA as described above. Multiple cytokines (**Table 2.1**) were detected and quantified using a custom-designed multi-array electrochemiluminescence detection assay (Meso Scale Discovery (MSD), Gaithersburg, MD, USA) as described previously (Coad et al., 2010). Briefly, 96-well multispot microtiter plates bearing a patterned array of capture antibodies to the different cytokines on the bottom of the wells were blocked with Diluent 7 buffer and shaken for 30 minutes. A master mix of recombinant cytokine standards (**Table 2.1**) was prepared and serially diluted in Diluent 7 buffer. Samples and standards (25µl per well) were added to the plate after removal of blocking buffer and incubated for 1 hour at RT with shaking. Afterwards the plate was washed three times with PBS with 0.05% Tween-20 (wash buffer) and 25µl per well detection antibodies conjugated to the ruthenium-based MSD Sulfo-TAG™ reagent and streptavidin Sulfo-TAG and incubated for 1 hour at RT while shaking. For each plate, 1.8µl streptavidin Sulfo-TAG™ (0.3µg/ml final) and 60µl of each antibody (each at 1µg/ml final) was made up to 3000µl of Diluent 8 and 25µl dispensed per well. The plates were washed three times and 150µl of MSD read buffer (diluted 1:1 with distilled water) added to each well. The Plates were then read with an enclosed CCD camera on a Sector Imager 6000 (MSD), datasets analysed on

the Discovery Workbench 3.0 Software and the samples quantified against standard curves.

Table 2.1. Cytokine standards for MSD multiplex assay

Cytokine	Supplier	Concentration (Top Standard)
IFN-γ	Pierce[a]	100ng/ml
IL-1β	AbD Serotec[b]	20ng/ml
IL-4	Pierce	2ng/ml
IL-6	IAH[c]	25U/ml
IL-10	IAH	30U/ml
IL-12	IAH	500U/ml
MIP-1β	MSD	10ng/ml

[a]Pierce, Thermo Fisher Scientific, Loughborough, United Kingdom, [b]AbD Serotec, Kidlington, United Kingdom, [c]Institute for Animal Health, Compton, United Kingdom

2.10 Statistical Analysis

Graphpad Prism 5.04 (Graphpad software, LaJolla, CA, USA) was used for graphical and statistical analysis. Analysis of variance (ANOVA) followed by a Bonferroni's post-test was used for the analysis of the effects of viral challenge or vaccination on antibody and cellular immune responses in the different treatment groups. Data was represented as means with standard deviation (SD) or standard error of means (SEM) shown to indicate the uncertainty around the estimate of the group means.

3 T CELL RESPONSES TO VIRAL PROTEINS FOLLOWING EXPERIMENTAL INFECTION OF CALVES WITH CYTOPATHIC BOVINE VIRAL DIARRHOEA VIRUS TYPE I

SUMMARY

The characterisation of the target of protective immune responses to BVDV is an important prerequisite in the design of next-generation vaccines. This study aimed to rationally address the development of more efficacious vaccines by characterising the kinetics and specificity of CD4 and CD8 T cell responses of cattle rendered immune to BVDV following recovery from infection. Four calves were inoculated with a low titre (10^4 $TCID_{50}$) of cytopathic BVDV and received a secondary high titre (10^7 $TCID_{50}$) inoculation 4 weeks later. PBMC were stimulated with live BVDV or synthetic peptide pools spanning the entire BVDV proteome and IFN-γ secretion assessed by ELISA or by flow cytometry. IFN-γ responses to BVDV peptide pools were not detected until 28 days after the second inoculation. The dominant responses were observed against peptide pools representing the E2 and NS3 proteins, and these responses were still detectable one year after experimental infection. Analyses to define the phenotype of these BVDV antigen-specific T cell responders, whilst not optimal, showed evidence of $CD4^+$ IFN-γ responses to E2 and NS3. IFN-γ secretion in response to *ex vivo* stimulation with BVDV, as measured by ELISA, was detected in all calves including the uninfected control. Flow cytometric analyses revealed that natural killer (NK) cells secreted IFN-γ in response to the virus, regardless of the animals' immune status. However, IFN-γ secretion by both CD4 and CD8 αβ T cells was significantly greater in the immune calves. This data supports the further evaluation of BVDV E2 and NS3 proteins as vaccine candidate T cell antigens.

3.1 INTRODUCTION

Studies of the immune response to bovine viral diarrhoea virus (BVDV) have suggested a role for both B and T cell responses in protection (Chase et al., 2004, Kapil et al., 2008, Howard, 1990, Potgieter, 1995). The characterisation of antibody and T cell targets of protective immune responses to BVDV is an important prerequisite in the design of next-generation vaccines.

There is good evidence for the role of T cell responses in BVDV immunity. Calves vaccinated in the presence of maternal antibodies, whilst unable to mount an effective antibody response, do generate memory T cells sufficient to protect against viral challenge (Endsley et al., 2003). Both CD4 and CD8 T cell responses have been shown to be evoked by BVDV (Rhodes et al., 1999), although mAb depletion of CD4 T cells but not CD8 or γδ T cells has been shown to increase the duration of virus shedding (Howard et al., 1992). These CD4 T cell responses have been shown to be directed principally against E2 and NS3 but also to other proteins such as the N^{pro}, C and E^{rns} proteins (Lambot et al., 1997, Collen and Morrison, 2000, Collen et al., 2002). A comparison of responses to all the different BVDV proteins in the course of natural or experimental infection has, however, not been conducted.

This study aimed at characterising the T cell responses to BVDV and to its constituent viral proteins following experimental infection with a BVDV-1a reference strain, Oregon C24V. The specific objectives were to characterise the kinetics and phenotype of T cell responses to BVDV proteins following experimental infection and to define the specificity of the BVDV specific T cell responses. The expected definition of T cell responses that are boosted upon re-challenge will serve as the populations for which we intend to target through vaccination and will be used

as tools to identify the relevant vaccine candidate antigens. Defining the specificities of the T cell responses elicited in our challenge model system will allow the identification of proteins or portions of proteins which could be evaluated for their vaccine potential.

3.2 RESULTS

3.2.1 Outcome of BVDV Infection

The experimental animals were assessed for the presence of BVDV antibody and antigen. Four of the calves were found retrospectively to be BVDV antigen negative but antibody positive prior to experimental infection indicating pre-exposure to BVDV while two were BVDV naïve (**Table 3.1**). This was unexpected as the animals were sourced as BVDV naïve calves from a reputable supplier. . Upon investigation tit was determined that the supplier had erroneously only pre-screened the calves for the presence of BVDV antigen and not antibody. The BVDV antigen ELISA is used to detect acutely infected and persistently infected (PI) animals, the latter of which are of great concern in BVD control, but this is not an appropriate assay to assess previous infection with BVDV. BVDV antibody ELISAs, which were conducted retrospectively, indicated that the animals had been previously exposed but uninfected at the point of experimental infection as determined by their antibody positive and antigen negative status at day 0. It was decided to proceed with the experiment, as it was still possible to assess the antigen specificity of T cell responses in these calves. Isolation and characterisation of the BVDV virus found to be present in the supplier's animals showed it to be closely related to BVDV Oregon C24V (APHA Mammalian Investigation Unit, personal communication), it was concluded that prior infection was unlikely to interfere with the assessment of T cell responses boosted by the experimental infection with BVDV Oregon C24V.

Clinical scoring and rectal temperatures monitored to determine the outcome of experimental infection and some evidence of a mild acute BVDV infection was observed in some of the animals. One of the pre-exposed infected animals (C09-

5016) showed evidence of dyspnoea and ocular discharge from 9 days post infection (dpi), while a second, calf C09-5018, had diarrhoea on 20-21 dpi, and the previously naïve animal that was experimentally infected (C09-5017) had ocular discharge between 10 and 17 dpi. Neither the naïve infected nor uninfected controls showed any significant clinical signs (**Figure 3.1a**). Median rectal temperatures for all the animals were normal (~38°C) throughout the observation period. Low levels of virus were detected by qRT-PCR from peripheral blood samples in animals C09-5017 and C09-5018, up to 49 dpi (**Figure 3.1b**). Virus was detected in the pre-exposed infected animals at 4 dpi, while virus was detected in the naïve infected animal at 11 dpi and peaked at 23 dpi. Viral load was higher post-infection for the naïve infected animal (C09-5017) than those that had been pre-exposed but both groups had similar virus levels after the high-dose infection (>28 dpi). The control animals showed no evidence of virus infection throughout. There were no differences in the mean white blood cell counts per μL (WBC/ μL) in the different animals after the primary and secondary infection (**Figure 3.1c**).

Table 3.1. BVDV antigen and antibody status of calves prior to experimental infection.

Calf Tag No.	Status post-challenge*	BVDV antibody (Antibody ELISA)	BVDV antigen (Antigen ELISA)
C09-5016	Pre-exposed infected	+	-
C09-5017	Infected	-	-
C09-5018	Pre-exposed infected	+	-
C09-5020	Pre-exposed infected	+	-
C09-5022[#]	Pre-exposed control	+	-
C09-5025[#]	Control	-	-

*All the animals were reported as BVDV antigen free prior to the experiment, but a number of the calves (n=3) in the group to be infected and one control calf were subsequently found to be pre-exposed to BVDV (antibody positive). [#]Controls.

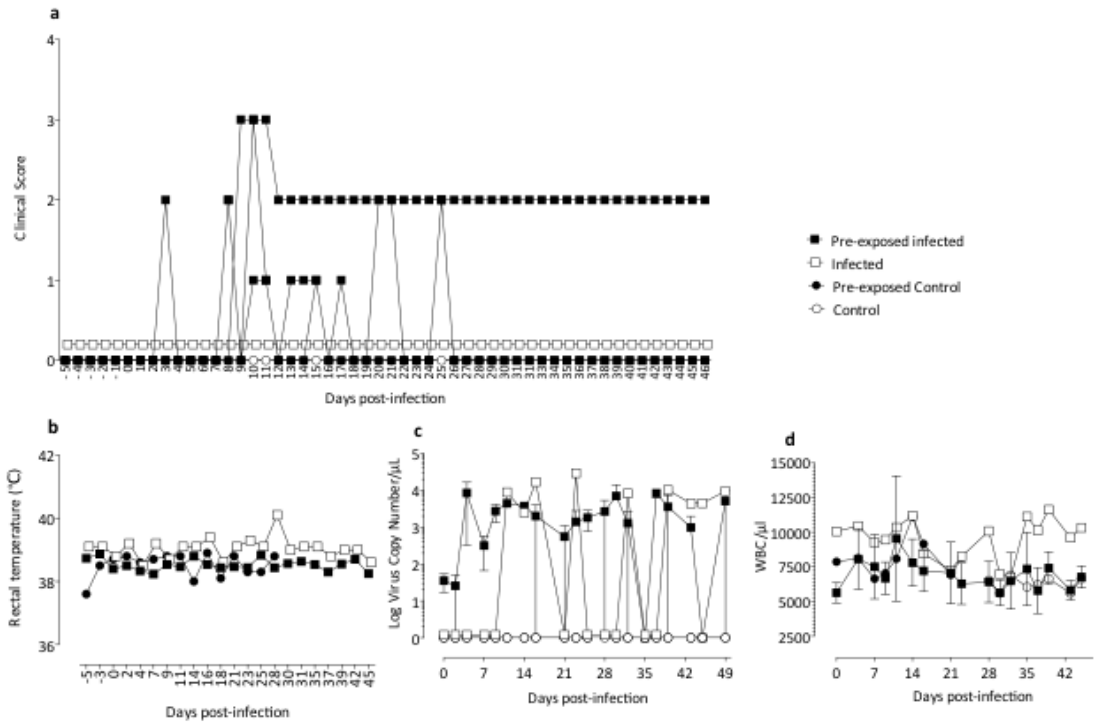

Figure 3.1. Outcome of primary (day 0) and secondary (day 28) experimental infections with BVDV-1a Oregon C24V. Calves pre-exposed to BVDV (pre-exposed infected, n=3) and a naïve calf (infected, n=1) were experimentally infected with BVDV-1a Oregon C24V, or uninfected control calf (control, n=1) were monitored for (**a**) clinical signs scored as the sum of 6 parameters, (**b**) rectal temperatures, (**c**) viraemia assessed by qRT-PCR and expressed as the BVDV RNA copy number/µl peripheral blood on a logarithmic scale (log) and (**d**) white blood cell counts were assessed by flow cytometry and expressed per µl blood (WBC/µL). Data represented as means ± SEM.

3.2.2 BVDV antibody responses

BVDV-specific antibodies were detected in the previously naïve animal inoculated with BVDV Oregon C24V from 21 dpi (**Figure 3.2a**), and levels continued to rise over the time-course studied up to 81 dpi. All the pre-exposed calves had high levels of BVDV specific antibody throughout the study period. The unexposed control animal was antibody negative. BVDV neutralising activity mirrored what was observed with total antibody with increase in titres from 21 dpi in the previously naïve animal while the previously exposed animals maintained fairly high titres (>640) from the onset of the experiment (**Figure 3.2b**). The unexposed control animal's sera did not exhibit any neutralising activity.

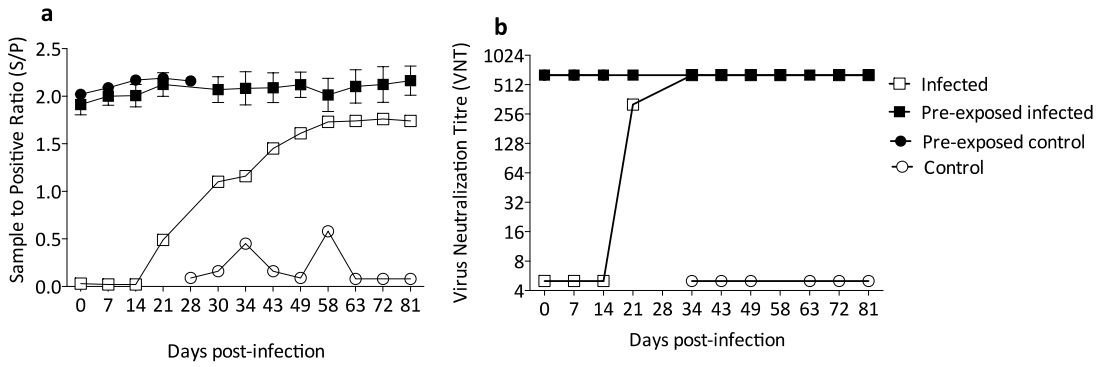

Figure 3.2. BVDV-specific antibody responses following primary and secondary experimental infection with BVDV-1a Oregon C24V. (**a**) BVDV-specific serum antibody and (**b**), serum virus neutralisation titres in previously naïve infected, (infected, n=1), pre-exposed infected (n=3), pre-exposed control (n=1) and a naïve calf (control, n=1) calves following the experimental infection.

3.2.3 Detection of BVDV-specific T cell IFN-γ responses

Ex vivo IFN-γ responses of PBMC to cytopathic BVDV Oregon C24V, as assessed by ELISA, showed responses by both infected and control calves (**Figure 3.3**). The responding cells were characterised by flow cytometry after surface staining for lymphocyte surface markers (CD3, a co-receptor expressed on all T lymphocytes, CD8α/β and CD4, markers for CD8 and CD4 T lymphocytes respectively, CD335, a marker specific for natural killer cells and γδ-TCR, a marker of gamma-delta T cells) and intracellular staining for IFN-γ (**Figure 3.3a**). A large proportion of the IFN-γ response in the control calf was from $CD4^{-}CD8^{-}$ cells while the infected animals showed larger proportions of $CD4^{+}$ and $CD8^{hi}$ IFN-γ^{+} cells (**Figure 3.3b**). One of the pre-exposed infected animals showed a higher response from $CD8^{lo}$ cells that needs to be verified. Further analysis of the phenotype of the responses showed that a large proportion of the $CD4^{-}CD8^{-}$ response was from $CD335^{+}$ NK cells but not from γδ-TCR^{+} cells (**Figure 3.3a**).

In order to assess whether the IFN-γ stimulation observed in the naïve control animal was due to the virus used in the stimulation, cytopathic BVDV Oregon C24V (C24Vcp), cells from all the experimental animals were stimulated with a cytopathic (cp) and non-cytopathic (ncp) C24V virus pairs, C24cp and C24ncp, as well as a pair from BVDV W5, a BVDV-1 UK field strain (W5cp and W5ncp). IFN-γ secretion was observed in the experimentally infected animals (C09-5016, 17, 18 and 20) after stimulation with all virus pairs as well as pokeweed mitogen (positive control) (**Figure 3.4**). IFN-γ reactivity in the naïve animal (C09-5025) was observed upon stimulation with both cytopathic and non-cytopathic C24V virus but not with the W5ncp and W5cp pair. We therefore believe that the stimulation of IFN-γ secretion

in the naïve animal is an effect specific to BVDV Oregon C24V regardless of the cytopathogenicity. This may be due to the attenuation of the virus for vaccination purposes but this effect was not investigated further in the context of this study.

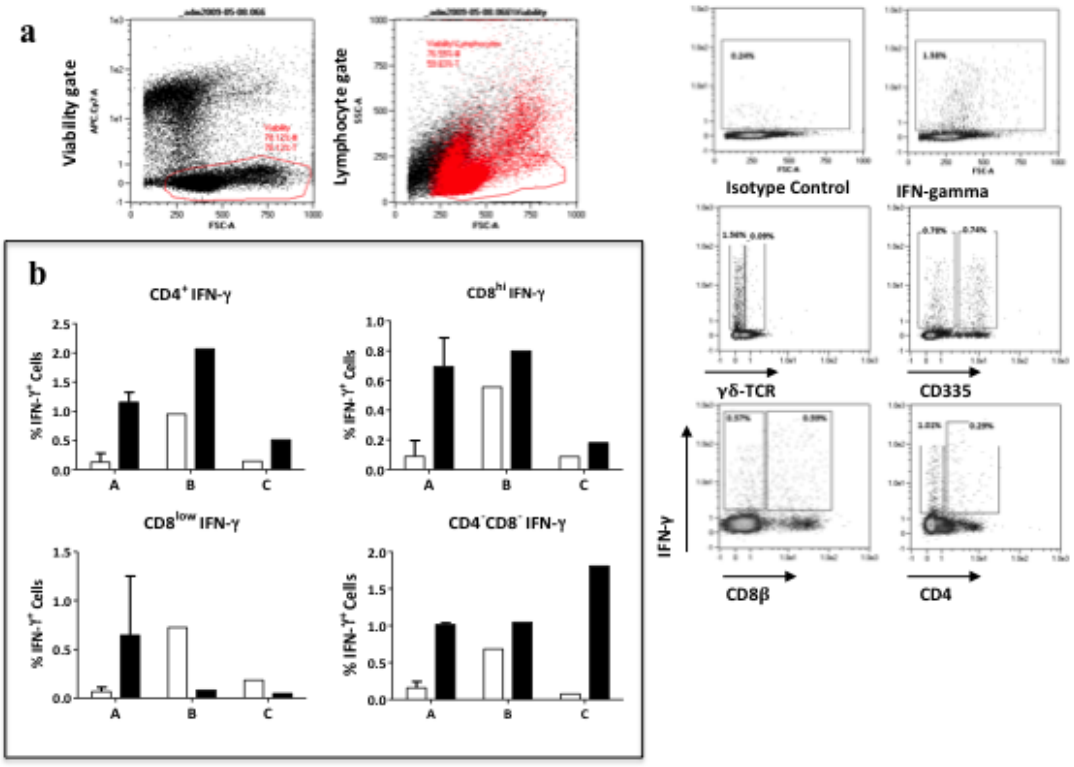

Figure 3.3. Phenotyping of BVDV specific IFN-γ responses by flow cytometry. (**a**) Representative dot plots of virus stimulated PBMC showing proportions of IFN-γ^+ cells from γδ T cell (γδ-TCR$^+$), NK cell (CD335$^+$), CD4$^+$ T cell or CD8β$^+$ T cell populations. The gating strategy involved viability and lymphocyte gating and assessment of IFN-γ^+ cells in contrast to isotype control. Gating out of doublets was not possible because this feature was not enabled in the early versions of the flow cytometry software. (**b**) Proportion of IFN-γ^+ γδ T cell (CD4$^-$CD8α$^-$), NK cell (CD4$^-$CD8α$^-$), CD4$^+$ T cell or CD8β$^+$ T cells following virus (closed bars) or mock (open bars) stimulation of PBMC from pre-exposed infected calves (A) and the infected calf (B) compared to the control calves (C).

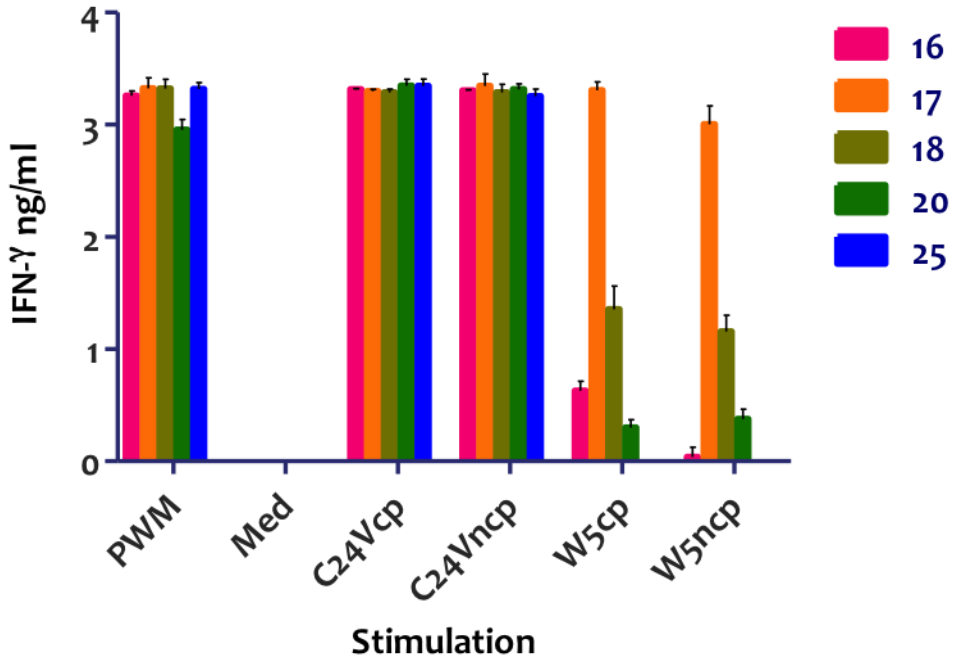

Figure 3.4 IFN-γ responses following stimulation with paired cytopathic and non-cytopathic BVDV strains. IFN-γ responses from infected (16, 17, 18 and 20) and control (25) calves following stimulation with Pokeweed Mitogen (PWM), Media (Med), Cytopathic (cp) and non-cytopathic (ncp) BVDV C24V and W5 viruses.

3.2.4 Quality control of BVDV peptide library

Reconstituted 16-mer peptides were pooled to represent the individual viral proteins of BVDV-1a Oregon C24V. Due to their large number, peptides representing NS3 and NS5B were each prepared in two pools. To assess any potential cytotoxic effects of the peptide pools and or the solvent used to reconstitute them, PBMC were cultured for 24 hours with peptide pools containing each peptide at 2μg/ml. Cultured PBMC were stained with near infra-red live/dead viability stain and analysed by flow cytometry to determine the proportion of live cells. The results showed that none of the peptide pools resulted in a loss of cell viability compared to cells cultured alone in complete RPMI (**Figure 3.5a**). The peptide pools were also found not to induce non-specific IFN-γ responses in PBMC from BVDV naïve animals, which were exposed to *Mycobacterium bovis* and therefore responded to tuberculin purified protein derivative (PPD) and to pokeweed mitogen (PWM) as assessed by IFN-γ ELISA (**Figure 3.5b**).

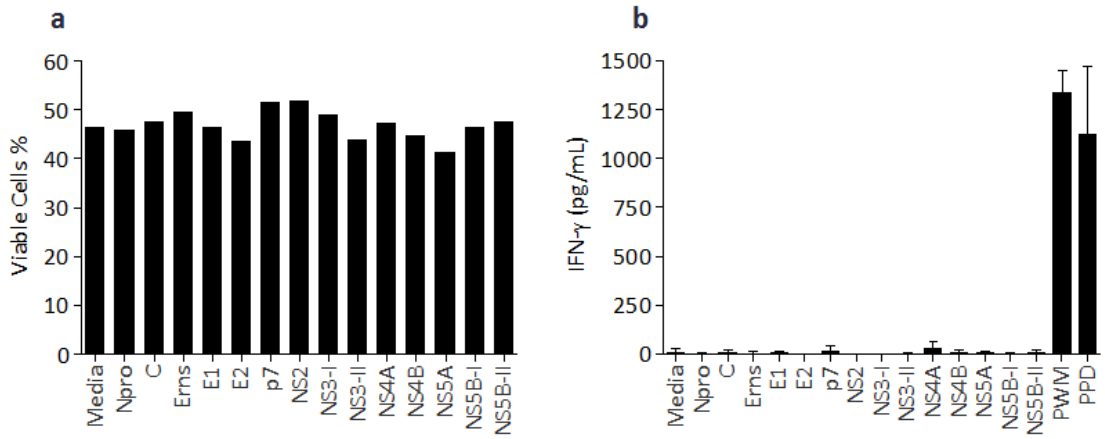

Figure 3.5. Quality control of BVDV peptide pools. (a) Proportion of viable cells after 72-hour culture of naïve PBMC with peptide pools or without peptides (media) as assessed by Live/Dead viability dye (n=1). (b) IFN-γ responses of PBMC from five BVDV naïve, tuberculin-reactor animals (n=4) to peptide pools, tuberculin PPD and PWM as positive controls.

3.2.5 Assessment of antigen-specificity of T cell responses

BVDV T cell responses to peptide pools were assayed by bovine IFN-γ ELISA and longitudinal responses from the experimentally infected animals up to 81 dpi are shown in **Figure 3.6**. Responses to peptide pools representing non-structural and structural proteins are shown in **Figures 3.6a and b**, respectively. The greatest responses were observed to the E2 and the NS3-II pool, with represents the C-terminal helicase domain of NS3, which were found to be statistically significant in comparison to the control ($p>0.01$, $p<0.05$ respectively) overall (**Figure 3.6c**). Lesser responses were additionally observed against pools representing the E^{rns}, NS2 and NS4B proteins. IFN-γ responses to peptide pools were detected 28 days after the secondary infection (56 dpi) and responses were still detectable at 153 dpi in the infected animals while the control did not mount a statistically significant (**Figure 3.6c**) IFN-γ response to any peptide pool. There were no significant differences in the peptide IFN-γ responses to peptide pools between the pre-exposed infected calves and the calf that was naive prior to experimental infection. The responses to the various peptides pools were observed even after 351 dpi but with greater breadth in the previously naïve calf (5017) compared to the pre-exposed calves (5016, 5017 and 5019). No responses were observed in the naïve control animal (5025) (**Figure 3.7**).

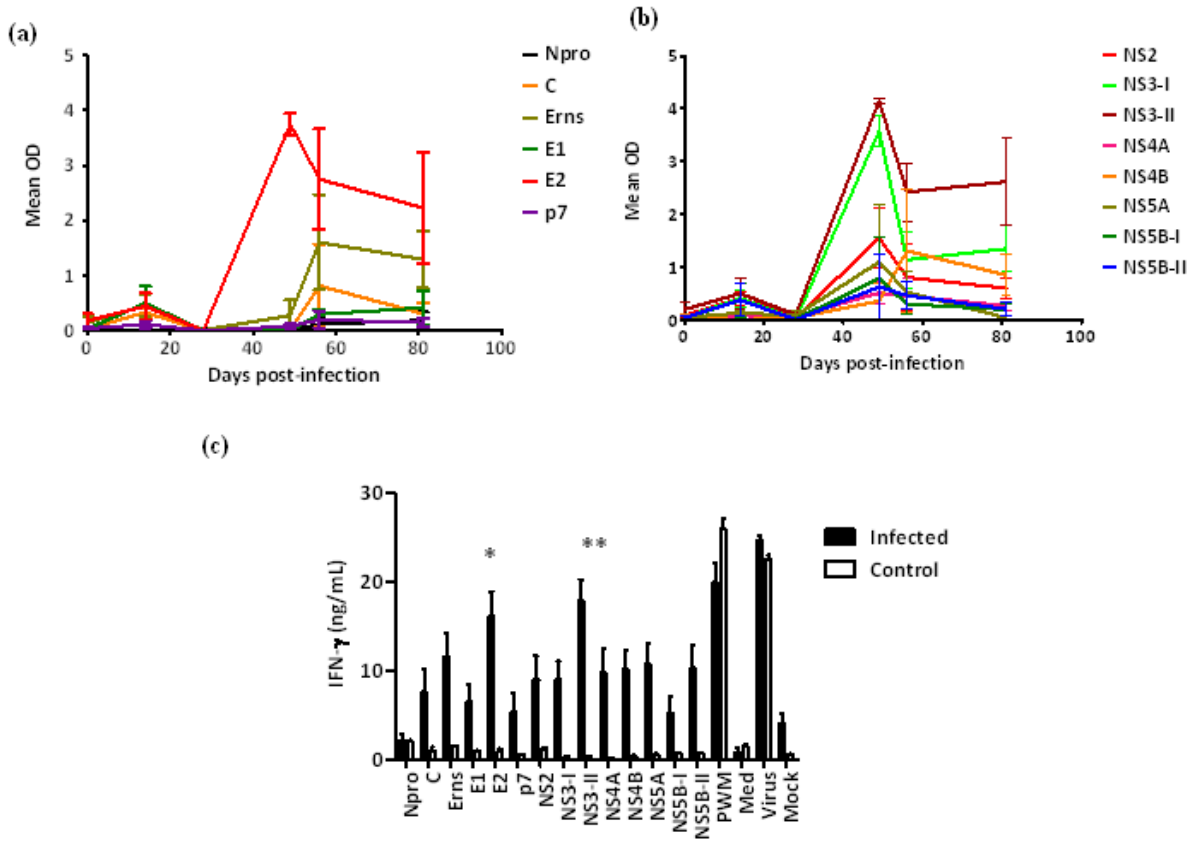

Figure 3.6. BVDV antigen-specific IFN-γ responses following primary and secondary experimental infections with BVDV Oregon C24V. Longitudinal IFN-γ responses of PBMC to peptide pool representing (**a**) structural and (**b**) non-structural proteins are presented as the mean media-corrected OD values (test OD minus media OD) for all the infected calves (n=4). (c) Responses to peptide pools and live BVDV (MOI=5) at 153 dpi in the infected (n=4) and uninfected control calf (n=1). PWM or complete RPMI (Med)/mock virus supernatant served as positive and negative controls respectively. Error bars represent SEM. (** = $p<0.01$, * = $p>0.05$ in the infected compared to the control)

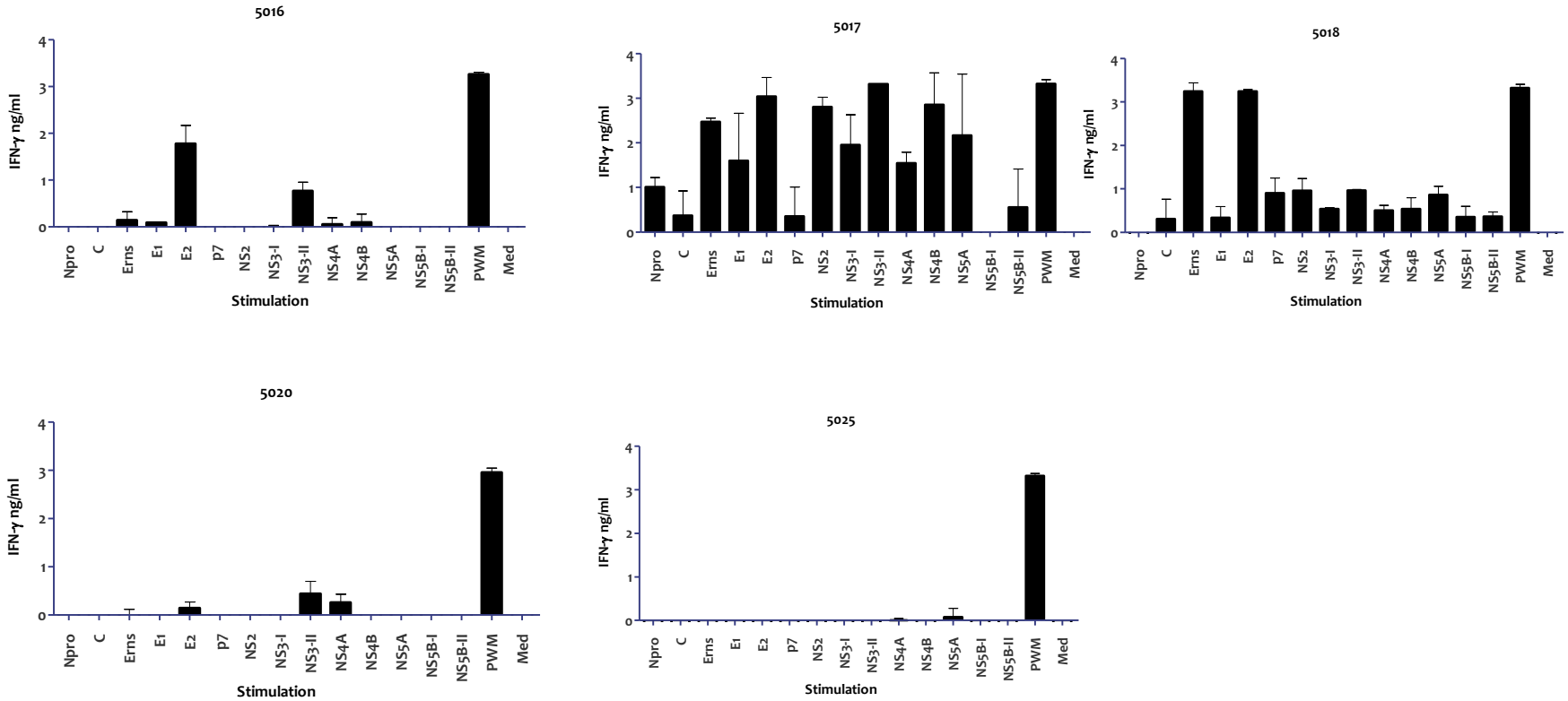

Figure 3.7. IFN-γ responses to BVDV peptide pools after one year (351 days post infection). Responses in pre-exposed infected (5016, 5018 and 5020), previously naïve infected (5017) and BVDV naïve (5025) calves.

3.2.6 Phenotype of BVDV Specific T cell Responses

Ex vivo responses of PBMC from the experimentally infected calves to peptide pools were not possible to resolve with the original protocol that included an overnight incubation with peptides followed by 4-hour incubation with brefeldin A. However, low frequency $CD4^+$ IFN-γ responses were seen for the E2 and NS3 peptide pools after optimisation of the assay to 2 hour incubation with peptides prior to 12 hour incubation with brefeldin A as shown in **Figure 3.8**. There were no observable CD8 T cell responses using this method. No systematic optimization of the intracellular cytokine staining (ICS) was conducted and we recommend further optimization of these assays to detect peptide-specific responses. It has been suggested that the duration of antigen-stimulation and subsequent brefeldin A incubation also alter the diversity of multifunctional T cells measured by intracellular cytokine staining (Kaveh et al., 2012).

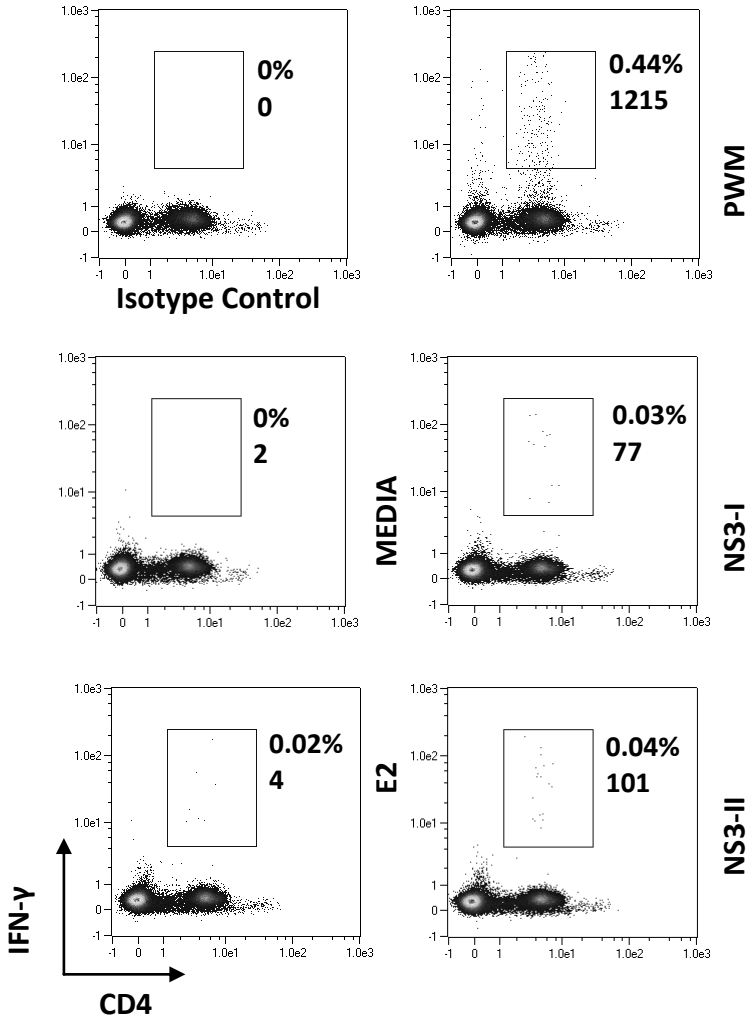

Figure 3.8. Phenotype of *ex vivo* IFN-γ responses to BVDV E2 and NS3 peptide pools. IFN-γ responses of PBMC from infected calf C09-5017 were assessed by flow cytometry following incubation of cells with brefeldin A. Viable lymphocytes were gated (gating strategy as shown in **Figure 3.4**) and the proportion and total numbers of cells of $CD4^+$ IFN-γ^+ cells as presented. PWM and media were used as positive and negative controls respectively while irrelevant mouse IgG_1 antibody (isotype control) was used to control for the IFN-γ staining.

3.3 DISCUSSION

Assessment of immune responses after recovery from an acute infection is thought to be a good model for the induction of immunity since it mimics acquisition of natural immunity and there is no bias expected in the antigens that are available for presentation to the immune system. Rechallenge of the animals with the homologous virus allows the assessment of which of the responses induced by the primary infection are effectively recalled and thus may constitute protective responses.

Although four out of the six experimental animals were found to have been pre-exposed to BVDV prior to inclusion in this study, it was possible to demonstrate BVDV specific IFN-γ responses following experimental infection. Serological typing and phylogenetic analyses of the prevalent BVDV strains from where the animals were sourced showed that they are closely related to BVDV-1a Oregon C24V (Mammalian Virus Investigations Unit, APHA). The effect of pre-exposure to BVDV is difficult to explore in this set up, as the nature of the previous exposure is not known. Pre-exposure has been reported to modulate immune responses in other related viral infections such as hepatitis C virus infection, where, sub-infectious exposure has recently been shown to suppress T cell responses following subsequent acute infection (Park et al., 2013). In this study, it was shown that previous exposure in rhesus monkeys to HCV resulted in diminished T cell responses following viral challenge.

The outcome of infection was mild/sub-acute as expected following infection with BVDV-1a Oregon C24V, which has previously been used as a live attenuated vaccine (Coggins et al., 1961). Antibody responses, as detected by ELISA, were seen

3 weeks after the low dose infection and may have contributed to the suppression of viraemia after the high dose infection. The higher peak in virus in the infected calf compared to the pre-exposed infected calves after the primary infection maybe due to pre-existing antibodies in the latter group.

Longitudinal analysis of *ex vivo* BVDV-specific T cell IFN-γ responses by ELISA showed clear responses to peptide pools after the second experimental infection, suggesting that the high dose virus infection was required for recruitment and expansion of antigen specific T cells as previously described (van Heijst et al., 2009). A low frequency of $CD4^+$ but not $CD8^+$ IFN-γ secreting cells to E2 and NS3 peptide pools was observed in PBMC from the infected calves possibly reflecting the low frequency of responders *in vivo*, a result that would need to be confirmed after further optimisation of the intracellular cytokine assay.

IFN-γ responses of PBMC, from both naïve and experimentally infected calves, after stimulation with cytopathic BVDV C24V, were shown to include non-specific/innate NK responses. There is a need to better characterize virus-specific responses from naïve versus infected animals and compare NK cell responses between non-cytopathic and cytopathic BVDV virus pairs. *Ex vivo* responses to BVDV peptide pools showed E2 and NS3 are the immunodominant proteins, while responses to other proteins such as E^{rns} and NS4B were also observed albeit not with statistical significance. However, to validate these findings, there is need to repeat this experiment with additional calves.

The determination of the fine-specificity of these responses was not done given the limited sample size, undetermined major histompatibility complex (MHC) background of the animals and previous exposure to BVDV. It is however hoped that

this study will inform further studies aimed at identifying minimal length peptides and epitopes in the various BVDV proteins that have been shown to be targets of T cell responses. This would employ the use of peptide matrix pools to identify individual peptides stimulating responses and to map T cell epitopes (Roederer and Koup, 2003) as well as the establishment of T cell lines in order to maintain and expand the frequency of antigen specific T cells (Hart et al., 2011b). The phenotype of responding T cells ($CD3^+CD8\beta^+$ or $CD3^+CD4^+$) can also be determined by either magnetic activated cell sorting (MACS) of specific cell populations prior to stimulation or by multiparametric flow cytometry. Staining for activation markers such as CD25 and CD26, and memory markers (CD45RO, CD62L) may also be useful in distinguishing cells in different activation states and distinguish naïve from central or effector memory cells. There is also a need to assay for additional cytokine responses by multiplex ELISA or intracellular staining for a broader array of cytokines such as TNF-α, IL-2 and IL-4, measure proliferative responses and cytotoxic activity by granzyme B or perforin staining or cytotoxicity assays with virus infected cells as targets.

Given that only a relatively small number of cattle will be utilised in this process, it is proposed to test the recognition of identified targets by T cells from a much larger group of cattle that have been exposed to BVDV in the field. This analysis would validate which of the identified antigens are the dominant targets and thus be good candidates for evaluation of their immunogenicity in vaccine experiments. Furthermore, an understanding of the fine specificity of these responses will enable an analysis of polymorphism affecting these epitope-encoding regions that has significant implications for subsequent subunit vaccine design. It also may

simplify potential vaccine formulations if only relevant portions of proteins are required to be expressed and formulated rather than the corresponding full-length proteins.

4 COMPARATIVE ANALYSIS OF IMMUNE RESPONSES FOLLOWING INFECTION WITH BOVINE VIRAL DIARRHOEA VIRUS-1 AND AN ASIATIC ATYPICAL BOVINE PESTIVIRUS

SUMMARY

Bovine viral diarrhoea virus (BVDV) exists in two recognized species, BVDV-1 and BVDV-2, and closely related atypical bovine pestiviruses have recently been described. While there is evidence of cross-protective immune responses between BVDV-1 and BVDV-2 virus strains in spite of antigenic differences, there is little information on the antigenic cross-reactivity between BVDV-1 and atypical bovine pestiviruses. The aim of this study was to assess whether antibody and T cells induced by one virus were capable of responding to heterologous virus and identify possible targets of cross-reactive or species-specific responses. Three to five month old calves were infected with BVDV-1a strain Horton-916 (Ho916), Th/04_KhonKaen (TKK), an Asiatic atypical bovine pestivirus strain, or co-infected with both. Antibodies against E2 were detected in all infected groups, but to a lower extent in the TKK infected animals. Homologous virus neutralization was observed in sera from both single virus infected and co-infected groups, while cross-neutralization was only observed in the TKK infected group to Ho916 virus. T cell IFN-γ responses to both viruses were observed in the TKK infected animals, whereas Ho916 infected calves responded better to homologous virus. Specifically, IFN-γ responses to NS3 were observed in all infected groups while responses to E2 were virus specific. Broader cytokine responses were observed with similar trends between inoculation groups and virus species. Overall, infection of cattle with TKK virus appeared to induce antibody and T cell responses that exhibited a greater degree of cross-reactivity and recognition of Ho916 virus that suggests that conserved targets of immune responses may be shared with a common archetypical virus ancestor.

4.1 INTRODUCTION

The *Pestivirus* genus within the *Flaviviridae* family of single stranded positive sense RNA viruses comprises four recognized species: Bovine viral diarrhoea virus 1 (BVDV-1) and 2 (BVDV-2), classical swine fever virus (CSFV) and border disease virus (BDV), (Simmonds et al., 2011). Additionally, five groups of unclassified pestiviruses have also been discovered: Tunisian sheep virus (TSV), pestivirus of giraffe, Pronghorn antelope virus, Bungowannah and atypical bovine pestiviruses, the last four of which have been proposed as additional *Pestivirus* species.

Pestiviruses are genetically and antigenically related with evidence of both cross-reactive monoclonal antibody (mAb) epitopes and serological cross-reactivity (Simmonds et al., 2011). Current classification of pestiviruses is based on genetic and serological relatedness as well as the host of origin. Phylogenetic analyses based on the 5' untranslated region (5'UTR), the amino-terminal proteinase, N^{pro}, and the major envelope glycoprotein, E2, sequences allow delineation of individual species. Genetic relatedness of pestiviruses has been shown to be consistent with antigenic relatedness as defined by binding assays with mAbs or serum cross-neutralization relative to the type virus of a particular species (Becher et al., 2003).

Atypical bovine pestiviruses, which form a monophyletic clade with a sister relationship to BVDV-1 and 2, have been detected in contaminated foetal bovine serum (FBS) and in natural infections in bovines (cattle and buffalo). These include: D32/00_HoBi, isolated from an FBS batch originating from Brazil (Schirrmeier et al., 2004); Brz buf 9, isolated from a buffalo in Brazil (Stalder et al., 2005); CH-KaHo/cont, a cell culture contaminant possibly from a South American FBS batch;

'Hobi'-like viruses detected in aborted bovine foetuses in Brazil (Cortez et al., 2006); Th/04_KhonKaen, detected from serum of a naturally infected calf in Thailand (Ståhl et al., 2007); SVA/cont-08, a viable pestivirus detected in a batch of FBS from South America (Liu et al., 2009b); and IZSPLV_To, isolated from FCS (Peletto et al., 2010). More recently, a 'Hobi'-like Pestivirus, Italy-1/10-1, has been associated with a severe respiratory disease outbreak and abortions in multiparous cows in Italy (Decaro et al., 2011b, Decaro et al., 2011a). While there is evidence suggesting that atypical bovine pestiviruses are already spread in cattle in South America (Cortez et al., 2006), southeast Asia (Ståhl et al., 2007) and recently in Europe (Decaro et al., 2011a), there is a need for broader surveillance to assess the extent to which they are spread in the cattle population worldwide and the possible impact on current BVDV control programs.

Genetic and antigenic diversity between bovine pestiviruses poses a significant challenge in BVDV diagnosis and vaccination (Bolin and Grooms, 2004). Diagnosis by detection of viral material (nucleic acid or antigen) or through quantification of virus specific immune responses can be hampered by the variability within these targets making it difficult to recognise divergent or atypical viruses. Furthermore, limited virus cross neutralization and the limited detection of atypical pestivirus antibody using commercial BVDV antibody ELISA (based on BVDV-1) is evidence of significant antigenic diversity between atypical pestiviruses and BVDV-1 and -2 (Schirrmeier et al., 2004, Ståhl et al., 2007, Bauermann et al., 2012). This also raises questions regarding the potential efficacy of existing BVDV vaccines against these novel viruses. Whilst clinical cross protection against BVDV-2 challenge has been reported using BVDV-1 based vaccines, vaccination failure due to inadequate protection from foetal infection, postnatal infection and virus shedding

is often attributed, in part, to virus diversity (Bolin and Grooms, 2004). In future, vaccines will have to be designed with responses to different BVDV-1 and 2 subgenotypes as well as atypical bovine pestiviruses in mind, by inclusion of the different virus genotypes or identification of targets of cross reactive immune responses, or by the use of species specific vaccines.

This study, conducted as part of an experiment comparing the infection kinetics of calves with a BVDV-1a virus (Horton 916), an Asiatic atypical bovine pestivirus (Th/04_Khon_Kaen) or co-infection with both viruses, aimed to assess whether antibody and T cell responses induced by one virus were capable of responding to the heterologous virus and to determine if the immunodominant proteins, E2 and NS3, identified in Experiment 1 (Chapter 3) are targets of cross-reactive or distinct species-specific responses.

This work was conducted as part of an EPIZONE Internal Call funded Project, ‘Comparative dynamics of BVDV (IC 6.8)’, a collaborative project between the Swedish University of Agricultural Sciences/National Veterinary Institute (SLU/NVI), Uppsala, Sweden, the National Veterinary Institute (PIWET), Puławy, Poland and the APHA, Surrey, UK. EPIZONE European Research Group (ERG) is a European network of veterinary research institutes working on epizootic animal diseases. I received a travel award to SLU/NVI and PIWET as the APHA collaborator and was involved in the planning and execution of the experiment and the immunological analyses.

4.2 RESULTS

4.2.1 Outcome of experimental infection

Three to five month old calves were infected with BVDV-1a strain Horton-916 (Ho916), Th/04_KhonKaen (TKK), an Asiatic atypical bovine pestivirus strain, or co-infected with both (n=5 per group). The clinical and virological outcome following inoculation with Ho916 and TKK or co-infection with both (Ho916/TKK) are reported elsewhere (Larska et al., 2012) (Appendix A) and summarised in **Table 4.1**. Whilst leucopoenia and lymphopoenia, assessed by complete blood cell counts, were observed to similar extents in all infected groups coinciding with the onset of viraemia, milder clinical signs were observed in the TKK group. Co-infection resulted in prolonged elevated temperatures in comparison to the singular infection with the Ho916 or TKK viruses. Similar courses of viraemia as determined by nucleic acid detection by qPCR and viral antigen detection by ELISA were determined in all three infected groups. Simultaneous detection of both viruses in blood and nasal swabs was observed in the co-infected group (Ho916/TKK). All inoculated groups seroconverted two weeks after inoculation as assessed by serum neutralisation of homologous virus.

4.2.2 Detection of E2 and NS3 specific antibodies by ELISA

All infected groups mounted a significant E2 specific antibody response from 14 dpi **(Figure 4.1a**) as determined by a competitive BVDV ELISA. Percent inhibition values in the Ho916 and Ho916/TKK groups exceeded the assay diagnostic cut-off for positivity from 14 dpi. While the E2 specific antibody levels in the TKK group increased from 14 dpi they were not above the cut-off until 21 dpi. The E2 specific

antibody levels of the Ho916 and Ho916/TKK groups were significantly greater than in the TKK group from 14 dpi. The Ho916 and Ho916/TKK groups showed NS3 antibody responses (**Figure 4.1b**) from 21 dpi compared to the control group, whilst the TKK group responses were significant compared to the controls from 28 dpi but did not exceed the diagnostic cut-off for assay positivity until 35 dpi. Ho916 group responses were significantly greater than the TKK group at 21 ($p<0.0001$) and 28 dpi ($p<0.001$), whereas, Ho916/TKK antibody levels were significantly greater than TKK between 14 and 35 dpi ($p<0.001$). There were no significant differences in NS3 responses between Ho916 and Ho916/TKK groups.

A serial dilution of sera (1:4 – 1:64) was assayed for both E2 and NS3 antibodies at 42 dpi in order to resolve possible quantitative differences in antibody levels that were obscured by assay saturation at the recommended sera dilution (**Figure 4.1c**). E2 antibodies were detected in Ho916 and Ho916/TKK groups at serum dilutions of 1:4 to 1:64, and responses in the TKK group were significantly lower, only being above the assay cut-off up to a dilution of 1:8. At the same time point, percent inhibition values for NS3 antibodies from all infected groups were above the cut-off up to the final dilution of 1:64 (**Figure 4.1d**).

Table 4.1. Outcome and kinetics of experimental infection with Ho916 and TKK viruses

	Ho916	**TKK**	**Ho916/TKK**
Parameter of infection	**Days post infection (dpi)**		
Pyrexia	7-9	7-10	7-12
Leukopoenia	2-7, 14*	5-9	2-7, 14*
Lymphopoenia	2-7, 14*	2-7	2-7, 14*
Clinical signs	5-14 (diarrhoea, coughing, nasal and ocular discharge)	7-21 (coughing, nasal and ocular discharge)	7-14 (diarrhoea, coughing, nasal and ocular discharge)
Viral antigen (E^{rns} Ag ELISA)	2-7	2-7	2-7
Viral RNA in PBMC (qPCR)	2-28	5-28	2-28**, 5-28***
Virus isolation from PBMC (IPX)	5-9	5-9	7-9
Virus RNA from nasal swabs (qPCR)	5-9, 21	2-21	5-14**, 2-21***
Virus isolation from nasal swabs (IPX)	5-7	5-7	5-9
Seroconversion (VNT)****	14	14	14

*Biphasic; **BVDV-1 specific real time RT-PCR (Baxi et al., 2006); ***atypical bovine pestivirus specific real time RT-PCR (Liu et al., 2008), **** Neutralization against BVDV-1d and TKK virus

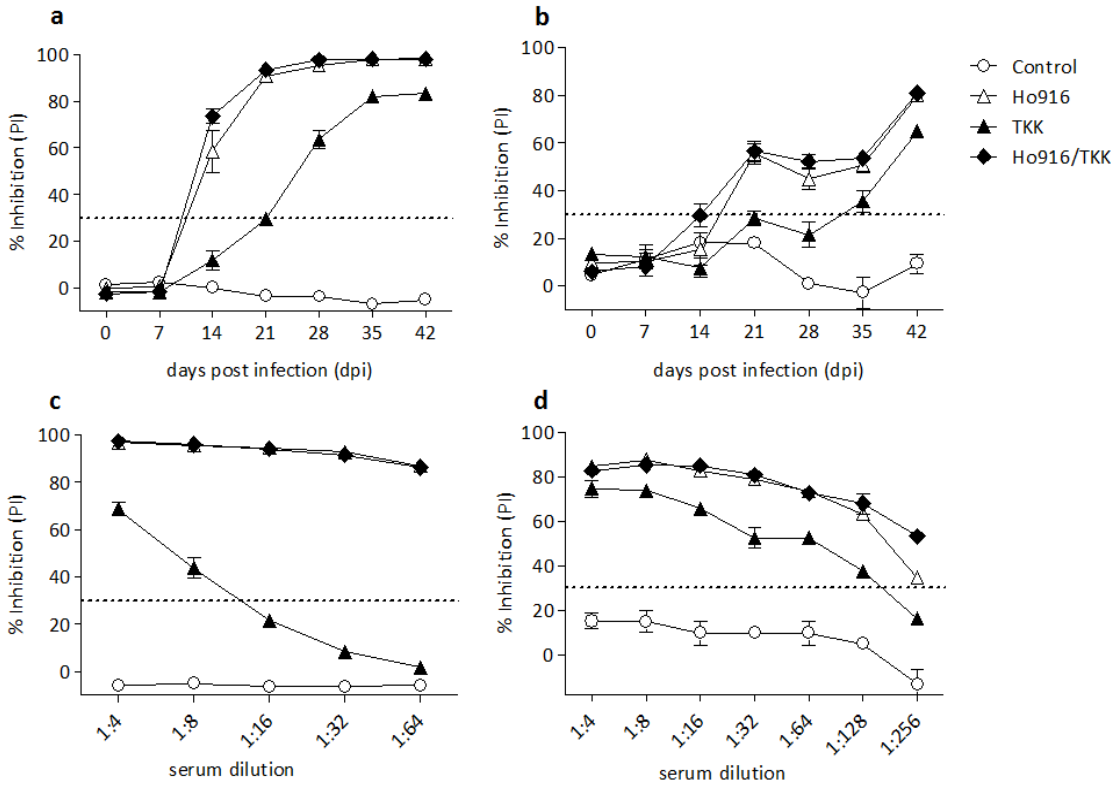

Figure 4.1. E2 and NS3 specific antibody responses of calves following experimental infection with BVDV-1 Ho916, atypical bovine pestivirus TKK, co-infection with both (Ho916/TKK) or uninfected controls. Longitudinal antibody responses were measured by competitive ELISA against BVDV E2 (**a**) and NS3 (**b**) proteins. Responses at 42 days post infection, were assessed using a serial dilution of serum (1:4 – 1:64) against E2 (**c**) and NS3 (**d**). Antibody levels were calculated as % inhibition (PI) of antigen specific mAb binding, and dashed lines denote positivity cut-off for the respective assays. Data represented as means ± SEM.

4.2.3 Measurement of virus-neutralising antibody titres

A comparison of homologous and heterologous neutralization activity of both viruses by sera from the different experimental groups was conducted in order to assess the antigenic relatedness of the viruses. Homologous neutralization of both Ho916 and TKK viruses was observed from 14 dpi in the single and co-infected groups (**Table 4.1**). However, neutralization of Ho916 virus by sera from TKK inoculated cattle only occurred at low titres at 42 dpi while neutralization of TKK virus by Ho916 inoculated animals' sera was observed from 21 dpi but also at low titre (Larska et al., 2012). Ho916 virus neutralization titres in both Ho916 and Ho916/TKK group sera were higher than the TKK group sera and a similar pattern was observed against BVDV-1a C24V but with lower titres (**Figure 4.2a**). In contrast, TKK virus was neutralized by TKK and Ho916/TKK inoculated group sera but not by the Ho916 inoculated animals' sera. Neutralization of BVDV-2 was observed at low titres in all inoculated groups with the lowest titres in sera from Ho916 inoculated animals. BDV was better neutralized by Ho916 and Ho916/TKK group sera compared to sera from the TKK inoculated animals. Assessment of cross-neutralisation, calculated as the percentage of heterologous versus homologous neutralization, showed that despite exhibiting weaker homologous neutralization, TKK group sera showed comparatively good neutralization of Ho916, C24V and BVDV-2. (**Figure 4.2b**) Ho916 group sera showed significant cross neutralization of C24V albeit with lower titres compared to homologous neutralization. While cross-neutralization of all other viruses by sera from the Ho916 inoculated animals was considerably less than homologous virus neutralization. Cross-neutralization of BDV-1 by sera from all inoculation groups was poor.

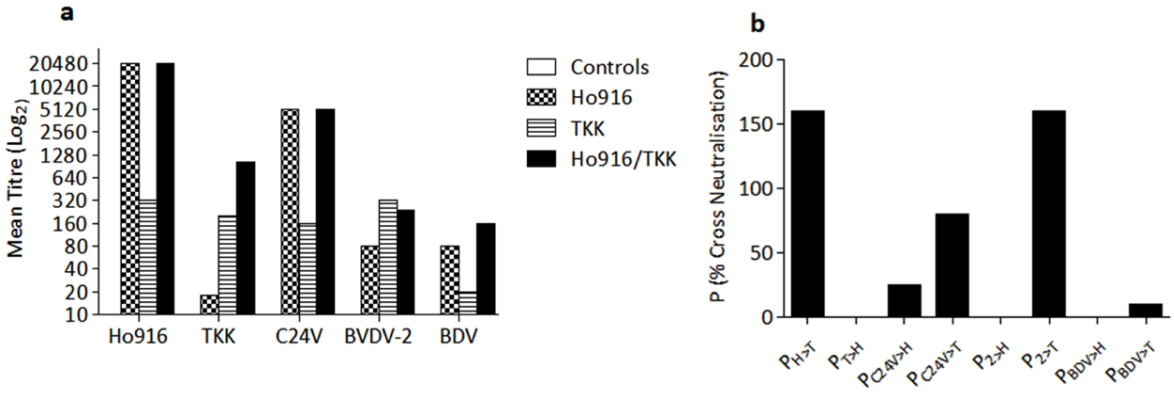

Figure 4.2. Homologous and heterologous virus neutralising antibody responses of calves experimentally infected with BVDV-1 Ho916, atypical bovine pestivirus TKK, co-infection with both (Ho916/TKK) or uninfected controls. (**a**) Neutralising titres of serum pools collected at 42 dpi from the different infection groups against Ho916, TKK, BVDV-1a Oregon C24V (C24V), BVDV-2 502643 (BVDV-2) and BDV-1 S137/4 (BDV). (**b**) Cross-neutralization of heterologous virus was calculated as a percentage of homologous neutralization of Ho916 virus by TKK inoculated cattle ($P_{H>T}$), TKK virus by Ho916 inoculated cattle ($P_{T>H}$) as well as cross neutralization of C24V (P_{C24V}), BVDV-2 (P_2), or BDV-1 (P_{BDV}) by either Ho916 or TKK sera.

4.2.4

BVDV sequence analysis

Sequence comparison of Ho916 and TKK translated E2 protein sequences revealed significant divergence between Ho916 and TKK (57.2% divergence, 59.2% identity) in comparison to Ho916 and the reference BVDV-1 C24V strain (14% divergence, 87.3% identity) (**Figure 4.3a**). The NS3 protein however was more conserved between the different viruses with 8.4% divergence, 90.5% identity between Ho916 and TKK and 1.4% divergence and 96.9% identity between Ho916 and C24V (**Figure 4.3b**).

4.2.5 Measurement of virus specific cytokine responses by IFN-γ ELISA and multiplex assay

4.2.5.1 IFN-γ responses

In comparison to uninfected controls, significant *ex vivo* PBMC IFN-γ responses were observed against Ho916 and TKK viruses from 28 dpi. PBMC from the Ho916 inoculated group stimulated with Ho916 virus showed significant IFN-γ responses on 35 ($p<0.05$) and 42 ($p<0.01$) dpi, whilst responses in the TKK group were only significant at 42 dpi ($p<0.01$) (**Figure 4.4a**). Responses to Ho916 virus in the Ho916/TKK group were less than in the other two infection groups and were not statistically significant. Responses to the TKK virus on the other hand, were significant in both the TKK and Ho916/TKK groups at 28 and 35dpi (**Figure 4.4b**). Responses to the TKK virus by PBMC from the Ho916 group were not statistically significant.

Responses to E2 peptide pools at 42 dpi followed similar trends to the responses to virus (**Figure 4.4c**). IFN-γ responses to the E2 peptide pool representing Ho916 virus (E2-Ho916) were observed in the Ho916 and Ho916/TKK groups but

not in the TKK group. Responses to E2-TKK peptide pool were seen in the TKK and Ho916/TKK groups albeit only statistically significant in the TKK group ($p<0.01$). Responses to the NS3 peptide pool (**Figure 4.4d**) were observed in all infected groups and were statistically significant in the Ho916 ($p<0.05$) and TKK ($p<0.001$) infected groups.

4.2.5.2 Detection of cytokines by multiplex assay

A number of cytokines and chemokines associated with cell-mediated responses (Coad et al., 2010) were assessed following *ex vivo* stimulation of PBMC with virus or peptides. These cytokines and chemokines were already available for detection using a custom multiplex ELISA assay (Coad et al., 2010). Broad responses were observed following virus (**Figure 4.5**) and peptide (**Figure 4.6**) stimulation. As with IFN-γ, the significance of cytokine levels in the different infection groups was assessed in comparison to the control group. TKK infected animals were observed to elicit a broad cytokine response following stimulation with homologous virus, as well as heterologous virus. Significant quantities of IL-4, IL-10, IL-12, IL-1β, IL-6 and MIP-1β were secreted by PBMC from TKK infected animals following TKK and Ho916 stimulation. With the exception of MIP-1β, which is secreted primarily by $CD8^+$ T cells, the other cytokines were not secreted to significant levels in the Ho916 infected animals following stimulation with the TKK virus. IL-12 secretion, typically associated with dendritic cells and macrophages, was observed in response to the Horton 916 virus in all infection groups. However, this was not the case with the TKK virus, which induced only a weak IL-12 response in the co-infected group that was not statistically significant. While IL-4 production was observed at lower levels in the co-infected animals in response to both viruses,

IL-10, IL-6 and MIP-1β were secreted in significant levels in this group following TKK stimulation. IL-1β responses in the co-infected animals were significant following TKK stimulation, and also observed following Ho916 stimulation, although not significantly so.

Significant IL-4 responses to the E2 peptide pool from the Ho916 virus (E2-Ho916) were observed in the Ho916 virus and co-infected groups and to the NS3 C24V peptide pool in the co-infected group but not against E2-TKK in any of the infected groups. IL-10 responses against NS3 and E2-TKK peptide pools were observed in the Ho916 infected group, and against E2-TKK peptide pool in the TKK infected group while the co-infected group had significant responses to both E2 peptide pools and NS3. IL-12 responses were only significant against NS3 in the co-infected group, while IL-1β responses were significant after stimulation with E2-TKK in the TKK and co-infected groups and to NS3 in all infected groups. IL-6 responses against E2-Ho916 were significant in the Ho916 group and to E2-TKK and NS3 in the TKK group. MIP-1β responses were significant in all infected groups to E2-Ho916 and NS3 and to E2-TKK in the TKK and co-infected groups.

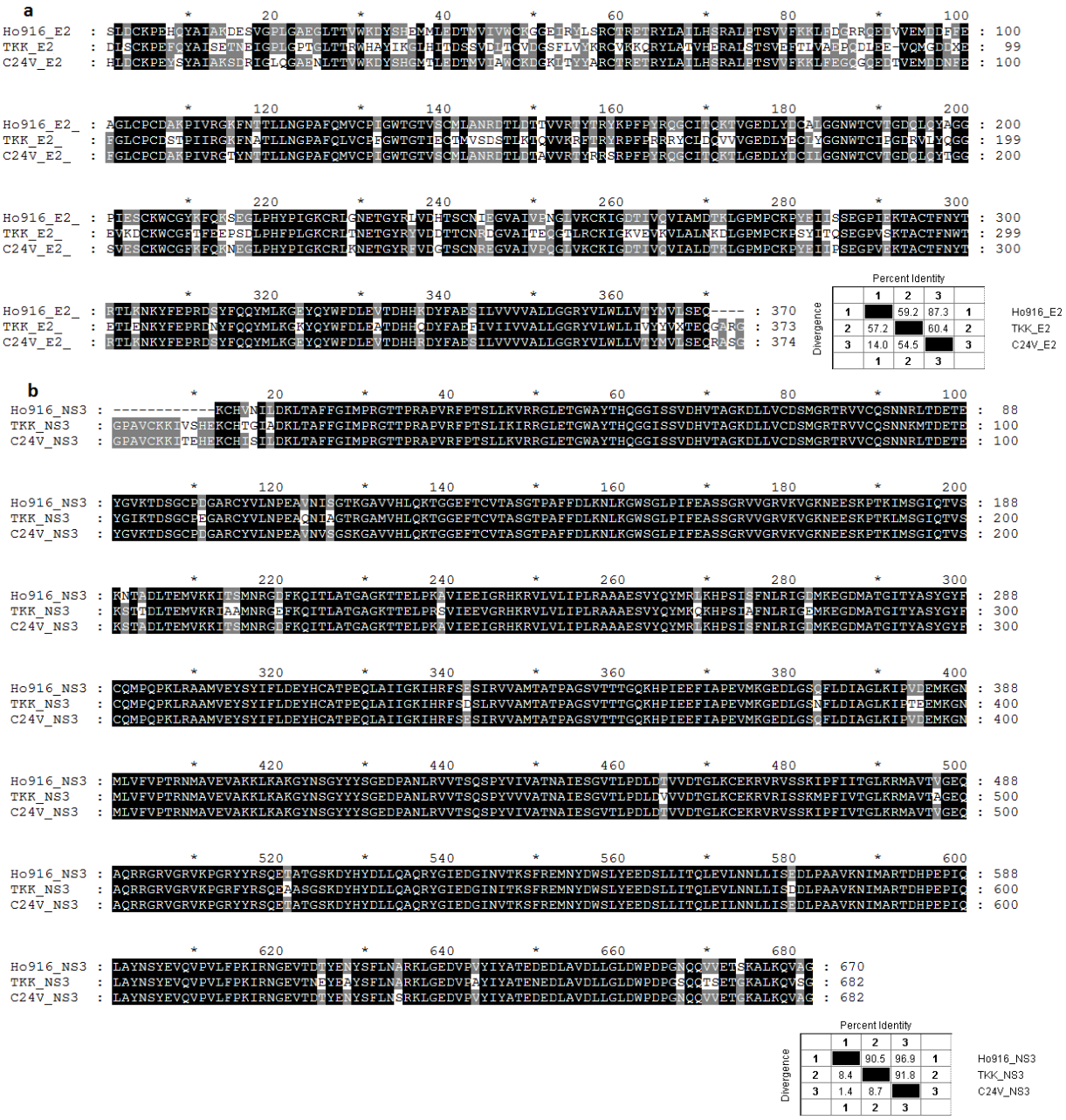

Figure 4.3. Alignment of translated E2 (a) and NS3 (b) protein sequences from BVDV-1 Ho916, atypical pestivirus TKK and BVDV-1 reference strain C24V. Sequences were aligned using *clustalw* and distances calculated from construction of phylogenetic tree (PAM250) on MegAlign (DNA Star Inc.). Sequence distances are shown in tables.

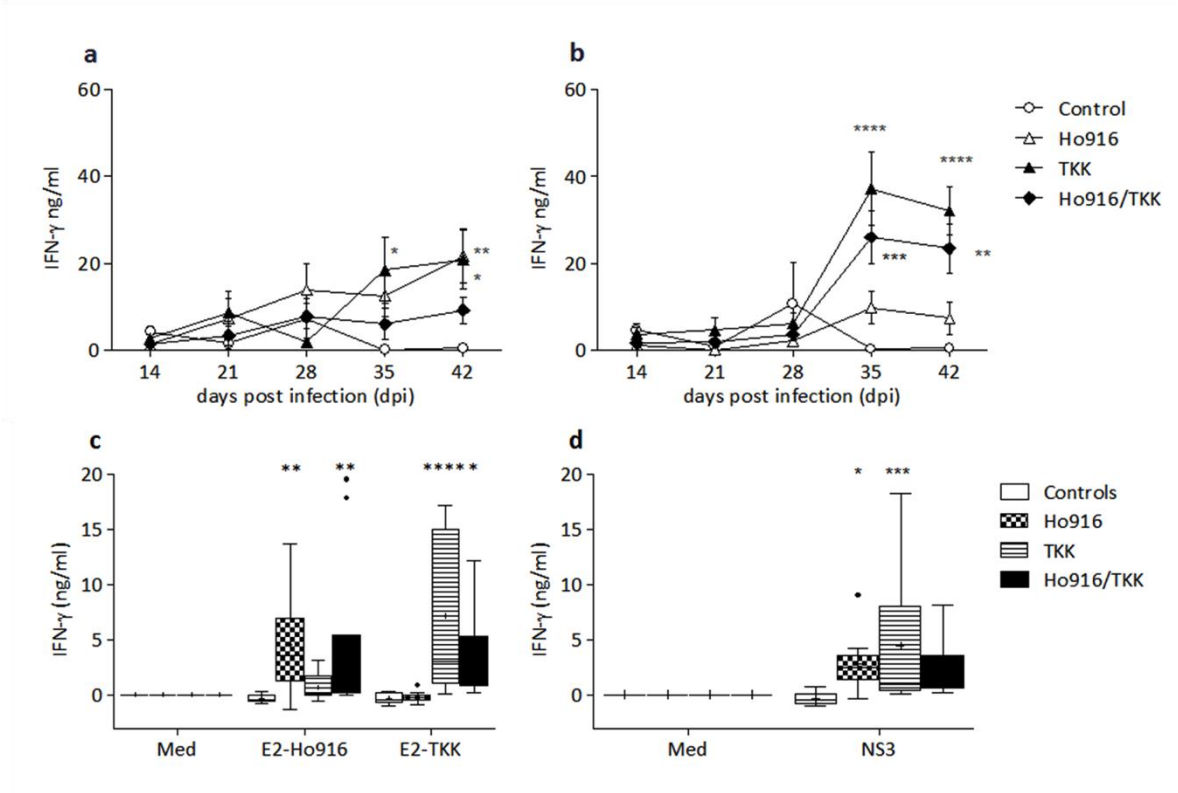

Figure 4.4. IFN-γ responses of calves following experimental infection with BVDV-1 Ho916, atypical bovine pestivirus TKK, co-infection with both (Ho916/TKK) or uninfected controls. Longitudinal PBMC IFN-γ responses following stimulation with Ho916 (**a**) and TKK (**b**) viruses were measured by ELISA and adjusted for mock stimulated background responses. PBMC isolated 42 dpi were stimulated with synthetic peptide pools representing E2 protein from Ho916 (E2-Ho916) and TKK (E2-TKK) (**c**) and NS3 protein from C24V (**d**), and IFN-γ production measured by ELISA and adjusted for background response of unstimulated cells. Data represented as means ± SEM (**a** and **b**) or box and whisker plots (**c** and **d**) and statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$).

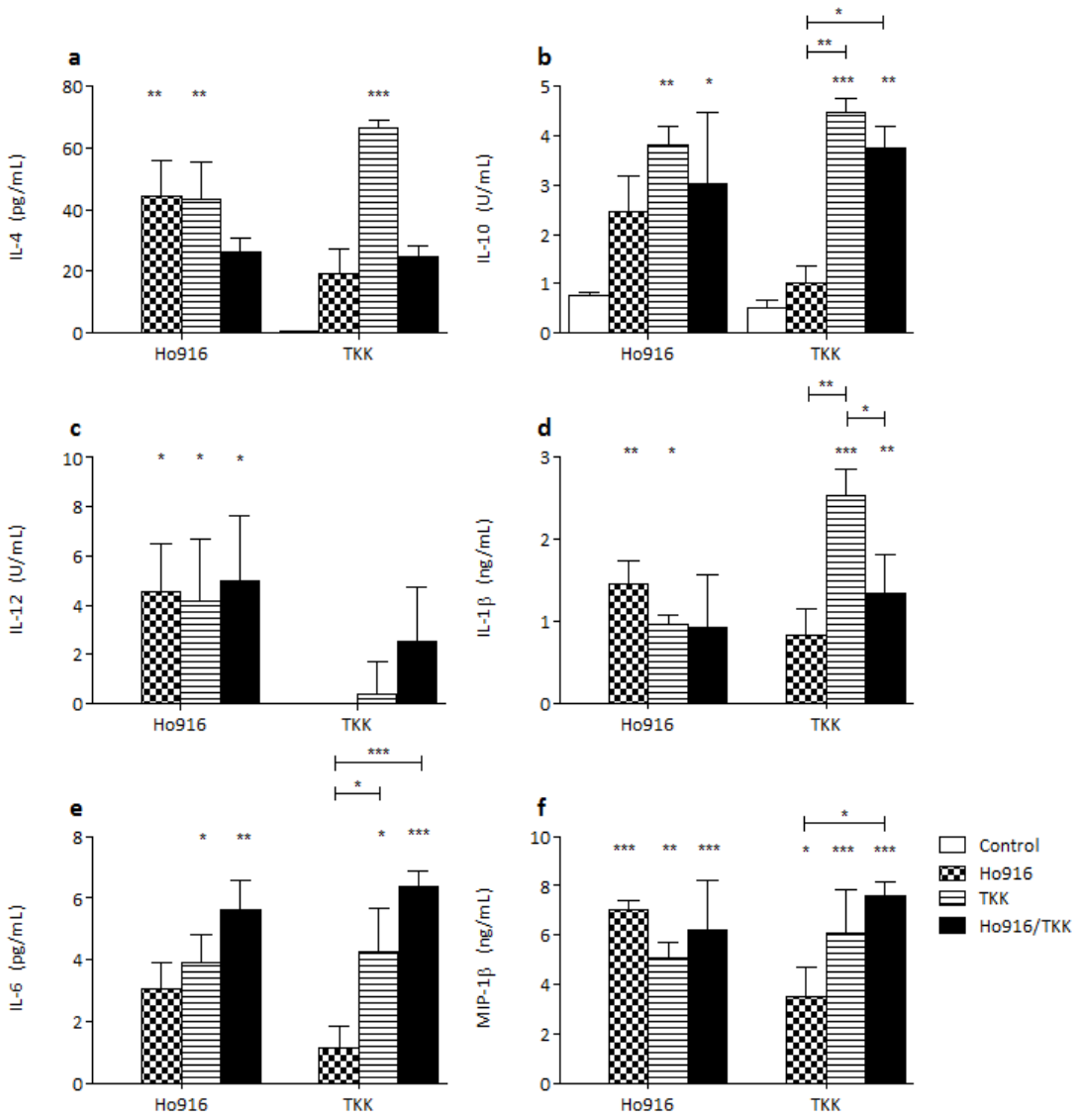

Figure 4.5. Assessment of virus-specific cytokine responses following experimental infection with BVDV-1 Ho916, atypical bovine pestivirus TKK, co-infection with both (Ho916/TKK) or uninfected controls. PBMC isolated at 42 dpi were stimulated with Ho916 or TKK viruses and secretion of IL-4 (a), IL-10 (b), IL-12 (c), IL-1β (d), IL-6 (e) and MIP-1β (f) measured by a multiplex assay. Data represented as means ± SEM and statistical significance denoted by, * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

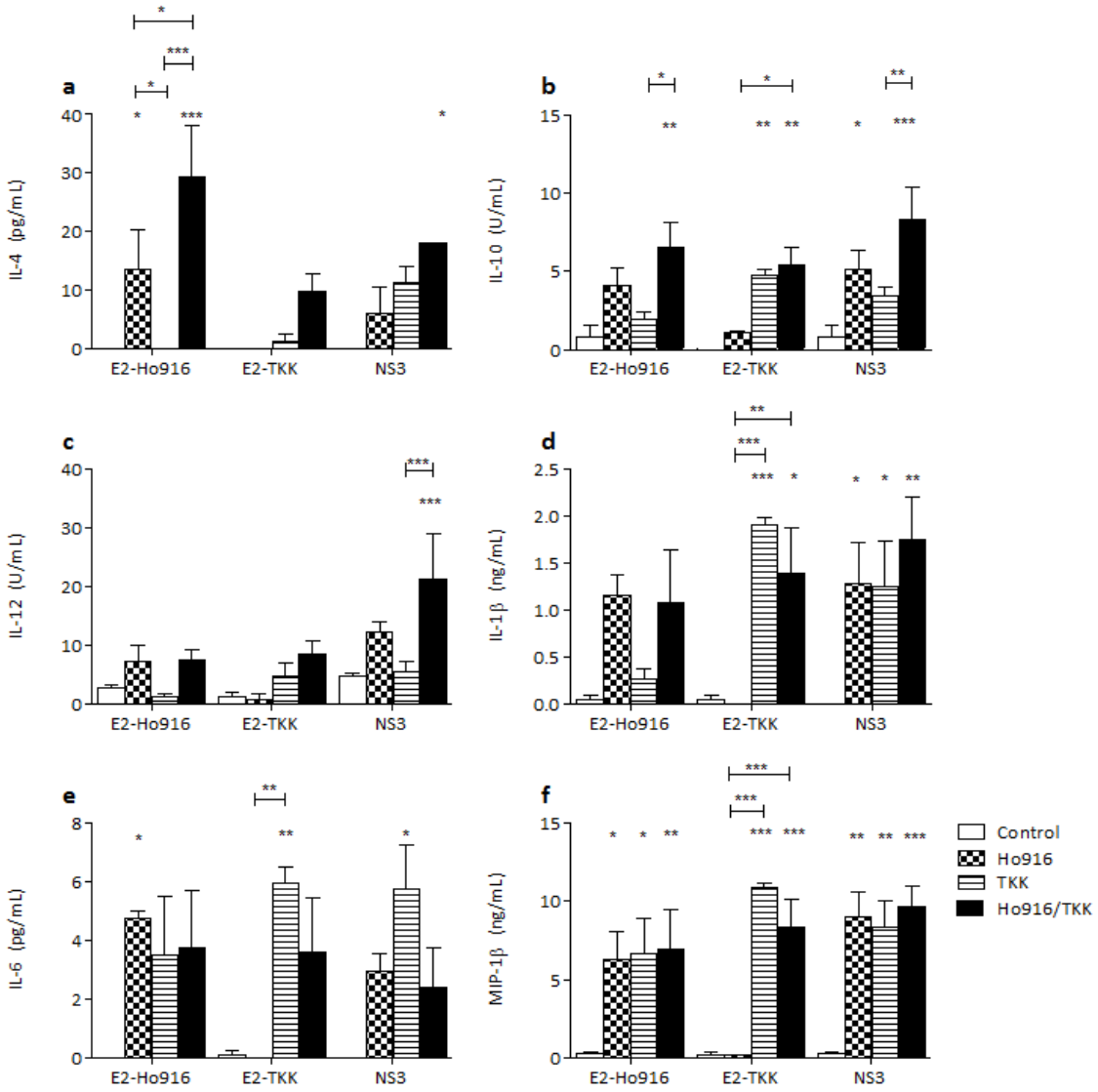

Figure 4.6. Assessment of antigen-specific cytokine responses following experimental infection with BVDV-1 Ho916, atypical bovine pestivirus TKK, co-infection with both (Ho916/TKK) or uninfected controls. PBMC isolated at 42 dpi were stimulated with peptide pools representing the Ho916 (E2-Ho916) and TKK (E2-TKK) E2 protein and NS3 protein from C24V and secretion of IL-4 (**a**), IL-10 (**b**), IL-12 (**c**), IL-1β (**d**), IL-6 (**e**) and MIP-1β (**f**) measured by a multiplex assay. Data represented as means ± SEM and statistical significance denoted by, * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$).

4.3 DISCUSSION

Recent disease outbreaks within the EU caused by atypical bovine pestiviruses have highlighted a group of viruses that have thus far received little attention. A recent study of atypical pestiviruses in FBS batches from different countries also suggests greater intercontinental spread/distribution than previously anticipated (Xia et al., 2011). Comparative analyses of antigenic relationships between atypical bovine pestiviruses and BVDV-1 and BVDV-2 point towards the need for improved diagnostics and reformulation of current vaccines for the detection and control of these new viruses (Bauermann et al., 2012). This study aimed to increase understanding of the implications of antigenic diversity on both T cell and antibody responses to BVDV and atypical pestiviruses.

In this study, both E2 and NS3 specific antibody responses were detected in all infected groups two to three weeks post infection albeit to a lesser extent in the Th/04_KhonKaen group. This raises questions about the sensitive detection of antibodies against atypical bovine pestiviruses, such as Th/04_KhonKaen, by diagnostic ELISA assays designed to detect antibodies typically against BVDV-1. Moreover, limited detection of antibodies to a European atypical bovine pestivirus, Italy-1/10–1, using a commercial BVDV-1-based ELISA, despite high neutralization titres has recently been described (Decaro et al., 2011a). In the present study, NS3 antibodies were better detected than antibodies against the less conserved E2 protein. NS3 antibodies were detected above the detection cut-off in all infected animals using a blocking ELISA kit (SVANOVIR® BVDV p80, Svanova Biotech AB) capable of recognizing antibodies against BVDV-1, 2 and 'HoBi-like' atypical bovine pestivirus according to the manufacturer. An alternative multispecies (BVDV and BDV) NS3 assay (Pourquier® ELISA BVD p80, Institut Pourquier, Montpellier,

France) which is known to use pan-pestivirus NS3 mAbs WB103 and peroxidase coupled WB112 (Edwards et al., 1988) as the capture and detection antibodies respectively. It has recently been proposed that diagnostics for BVDV and atypical pestiviruses should be based on NS2/3 or E^{rns} which bear good cross reactive targets while assays targeting E2 may allow discrimination of pestivirus species (Bauermann et al., 2012). This is in agreement with our observation of greater cross-reactivity in responses against NS3 relative to responses to E2 which were generally virus strain-specific.

Here, homologous neutralization was observed in all infected groups, yet cross-neutralization was only observed against the Horton 916 virus in the Th/04_KhonKaen infected group, whose sera was able to neutralize the Horton 916 virus almost to the same extent as the homologous virus. The relatively low homologous neutralizing antibody titres against Th/04_KhonKaen however, are suggestive of an inferior antibody response compared to the more robust titres in the Horton 916 infected and the co-infected groups. Similar patterns were observed with BVDV-1a NADL, routinely used in virus neutralization tests, while another atypical Pestivirus, D32/00_HoBi virus had similar neutralization patterns as the Th/04_KhonKaen virus. Patterns of cross neutralization of other bovine pestiviruses suggest that BVDV-1 is more similar antigenically to BDV-1 than atypical bovine pestiviruses, while closer similarity in neutralization was observed against BVDV-2. Limited cross neutralization activity, alluding to significant antigenic differences between BVDV-1 and 2 and atypical bovine pestiviruses have been previously reported (Schirrmeier et al., 2004, Ståhl et al., 2007).

A degree of cross-reactivity of T cell responses in Th/04_KhonKaeninfected animals after re-stimulation with Horton 916 virus was observed. However, IFN-γ

responses to Th/04_KhonKaen however, were not observed in the Horton 916 infected group. IFN-γ responses to E2 peptide pools were virus species specific whilst responses to the more conserved NS3 target appear to be maintained, to different extents, in all the infected groups.

Analysis of broader cytokine responses showed differential induction of several cytokines in the infection groups suggestive of a possible modulation of responses to either of the viruses. The lack of cytokine responses in Horton 916 infected animals following heterologous virus stimulation may hint at loss of T cell epitopes that may otherwise elicit cross-reactive responses. Broader cytokine responses however, were observed, in the Th/04_KhonKaen infected animals. IL-12 production, which is important in inducing and maintaining Th1 cell responses, was observed in response to the Horton 916 virus but not the Th/04_Khon_Kaen virus. This may, in part, explain the differences in T cell responses to the two viruses but this observation needs to be investigated further. Cytokine responses to E2 and NS3 peptide pools followed similar trends to IFN-γ. Responses to E2-Ho916 were not observed in the TKK infected group, with the exception of MIP-1β and to some extent, IL-6. Similarly, responses to E2-TKK were, by large, absent in the Horton 916 group, while as observed with IFN-γ, a number of cytokine responses were observed to the NS3 peptide pool in all the infected groups. These observations, taken together, point towards E2 responses being virus specific, with responses to the NS3 antigen being better conserved between the viruses. However, it is not clear what implications these differences in cytokine responses may have had, if any, on clinical and virological outcomes of infection with these viruses. This may be investigated better by transcriptomic profiling of host responses following infection

with the different viruses to elucidate differences in induction of host responses that may result in the different cytokine profiles.

The limited T cell and antibody immune reactivity of Ho916 inoculated animals to Th/04_KhonKaen suggests that animals vaccinated with BVDV-1-based vaccines may not be protected against atypical bovine pestiviruses. Alignment of translated E2 and NS3 sequences from BVDV-1 Horton 916, a reference BVDV-1 Oregon C24V and Th/04_KhonKaen confirmed greater genetic diversity in the E2 sequences compared to the NS3 sequences. E2 antigenic variation is pronounced among BVDV viruses and is thought to contribute to vaccine failure. Domains associated with neutralizing epitopes have been mapped to a hypervariable region located in the N-terminus of the E2 protein (Paton et al., 1992, Deregt et al., 1998). The extent of variation in E2 is better appreciated amongst BVDV-1 viruses because of the availability of a large sequence dataset; there is however a need to generate more E2 sequence data from atypical pestiviruses to enable a more robust comparison. NS3, the most conserved pestivirus protein, elicits antibodies that are not strongly neutralizing but are broadly recognized (Donis et al., 1991, Bolin and Ridpath, 1989).

BVDV subunit vaccines studies in cattle have focused on E2 or NS3 antigens, either as protein or plasmid encoding antigen DNA (Nobiron et al., 2003, Young et al., 2005, Thomas et al., 2009). DNA vaccination in general did not result in a robust immune response, whilst E2 protein on its own only conferred partial protection. The combination of E2 protein and the more conserved antigen, NS3, in a vaccine formulation has been suggested as a means to enhance protective immune responses against BVDV (Brownlie et al., 2010) and the evaluation of a nanoparticle vectored E2 and NS3 protein formulation is presented in Chapter 5.

There is currently discussion on whether atypical bovine pestiviruses should be recognized as a third BVDV species and if so, what implications this might have for currently licensed BVD diagnostics and vaccines as well as for the BVDV control programmes and eradication status of countries and regions. There is increasing evidence of the clinical and epidemiological relevance of these emerging viruses with clinical disease following experimental infection in cattle and sheep and reports of natural infections in Southeast Asia, South America and Europe. Antigenic variation between BVDV-1 and 2 species has already led to the inclusion of BVDV-2 strains in some vaccines (Ridpath, 2005), and this needs to be considered in the case of atypical bovine pestiviruses. Vaccine efficacy studies should also include challenge with heterotypic BVDV isolates as well as novel antigenically distinct variants (Kapil et al., 2008). Our findings support the consideration of atypical bovine pestiviruses in the development of diagnostics and vaccines, as well as the recognition of their clinical and epidemiological relevance.

Finally, it has been suggested that vaccines using reconstructed or archetypical/parental virus species or strains may be more broadly protective as the likelihood of conserved immune targets is greater than in viruses further down the evolutionary tree (Ducatez et al., 2011, Weaver et al., 2011). This is especially the case for RNA viruses with high mutation rates and significant genetic diversity (Lauring and Andino, 2010). While it is technically feasible to combine genetic variants of a single antigen (Liang et al., 2007), a recent influenza study has shown that a computationally optimized hemaglutinin-based virus-like particle (VLP) vaccine has better breadth of antibody and protective efficacy than a VLP containing a polyvalent mixture of HA proteins from multiple clades (Giles et al., 2012). Given

the vast amount of genetic and phylogenetic information available, a similar approach should be considered for future BVDV vaccines.

In conclusion, this study highlights the impact antigenic diversity among bovine pestiviruses may have on both T cell and antibody responses. It is hoped that this work will aid in the development of more broadly efficacious vaccines, as well as improved diagnostics against bovine pestivirus infections.

5 EVALUATION OF THE IMMUNOGENICITY AND EFFICACY OF BIODEGRADABLE NANOPARTICLE VECTORED BOVINE VIRAL DIARRHOEA VIRUS ANTIGENS

SUMMARY

The effectiveness of vaccination as a tool for control of BVDV has been hampered by inadequacies of existing vaccines. The development of subunit vaccines has focused on the polymorphic E2 protein, a dominant target of neutralising antibodies and CD4 T cell responses, with good efficacy albeit limited to homologous viral challenge. The inclusion of a better conserved antigen such as NS3 protein, a target of both T cell and antibody responses, has been proposed for induction of broader immune responses. This study aimed to test the vaccine potential of E2 and NS3 antigens formulated in poly (D, L-lactic-co-glycolic acid) (PLGA) nanoparticles adjuvanted with polyinosinic:polycytidylic acid (poly(I:C)) (Vaccine NP) in comparison to ovalbumin and poly(I:C) formulated in a similar manner (Control NP), and an inactivated BVDV vaccine (IAV). Six to eight month old calves (n=6 per group) were inoculated subcutaneously with each of the preparations, received a similar boost vaccination after three weeks, and were challenged with $10^{6.7}$ $TCID_{50}$ of BVDV-1a Horton 916 after a further three weeks. Virus neutralizing activity and E2 specific antibodies were observed in both Vaccine NP and IAV groups although significant NS3 antibodies were not induced in the Vaccine NP group until after viral challenge. IFN-γ, MIG/CXCL9 and proliferative responses were observed following *ex vivo* PBMC stimulation with E2 and NS3 proteins in both vaccinated groups after challenge infection. IFN-γ responses were also observed to the challenge virus in all groups after resolution of infection, but only the Vaccine NP and IAV groups responded to a divergent atypical bovine pestivirus, Th/04_KhonKaen. Reduced clinical symptoms and virus load were observed in the vaccinated groups compared to the Control NP group. While the protection afforded by both vaccines was not complete and the performance of the Vaccine NP appeared inferior to the IAV vaccine, particulate delivery of subunit antigen presents a customisable mode of antigen delivery that can be improved.

5.1 INTRODUCTION

Bovine viral diarrhoea virus (BVDV) is an economically important pathogen that is endemic in cattle around the world. BVDV has a profound impact on the cattle industry as a result of productive and reproductive losses (Houe, 1999). The main strategies for the control of BVDV have included the identification and removal of persistently infected (PI) calves, believed to be the major reservoirs of infection, increased biosecurity and vaccination (Deregt, 2005).

Vaccination plays a significant role in controlling BVDV infection, but the inadequacy of existing vaccines has hampered eradication efforts (Kelling, 2004). Modified live attenuated vaccines, although effective, have some shortcomings such as the requirement of cold storage and a limited shelf life, safety concerns in pregnant cattle, immunosuppression and possible reversion to virulence or genetic recombination with field viruses (van Oirschot et al., 1999). Inactivated vaccines whilst safer to use, require frequent immunisation and often fail to provide foetal protection. It has been proposed that inactivated BVDV vaccines lack efficacy because of their failure to induce potent T cell responses (Endsley et al., 2003). Collen and Morrison (2000) have further speculated that this failure is due to inactivated preparations lacking non-structural viral proteins that have been shown to be targets of the T cell responses of immune cattle.

Attempts to develop subunit vaccines have focused on the structural envelope protein E2, which is a dominant target of neutralising antibodies and a target of CD4 T cell responses (Donis et al., 1988). DNA vaccination with an E2 plasmid leads to neutralizing antibodies to homologous virus but not to heterologous virus prior to challenge (Harpin et al., 1999, Nobiron et al., 2003, Liang et al., 2005). Unlike in

mouse models, DNA vaccination in large animals has typically been shown to be of low efficacy and requires large doses to achieve strong immune responses (Brun et al., 2011). Vaccination using an E2 DNA prime-protein boost regime (Liang et al., 2006) or Quil-A adjuvanted E2 protein (Thomas et al., 2009) has been shown to have improved efficacy, but immunity to heterologous viruses is still hampered by the high antigenic variability of this target (Liang et al., 2008).

In addition to the use of adjuvants and/or cytokines such as IL-2, IL-1β and GM-CSF to improve E2 vaccine efficacy, the inclusion of better conserved targets such as non-structural viral proteins has been suggested (Reddy et al., 1993). The non-structural protein, NS3, is the most conserved pestivirus protein (Collett, 1992) and has also been considered as a BVDV vaccine candidate. The NS3 protein has multiple enzymatic activities (Grassmann et al., 1999) and functional constraints on this protein may counterbalance immunologic pressure thus allowing it to be a common target for T cell and antibody responses. DNA vaccination using an NS3 expressing plasmid, however, only showed partial protection in calves (Young et al., 2005). It has been proposed that combining recombinant E2 and NS3 protein in a vaccine formulation may increase the efficacy of E2 subunit vaccines and also improve heterologous protection by inclusion of a target of conserved responses (Brownlie et al., 2010).

Biodegradable nanoparticles synthesised with innocuous polymers such as poly(D, L-lactic-co-glycolic acid) (PLGA) have been proposed as vehicles for the delivery of vaccine antigens for human and veterinary applications (Peek et al., 2008, Scheerlinck and Greenwood, 2006). In addition to functioning as antigen reservoirs and protecting antigen from degradation in the extracellular or endosomal

environments, particulate delivery has been reported to enhance cross-presentation of exogenous antigen by MHC class I molecules to $CD8^+$ T cells (Shen et al., 2006). PLGA particles have also been shown to activate the inflammasome in antigen presenting cells and therefore enhance innate and adaptive responses (Sharp et al., 2009). Furthermore, defined immunostimulatory molecules such as Toll-like receptor (TLR) agonists and cytokines, as well as antibodies or ligands targeting the particles to particular antigen-presenting cells such as dendritic cells can be rationally encapsulated or coated onto the surface of the nanoparticles (Elamanchili et al., 2004). Co-delivery of TLR agonists and antigen in biodegradable nanoparticles has been shown to enhance both T cell and humoral responses compared to inoculation of soluble antigen and TLR agonists (Kasturi et al., 2011, Diwan et al., 2002).

A variety of TLR agonists have been evaluated for their potential to adjuvant vaccines and of particular significance for anti-viral vaccines is polyinosinic: polycytidylic acid (poly(I:C)), a synthetic double stranded RNA analogue that is an agonist for endosomal TLR-3 and the cytosolic receptors retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA-5) and DNA-dependent protein kinase catalytic subunits **(**DNA-PKcs**)** (Meylan and Tschopp, 2006). Poly(I:C) is an innate inducer of IL-12 and type I IFN thus enhancing DC maturation and B cell activation resulting in enhanced cross-presentation and potent T cell and antibody responses (Steinhagen et al., 2011).

This study aimed to assess the immunogenicity and protective efficacy of E2 antigen-coated PLGA nanoparticles encapsulating NS3 antigen and poly(I:C) after a homologous prime-boost vaccination and challenge with a BVDV field strain. Antigen specific T cell and antibody responses after vaccination and protection after

viral challenge were directly compared against those induced by a commercial inactivated BVDV vaccine.

5.2 RESULTS

5.2.1 Immune responses following vaccination and challenge

5.2.1.1 Antibody responses

Six to eight month old calves (n=6 per group) were inoculated subcutaneously with E2 and NS3 antigens formulated in PLGA nanoparticles adjuvanted with poly(I:C) (Vaccine NP) in comparison to ovalbumin and poly(I:C) formulated in a similar manner (Control NP), and an inactivated BVDV vaccine (IAV). The calves received a similar boost vaccination after three weeks, and were challenged with $10^{6.7}$ $TCID_{50}$ of BVDV-1a Horton 916 after a further three weeks. Antibody responses following vaccination and challenge were assessed by measuring BVDV specific antibody responses, using a commercial indirect ELISA kit, by assessing virus neutralization titres and by competition antibody ELISA against E2 and NS3 proteins. BVDV specific antibody responses were determined as percent positivity (PP) values of serum antibody binding to plates coated with crude BVDV antigen (**Figure 5.1a**). Both the IAV and Vaccine NP groups seroconverted after the booster vaccination at 28 days post vaccination (28 dpv), with the IAV group having significantly higher responses than the Vaccine NP group up to the point of viral challenge (41 dpv). Following challenge, the Control NP group become antibody positive after two weeks (58 dpv) but antibody levels were significantly lower than the IAV and Vaccine NP groups. The IAV group was still significantly higher than the Vaccine NP group at 58 dpv.

Virus neutralization titres (VNT) followed similar trends to that of total BVDV antibody, with neutralization of the challenge virus, BVDV-1a Horton 916,

being observed at the point of seroconversion (28 dpv) (**Figure 5.1b**). VNT values appeared higher in the IAV group compared to the Vaccine NP group prior to challenge and were significantly greater at 58 dpv. After challenge (58 and 65 dpv), VNT titres from both vaccinated groups were significantly higher than in the Control NP group.

E2 and NS3 specific antibody levels were determined as percent inhibition (PI) values. E2 antibody values in all groups were in agreement with those observed by the BVDV antibody ELISA and VNT (**Figure 5.2a**). In contrast, NS3 antibodies were only observed at a level considered 'positive' by the assay manufacturer in the IAV group after the boost vaccination but not in the Vaccine NP or Control NP groups until two weeks after challenge (58 dpv) (**Figure 5.2b**). NS3 antibodies appeared to be mounted following boost in the Vaccine NP group but were not above the assay cut-off for positivity. NS3 antibody levels after challenge in the Vaccine NP group were lesser than in the IAV group but greater levels than in the control NP group. Differences in antibody levels were resolved by a two-fold serial dilution of pooled sera from the two vaccine groups prior to challenge (41 dpv) for E2 and after challenge (58 and 65 dpv) between all three groups for both E2 and NS3 antibodies. Prior to challenge (41 dpv), E2 antibodies in the Vaccine NP group were above the assay cut-off at 1/32 dilution, while the IAV group was detected at 1/64. After challenge, both vaccinated groups had detectable amounts of antibody up to 1/512 with the IAV group levels significantly greater than in the Vaccine NP group at both 58 and 65 dpv. NS3 antibodies were significantly greater in the vaccinated animals than the Control NP group at all dilutions tested (1/4 – 1/512) at 58 and 65 dpv, and greater in the IAV group than at Vaccine NP group at 1/512 at 58 dpv, and from 1/32

at 65 dpv (**Figure 5.2c**). Ovalbumin antibody responses were observed in the Control NP (Ovalbumin and Poly (IC) encapsulated in PLGA) group (n-6) pre-boost (21 dpv) and following challenge (day 42 dpv) but not pre-vaccination (0 dpv) or in the Vaccine NP group (**Figure 5.3**).

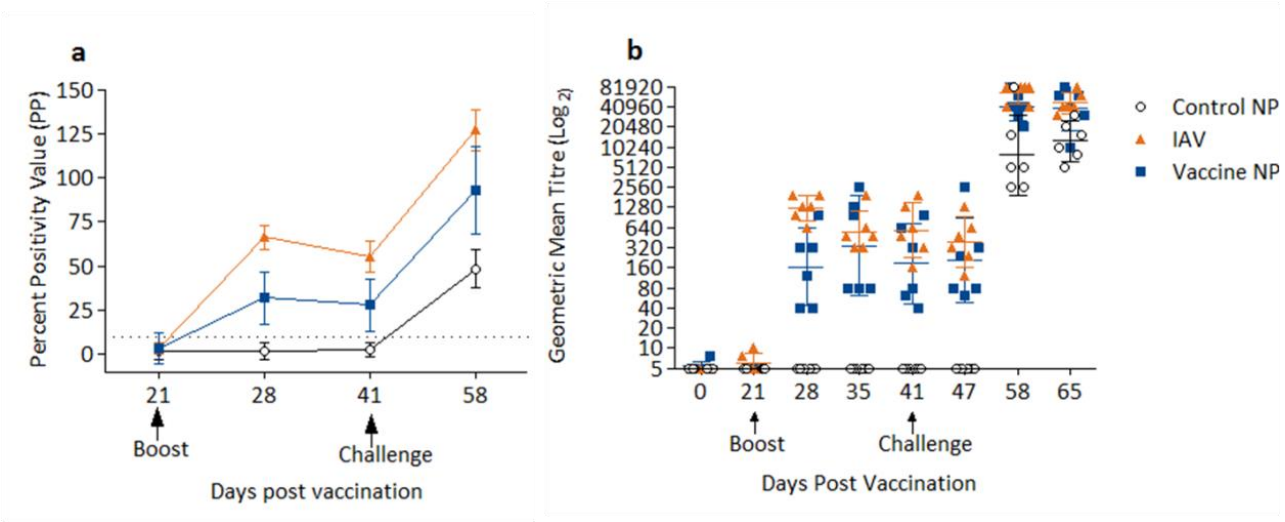

Figure 5.1. BVDV specific antibody responses following vaccination and challenge. (**a**) Total BVDV antibody responses determined by indirect ELISA as percent positivity (PP) after booster vaccination (21 dpv) and challenge (41 dpv). PP values greater than 10% were considered positive. Data shown as group means±SD. **(b**) Virus neutralization titres following priming (0) and booster (21 dpv) vaccination, and challenge (41 dpv) in the different groups shown as scatter plots with geometric means and 95% confidence interval.

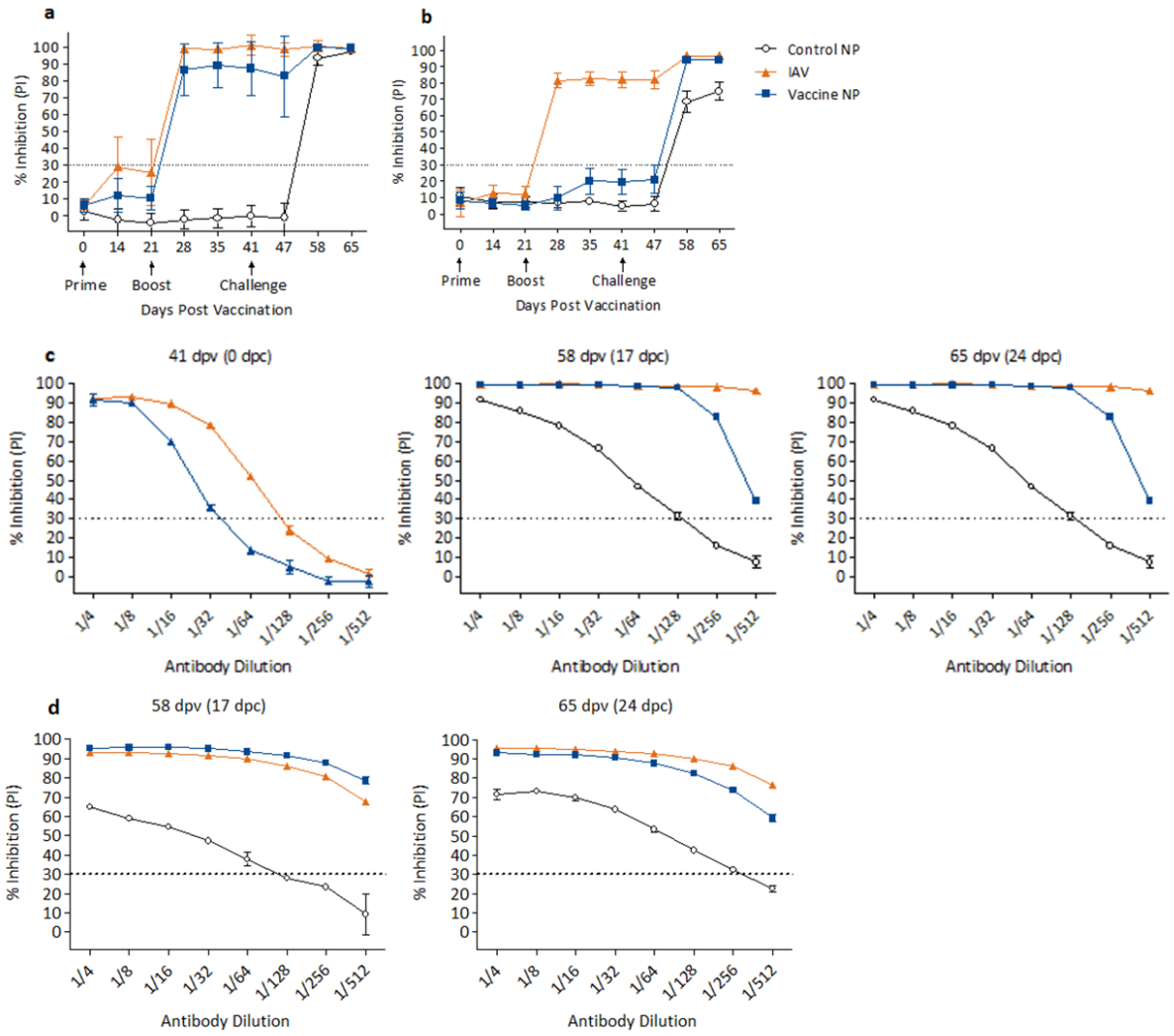

Figure 5.2. E2 and NS3 specific antibody responses following vaccination and challenge. E2 (**a**)and (**b**) NS3 antibodies as measured by blocking ELISA and given as mean percent inhibition (PI) values for the different experimental groups after prime (0 dpv) and boost (21 dpv), and following viral challenge (41 dpv). Antibody levels in both assays were considered positive if greater than 30%. A two-fold dilution of pooled serum from the different groups was assessed for E2 antibodies (**c**) between the IAV and Vaccine NP groups at the day of challenge (41 dpv) and between the three groups after challenge, at 58 dpv (17 dpc) and 65 dpv (24dpc). A similar dilution was assessed for NS3 antibodies (**d**) between the three groups at 58 and 65 dpv. Data is shown as means±SD.

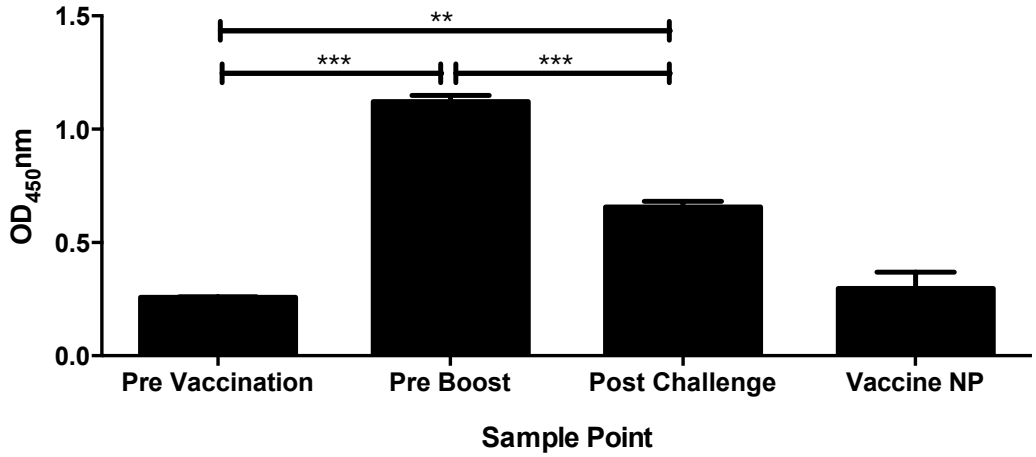

Figure 5.3 Ovalbumin-specific antibody responses in the control NP group. Ovalbumin specific antibody responses were assessed by ELISA using sera from the Control NP (Ovalbumin and Poly (IC) encapsulated in PLGA) group (n-6) pre-vaccination (0 dpv), pre-boost (21 dpv) and following challenge (day 42 dpv) . Data are presented as means ± SEM and statistical significance denoted by, ** ($p<0.01$) and *** ($p<0.001$).

5.2.1.2 T cell responses

T cell responses were assessed by measuring IFN-γ, proliferation and MIG/CXCL9 levels following *ex vivo* stimulation of PBMC with the challenge virus or E2 and NS3 proteins. IFN-γ and proliferation were measured as they are established markers of T cell responses. MIG/CXCL9 was selected as it has been reported to be a monokine induced by IFN-γ that serves as an amplified and functional measure of the IFN-γ response (Berthoud et al., 2009). IFN-γ was secreted at significant levels following virus stimulation after challenge in both of the vaccinated groups. The IAV group had significant responses from 51 dpv (10 dpc), while significant responses were not observed in the Vaccine NP group until 61 dpv (20 dpc) (**Figure 5.4a**). The Control NP animals did not exhibit significant IFN-γ responses to the virus in this time-frame. IFN-γ responses to E2 protein followed similar trends with significant responses observed in both vaccinated groups from 10 dpc, while the control animals did not appear to mount any response (**Figure 5.4b**). Responses to NS3 on the other hand were observed to significant levels in the IAV group following boost vaccination (28 dpv) and following challenge (44 dpv/3 dpc) (**Figure 5.4c**). Significant NS3 responses in the Vaccine NP were only observed after 51 dpv/10 dpc) but not in the Control NP group.

After resolution of infection (65 dpv/24 dpc), IFN-γ, MIG/CXCL9 and proliferative responses were assessed following *ex vivo* stimulation of PBMC with virus or proteins. IFN-γ was secreted at significant levels in response to both E2 and NS3 protein in the Vaccine NP group and with higher means in the IAV group but with no statistical significance compared to the Control NP group (**Figure 5.5a**). Whilst some proliferative responses were observed to both proteins in the IAV

group, these responses were only significant in the Vaccine NP group (**Figure 5.5b**). By this time point, IFN-γ responses to the challenge virus were observed in all three experimental groups, with trends towards lesser magnitude responses in the IAV group but without statistical significance (**Figure 5.5c**). Responses to an atypical bovine pestivirus, however, were significantly greater in the Vaccine NP group compared to the Control NP group. MIG responses were observed in all the groups in response to the challenge virus, but only in the IAV and Vaccine NP groups following stimulation with E2 and NS3 protein (**Figure 5.5d**). T cell responses to ovalbumin were not observed.

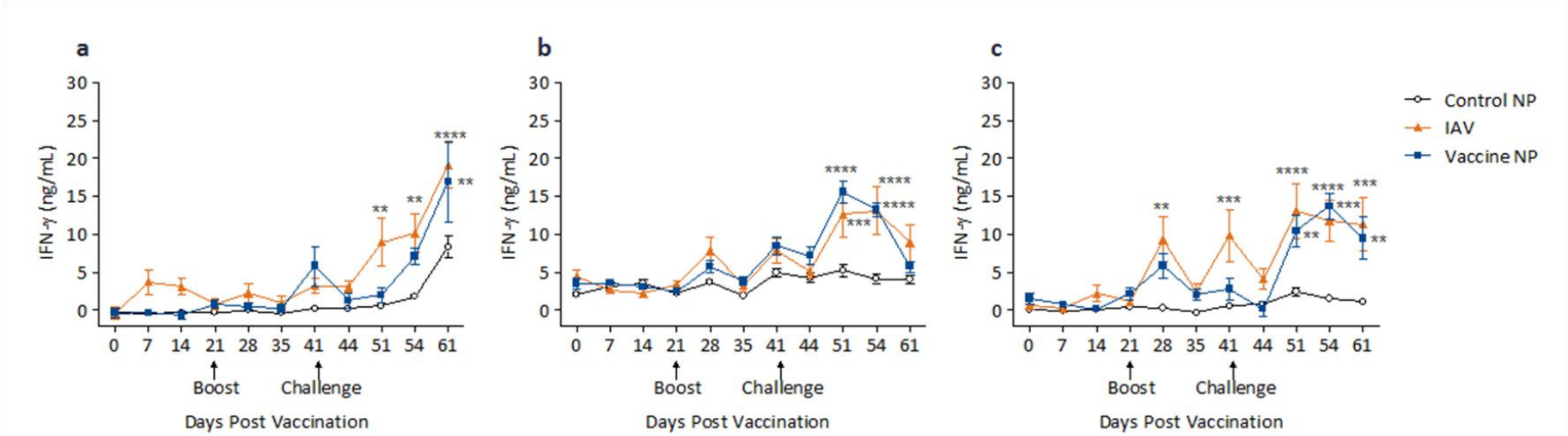

Figure 5.4. Longitudinal IFN-γ responses following vaccination and challenge. Antigen specific PBMC IFN-γ responses following stimulation with BVDV-1a Horton 916 virus (**a**), E2 (**b**) and NS3 (**c**) proteins were measured by ELISA and adjusted for mock (uninfected cell culture cryolysate) stimulated or media background responses. Data represented as means ± SEM and statistical significance is denoted by ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$). N=6 per group.

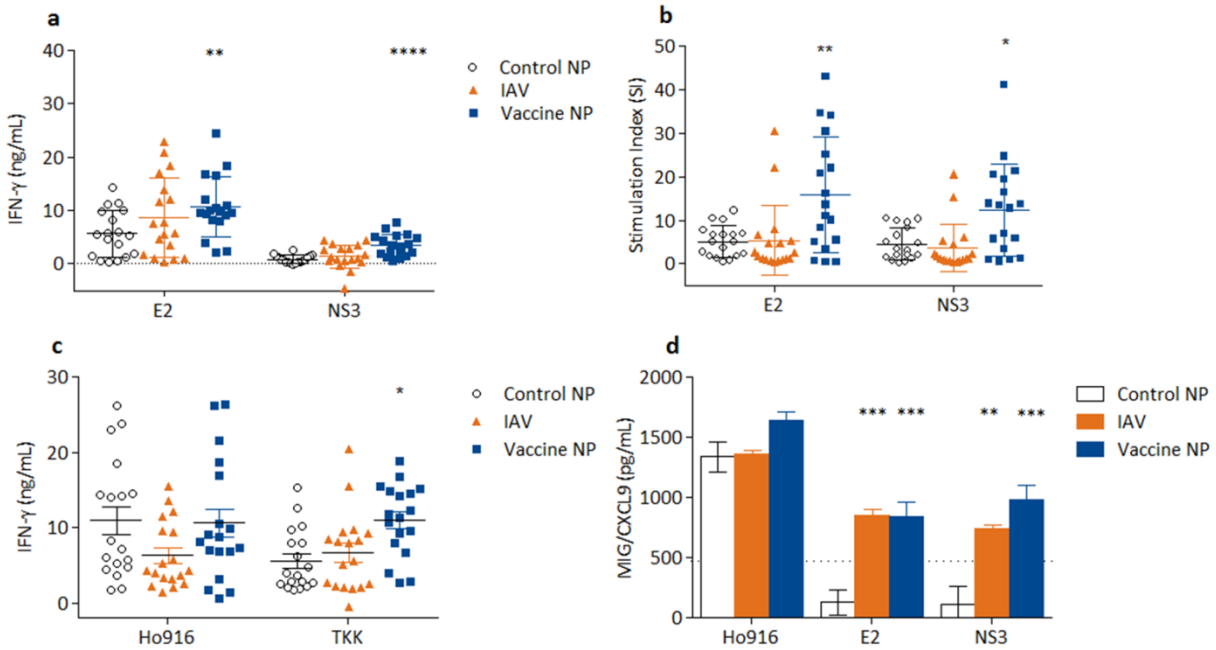

Figure 5.5. T cell responses after resolution of viral challenge infection. (**a**) IFN-γ responses and (**b**) proliferative responses following *ex vivo* stimulation of PBMC with E2 and NS3 proteins at 65 dpv (24 dpc) (**c**) *Ex vivo* responses following stimulation with the challenge virus (Ho916) or a genetically divergent atypical bovine Pestivirus, Th/04_KhonKaen (TKK). (**d**) MIG/CXCL9 responses in pooled supernatants from the different groups stimulated with Ho916 virus, E2 or NS3 proteins. All stimulations were performed in triplicate in the individual animals (n=6 per group) apart from the MIG ELISA which was used duplicate wells of pooled group samples. Data represented as means ± SEM and statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$).

5.2.2 Outcome of viral challenge

5.2.2.1 Clinical scoring, temperatures and haematological parameters

All the animals in the three experimental groups were challenged by intranasal inoculation with $10^{6.7}$ $TCID_{50}$ of BVDV-1a Horton 916 virus. The clinical picture observed following challenge was mild with relatively few signs and symptoms observed between 3 and 10 dpc (**Table 5.1**). Three out of six animals in the Control NP group experienced mild signs of disease ranging from coughing, nasal and ocular discharge, and with evidence of diarrhoea in the pen. Nasal and ocular discharge were observed as distinct clinical signs in two different control animals between 8 and 10 dpc. Slightly altered ocular discharge was observed in one out of six animals in the IAV group. Diarrhoea was observed as a distinct clinical sign in one out six animals in the Vaccine NP group while another animal was observed to have persistent diarrhoea prior to viral challenge. No significant differences in weight gain were observed between the groups following vaccination and challenge. Mild transient pyrexia (rectal temperature above 40°C) was observed in all the groups after viral challenge (**Figure 5.6a**). One of the Control NP animals had an elevated temperature (40.6°C) at 2 dpc, while a number of animals from each group had fever between 7 and 8 dpc. Fewer animals in the IAV group had rectal temperatures above the normal range (38.6 ±1.6°C) with mean temperatures significantly lower than in the Control NP and Vaccine NP groups at 7 dpc. Three out of six animals in the Vaccine NP group had fever at both 7 and 8 dpc, while only one and three animals had fever at 7 and 8 dpc, respectively in both the Control NP and IAV groups.

Transient leukopoenia (**Figure 5.6b**) was also observed between 3 and 8 dpc in all the groups, with mean leukocyte counts in the IAV group being significantly greater than in the in the Control NP group at 8 dpc. Thrombocytopenia (**Figure 5.6c**) was observed in all groups around 3 dpc with no significant differences between groups. Differential cell counts were also assessed by flow cytometry following gating of leukocyte subpopulations on the basis of CD45 staining and differences in side scatter. Lymphocytopenia mirroring the general decrease in leukocytes numbers was observed in all groups without any significant differences in the means (**Figure 5.7a**). Monocytes exhibited a slight increase in number (**Figure 5.7b**), while neutrophils exhibited an initial slight decrease followed by a sharp increase in number at 8 dpi, which was greater in the IAV and Vaccine NP groups relative to the Control NP group, albeit with no statistical significance (**Figure 5.7c**). A transient decrease in the number of eosinophils was also observed in all the groups (**Figure 5.7d**). The reference normal range of the different populations are shown as suggested in Schalm's Veterinary Haematology for 6 months old cattle (Weiss and Wardrop, 2010).

Table 5.1. Clinical scores after BVDV challenge

	dpc	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
	Calf																	
Control NP	66					1		1		**1**,2	2	2	0.5*					
	67								1				0.5					
	68												0.5					
	69												0.5					
	70												0.5					
	71									2	2	2	0.5					
IAV	72																	
	74																	
	79																	
	80																	
	81																	
	83					1												
Vaccine NP	73																	
	75																	
	76																	
	77**	2	2	2	2	2	2	2	2	2	0	0	0	0	0	0	2	2
	78										2	2						
	82																	

Parameters: Depression, Ocular discharge, Nasal discharge, **Cough**, Dyspnoea, Inappetance, and Diarrhoea

Scored as: Normal (0), Slightly altered (1), A distinct clinical sign (2) or Severe symptom (3).

*0.5- evidence of diarrhoea in the pen

** Animal 77 had persistent diarrhoea not associated with BVDV infection

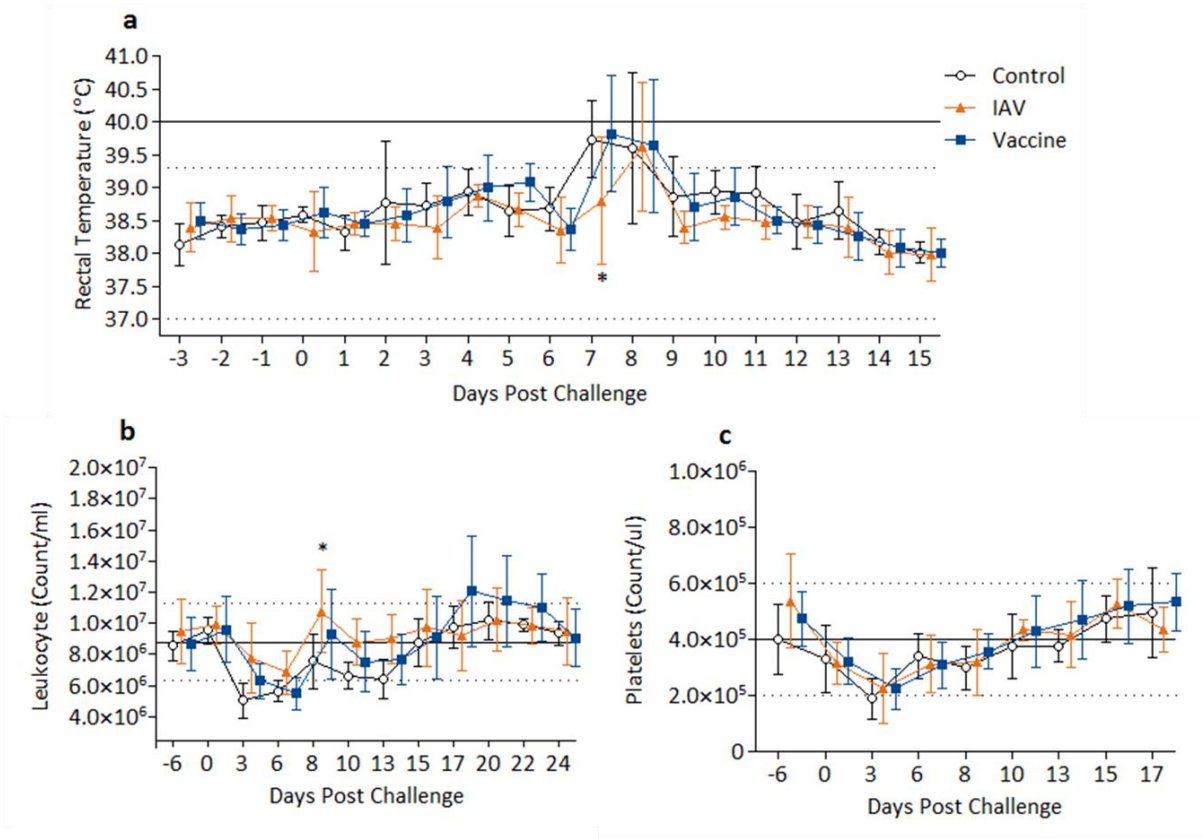

Figure 5.6. Rectal temperatures and haematological parameters following BVDV challenge. (**a**) Mean rectal temperatures in experimental animals following challenge. Horizontal dotted lines denote normal temperature range while the solid line denotes fever. (**b**) Leukocyte and (**c**) platelet counts following BVDV challenge. Data shown as group means ± SD. Horizontal dotted lines denote normal cell count range while the solid line denotes the average (Weiss and Wardrop, 2010).

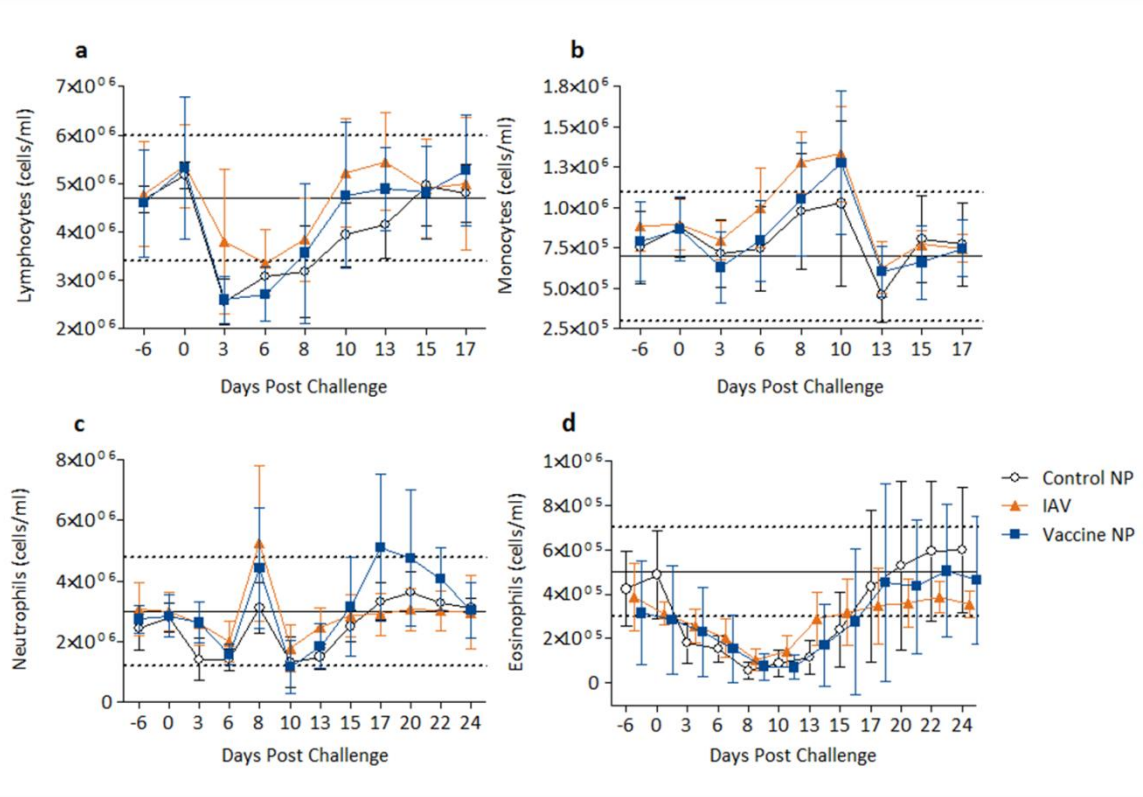

Figure 5.7. Differential leukocyte counts following BVDV challenge. Changes in (**a**) lymphocytes, (**b**) monocytes, (**c**) neutrophils and (**d**) eosinophils in peripheral blood following BVDV challenge as assessed by flow cytometry. Data shown as group means±SD. Solid horizontal lines indicate approximate normal values while the dotted lines indicate the normal range for the respective populations (Weiss and Wardrop, 2010).

5.2.2.2 Detection of viral antigen and nucleic acid

BVDV antigen was detected by E^{rns} antigen ELISA (**Figure 5.8a**). Viral antigen above the assay cut-off for positivity (0.3) was detected in the Control NP and Vaccine NP groups between 6 and 8 dpc. Mean values of the viral antigen were significantly less in both the IAV and Vaccine NP groups compared to the Control NP group, and less in the IAV compared to the Vaccine NP group at both time points. Detection of viral RNA by qRT-PCR (**Figure 5.8b**) mirrored antigen levels with viral RNA being detected in the Control NP group between 3 and 20 dpc and significantly greater levels observed at 9 dpc compared to the two vaccinated groups. The duration in which viral RNA was detected was shorter in the IAV and Vaccine NP groups (up to 13 dpc and 15 dpc, respectively).

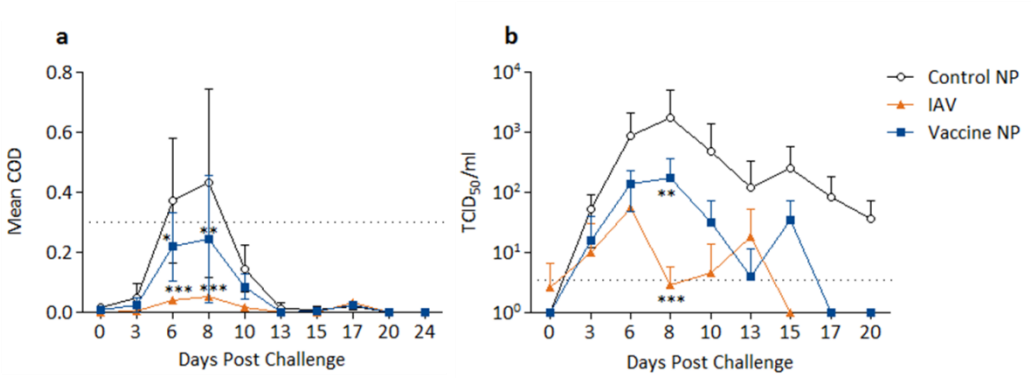

Figure 5.8 Detection of viral antigen and nucleic acid following challenge with BVDV-1a Horton 916. (**a**) BVDV E^{rns} antigen detection in blood by ELISA. The dotted line shows the assay cut off for antigen positivity (COD>0.3). (**b**) Viral RNA as quantified by qRT-PCR shown as equivalent $TCID_{50}$ values. The dotted line shows the lower detection limit ($10^{0.55}$ $TCID_{50}$). Data shown as means±SD and statistical significance is denoted by * ($p<0.05$), ** ($p<0.01$) and *** ($p<0.001$) compared to the control group.

5.3 DISCUSSION

This study aimed to evaluate vaccination with recombinant BVDV antigens, E2 and NS3 in a poly(I:C) adjuvanted PLGA nanoparticle formulation compared to a commercial inactivated vaccine. Antibody responses as detected by antigen capture ELISA and virus neutralization titres (VNT) were detected in both vaccine preparations after boost inoculation and at higher levels than the control animals after viral challenge. Higher levels of total BVDV specific antibody were observed in the inactivated vaccine group than the E2 and NS3 protein vaccinated animals, possibly due to the presence of antibody to other BVDV antigens such as E^{rns} in the former. E2 antibodies in the vaccinated animals, and in the control animals after challenge, were highly correlated with VNT as expected (Deregt et al., 1998).

In contrast to the to the inactivated virus vaccine, significant levels of NS3 antibodies were not detected after vaccination with the E2 and NS3 nanoparticulate formulation but were detected to higher levels compared to the control animals following BVDV challenge. To rule out a limited detection of antibody to the NS3 helicase in the competition ELISA, an antigen-capture ELISA was setup with the helicase antigen and the antibodies used in the commercial kit. It was confirmed that these antibodies were able to recognize and therefore compete with serum antibody against the NS3 helicase domain. The lack of a strong antibody response against encapsulated NS3 antigen is interesting since antibody responses to ovalbumin were detected in control animals vaccinated with an equivalent quantity of encapsulated antigen. It may be speculated that the dose of NS3 administered, although equivalent to ovalbumin, was not sufficient to elicit a robust antibody response, or there may have been a loss of conformation of NS3 protein due to *E. coli* expression or during nanoparticulate formulation.

Partial E2 and NS3 antigens were used in this study for practical purposes, primarily ease of expression. The E2 antigen was without the C terminus that includes 30 hydrophobic amino acids, which could function as a transmembrane anchor for E2. It is therefore able to be secreted from cells that express it unlike the full length protein which remains cell associated (Rumenapf et al., 1993). The NS3 protein consisted of the C-terminal helicase region without the protease domain that has been shown to induce apoptosis related to increased caspase activity (St-Louis et al., 2005, Gamlen et al., 2010). These partial proteins however, have been previously shown to be the target of both T cell and antibody responses (Paton et al., 1992, Deregt et al., 2005, Collen et al., 2002). Whilst the use of partial-length proteins is justified for practical purposes, it may be important to express and evaluate responses to full-length antigens in the future.

In this study, T cell responses were also observed in animals vaccinated with the inactivated vaccine and the BVDV subunit nanoparticulate formulation. IFN-γ responses to NS3 were detected in the inactivated vaccine before viral challenge, while responses to E2 and the challenge virus were found to be more robust than in the control animals following BVDV challenge. After challenge and resolution of infection, the nanoparticulate vaccine group appear to mount better IFN-γ, monokine induced by gamma (MIG/CXCL9) and proliferative responses than observed in the inactivated vaccine group. Better proliferative responses to the nanoparticulate vaccine may hint at improved central memory responses, while MIG/CXCL9 responses are associated with vaccine-induced T cell responses (Berthoud et al., 2009). Responses to the challenge virus were detected in all groups by 24 dpc, although responses to an atypical bovine Pestivirus, Th/04_KhonKaen, were detected to a greater level in the nanoparticulate vaccine and inactivated vaccine groups

compared to the control group perhaps indicating a better quality response which may be more broadly protective. T cell responses to ovalbumin were not detected in this study but have been reported previously following vaccination of cattle with ovalbumin in IFA (Nonnecke et al., 2012).

While antibody responses to the nanoparticle formulation were observed to be inferior to the inactivated vaccine, the advantage of this method of delivery is that it is highly adaptable with scope for improvement. Responses may be enhanced by use of better adjuvants or combinations of adjuvants that stimulate broader immune responses. Also, combinations of TLR agonists may act synergistically in triggering the desired T cell or humoral immune responses (Napolitani et al., 2005) A recent study, demonstrated enhanced antibody responses to synthetic nanoparticles containing antigen and TLR-4 and TLR-7 ligands compared to nanoparticles containing antigen and individual TLR ligands (Kasturi et al., 2011). Another consideration for enhanced responses would be the use of a heterologous prime-boost regime. An E2 DNA/protein prime/boost strategy has shown enhanced immunogenicity and efficacy compared to homologous prime and boost with the protein or DNA constructs (Liang et al., 2006).

Neither the E2 and NS3 nanoparticulate formulation nor the inactivated virus vaccines evaluated in this study were able to confer sterile immunity. However, decreases in viral load (both peak and duration of viraemia), as shown by decrease in both viral antigen and RNA, as well as reduced clinical scores in both vaccinated groups were evidence of a degree of protection. Nevertheless, no significant differences in haematological parameters or rectal temperatures were observed between the vaccinated and control groups. The failure of vaccination to prevent transient leukopenia associated with BVD may be disadvantageous to the animal in

spite of recovery from acute infection as this is thought to render animals susceptible to secondary pathogens (Bolin, 2002).

The transient leucopoenia and elevated rectal temperature appear to be a hallmark of an innate mechanism of post-challenge virological control and not necessarily a consequence of virus induced pathology. It is thought that that these responses play a role in virus clearance in addition to pre-existing adaptive responses in the vaccinated animals, and in the control animals, albeit, after a longer period which may present a chance to shed and transmit virus to other naïve animals. The transient leucopoenia associated with early viral infection has been shown to be dependent on type I IFN receptor signaling. Type I IFN (α/β) responses to viral infection or treatment with TLR agonists poly(I:C) and resiquimod (R-848) have been shown to cause lymphopenia by regulating lymphocyte recirculation (Kamphuis et al., 2006). Early IFN-α and γ responses have been shown to be induced following infection with noncytopathic BVDV (Charleston et al., 2002) and it is possible that the early transient leucopoenia may be associated with secretion of these cytokines in response to viral infection.

Elevated temperatures in this study were also shown to coincide with the transient increase in neutrophils. Interestingly, the vaccinated animals, which control the virus better, appear to have higher levels of neutrophils than the control animals. A similar transient increase in neutrophils after viral challenge that is enhanced in vaccinated animals has been observed following an E2 DNA-prime, protein-boost vaccination and BVDV-1 and -2 challenge (Liang et al., 2008). Neutrophils play an important role in virus control and their function is found to be suppressed in cattle persistently infected with BVDV, an observation that was reversed by treatment with recombinant IFN-α and γ (Brown et al., 1991, Steinbeck and Roth, 1989). It may be

speculated that the reduced viral load in the vaccinated animals unmasks this suppression resulting in a transient neutrophilia which may aid in clearance of the virus.

Acute BVDV type I infection, which represent the majority of BVDV infections, results in mild disease of short duration characterized by fever, increased respiratory rate, diarrhoea and leukopenia (Baker, 1995). The challenge virus used in this study, BVDV-1 strain Horton 916, was associated with severe fatal disease in the field and has been previously used in an experimental infection at a titer of $10^{6.7}$ $TCID_{50}$ with mild clinical disease and leucopoenia (Rebecca Strong, APHA, personal communication). The reproduction of symptomatic acute BVDV infection in immunocompetent animals by experimental intranasal challenge is difficult, even in the case of virulent field viruses, and better challenge models need to be explored.

Bovidec®, the inactivated vaccine in this study, is used for active immunisation of female breeding cattle to prevent transplacental infection, and in calves from 4 months of age (after the decline of maternal antibody) to prevent viraemia and viral shedding. Systemic vaccination with Bovidec has been shown to protect against respiratory challenge (Howard et al., 1994) and against foetal infection (Brownlie et al., 1995). Whilst somewhat lesser responses were observed to the nanoparticulate formulation in this study, similarities in induction of neutralizing antibody and T cell reactivity makes this means of delivering subunit antigens a prospect worthy of improvement.

In conclusion, whilst the inactivated vaccine performed better in this study, the nanoparticulate vaccine formulation offers a promising mode of vaccination that can be improved by use of better adjuvants, greater consideration on location of antigen (coated or encapsulated), as well as targeted delivery of antigen and adjuvant

to dendritic cells. In addition to the adaptability of this method of antigen delivery, other advantages such as the relative safety and capacity for differentiating infected from vaccinated animals (DIVA) are offered by the use of subunit antigens. It is hoped that the data generated will inform the design of improved vaccines against BVD.

6 GENERAL DISCUSSION

An understanding of the immune responses that contribute to protection is critical to a rational approach to vaccine development, and involves an empirical evaluation of the immune mechanisms induced by infection or vaccination. This includes an appreciation of the main antigenic targets, the phenotype, functional mechanisms and kinetics of these responses. Effective vaccines have been shown to elicit antigen-specific antibody responses by plasma cells, as well as both B cell and T cell memory responses (Amanna and Slifka, 2011). This study endeavoured to generate data on T cell responses to viral proteins and utilize this for the design of a subunit antigen vaccine against BVDV.

The first part of the study aimed to rationally identify the main targets of T cell responses to BVDV by screening synthetic peptides representing the BVDV proteome. While a number of proteins have been shown to be targets of CD4 responses (Collen and Morrison, 2000), no systematic study has been conducted to assess responses to all proteins simultaneously. In this study, we attempted to characterize T cell responses to BVDV by assessing IFN-γ responses to synthetic overlapping peptide pools representing the different viral proteins and spanning the BVDV proteome. A similar approach has previously been utilized in the case of related CSFV in pigs with a peptide library representing 82% of the proteome (Armengol et al., 2002). In that study, proliferative and IFN-γ responses were observed to a number of peptides on the E^{rns}, E1, E2 , NS2-3, NS4A, NS4B and NS5A proteins, with a CSFV specific $CD8^{+}$ cytotoxic T lymphocyte (CTL) and a helper T cell epitope identified on NS2-3 protein.

This work was largely limited to assessing immune responses to peptide pools representing individual BVDV proteins with no significant attempt to define individual antigenic peptides. This was primarily due to the limited number of

animals (four) and also, the minimal knowledge of the MHC-II haplotypes of the animals. A single CD4 antigenic peptide was mapped on E2 protein for exploratory purposes. Interestingly, only a few viral CTL epitopes have been mapped in cattle, which were identified from the foot and mouth disease virus (FMDV) (Guzman et al., 2008), indicating that this is an aspect that needs further exploration to generate more detailed data. CTL epitopes have also been successfully mapped using synthetic peptides against visna/maedi virus in sheep following infection and a DNA-prime-MVA-boost vaccination (Wu et al., 2008). To date, the development of vaccines against rapidly evolving RNA viruses and other complex pathogens using synthetic peptides has not been fruitful due to poor immunogenicity as a result of immune escape caused by mutation. The MHC restricted nature of these responses and the potential antigenic polymorphism of these targets between different strains are further drawbacks to such a reductionist approach (Naz and Dabir, 2007). However, definition of T cell epitopes allows the development of tools such as MHC glycoprotein-peptide tetramers (Altman et al., 1996) which may be of use in better characterizing responses following infection and/or vaccination. Also, more recently, regulatory or pathogenic epitopes have been described whose exclusion from vaccine antigens can increase their efficacy (Welsh and Fujinami, 2007).

The proper mapping and definition of specificities and MHC restriction of antigenic determinants/epitopes often requires MHC homozygous animals or at the very least, MHC defined animals which requires the capacity for molecular characterization of both MHC class I and II alleles (Collen et al., 2002). Also, tools such as homologous transformed antigen presenting cells (Hart et al., 2011a), antigen specific T cell lines (Glass and Spooner, 1990) and xenogeneic antigen presenting cell lines transfected with single MHC alleles (Gaddum et al., 1996a) can be useful

for the determination of the fine specificity of T cell responses. Computational prediction of epitopes to bovine pathogens has been aided by definition of peptide binding patterns/motifs (Hegde et al., 1995, Bamford et al., 1995, Gaddum et al., 1996b) as well as the development of a bovine leukocyte antigen (BoLA) epitope mapping algorithm/matrix (De Groot et al., 2003b, De Groot et al., 2003a). An attempt at computational prediction of bovine MHC class I restricted CTL BVDV epitopes has been made, although none of the candidate epitopes have been validated to date (Srikumaran and Hegde, 1997).

The second part of this study looked at the effect of genetic and antigenic diversity, and the emergence of new variant viruses on immune responses, which may have consequences for the efficacy of vaccination programmes. Evolutionary changes in BVDV are responsible for emergence of genetic and antigenic diversity (Goens, 2002). This has so far lead to the emergence of two recognized BVDV species as well as a third putative species, and numerous subgenotypes within the species. Evidence of positive selection on BVDV E2 glycoprotein has been observed, that may be driven by immune pressure or host tropism (Tang and Zhang, 2007). While surface antigens are likely to be subject to the most variability as a result of immune pressure as well as host and cell tropism, non-structural proteins are likely to be better conserved as a result of the limited exposure. Various non-structural proteins, such as BVDV NS3, have been shown to be targets of immune responses that may contribute to protection. It was, therefore, hypothesised that combination of NS3 with the immunodominant surface glycoprotein E2 would enhance the efficacy of subunit vaccines and reduce the likelihood of immune escape as a result of the antigenic diversity observed to the latter antigen. While the antigenic closeness of vaccine and prevalent field viruses may be crucial for vaccine efficacy, heterogeneity

from previously encountered strains may aid avoidance of 'original antigenic sin' (Francis, 1960), a phenomenon in which a secondary response is focused to only the cross-reactive targets in the primary exposure, which may aid immune escape (Lambert et al., 2005). The possible effect of previous exposure or vaccination will also need to be explored in vaccination against BVDV. This study showed that the extent of cross-reactivity varies between antigens depending on the degree of conservation across different virus strains. Selection of conserved antigens may, therefore, overcome possible interference due to pre-exposure to variant antigens, as well as provide cross-protection.

The final part of this study looked to assess the immunogenicity of selected BVDV antigens, E2 and NS3, in a vaccination and challenge study. While the PLGA nanoparticles delivery model proved promising, questions remain regarding the dosage and quality of the antigen used, as well as the choice of adjuvant. The importance of antigen quantity in a vaccine dose for efficacy is known for E2 (Bruschke et al., 1997, Thomas et al., 2009) but is yet to be reported for NS3. The means of expression of recombinant protein is crucial, as it will determine both the quantity required and antigenic authenticity of the protein. While functional authenticity may not be critical for T cell responses and linear B cell epitopes, B cell recognition of conformational epitopes will rely on the protein being produced as close to its native structure as possible. The choice of expression system has traditionally relied on convenience, yield and functional authenticity. In addition to high yields, baculovirus insect cell expression produces proteins with similar post-translational modification to mammalian cells. However, it has been shown that mammalian expressed E2 protein is required at considerably lesser quantities than baculovirus expressed protein to elicit comparable immune responses and protection

from viral challenge (Thomas et al., 2009). The loss of glycosylation sites on CSFV E2 and E^{rns} baculovirus derived proteins has been shown to affect antibody responses but not necessarily protection from lethal challenge suggesting that T cell responses may contribute significantly to this (Gavrilov et al., 2011). Interestingly, truncated E2 protein expressed in cultivated tobacco (*Nicotiana tabacum*) plants to avoid animal contaminants has been tested successfully for antigenicity and immunogenicity in guinea pigs and is being considered as a next generation vaccine (Nelson et al., 2012). The performance of *E. coli* derived NS3 in our study appears to result in inferior antibody responses compared to native NS3 contained in the inactivated vaccine preparation suggesting that antigenic authenticity may be just as important for antibody responses to non-structural proteins.

A number of BVDV vaccine studies have been conducted in mice, cotton rats, guinea pigs and other models (Chase et al., 2004). The selection of appropriate models however needs better consideration. Sheep, which are also susceptible to BVDV infection, are suitable models in vaccination studies but like in cattle, care has to be taken to ensure that they are border disease virus (BDV) free prior to challenge. Correlations have been established between bovine, and ovine and guinea pig models following the evaluation of the immunogenicity of experimental vaccines concurrently (Fernandez et al., 2009). Subsequently, immunogenicity of an E2 subunit vaccine has been determined in guinea pigs on the basis of the association found between responses in this experimental model and the natural bovine host (Pecora et al., 2012). Prevention of foetal infection is considered to be the gold standard for BVDV vaccines, but it is not a rational model for primary immunogenicity and efficacy testing. Systemic multiple site inoculation of calves, albeit artificial has been shown to induce more overt clinical disease than traditional

respiratory (intranasal) challenge (Polak and Zmudzinski, 2000) and may be a useful modification to current viral challenge protocols. Another challenge model that could be explored could be transmission via in-contact persistently infected animals which is a likelier mode of transmission in herds than via transiently infected animals (Thurmond, 2008).

Modified live virus vaccines, although reported to cause transient leukopoenia after inoculation, are thought to stimulate better T cell and antibody responses than inactivated vaccines and hence confer sterile protection with no viraemia, fever or haematological changes observed after challenge (Fulton, 2008). While we were unable to assess immune responses to a modified live vaccine in this study, this would be the ideal comparison, as this appears to be the gold standard of vaccination, albeit with significant safety concerns.

T cells have been shown to play a significant role in adaptive immune responses to infectious diseases including BVDV and it is important to have an understanding of the phenotype and function that are reflective of effective T cell responses. This will involve assessing the quantity and quality, as well as the kinetics/synchrony of immune responses and the effect on memory and protection (Seder et al., 2008, Pantaleo and Harari, 2006). The need for broader, more in-depth evaluation of immune responses to BVDV vaccines has been recognized. Reber et. al. (2006) measured VNTs, lymphocyte proliferation, cytokine mRNA expression, intracellular cytokine production and release of IFN-γ following vaccination with modified live or killed BVDV vaccines. More in-depth analyses, however, have not been conducted to characterize the specificity and phenotype of T cell responses to BVDV. Different CD4 T cell memory populations have been previously described on the basis of CD45RO, CD62L and CCR7 staining in cattle (Totte et al., 2010).

Bovine antigen specific memory $CD8^+$ cells induced by BCG in cattle have also been defined as $CD45RO^+$ (Hogg et al., 2009). Furthermore, protocols for the identification of polyfunctional bovine T cells populations by intracellular detection of IL-2, TNF-α and IFN-γ have recently been published (Whelan et al., 2011) and could be utilised in the assessment of vaccine induced responses. Secretion of other cytokines such as MIP-1β and functions such as proliferation, cytotoxic activity (direct killing, degranulation or perforin expression) are also crucial in characterizing the functional profile of T cells.

An alternative read out for immunogenicity is monokine induced by gamma (MIG/CXCL9). MIG is an inflammatory chemokine secreted primarily by $CD14^+$ monocytes and macrophages, as well as neutrophils, B cells and eosinophils that is induced by IFN-γ (Whiting et al., 2004). MIG is important in the recruitment of activated T cells to the site of infection, and serves as an amplified (more sensitive) and functional measure of IFN-γ activity (Berthoud et al., 2009). The availability of a bovine MIG assay used in this study can be further exploited to better characterize vaccine-induced responses. In this study, MIG proved to be a robust readout comparable to IFN-gamma, and therefore a useful tool in the assessment of T cell responses to BVDV and to other bovine pathogens/vaccines.

The measurement of antibody responses has traditionally been the principle way of assessing immune responses to BVDV. Conventional BVDV antibody ELISAs are based on crude antigen prepared from BVDV infected cells usually by treatment with detergents and other inactivation reagents that may interfere with the native antigen structure. The use of recombinant antigens expressed in bacterial or eukaryotic systems provides an alternative, albeit more expensive, source of antigen for testing for BVDV specific antibodies in serum. These enable the discrimination

of antibodies against different virus proteins (Chimeno Zoth and Taboga, 2006) and in the case of subunit or marker vaccines, may enable differentiation between infected and vaccinated animals (DIVA) (van Oirschot, 1999). Furthermore, other means of assessing antibody responses need to be applied in monitoring humoral responses to BVDV. While neutralizing antibodies are seen primarily to E2 protein, non-neutralizing antibodies are elicited to other targets such as E^{rns}, E1 and NS23 that may also be protective. A number of alternative assays may be applied to dissect other aspects of antibody responses such as affinity, avidity, complement activity, antibody-dependent cellular cytotoxicity or cell mediated virus inhibition (ADCC/ADCVI) (Baum, 2010). There is also a need to distinguish long-lasting antibody-secreting plasma cells that constitute protective memory responses with long lived reactive memory B cells that are triggered in response to recurrent antigenic challenge (Dorner and Radbruch, 2007).

Innate immune responses to pathogens are known to shape the magnitude, quality and durability of adaptive responses capable of eliminating infection and conferring long term protective immunity (Pulendran and Ahmed, 2006). Adjuvants including agonists for pattern recognition receptors such as TLR ligands are known to activate innate immunity and create the right environment for robust adaptive responses. In humans, PolyICLC, an RNase-resistant analogue of Poly(I:C) stabilized with poly-lysine, has been shown to induce expression of genes encoding TLR7 and TLR4, the RIG-I and MDA-5 viral sensors, transcriptional factors involved in type I IFN responses (IRF7, IRF5 and IRF1) in a similar fashion to a protective replication-competent viral vaccine *in vivo* (Caskey et al., 2011). Live viral vaccines however, induce broader responses, an effect that could perhaps be mimicked by the combination of multiple adjuvants. It is possible that the

nanoparticulate formulation assessed in this study may be improved by testing with more potent adjuvants to elicit the desired responses.

It is also important to establish absolute or relative quantities of an immune response as strongly associated with a desired outcome (correlates of protection) or suitable *in vitro* surrogates that could be employed to measure vaccine efficacy (Qin et al., 2007). Predictive correlates of vaccine efficacy are important in vaccine development as they could partially eliminate the need for elaborate and costly vaccination and challenge studies. For this purpose, highly precise immunological assays and empirical validation of these correlates are necessary. *In vitro* surrogate tests may also suffice as predictors of protection by vaccination if the correlates of protection are unknown or difficult to measure. While both T and B cell responses have been shown to contribute to protective immunity against BVDV, specific correlates of protection have not been defined. The establishment of such correlates may require studies of natural infection, vaccination trials or experimental infection in cattle or relevant animal models (Amanna and Slifka, 2011). It is well established that the presence of neutralizing antibody in blood correlates with protection afforded by BVDV vaccination and thus serum antibody titres are a useful indicator of vaccine efficacy. (Saliki and Dubovi, 2004). However, no definitive quantitative correlate of protection has been described. Following experimental infection with a highly virulent BVDV-2 strain, virus neutralizing titres of 256 or less were shown not to protect from fever and systemic virus spread, while calves with titres lower than 16 developed clinically severe disease (Bolin and Ridpath, 1989). A second study showed that titres up to 64 were unable to protect from transmission of BVDV-1b following exposure to persistently infected calves (Fulton et al., 2005). Titres required to confer cross protective virus neutralization between BVDV genotypes

have not been established, but titres following vaccination with inactivated BVDV-1a and 2a were shown not to be able to protect against infection with BVDV-1b (Grooms et al., 2007). While our study did not attempt to establish any correlates of protection, this should be viewed as a critical aspect of assessment of existing vaccines and development of improved vaccines.

Finally, systems biology approaches could be employed to better characterise responses following BVDV infection or vaccination. BVDV has also been shown to differentially modulate toll-like receptors, cytokines and co-stimulatory molecule gene expression by RT-qPCR (Lee et al., 2008). A proteomics/mass spectroscopy approach revealed that the expression of proteins related to antigen presentation (Lee et al., 2009) and protein kinases involved in anti-viral mechanisms are modulated in BVDV infected monocytes (Pinchuk et al., 2008). Microarrays have also been used to differentiate the effects of infection with cp and ncp BVDV (Werling et al., 2005). A global approach may be useful in providing a better understanding of immune responses following infection and vaccination and therefore accelerate vaccine development by identifying predictors of immunogenicity and previously unknown mechanisms that underlie protective responses (Trautmann and Sekaly, 2011).

It is hoped that the data generated in this study will provide a background for the better understanding of immune responses to BVDV and in the development of improved vaccines to support control and eradication. In summary, we have outlined a proteome-wide method of screening and selecting vaccine candidate T cell antigens, assessment of the extent of cross-reactivity by the defined antigens, and evaluated a formulation of these antigens *in vivo* in comparison to an existing vaccine. We hope that lessons learnt in the course of this study, and discussion

points arising from it will aid in the advancement of rational vaccine development strategies to aid BVDV control and eradication.

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APPENDICES

APPENDIX A: PUBLICATIONS

Kinetics of single and dual infection of calves with an Asian atypical bovine Pestivirus and a highly virulent strain of bovine viral diarrhoea virus 1

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Kinetics of single and dual infection of calves with an Asian atypical bovine pestivirus and a highly virulent strain of bovine viral diarrhoea virus 1

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ABSTRACT

Atypical bovine pestiviruses related to bovine viral diarrhoea virus (BVDV) have recently been detected in cattle from South America, Asia and Europe. The purpose of this study was to compare the clinical and virological aspects of dual infection with BVDV-1 (Horton 916) and an Asian atypical bovine pestivirus (Th/04_KhonKaen) in naïve calves, in comparison to single infections. Milder clinical signs were observed in the animals infected with single Th/04_KhonKaen strain. Leukocytopenia and lymphocytopenia were observed in all infected groups at a similar level which correlated with the onset of viraemia. Co-infection with both viruses led to prolonged fever in comparison to single strain inoculated groups and simultaneous replication of concurrent viruses in blood and in the upper respiratory tract. Following the infections all the calves seroconverted against homologous strains. Atypical pestiviruses pose a serious threat to livestock health and BVDV eradication, since they may have the potential to be widely spread in cattle populations without being detected and differentiated from other BVDV infections.

1. Introduction

Bovine viral diarrhoea virus (BVDV) belongs to the *Pestivirus* genus of the *Flaviviridae* family and includes two recognized species: BVDV-1 which is more dominant in Europe, and BVDV-2 which is more common in North and South America, but whose transmission into European herds has been described in the last two decades [1]. Interestingly, retrospective studies have revealed that BVDV-2 had been detected previously in a pig in Europe in the late 1970s and was considered as an atypical classical swine fever virus strain [2]. Recently, a novel pestivirus named D32/00_'HoBi' was discovered in a batch of foetal bovine serum (FBS) originating from Brazil [3], and was proposed as a representative of a possible novel pestivirus species [4]. Additional closely related viruses have been found in FBS of South American origin [5,6] and more recently viruses associated with natural infections in cattle have been reported [7–9]. Phylogenetic analysis of the 5′UTR sequences of these novel virus strains has shown that these

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viruses are genetically distinct from the defined four recognized pestivirus species. Within this potential species named 'atypical pestiviruses', strains from South America and Europe cluster together, with the Th/04_KhonKaen strain, isolated from a naturally infected calf in Thailand, being the most divergent member and forming a potential subgroup [4,10].

Atypical bovine pestiviruses may have been more widely spread than previously thought with isolates recently identified in Asia and Europe in addition to South America [7,8,10]. A likely scenario is the introduction of these viruses, via importation of infected animals, semen or contaminated vaccines, into herds that are already infected with BVDV-1 or BVDV-2. Dual BVDV infection is only well documented in persistently infected (PI) animals [11,12]. However, little is known about the clinical aspects of disease in cattle that are acutely infected with different pestivirus strains simultaneously. Depending on the host factors and virus strains the clinical manifestation of BVDV infections generally vary from most common asymptomatic or mild to severe, including reproductive failure, poor reproductive performance, increased susceptibility to secondary infections due to the immunosuppressive effect of BVDV, congenital abnormalities, and the birth of PI calves.

The majority of cases of infections with atypical pestiviruses related to 'HoBi' have been reported in cattle [3–8,13], potentially the natural host species, although these viruses have been shown to infect other members of the group *Artiodactyla*, including buffalo [6]. The contamination of FBS with BVDV is a common problem [14–16] raising concerns for the safety of vaccines [17] and plasma-derived bio-products [18], and for the validation of diagnostic tests utilizing cell cultures [19]. Genetic and antigenic dissimilarity of novel atypical pestiviruses and limitations of current diagnostic methods to efficiently detect and/or differentiate between the different pestivirus species [20] provokes the search for suitable assays [21,22]. The conventional RT-PCR protocol [23] used by many laboratories to control field samples as well as test batches of FBS used in cell culture propagation has been shown to be insufficient for the detection of several atypical bovine pestiviruses [9].

This work was designed to study the possible outcome of infection with a novel atypical Asian pestivirus in European naïve calves in comparison to BVDV-1 infection. Dual infection was also carried out to simulate the introduction of atypical pestivirus into a cattle herd with concurrent BVDV-1 infection. We looked at the dynamics of infection, the development of immune responses, the possible interference of these different bovine pestiviruses at the level of individual animals, and the performance of BVDV diagnostic assays to detect infection with atypical pestivirus.

2. Materials and methods

2.1. Experimental design

2.1.1. Virus strains

Two pestiviruses were used in this study: an atypical bovine pestivirus strain: Th/04_KhonKaen that was recovered from a serum sample of a dairy calf in Thailand [8,10] and a BVDV-1a strain Horton 916 (Ho916) isolated from an acute fatal outbreak of BVD infection in adult cattle in the UK in 1993 [24]. Both non-cytopathogenic (NCP) viruses were propagated for approximately 6 passages and titrated in foetal bovine turbinate (BT) cells of low passage. The specificity of virus isolation was confirmed by immunoperoxidase assay according to the protocol described below. The contamination of the inocula with other pestiviruses was excluded by sequencing of 5′UTR amplicons generated using the 324/326 primers [23] and using the real-time RT-PCR assays specific for atypical pestiviruses [22] and BVDV-1 [25]. After a freeze–thaw and centrifugation procedure the clarified supernatant was aliquoted into 10 ml inoculum doses and kept at −70 °C until required.

2.1.2. Animals

Twenty clinically healthy Holstein male calves 3–5 months old obtained from a commercial farm were randomly placed in four separate containment units (5 calves per unit) in biosafety level 3 (BSL3) animal facilities. Prior to inoculation, the calves were negative for the presence of virus antigen and specific antibodies by the Herd-Chek BVDV Antigen/Serum ELISA and the IDEXX Antibody ELISA (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland) respectively, which are both routinely used for the detection of BVDV-1 and BVDV-2. No commercial tests are validated or available to detect atypical pestiviruses. The assays used have been reported previously as being able to detect atypical pestiviruses [8,9,26]. Additionally, buffy coats separated from whole blood collected from the calves before the experiment were confirmed negative by routine RT-PCR for the following viruses: BVDV-1, BVDV-2, atypical pestiviruses, bovine leukaemia virus (BLV), bovine herpes virus types 1 and 5 (BoHV-1 and BoHV-5), bovine respiratory syncytial virus (BRSV) and bovine foamy virus (BFV) [22–24].

2.1.3. Experimental design

After a week of adaptation including preventive antibiotic treatment, the calves were divided into 4 groups of 5 calves each: Group I, inoculated with Ho916 (BVDV-1); Group II, inoculated with Th/04_KhonKaen (atypical bovine pestivirus); Group III, inoculated with a mixture of Ho916 and Th/04_KhonKaen. Each animal was inoculated with a dose of 10^5 $TCID_{50}$ of the appropriate virus or viruses in 10 ml inoculum by intranasal (5 ml) and intramuscular (5 ml) routes simultaneously. Group III calves were inoculated with 10^5 $TCID_{50}$ of each strain (corresponding to $10^{8.3}$ and 10^8 BVDV-1 RNA copies and atypical pestivirus RNA copies per ml) in 10 ml inoculum by the same routes. The inocula were back titrated after inoculation. A control group was administered with 10 ml of sterile Eagle's minimal essential medium (EMEM) in the same way as the calves inoculated with viruses. Clinical examinations including rectal temperatures were carried out daily until 45 days after inoculation. The animals were handled and euthanized according to the protocol approved by the

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Ethics Committee for Animal Experiments by the Ministry of Science and Higher Education in Poland (No. 79/2010).

2.1.4. Sample collections

Samples including non- and EDTA-stabilized whole blood and nasal swab samples were collected in vacuum tubes (Greiner Bio-One) and in swab tubes containing 1 ml of viral transport medium consisting of Hank's balanced salts, bovine albumin, L-cysteine, HEPES Buffer and antibiotics (Copan Diagnostics Inc.), respectively, both pre- and post-inoculation days 0, 2, 5, 7, 9, 14, 21, 28, 35 and 42. The total white blood cell counts (WBC), platelets (PLT), lymphocytes (LYM), monocytes and some eosinophil granulocytes (MID) plus neutrophil, eosinophil and basophil granulocytes (GRA) were analysed in EDTA-stabilized blood samples using a Celoscope-AutoCounter AC 920 (Swelab Instrument AB, Sweden) within 2–3 h of collection. Buffy coat was separated by density centrifugation using Bicoll (density 1077 g/ml, Biochrom, Germany) and frozen at −70 °C until tested.

2.2. Virus detection

2.2.1. Virus isolation (VI)

The presence of virus in blood and nasal discharges was tested by virus isolation in bovine turbinate cells (BT) followed by immunoperoxidase (IPX) staining [27]. In brief, 10 μl of freeze–thawed peripheral blood mononuclear cells (PBMC) sample or nasal swab material was placed in quadruplicate wells of a 96-well microplate to adsorb for one hour at 37 °C on one-day-old BT monolayer. Plates were then washed, overlaid with Eagle's MEM supplemented with Tricine, L-glutamine, penicillin–streptomycin (PEST) and 5% gamma-irradiated FBS of Australian origin and free of pestiviruses and specific antibodies (PAA Laboratories GmbH, Pashing, Austria), and incubated at 37 °C in a 5% CO_2 atmosphere for 3–4 days. Following fixation with 20% acetone, the cells were incubated with polyclonal BVDV antiserum (VLA, Weybridge, UK) and subsequently with rabbit anti-cow serum conjugated with horseradish peroxidase (HRP). After adding a solution of 3-amino-9-ethylcarbazole (AEC) and N,N-dimethylformamide (HRP substrate), the plates were examined microscopically for the presence of virus specific staining within the cytoplasm. Mock-inoculated BT cells served as the negative control for IPX staining.

2.2.2. Antigen detection

The HerdChek BVDV-Ag/Serum Plus ELISA (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland) was used to detect BVDV E^{rns} glycoprotein in serum and nasal swab samples. The manufacturer described the kit as 100% specific and 100% sensitive and that it efficiently detects BVDV-1 as well as BVDV-2 strains, and has also been shown to detect atypical pestiviruses [8,9]. The test was performed according to the manufacturer's instructions. All samples with a corrected optical density (COD) values equal to or above 0.3 were regarded as positive.

2.2.3. Real-time RT-PCR

Total RNA extraction was performed using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Two different real-time RT-PCR assays were used to detect atypical bovine pestiviruses [22] and BVDV-1 [25]. Both assays were performed in a Rotor-Gene 3000 real-time PCR machine (Corbett Research) using the one-step QuantiTect Probe RT-PCR kit (Qiagen, Hilden, Germany) in a reaction containing 2 μl of RNA (corresponding to 20 μl of unpurified sample), 0.6 μM of each primer and 0.2 μM of the TaqMan probe. The RT-PCR cycling profile included RNA transcription at 50 °C for 30 minutes (min), activation of DNA polymerase at 95 °C for 15 min and 45 cycles of 95 °C for 20 seconds (s), 60 °C for 60 s. Positive and negative extraction controls and RT-PCR controls were included in each run. The RT-PCR efficiency and sensitivity were controlled using serial dilutions of either a Th/04_KhonKaen cRNA standard for the atypical bovine pestivirus RT-PCR or a NADL cRNA for the BVDV-1 specific RT-PCR using methods described previously [22]. The atypical bovine pestivirus RT-PCR limit was 200 viral RNA copies per reaction and for the BVDV-1 specific RT-PCR 2 viral RNA copies per reaction.

2.3. Serological testing

2.3.1. Antibody ELISA

Serum samples were tested for antibodies against BVDV with the indirect HerdChek® BVDV Antibody ELISA (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland) according to the manufacturer's instructions. This test was initially validated for BVDV-1 and BVDV-2 infections although subsequent studies have found that this assay also detects antibodies to atypical pestivirus [26]. The samples with COD values equal to or above 0.3 were classified as positive.

2.3.2. Virus neutralization test (VNT)

To quantify neutralizing antibodies against homo- and heterologous BVDV strains, sera were tested against BVDV-1d strain DK258 in MDBK-cells [28] and Th/04_KhonKaen strain in BT cells. Triplicate of twofold dilutions of heat-inactivated sera (from 1:10 up to 1:1280) was incubated with approximately 100 $TCID_{50}$ of each virus strain at 37 °C for 1 h. Cells were then added and further incubated at 37 °C in an atmosphere of 5% CO_2 for 3–4 days. The cells were fixed and stained using the IPX protocol described above. The neutralizing titres were calculated using the Spearman–Kärber method.

2.4. Statistical analysis

The results were expressed as mean values ± SEM. A one-way ANOVA test was used to analyse the data. Statistical significance between groups was determined by repeated measures ANOVA followed by Tukey's HSD (Honestly Significant Difference) and Dunnett's test. Tukey's test, based on the studentized range statistic, calculates a critical value to evaluate the significance of the difference between two pairs of means which is then compared to the Tukey critical value. If the obtained difference is larger than the Tukey value, the comparison is significant. The

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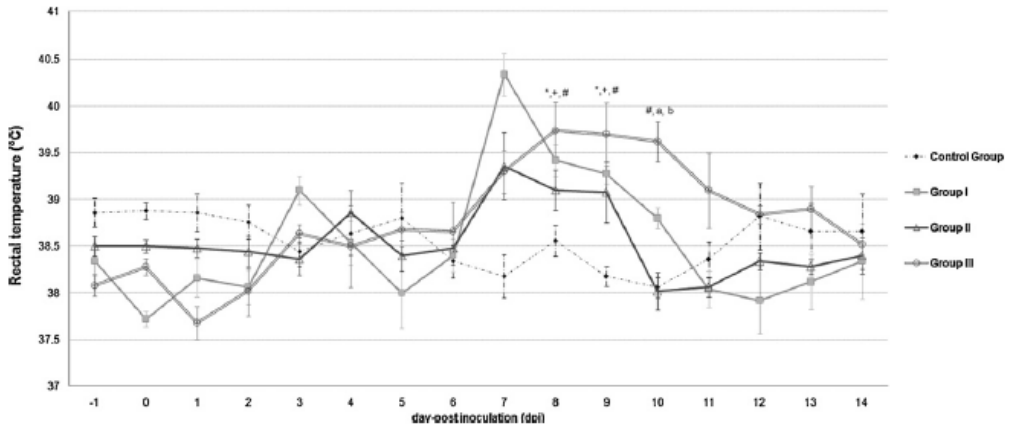

Fig. 1. Kinetics of mean rectal temperature measurements (°C) in calves inoculated with BVDV-1 strain Ho916 (group I); Th/04_KhonKean (Group II); mixture of both viruses (Group III); and uninfected control group (control group). The vertical bars represent standard error of mean (SEM). Statistically significant variation ($p < 0.05$) between Group I (*), Group II (+) and Group III (#) with respect to control group was analysed using two-sided Dunnett's test, while the differences between all the groups of inoculated calves (a, I versus III; b, II versus III; c, I versus II) were studied using Tukey (HSD) test.

Dunnett's test was used for comparison of virus inoculated groups with the mock-inoculated, control group. Differences were considered statistically significant when the p value was below 0.05.

3. Results

3.1. Clinical and haematological observations

The animals in the control group remained clinically healthy and had no fever throughout the study period. In all BVDV exposed calves bilateral conjunctivitis (pink eye), mucous, ocular and nasal discharge and cough of moderate intensity were the first signs of disease observed at 7 days post-inoculation (dpi) which correlated with the increase of average body temperature (Fig. 1). In Groups I and II, peak pyrexia was observed at 7 dpi lasting 2–3 days with the Group II temperatures remaining lower than Group I calves. In Group III a prolonged fever (lasting 5 days) was observed. The clinical signs observed in Groups I and III included moderate watery diarrhoea, depression, slight dehydration, wet and spontaneous cough and dyspnoea between 7 and 14 dpi, coupled with bilateral mucous to mucopurulent nasal and ocular discharge that lasted until 3 weeks post infection. The clinical manifestation of the disease in animals in Group II was slightly milder, without diarrhoea, and limited to bilateral moderate conjunctivitis, watery-mucoid nasal and ocular discharge, occasional dry coughing and slight depression for around two weeks after the inoculation. All the calves from the three BVDV exposed groups developed skin trichophytosis after 28 dpi which was treated with enilconazole solution wash (Imaverol, Janssen Pharmaceutica NV, Beerse, Belgium) to ease animal discomfort.

The blood cell counts in the control group remained unchanged with the level of fluctuation decreasing with time as the calves acclimatized to the environment and sampling procedures. A biphasic decrease of WBC between 2 and 5 dpi and again at 14 dpi (Fig. 2A) was connected to the decrease in lymphocyte (LYM) numbers (Fig. 2B) in Group I and Group III. The mean minimum WBC for those groups was 6.7×10^9/l (decrease of 46.5% from the pre-inoculation value) recorded at 5 dpi and 6.5×10^9/l at 7 dpi (decrease of 41.0%), respectively. The mean LYM dropped to the minimum value of 3.6×10^9/l (decrease of 49.3% from the pre-inoculation values) at 5 and 7 dpi, and 3.4×10^9/l (decrease of 43.3%) at 5 dpi, respectively. Leukopenia correlated to lymphocytopenia was also observed in the Group II calves; however animals started recovering earlier than in Group I, reaching the levels comparable to the control group at 14 dpi.

The platelet counts showed only minor fluctuations within normal range in and between the groups, except for 7 dpi when the decrease in PLT values in Group II and Group III was statistically significant ($p < 0.01$) with respect to the control group. Similarly significant decrease in PLT value was maintained in Group III until 9 dpi. The monocyte counts (MID), neutrophils and granulocytes (GRA) as well as the other haematological parameters remained stable within normal ranges during the experiment in all animal groups (figures not shown).

3.2. Viraemia and virus shedding

Blood and nasal swabs samples assessed for virus presence were negative in all calves prior to inoculation and remained negative in the control group during the experiment. Virus isolation (VI) in BT cells was positive from buffy coats collected from Group I calves at 5 and 7 dpi and from Groups II and III between 5 and 9 dpi (Table 1). Virus isolation was possible in nasal swabs for a short duration from Groups I and II between 5 and 7 dpi, while in Group III virus shedding lasted until 9 dpi.

Detection of BVDV by antigen ELISA in serum was comparable to virus isolation (Table 1). Antigen was detected in

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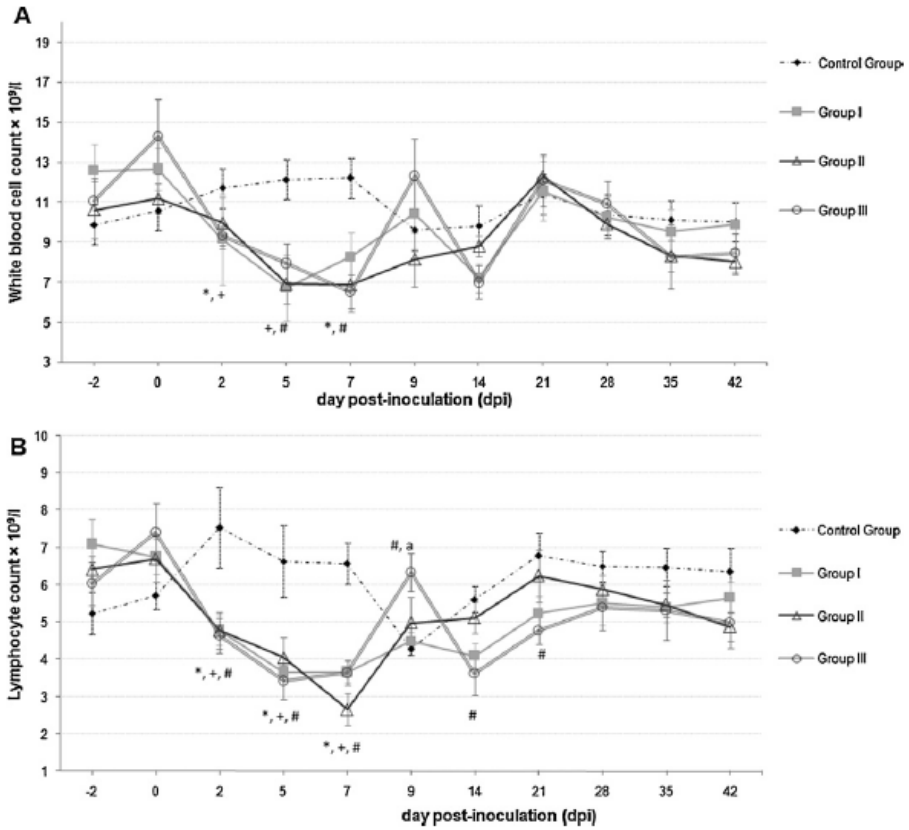

Fig. 2. Mean values of total white blood cell count (WBC) (A) and lymphocyte count (LYM) (B) in uninfected calves (control group) and experimentally infected with BVDV-1 strain Ho916 (Group I), Th/04_KhonKean (Group II) and mixture of both viruses (Group III). The error bars indicate ±SEM. Statistically significant variation (p-value < 0.05) between Group I (*), Group II (+) and Group III (#) with respect to control group were analysed using two-sided Dunnett's test, while the differences between all the groups of inoculated calves (a, I versus III; b, II versus III; c, I versus II) were studied using Tukey (HSD) test.

the blood of Groups I, II and III calves as early as 5 dpi with a peak at 7 dpi, except for one animal from Group II which was negative at 9 dpi (COD = 0.09) (Fig. 3). While no significant differences were found in the COD values (ranging from 2.4 to 3.5) between Groups I and III, the COD values in serum of calves from Group II were significantly lower ($p < 0.0001$) between 0.7 and 1.9 at 7 dpi. The antigen ELISA COD values for nasal swabs were slightly elevated between 5 and 9 dpi, but were still below the cut off in all inoculated groups.

The results of strain specific real-time RT-PCR for blood and nasal swabs tested in all groups are presented in Fig. 3. Viral RNA was detected in blood of Group I calves two days after the inoculation and remained in some calves until 28 dpi. Atypical pestiviral RNA was detected in blood of Group II calves from 5 dpi until 9 dpi with the exception of two calves which remained positive in real-time RT-PCR until 28 dpi. Interestingly, the blood of the calves from Group III was positive for viral RNA of both viruses between 2 and 28 dpi. BVDV-1 RNA was present in nasal swabs of inoculated animals between 5 and 14 dpi and again in one animal at dpi 21, while RNA from atypical BVDV was detected from 2 until 21 dpi.

3.3. Serological findings

Antibody ELISA testing (Fig. 4) revealed the presence of BVDV antibodies in all animals from Groups I and III starting from 21 dpi, while only two out of five calves from Group II were positive on that day. The rest of animals became serologically positive at 28 dpi.

None of the samples from the control group gave positive results in the VNT and Ab ELISA. Group I calves (Fig. 4A) seroconverted against homologous BVDV-1 at 14 dpi with a mean titre of 15.6 which increased with time, reaching a mean VN titre of 601 at the end of the experiment. The mean antibody titres against Th/04_KhonKaen virus (Fig. 4B) in sera of calves infected with the homologous strain rose from 54 at 14 dpi to 1763 at 42 dpi. The titres calculated for those groups in VNT using heterologous strains were significantly lower ($p < 0.05$). The calves from Group I seroconverted to atypical pestivirus at 21 dpi and later

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Table 1
Results of BVDV isolation in BT cells from PBMC and Ag ELISA of serum from individual animals of BVDV infected groups. Positive results in Ag ELISA are indicated by grey shading, while positive results in Ag ELISA in serum and positive results in VI in buffy coat were marked by diagonal lines. The numbers apply to individual COD values in Ag ELISA. ✖ indicate the positive result of VI in BT cells from nasal swabs.

Group	Animal ID	Day post-inoculation (dpi)						
		0	2	5	7	9	14	21 - 42
I	6	0.07	0.06	0.86	2.89 ✖	0.83	0.07	0.06
	7	0.07	0.06	1.19 ✖	3.51 ✖	2.17	0.08	0.06
	8	0.06	0.07	0.28	2.74 ✖	0.74	0.08	0.07
	9	0.06	0.07	1.90	2.39 ✖	0.74	0.09	0.08
	10	0.06	0.07	0.52	3.02 ✖	0.25	0.07	0.07
	11	0.06	0.06	1.01 ✖	1.90 ✖	0.12	0.07	0.07
II	12	0.06	0.06	0.55	1.71 ✖	0.30	0.07	0.08
	13	0.08	0.07	0.43 ✖	1.98	1.01	0.08	0.07
	14	0.07	0.06	0.24	0.73	0.32	0.07	0.07
	15	0.06	0.06	0.25	0.93	0.09	0.06	0.07
III	16	0.06	0.07	1.02	3.20 ✖	1.31 ✖	0.09	0.08
	17	0.06	0.08	0.48 ✖	2.96	1.32	0.09	0.09
	18	0.06	0.07	0.79	2.96 ✖	1.92 ✖	0.09	0.06
	19	0.07	0.07	1.45	3.33 ✖	0.91	0.07	0.06
	20	0.07	0.07	1.00	2.92 ✖	1.23	0.10	0.07

fluctuated from below the detection limit at 28 dpi, increasing at 35 dpi to a mean of 112 and again decreasing to mean titre of 27 at 42 dpi. In the case of the Group II calves, only the 42 dpi sera cross-neutralized BVDV-1 strain at a mean titre of 17. The calves inoculated simultaneously with both viruses (Group III) seroconverted to both strains at 14 dpi, reaching the highest mean titres of 504 against BVDV-1 and 1354 against Th/04_KhonKaen virus at 42 dpi.

4. Discussion

In the present study, single infection of animals with atypical bovine pestivirus Th/04_KhonKaen strain resulted in mild respiratory disease and transient pyrexia which is consistent with previous experimental studies using D32/00_'HoBi' virus [3,29]. In contrast, the BVDV-1-infected calves developed a biphasic pyrexia with a more severe disease including watery diarrhoea, albeit a less severe clinical presentation than observed in the original field infection [24] which is routinely reported [30,31]. Th/04_KhonKaen was less pathogenic than Ho916, but clearly has the potential to cause clinical disease comparable to most BVDV strains which cause asymptomatic or mild clinical infections in cattle in field conditions [32]. The severity of illness is usually enhanced in calves deprived of passive immunity and/or when other pathogens are involved [33,34]. Severe respiratory disease in calves infected with Hobi-like pestivirus in Italy with mortality caused by bronchopneumonia in 8% of cases was recently described [7]. In the dual infection group, calves had clinical presentation similar to that of single BVDV-1 infection, thereby making any possible synergistic effect unlikely.

Leukopenia which is a representative element of BVDV immune evasion responsible for increased susceptibility of BVDV infected cattle to secondary infections [33,34] was observed in this study in all inoculated groups at a similar level. Under the assumption made previously (reviewed by Kapil et al. [35]) that BVDV virulence is associated with the degree of leukopenia, we may conclude that Th/04_KhonKaen virus impairs the immune system at a similar level to the Ho916 strain. Close correlations between the presence of virus in blood and leukocyte decline were observed in all inoculated groups. Thrombocytopenia, which is reported to be more common in the infections caused by specific BVDV-2 strain [36], was observed temporarily in calves inoculated with Th/04_KhonKaen (Group II) and in animals inoculated with the mixture of viruses (Group III).

Similar kinetics of viraemia was observed in both Th/04_KhonKaen and Ho916 infected groups. Viral antigen

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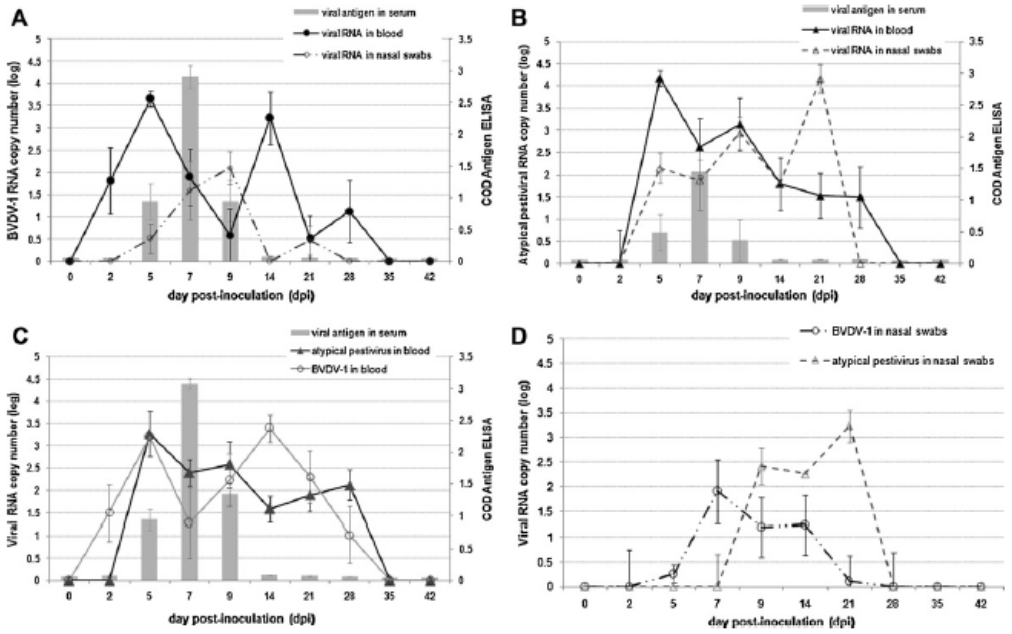

Fig. 3. Kinetics of detection of viral RNA in blood (PBMCs) and nasal swabs by BVDV-species-specific real-time RT-PCR [22,25] and BVDV antigen in serum by HerdChek BVDV Ag ELISA (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland). The values correspond to the log transformed mean of viral RNA copy numbers per reaction and mean corrected optical densities (COD). The results are summarized by groups: I, Ho916 infected calves (A); II, Th/04_KhonKaen infected calves (B); II, calves co-infected with Ho916 and Th/04_KhonKaen (C for blood and D for nasal swabs). The error bars indicate ±SEM. The sensitivities of the real-time RT-PCR were 200 copies per reaction set using Th/04_KhonKaen cRNA [22] and 2 copies per reaction set using BVDV-1 standard with specific primers and probe [25] in our experimental conditions. The values of COD of antibody ELISA > 0.3 were considered positive (according to the manufacturer's instruction).

was detected in all groups between 2 and 7 dpi using the HerdChek BVDV Antigen/Serum ELISA. However the COD values for the calves inoculated only with Th/04_KhonKaen were significantly lower than expected. The same antigen ELISA kit permitted the identification of a calf in a herd in Thailand [8] from which Th/04_KhonKaen virus was recovered previously [10]. This antigen ELISA has been developed and validated for the detection of BVDV-1 and BVDV-2, and so the low COD levels obtained when analysing serum samples from Th/04_KhonKaen infected cattle may indicate a reduced ability of this diagnostic assay to reliably detect atypical pestivirus. However, considering that the antigen ELISA is developed rather for PI detection, the performance of the test in detecting atypical pestivirus can be enhanced when antigen concentration is higher.

While detection of the virus by antigen ELISA and virus isolation test seemed to be impaired by neutralizing antibodies in the late stages of infection, specific viral RNAs were detected by the highly sensitive real-time RT-PCR assays until 28 dpi in calves infected with Th/04_KhonKaen virus and in the groups with mixed infection and until 21 dpi in calves infected with Ho916. The viraemia was followed a few days later with nasal shedding in all inoculated groups what shows the potential for transmission via the oro-nasal route. In previous studies with BVDV-1 Ho916 strain, 'in contact' animals became infected probably through transmission of virus from infected animals via the oral route [Dr. R. Strong, unpublished data]. The significantly more pronounced higher levels of Th/04_KhonKaen RNA in the upper respiratory tract compared to Ho916 suggest the potential for a greater transmission rate of this atypical pestivirus to spread via the direct contact with acutely infected animals. All inoculated groups of calves in this study seroconverted against homologous strains on 14 dpi. Seroconversion was correlated to the clearance of the virus from blood and the termination of virus nasal shedding. The detection of the antibodies, however, was delayed by a week in the indirect antibody ELISA in Groups I and III, while in Group II calves, infected only with Th/04_KhonKaen virus, antibodies were not detected until four weeks post-inoculation. Other studies with atypical pestivirus have also raised questions regarding the specificity of commercial antibody ELISAs to accurately detect antibodies to these novel pestiviruses. Decaro et al. [29] found positive antibody response in calves experimentally inoculated with the Italian atypical pestivirus at 14 dpi simultaneously in VNT and ELISA, but the values were just above the cut-off. Seroconversion post intra-uterine infection of cows with D32/00_'HoBi' was delayed until between 27 and 41 dpi and this was proposed to be potentially due to the route of infection and also the sensitivity of the commercial ELISA test used [26]. The antibodies against atypical pestivirus were

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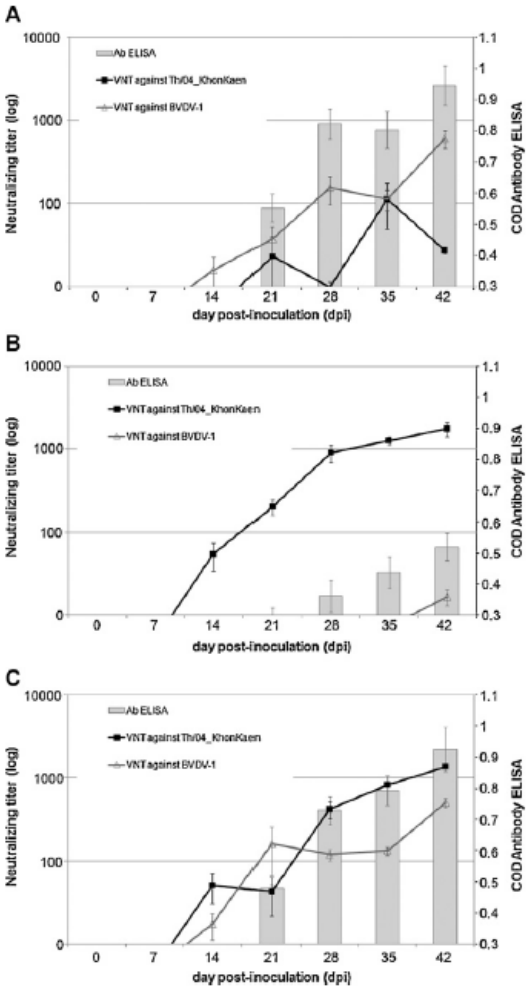

Fig. 4. Dynamics of antibody response measured by indirect Ab ELISA (IDEXX Switzerland AG, Liebefeld-Bern, Switzerland) and virus neutralization tests (VNT) in calves from Group I (A), Group II (B), and Group III (C). The values correspond to means calculated for corrected optical density (COD) in Ab ELISA and means of antibody titres calculated using Spearman–Kärber method. The error bars indicate ±SEM. The axis minimum values were set on the vales of corresponding threshold of the test used: for Ab ELISA 0.3 COD and for VNT serum dilution 10.

detected even later or were missed when tested in milk samples. The reduced ability of available antibody ELISAs to either detect or accurately quantify levels of antibodies to atypical pestiviruses highlights the need for either the validation and/or development of tests for these novel pestiviruses.

The serological cross-neutralization between BVDV-1 and atypical bovine pestiviruses is rather limited which may possibly complicate serological diagnosis and cause failure of available BVDV vaccines against these novel viruses. However, the differences in neutralizing properties enable also the typing of pestivirus strains circulating in the herd [3,8,9]. VNT results however showed rather limited cross-neutralization between BVDV-1 and atypical bovine pestiviruses. The BVDV-1 antibodies were detected in inoculated calves only just at the end of experiment at 42 dpi, while the Th/04_KhonKaen neutralizing antibodies were detected in Ho916 animals first at 21 dpi, however later fluctuated significantly between the detection limit up to the mean of 111.

Co-infection with two BVDV strains resulted in simultaneous Th/04_KhonKaen and Ho916 viraemia showing that BVDV-1 and atypical bovine pestivirus can multiply in individual animals at the same time. To the best of our knowledge, this is the first study describing dual infection with BVDV-1 and atypical bovine pestivirus. The long term importance of the interplay between the BVDV strains may be different as the nasal virus excretion from BVDV-1 infected calves was reduced compared to Th/04_KhonKaen in both single and mixed infections. Dual acute infections with BVDV have not yet been studied to a great extent. In previous studies, infection of pregnant heifers with a mixture of BVDV-1 and BVDV-2 strains led to the birth of seronegative calves persistently infected (PI) with both BVDV species [37]. In contrary, another experiment [38] showed that most cases of dual transplacental infections resulted in systemic infection of newborn calves with only BVDV-2 strain, while BVDV-1 was cleared. In a study by Nuttall et al. [39] the cytopathogenic NADL strain inhibited the replication of the co-infecting non-cytopathogenic pestivirus from FBS resulting in less severe disease. The mechanism was described in vitro as superinfection exclusion when one BVDV strain was inhibiting another BVDV strain by blocking its cell entry and RNA replication [40]. In our study the co-infection of calves with two different BVDV species resulted in undisturbed replication of the viruses. Successful dual infection with BVDV-1 and atypical pestivirus without signs of mutual suppression may indicate that the viruses may use different receptors or that the pathways of the infection are regulated independently. This requires further investigation. Whether natural co-infections with different BVDV strains occur has not yet been demonstrated, however coexistence of different genetic and antigenic BVDV variants is described in epidemiological studies [41,42]. Of concern is the potential of another bovine pestivirus being introduced into the cattle population with circulating BVDV-1 or BVDV-2 which has also been described previously [8,17]. The potential of emergence of recombinant viruses through dual acute infection should also be considered in the future [11].

In conclusion, Asian atypical bovine pestivirus in an experimental study setting caused mild clinical signs in European naïve calves resembling most acute BVDV infections. The kinetics of infection including the degree of leukopenia was very similar to the effects of infection with European BVDV-1 strain which originated from a severe outbreak in adult cattle. We believe that atypical bovine pestiviruses present considerable risk to the cattle health and management, considering the growing evidence of their occurrence in geographically distant cattle populations and the existence of the diagnostic gap in current detection methods.

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APPENDIX B: MEETINGS, PRESENTATIONS AND TRAINING COURSES

MEETINGS AND PRESENTATIONS

5th Annual Epizone (European Network of Excellence of Epizootic Disease Diagnosis and Control) Meeting, Arnhem, The Netherlands – 11th - 14th April 2011 - Oral presentation (IC 6.8 project meeting) and Poster presentation (cited)

Festival of Research, University of Surrey, Guildford, Surrey, UK – 5th July 2011 - Poster presentation

Festival of Research, University of Surrey, Guildford, Surrey, UK – 6th July 2010 - Poster Presentation (Received best poster prize in Infection and Immunity theme)

5th ENII EFIS/EJI Summer School in Advanced Immunology, Capo Caccia, Sardinia, Italy. – 9th -16th May 2010 - Poster presentation

Research Student Day, Royal Dick School of Veterinary Studies, R (D) SVS, University of Edinburgh, Edinburgh – 1st April 2009 – Poster presentation

TRAINING COURSES AND WORKSHOPS

The Viva Examination, Postgraduate Skills Development Programme (PGSDP), University of Surrey, 20th March 2012

Project Management course, Wagenigen Business School, Amsterdam, The Netherlands - 27th – 28th September 2011

Epizone Short Term Mission, National Veterinary Institute, Puławy, Poland, 16th August – 17th September 2010

Getting Published, PGSDP, University of Surrey, 21st May 2010

5th ENII EFIS/EJI Summer School in Advanced Immunology, Capo Caccia, Sardinia, Italy. – 9th -16th May 2010

Academic Writing: Reporting on Your Progress, PGSDP, University of Surrey, 9th March 2010

PhD Project Management, ULearn, University of Surrey, March 2010

Good Practice in PhD Research , College of Medicine and Veterinary Medicine, University of Edinburgh, March 2009

Postgraduate Essentials, Transkills, University of Edinburgh, October 2008