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# Characterization of recombinant BVDV-2 vaccine prototypes based on packaged replicons and replication competent deletion mutants

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Meiner Familie

„quo fata ferunt“

(Bermuda)

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# 1. Introduction

*Bovine viral diarrhea virus* (BVDV) is grouped in the genus *Pestivirus* in the family *Flaviviridae* (Mayo, 2002). BVDV was first described in the United States, where it was isolated as the causative agent of a diarrhea in cattle in 1946 (Olafson et al., 1946). It has a single-stranded RNA genome of positive orientation, approximately 12.4 kb in size, which codes for structural and non-structural proteins. Two species, BVDV-1 and BVDV-2, are delineated (Ridpath et al., 1994; Harpin et al., 1995) due to marked genetic and antigenic differences. Within both, there are two biotypes, cytopathic (cp) and noncytopathic (ncp), characterised by their effect on cultured cells (Lee and Gillespie, 1957). BVDV occurs worldwide and despite the development of different vaccines and eradication programs, it still causes pronounced economic losses in the cattle industry.

Most infections are subclinical. Animals with clinical manifestations show respiratory, gastrointestinal or reproductive symptoms. Infection of seronegative cows during pregnancy can result in diverse disorders and, as a special feature of this disease, in the generation of persistently infected (PI) offspring when the dam is infected with a non-cytopathic strain of either species during the first 120 days of pregnancy (Moennig and Liess, 1995). These BVDV carriers can be inconspicuous at birth but continuously shed high amounts of virus and are the most important factor in virus spread (Houe, 1999).

Therefore, the primary aim of BVDV vaccination is to prevent fetal infection and the birth of BVDV carriers. To be an effective tool in BVDV control, a vaccine must fulfill high requirements (Beer et al., 2000; Fulton et al., 2003), and no currently commercially available vaccine meets all of them. Several modified-live virus (MLV) vaccines and inactivated vaccines are licensed for use in Germany. Modified-live virus vaccines are considered efficacious in inducing protective immunity, but their safety concerning viremia and vaccine virus shedding is a matter of controversy. The available inactivated vaccines are safe, but their efficacy is not satisfactory. To control BVDV infections, there is a need for better, safer vaccines. Different attempts using genetically modified variants seek to combine advantages while diminishing disadvantages. In the work presented here, two approaches for attenuated and efficacious BVDV-2 mutants derived from a recently constructed BVDV-2 full-length cDNA clone (Mischkale et al., 2009) were tested *in vivo*. Newly generated virus derived from the full-length clone was also tested for virulence compared to the parental wild type strain.

# 2. Bovine viral diarrhea virus (BVDV) - Literature review

## 2.1. Taxonomy and molecular characteristics

The family of *Flaviviridae* comprises three different genera: *Flavivirus*, *Hepacivirus* and *Pestivirus*. Four species are included in the genus *Pestivirus* (Mayo, 2002) which are non-zoonotic animal pathogens: Classical swine fever virus (CSFV), Border disease virus (BDV), and BVDV-1 and BVDV-2. Liu et al. (2009) suggested a new classification, including the introduction of a third BVDV species, BVDV-3, encompassing atypical bovine pestiviruses. Subgroups were often described but the significance is a matter of debate. European BVDV-1 strains have been divided into 11 subgroups (Vilcek et al., 2001), while BVDV-2 strains were segregated into only 2 subgroups (Becher et al., 1999a; Flores et al., 2002) namely BVDV-2a and -2b.

All members of the *Flaviviridae* family have a genome of approximately 12.4 kb in conserved organization with one open reading frame (ORF) flanked by untranslated regions (UTR) at the 5' and 3' end. The structural proteins are encoded in the 5' region while the non-structural genes lie at the 3' end. BVDV replicates in the cytoplasm. The viral proteins are translated into one single polyprotein, and co- and post-translational cleavage by viral and cellular proteinases is necessary (Collett et al., 1988; Lackner et al., 2004) (Fig.1). The order of the individual proteins is as follows: $N^{pro}$ – C – $E^{RNS}$ – E1 – E2 – p7 – NS2/3 – NS4a – NS4b – NS5a – NS5b.

Unique to the pestiviruses are the $N^{pro}$ and the $E^{RNS}$ proteins (Ridpath and Bolin, 1995, 1997). $N^{pro}$ functions as an autoproteinase and $E^{RNS}$, a glycoprotein of the envelope, has an intrinsic RNase function (Schneider et al., 1993; Hulst et al., 1994).

The spherical BVDV virions (40 to 60 nm in diameter) have a lipid envelope derived from the host cells. This envelope makes them susceptible to detergent and solvent inactivation.

BVDV-1 and -2 have different antigenic and genetic profiles (Ridpath et al., 2000; Fulton et al., 2003). This pronounced variation has an impact on BVDV detection and control. Today, comparing the sequence of the 5'UTR region is a widely used method for classification as this is the best-conserved region of the pestivirus genome (Becher et al., 1997; Ridpath et al., 2000; Beer et al., 2002), followed by $N^{pro}$, parts of the $E^{RNS}$ and E1 protein. Differentiating PCR-tests target its two variable regions. The most variable protein in the ORF is E2, BVDV's major immunogen (Donis et al., 1988; Bolin and Ridpath, 1989).

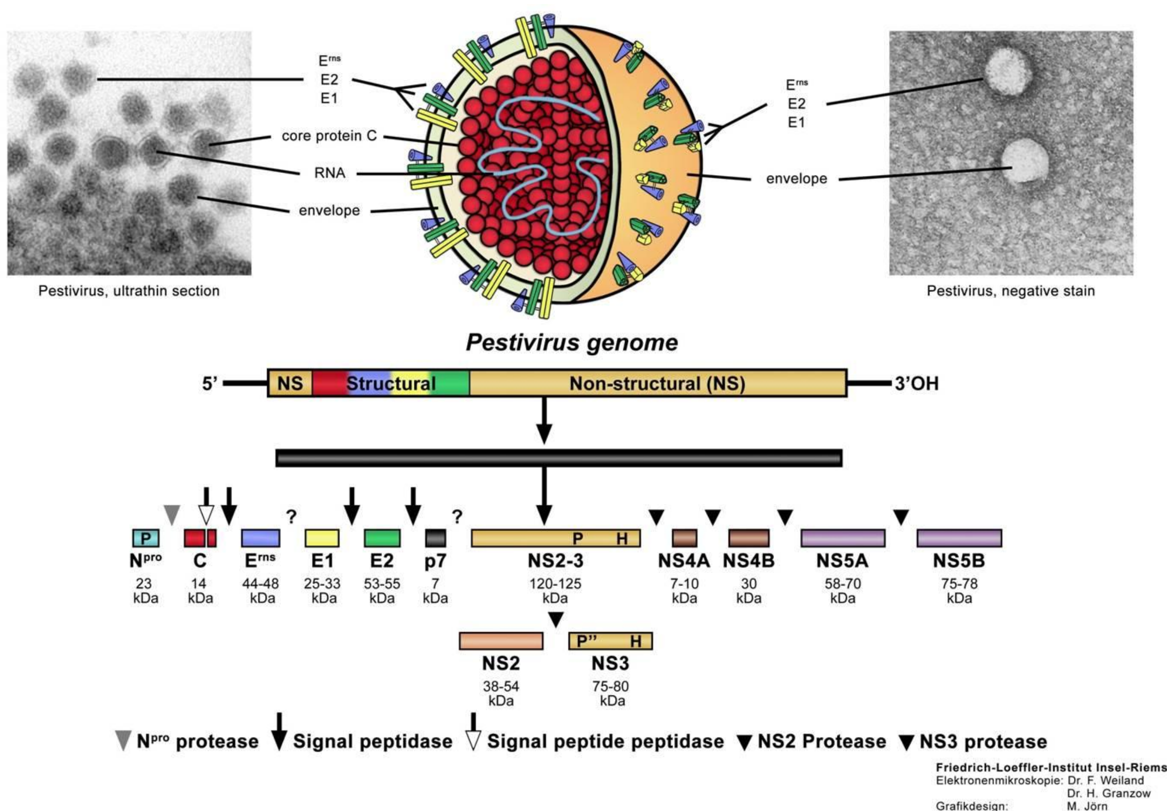


Fig.1 Virion morphology of BVDV, genome organization, co/post-translational cleavage sites and mechanisms of the single proteins (source: H.Granzow; FLI; Insel Riems).

Both species can be further subdivided into two biotypes – cytopathic (cp) and noncytopathic (ncp) – based on the phenotype when propagated in cell culture (Lee and Gillespie, 1957; Gillespie et al., 1960). Cytopathogenicity does not correlate with a strain's virulence *in vivo*, as most if not all strains of high virulence are ncp isolates (Corapi et al., 1990; Carman et al., 1998; Ridpath et al., 2000; Liebler-Tenorio et al., 2002). Furthermore, cp BVDV amplifies viral RNA at pronouncedly higher levels than the ncp counterparts (Kümmerer and Meyers, 2000; Glew et al., 2003).

The single-stranded positive-sense RNA genome is prone to mutations as the RNA-dependent RNA polymerase has no proofreading function. This leads to genetic drift and in time to the development of different genotypes. In RNA viruses, strong replication with high error rates creates a virus population resembling an inhomogenous cloud of mutants, "quasispecies" (Eigen, 1993) grouped around a most frequent sequence (Becher et al., 1999b; Moya et al., 2000). This is one aspect of viral immunoevasion (Bolin et al., 1991). On the other hand, a stabilization of the genome has been described in PI animals (Hamers et al., 1998, 2001) and for herd-specific strains (Paton et al., 1995).

Receptor-mediated endocytosis involving cell surface proteins (heparin surface proteoglycans and low density lipoprotein receptor [LDLR]; Iqbal et al., 2000; May et al., 2003) and viral

envelope proteins ($E^{RNS}$ and E2) leads to binding of and entry into the host cell (Agnello et al., 1999). Viral RNA is unpacked in the cytoplasm, where it immediately acts as mRNA directing the translation of viral proteins, initiated by the internal ribosome entry site (IRES) in the 5' UTR that mediates binding of the correct initiation codon to the ribosome (Pestova et al., 1998; Pestova and Hellen 1999). Recruitment of cellular factors is necessary for a successful translation of BVDV polyprotein. After initial translation a stem loop formation at the far end of the 5' UTR switches the viral RNA from mRNA to a template for RNA replication (Behrens et al., 1998; Li and McNally, 2001) by the replicase complex (assembled nonstructural viral proteins and cellular components). Maturation takes place in intracellular vesicles and only mature particles are released by exocytosis (Heinz et al., 2000; Grummer et al., 2001).

Untranslated regions (UTR) at the 5' and 3' ends: The well-conserved 5'UTR serves as internal ribosome entry site (IRES) that mediates ribosomal attachment to the translation initiation codon. The 3' UTR encodes for critical replication structures initializing e.g. negative strand synthesis (Yu et al., 1999; Fields et al., 2001).

The single proteins C, $E^{RNS}$, E1 and E2 are structural components of mature virions. The Capsid protein (C) forms the capsid, packing the RNA, and is relatively conserved. Heinz et al. (2000) state that it codes for an internal signal sequence directing translocation of the structural glycoproteins to the endoplasmatic reticulum. Glycoprotein $E^{RNS}$, as a part of the lipid envelope, has ribonuclease activity and forms disulfide-linked homodimers sometimes associated with E1E2 heterodimers. It can also be found in a free, soluble form in infected cells *in vitro* (Rümenapf et al., 1993). Neutralizing antibodies (nab) against $E^{RNS}$ are formed upon infection (König et al., 1995) but their role in disease control is still a matter of controversy. $E^{RNS}$ can be found as a precursor protein together with E1 ($E^{RNS}$-E1), which is stable but not essential for virus formation (Wegelt et al., 2009).

Glycoproteins E1 and E2 are components of the viral envelope as well and form heterodimers (Weiland et al., 1990). E2 is the immunodominant protein of BVDV with several neutralizing epitopes, and its high antigenic variation can contribute to vaccine failure (Bolin and Ridpath, 1989; Ridpath et al., 2000; Van Campen et al., 2000). Cleavage of structural proteins is mediated by cellular signal peptidases (Rümenapf et al., 1993; Elbers et al., 1996; Wegelt et al., 2009).

The first non-structural protein in the ORF is $N^{pro}$ that functions as an autoprotease and as an inhibitor of the host's innate immune system (Hilton et al., 2006; Ruggli et al., 2003, 2005). The role of the small p7 protein in the replication and assembly process of BVDV is still

putative, but it is discussed as viroporin in other members of *Flaviviridae* (Hepatitis C virus; Cook and Opella, 2009) and in BVDV (Reimann et al., 2009). NS2/3 has a serine protease function residing in the NS3 portion, and cleaves the nascent non-structural proteins from the polyprotein. Its additional roles as RNA helicase and RNA-activated NTPase (Warrener and Collett, 1995) are essential for viral viability (Gu et al., 2000). Antibodies against NS2/3 or NS3 are formed but have no neutralizing abilities. NS4a acts as co-factor for the serine protease function of NS2/3. NS4b and NS5a are suspected to play a role in the replicase complex, but the exact functions are unknown. NS5a interacts with cellular elongation factor 1 α which also binds to the secondary structure of the 3' UTR. This may be used for correct positioning and/or orientation of the RNA template for replication. NS5b provides the RNA polymerase function for viral replication (Lai et al., 1999).

## 2.2. Distribution, economical aspects and control programs

BVDV is a global pathogen of cattle. Both species have been reported in Europe (van Rijn et al., 1997; Wolfmeyer et al., 1997; Vilcek et al., 2002) and the Americas (Pellerin et al., 1994; Canal et al., 1998; Jones et al., 2001). The prevalence of BVDV antibody positive animals in Germany varies between 42 and 55% while the herd prevalence is clearly higher with 67 to 97% (Schirrmeier; personal communication).

There are noticeable differences in the prevalence of BVDV-2, which was initially isolated in the U.S. and Canada in the early 1990s (Corapi et al., 1989, 1990b; Carman et al., 1998). BVDV-2 isolates are rarely found in Europe (Wolfmeyer et al., 1997; Vilcek et al., 2002; Drew et al., 2002), but comprise 24 to 48% of BVDV isolates in North America (Carman et al., 1998; Bolin and Ridpath, 1998; Fulton et al., 2000a; Ridpath, 2005). Beer and Wolf (1999) identified 6.5% of field isolates (1993-1997), Wolfmeyer et al. (1997) 11% of tested German field strains (1992-1996) as BVDV-2 while a more recent investigation claims 14.3% (Cedillo Rosales S., 2004).

Persistently infected (PI) animals are estimated to comprise up to 2% of the cattle population (Bolin et al., 1985a; Howard et al., 1986; Houe et al., 1995a, 1995b; Beer and Wolf, 1999; Wittum et al., 2001; Moennig und Greiser-Wilke, 2003), depending on the country and situation in the field. Distribution of BVDV-1 and -2 among PI animals found in field studies in the U.S. seems to be equal (Wittum et al., 2001). PI cases are often clustered: while in the majority of herds no persistent infections are present, in some affected herds there are several PI animals (Bolin, 1990).

BVDV causes continued substantial economic losses in the cattle industry worldwide (Duffel et al., 1986; Houe and Heron, 1993; Innocent et al., 1997). Reproductive dysfunctions and losses through abortions, reduced conception rates and birth of weak offspring present the main economic impact of BVDV infection (Kirkbride, 1992; Dubovi, 1994; Moennig and Liess, 1995; Rüfenacht et al., 2001), closely followed by production losses through decreased weight gain and milk production and secondary infections.

The complex pathogenesis and diverse clinical manifestation of BVDV along with its genomic diversity complicate effective control. As one of the first countries, Sweden introduced a national BVDV program in 1993 that is used today as a template for similar programs in many other countries (Moennig and Greiser-Wilke, 2003). In herds, freedom from BVDV is confirmed by bulk milk screening and maintained without vaccination (Bitsch and Ronsholt, 1995). In countries with high BVDV prevalence and intensive trade, the aim should be to minimize infection pressure (Moennig and Greiser-Wilke, 2003), while the identification and elimination of PI animals is of utmost importance in any scenario (Schelp and Greiser-Wilke, 2003).

The reintroduction of BVDV after elimination of PI animals must be prevented by good management and vaccination. In 2004, BVDV was made a notifiable animal disease in Germany (Anonymous, 2004). The "Verordnung zum Schutz der Rinder vor einer Infektion mit dem Bovinen Virusdiarrhoe-Virus" (BVDV-Verordnung) (Anonymous, 2008) was passed in December 2008 and will be implemented from January 2011. This includes the eradication of persistently infected animals detected by compulsory testing, certification of herds without PI animals and protection against BVDV reintroduction by restricting trade to certified non-PI cattle. In addition, vaccination is recommended to avoid the scenario of a seronegative, highly susceptible population.

BVDV freedom in Germany can only be realized in the long term and vaccination plays an essential part in the program by reducing the infection pressure and the number of susceptible animals. To this end, vaccines should contain at least one strain of both species (Fulton et al., 2003).

## 2.3. Transmission and disease

Horizontal transmission over various routes is described as BVDV is shed in most excretions and secretions. Amount and duration of virus shedding and viremia vary, depending on strain virulence (Bolin and Ridpath, 1992) and immune status. Sources are often PI animals and less likely transiently infected cattle (Niskanen et al., 2000). Direct contact, artificial insemination (Paton et al., 1990; Kirkland et al., 1994), natural service (Wentink et al., 1989), embryo transfer and milk are described as routes of infection together with iatrogenic transmission (Lang-Ree et al., 1994; Houe, 1995) and contamination of biologicals (e.g. FCS, vaccines). Vertical transmission plays an important role in keeping the virus in a population. Acute infection of the dam with an ncp strain of either species or vaccination with an MLV vaccine containing an ncp strain in the first 90 days of gestation can lead to persistently infected offspring. Interspecies transmission among domestic and wild ruminant species is also an important aspect for BVDV control programs (Loken, 1995; Nettleton and Entrican, 1995).
BVDV is transmitted primarily through aerosols. Virus progeny created by replication in epithelial cells of the nasal mucosa and draining lymph nodes is spread by circulating lymphocytes. With the blood the virus reaches all other organs causing multiple systemic effects after an incubation period of 5 to 7 days. The majority of infections stays subclinical or mild (Ames, 1986). When clinical disease is manifest, typical signs can be observed: a biphasic pyrexia, leukopenia, anorexia associated with depression, decrease in production (milk, weight gain) (Moerman et al., 1994), reproductive disorders (Archbald et al., 1979; Kirkland et al., 1994; Kafi et al., 2002), as well as respiratory and intestinal illness (Bolin and Ridpath, 1992; Bruschke et al., 1998; Hamers et al., 2000; Stoffregen et al., 2000; Liebler-Tenorio et al., 2002, 2003a, 2003b). Highly virulent strains can spread to the bone marrow (infecting myeloid cells and megakaryocytes), and often show a wider distribution later in the infection. Infections with ncp strains in general are cleared later than cp virus (Spagnuolo-Weaver et al., 1997). Recovery and repair of lesions may take up to two weeks, even longer for more virulent strains (Liebler-Tenorio et al., 2002, 2003b).
Two patterns are observed: acute infection varying in severity with a transient virus shedding in immune-competent animals and possible transplacental transmission and persistent infection of the fetus. Species, biotype and virulence of the strain determine the outcome of infection as do immune status and competence, overall health and age of the host.
A particularly severe form of acute BVD (Carman et al., 1998; Flores et al., 2000; Liebler-Tenorio et al., 2002), termed hemorrhagic syndrome, is mainly associated with ncp BVDV-2

strains. It is characterized by fast progression, high fever, leucopenia and marked thrombocytopenia leading to petechiation of mucosal surfaces epistaxis and bloody diarrhea with high letality (Corapi et al., 1990).
BVDV infection plays a role in the bovine respiratory disease complex (Potgieter et al., 1984; Castrucci et al., 1992; Fulton et al., 2000b; Shahriar et al., 2002; Callan and Garry, 2002) owing to its immunosuppressive effect. Extensive lymphoid depletion of both B- and T-lymphocytes (Bolin et al., 1985b; Beer et al., 1997) and diminished leukocyte functionality (Bruschke et al., 1997; Ellis et al., 1988) are a consistent finding, more pronounced in extent and duration with virulent strains (Kelling et al., 2002).
In addition to clinical disease of the dam, first-time infection of pregnant animals can lead to viremia and transplacental infection causing early embryonic death, abortion or congenital defects (Done et al., 1980; Duffel and Harkness, 1985; McGowan et al., 1993; Sanderson and Gnad, 2002; Grooms et al., 2002). Both species and both biotypes can cross the placental barrier (Vanroose et al., 1998; Brock and Chase, 2000; Wittum et al., 2001). Infection with non-cytopathic strains between days 90 and 120 of gestation may lead to fetal immunotolerance to BVDV (Brownlie et al., 1998). Pregnancy can be maintained and the dam gives birth to a persistently infected animal, virus positive and seronegative before colostral uptake. PI animals are carrying a higher risk of severe illness as they are more predisposed to other infections (Roth et al., 1981, 1986; Werdin et al., 1989; Munoz-Zanzi et al., 2003). The induction of persistent infections by ncp strains was attributed to an inhibition of interferon (IFN) type I production (Charleston et al., 2001).
Superinfection of a persistently infected animal with a homologous or antigenically closely related (Howard et al., 1987) cytopathic strain by either natural infection, vaccination with an MLV vaccine (Ridpath and Bolin, 1995) or de novo mutation of the persisting virus causes “mucosal disease” (Ramsey and Chivers, 1953; Tautz et al., 1998). Affected animals develop typical mucosal lesions and severe diarrhea, and usually die due to dehydration or septic infections within 3 to 10 days. Mortality reaches approximately 100% (Tautz et al., 1994).

## 2.4. Diagnosis

The differences in genotype, antigenic profile, virulence and biotype have an impact on detection and control. Clinical signs, which are highly variable, may only lead to a presumptive diagnosis which has to be confirmed in the laboratory.

Infection can be assessed directly by antigen detection. PI animals have very high virus loads in almost every tissue, so some methods and sample types for their detection are of limited use in transiently infected animals with low, inconsistent amounts of antigen. Virus isolation in highly susceptible cell culture systems visualized by immunofluorescence or immunoperoxidase staining is the most reliable method ("gold standard") to detect viral antigen. Monoclonal antibodies (mab) must be chosen carefully due to cross-reactivity between strains and species (Ridpath et al., 1994). RT-PCR (reverse transcription polymerase chain reaction) detecting nucleic acids is a highly sensitive and rapid alternative to other BVDV detection methods. It can also be used in pooled samples like bulk milk or blood. BVDV's high variability requires careful primer design (Ridpath et al., 1993) to detect all strains. Differential PCR assays can distinguish between species and sub-genotypes (Ridpath and Bolin, 1998: BVDV-1a, -1b, -2), and contribute to identification and grouping of strains together with sequencing. Antigen-capture enzyme-linked immunosorbent assays (ELISA) using monoclonal antibodies e.g. against NS2/3 or $E^{RNS}$ to detect the majority of strains are commercially available (Gottschalk et al., 1992; Brinkhof et al., 1996; Kampa et al., 2007). Virus in tissue sections is visualized using immunohistochemistry (Thür et al., 1996).

Antibody detection is an indirect measure of infection. BVDV proteins inducing neutralizing antibody development in the host are predominantly E2 and to a minor extend $E^{RNS}$. Antibogies against NS3 are also formed, but have no neutralizing abilities. They are produced after natural infection, vaccination or can be acquired by passive transfer over colostrum. Reference strains from both species are required and paired samples are useful to distinguish between acutely and persistently infected animals. The virus neutralization assay is the "gold standard" (Rossi and Kiesel, 1971) due to its high sensitivity, high specificity and good correlation with protective immunity (Fulton et al., 1997). ELISA systems detecting antibodies employ whole virus antigen, nonstructural proteins, peptides and mab. An $E^{RNS}$ peptide based ELISA for species-independent detection of pestivirus antibodies was developed by Langedijk et al. in 2001. Further ELISA systems are commercially available detecting NS3 or E2 antibodies. Improvements are possible using defined antigens (Haines and Ellis, 1994).

## 2.5. Immunology

BVDV has a predilection for infecting immune cells (Sopp et al., 1994), and is in some cases able to evade immune recognition through its antigenic plasticity. $E^{RNS}$, E2 and NS2/3 (NS3) induce antibody responses and E2 is the immunodominant protein, leading to production of neutralizing antibodies (Donis et al., 1988; Weiland et al., 1989). Active immunity in virus infections is based on the development of a humoral and cellular response, with viral replication apparently essential (Zinkernagel, 1994) for the latter. Maximal antibody levels against BVDV are reached 10-12 weeks post infection (Howard et al., 1992), slowly declining afterwards. Passive immunity is acquired by the colostral transfer of maternal antibodies within the first 24 to 48 hours of life and decreases from 4-6 months of age (Munoz-Zanzi et al., 2002; Ridpath et al., 2003). High titers of maternal antibody may block the development of an active immune response while T-cell responses improve vaccination efficacy (Endsley et al., 2003, 2004). These are known to play an important role in immunity acquired against BVDV (Beer et al., 1997; Rhodes et al., 1999; Collen et al., 2002).
Cytokines mediate reactions of individual parts of the immune response by influencing cellular function (activation, deactivation). BVDV infection of macrophages leads to a decrease in chemotaxis (Ketelsen et al., 1979) and reduced production of tumor necrosis factor-α (TNF-α) (Adler et al., 1996, 1997), which is a modulator of many other cytokines (Chase et al., 2004). Interferon (IFN) is the most important cytokine in innate defense mechanisms to limit infection. BVDV targets interferon regulatory factor-3 (IRF-3), an activator of IFN transcription, causing its proteasomal degradation. Ruggli et al. (2003) determined the role of the $N^{pro}$ protein of pestiviruses in this process. Interference with both cytokines probably contributes to the immunosuppression observed in BVDV infections.

## 2.6. Vaccination

A number of vaccines are licensed for use in cattle in Germany, some with the label claim of "fetal protection". Two different types of vaccines are registered: Modified-live virus (MLV) vaccines lead to a protective humoral and cellular immune response after one-time application, mediating long-lasting immunity. On the other hand, MLV vaccines carry the risk of vaccine virus viremia and shedding due to systemic propagation in the vaccinated animal (Cortese et al., 1997; Fulton et al., 2003) and should not be used in pregnant animals.

Additionally, they can trigger MD, cause immunosupression, reproductive disorders (Thierauf, 1993) or even disease similar to field infection after vaccination. The other type are killed or inactivated vaccines. Killed vaccines are only weakly immunogenic and high amounts of antigen with adjuvants are needed, increasing production costs. They are considered safe as no viremia or shedding is possible, but immunity is not complete especially against heterologous strains. Therefore, a two-step vaccination scheme has been endorsed, comprising vaccination with an inactivated vaccine followed by a booster immunization with a modified-live vaccine before breeding to reduce viremia and shedding of the latter (Hofmann, 1998).

Routine vaccination is helpful in reducing the number of PI animals in a population. Understanding the antigenic and genetic diversity of BVDV and the high rate of genetic insertion and recombination events (Fields et al., 2001) is important for the evaluation of advantages and limitations of BVDV vaccines. Early vaccines merely reduced the extent of clinical disease. The standards a vaccine has to meet to qualify for a label claim of "providing fetal protection" have been designed only recently (Anonymous, 2001). These vaccines reduce the risk of generating new PI animals that are responsible for sustained virus circulation in a herd. But protection against heterologous challenge may be incomplete (Bolin et al., 1991; Van Campen et al., 2000) depending on the homology between the vaccine strain and the field strains to which the animals are exposed. If homology is low, cross-protection is usually poor. Numerous studies (reviewed by Van Oirschot et al. [1999] or Bolin [1995]) have been carried out to determine the efficacy of BVDV-1 vaccines against BVDV-2 challenges (Beer et al., 2000; Makoschey et al., 2001; Ficken et al., 2006), even in pregnant cattle (Brock and Cortese, 2001). The overall conclusion was that only including both species in vaccines can reduce the risk of an infection with BVDV-1 and BVDV-2. A vaccine containing only BVDV-1 had little to no protective effect against a BVDV-2 challenge (Ficken et al., 2006). Dubovi (1992) even recommended the use of multiple strains in one vaccine for a better and broad immunity as protection through vaccination increases with strain homology. For registration, vaccines have to meet safety, quality (purity and potency) and efficacy requirements (Anonymous, 1998). For example vaccines have to be tested in vaccination-challenge studies under experimental conditions. Virulent strains must be used for challenge, but the strain itself is not prescribed. Transmission experiments must be conducted before proceeding with field trials (Van Oirschot et al., 1999). Recombinations are an important issue for new vaccine candidates, as they have been shown to happen between persisting strains and vaccine strains (Becher et al., 2001), BVDV-1 and BVDV-2 (Ridpath

and Bolin, 1995), ncp and cp strains (Ridpath and Bolin, 1995) as well as between BVDV and the host cell genome (Meyers et al., 1998; Becher et al., 2002).

### 2.6.1. New BVDV vaccines using recombinant technologies

New vaccine developments should strive to combine the advantages of both types of vaccines and minimize their disadvantages. Subunit vaccines using the E2 glycoprotein were used experimentally against CSFV and BVDV (Bolin and Ridpath, 1996; Bruschke et al., 1999; de Smit et al., 2000, 2001; Thomas et al., 2009) as were DNA vaccines with a herpesviral promoter in mice and cattle (Harpin et al., 1997, 1999; Liang et al., 2008) and vector-based E2 vaccines (Kweon et al., 1999; Schmitt et al., 1999). Widjojoatmodjo et al. (2000), van Gennip et al. (2002) and Reimann et al. (2007) described the use of replicons as a promising approach. Naturally occuring replicons are “defective interfering particles” (pestivirus prototype: DI9; Tautz et al., 1994), i.e. viral genomes with deletions in the structural protein genes which replicate effectively but need the support of a co-infecting helper virus (Huang and Baltimore, 1970) to generate infectious progeny. After selective deletions in infectious full-length clones *in vitro* (Behrens et al., 1998; Moser et al., 1999; Reimann et al., 2003) (example: Fig.3), genetically engineered replicons can be complemented in-trans using helper cell lines (Reimann et al., 2003, 2007) or co-replicating intact or defect genomes (Varnavski and Khromykh, 1999). Generated “pseudovirions” (DISC [defective in second cycle] virions, virus-replicon particles [VRP] [Maurer et al., 2005] or pseudo particles [Liang et al., 2009]) are one-time infectious particles. Infection of a non-complementing cell leads to replication and expression of viral genes on a scale resembling natural infection but without infectious progeny, suggesting replicons as very safe attenuated vaccine candidates. BVDV-1 replicons lacking a part of the small structural Capsid protein already proved to be successful against a heterologous BVDV-1 challenge (Reimann et al., 2007).

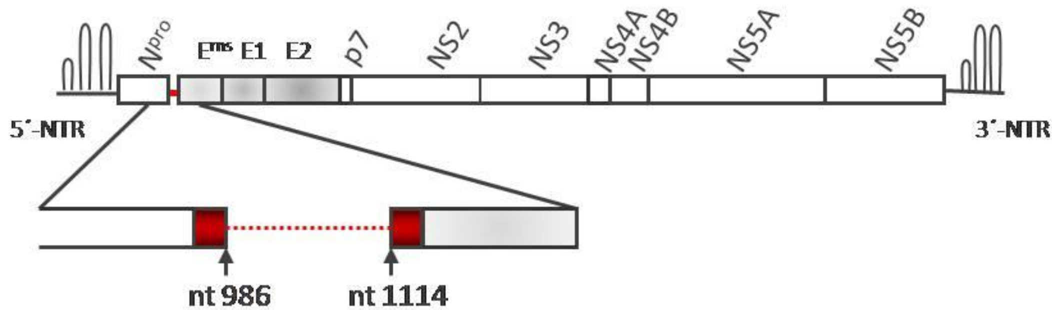


Fig.3 Example for the construction of a replicon based on a full-length cDNA clone; partial deletion of the Capsid protein encoding region still enabling effective cleavage between single proteins

Another approach is attenuation by knocking-out viral virulence factors such as the non-structural protein and autoproteinase $N^{pro}$. This protein is involved in the suppression of the host's innate immune system (Ruggli et al., 2003) and could have an important function in the establishment of persistent infections (Tratschin et al., 1998; Ruggli et al., 2003, 2005; Gil et al., 2006). It is dispensable for viral replication (Tratschin et al., 1998) and growth *in vitro* so deletion mutants (example: Fig.4) can be propagated on conventional cell lines. Pestivirus virions lacking $N^{pro}$ are clearly attenuated *in vivo* (Mayer et al., 2004), and were tested safe and efficacious for BVDV-1 (König, unpublished) and CSFV (Mayer et al., 2004).

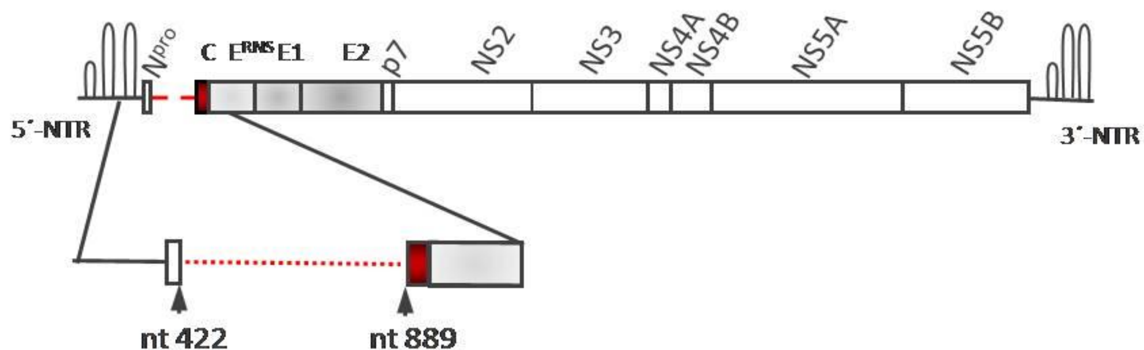


Fig.4 Example for the construction of an $N^{pro}$-deletion mutant based on a full-length cDNA clone; partial deletion of the $N^{pro}$ protein encoding region leaving the IRES domain functional.

For a chimeric pestivirus, the possibility of differentiating between vaccinated and infected animals (DIVA) has recently been shown (Reimann et al., 2003; Koenig et al., 2007; Leifer et al., 2009b) and the same principles can also be used for $N^{pro}$ deleted constructs.

However, all these approaches require functional full-length cDNA clones, which have been constructed for BVDV-1 (strains CP7, NADL and Oregon; Meyers et al., 1996; Vassilev et al., 1997; Mendez et al., 1998; Kümmerer and Meyers, 2000) and BVDV-2 (strain ncp NY93 and 890) (Meyer et al., 2002; Mischkale et al., 2009).

## 2.7. Objectives:

Currently no vaccines licensed in Germany offer cross-protection against BVDV-2 infections. Therefore, two different approaches of attenuating a virulent BVDV-2 full-length cDNA clone for enhanced safety and effective immune response were tested.
Virus (v890FL) derived from the infectious full-length clone of BVDV-2 strain 890 (p890FL; Mischkale et al., 2009) and genetically engineered deletion mutants were characterized *in vivo* in two independent animal trials. First, v890FL was compared to its parental strain in an infection study (trial A – see Fig.5). Two groups of cattle were intranasally infected with the same $TCID_{50}$ of each virus stock (v890FL or 890 wildtype respectively) and were monitored for signs of clinical disease, viremia and nasal virus shedding.

Fig.5 Design of trial A – *in vivo* comparison of virus derived from the constructed BVDV-2 full-length clone v8900FL to BVDV-2 wildtype strain 890.

BVDV-1 and BVDV-2 deletion mutants, each lacking a major part of the $N^{pro}$ gene (BVDV-1$\Delta N^{pro}$ / v890$\Delta N^{pro}$), as well as BVDV-2 pseudovirions derived from a Capsid protein deletion mutant (replicon; v890$\Delta$C) were tested against a heterologous, virulent

BVDV-2 challenge infection (trial B – see Fig.6). Pseudovirions were handled like killed vaccines and were administered twice, 25 days apart. Animals were vaccinated only once if receiving an $N^{pro}$ deletion mutant. The four immunized groups of cattle (v890ΔC, v890Δ$N^{pro}$, BVDV-1Δ$N^{pro}$, combination of v890Δ$N^{pro}$ and BVDV-1Δ$N^{pro}$) and a naïve control group were challenged with a virulent German BVDV-2 field strain. Safety of the mutants (post vaccinational disease, vaccine virus viremia and shedding) was evaluated as was their efficacy protecting against the challenge infection.

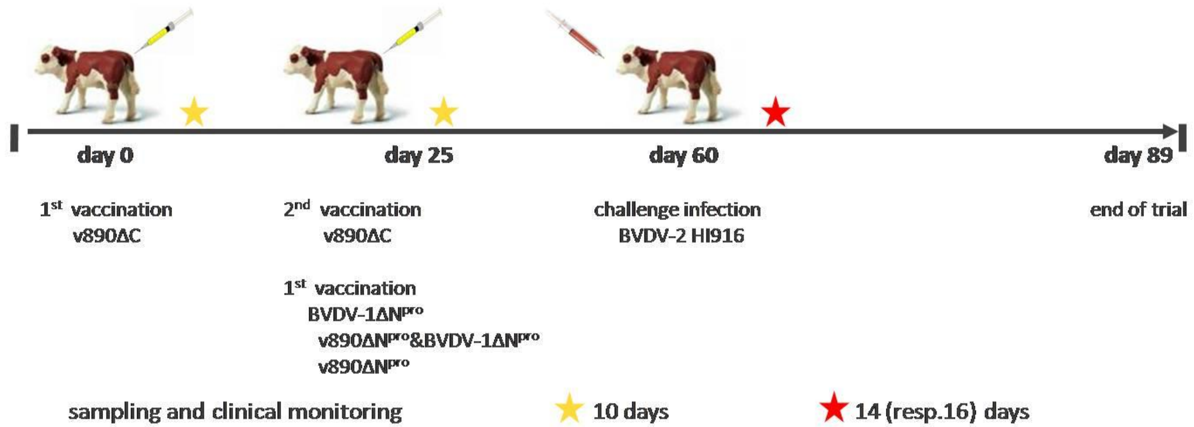


Fig.6 Design of trial B – vaccination-challenge trial – safety of selected mutants and efficacy against a heterologous BVDV-2 challenge were investigated.

## 3. Results - Publications

### 3.1. Publication 1

K. Mischkale, I. Reimann, J. Zemke, P. König, M. Beer

**Characterisation of a new infectious full-length cDNA clone of BVDV genotype 2 and generation of virus mutants**

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# Characterisation of a new infectious full-length cDNA clone of BVDV genotype 2 and generation of virus mutants

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**Abstract**

Based on their genomic sequences, two genotypes of Bovine viral diarrhea virus (BVDV) can be differentiated, BVDV type 1 (BVDV-1) and BVDV type 2 (BVDV-2). The complete genomic sequence of the highly virulent BVDV-2 strain 890 was cloned as cDNA to establish the infectious cDNA clone p890FL. In *vitro*-synthesised full-length RNA of p890FL was transfected into bovine cells and infectious virus could be recovered (v890FL). In *vitro,* recombinant v890FL showed similar growth characteristics as wild type virus 890WT. However, infection experiments in calves revealed an attenuation of recombinant v890FL in comparison to the parental isolate. Both leukocytopenia and fever were less pronounced in v890FL-infected calves. Nevertheless, viremia and virus shedding were comparable between recombinant and parental BVDV 890. Furthermore, mutants with partial deletions of the genomic region encoding for the autoprotease $N^{pro}$ (p890Δ$N^{pro}$) or the capsid protein (p890ΔC) were constructed and characterised. In order to generate pseudovirions, replicon v890ΔC was efficiently *trans*-complemented on a helper cell line. In summary, the newly developed construct p890FL represents the first infectious full-length cDNA clone for the BVDV-2 strain 890 and offers a useful tool for further studies on the pathogenesis of BVDV-2 and the development of novel recombinant BVDV-2 specific vaccine candidates.

**Keywords**: Bovine viral diarrhea virus type 2; pestivirus; infectious pestivirus clone

## 1. Introduction

The Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* within the family *Flaviviridae*. BVDV is closely related to the classical swine fever virus (CSFV) and the ovine border disease virus (BDV) (Fauquet et al., 2005). The pestiviral genome consists of a single stranded positive-sense RNA with a length of about 12.3 kb. It contains one large open reading frame (ORF), which is flanked by non-translated regions (NTR) on both genome termini. The single ORF is translated into one polyprotein, which is co- and post-translationally processed into the mature proteins $N^{pro}$, C, $E^{rns}$, E1, E2, p7, NS2/3, NS4a, NS4b, NS5a and NS5b by viral and cellular proteases (Collett et al., 1988; Lackner et al., 2004). In cell culture, two BVDV biotypes have been described: cytopathogenic (cp) and non-cytopathogenic (ncp). While the cp biotype induces apoptosis and cell death (Zhang et al., 1996), the ncp biotype leads to a persistent infection of cell cultures (Donis and Dubovi, 1987). Since the late 1980s, a new type of BVDV infections with severe thrombocytopenia associated with hemorrhagic syndrome in cattle has been described in Northern America (Pellerin et al., 1994; Rebhun et al., 1989; Ridpath et al., 1994). In Europe, first observations of hemorrhagic syndrome associated with BVDV were reported in the early 1990s (Broes et al., 1992; Lecomte et al., 1996; Thiel, 1993). Analysis of different isolates resulted in classification of BVDV into genotype 1 and 2. Because of their genetic, antigenetic and phylogenetic marked differences, the isolates mentioned above were classified as BVDV genotype 2. The highly virulent strain 890 was isolated by Ridpath et al., 1994. Furthermore, vaccination against BVDV-1 provided only partial protection from BVDV-2 infections and most monoclonal antibodies against BVDV-1 failed to detect BVDV-2 (Bolin et al., 1991; Ridpath et al., 1994). The ncp BVDV-2 strain 890 was the first BVDV-2 to be completely sequenced (GenBank accession no. U18059). In comparison to other ncp pestiviruses the ORF is elongated due to an insertion of 228 nucleotides in the genome segment encoding for

the non-structural protein NS2 (Ridpath and Bolin, 1995). Here, we describe the establishment of an infectious BVDV-2 cDNA clone of strain 890 as well as selected deletion mutants, allowing further studies concerning BVDV pathogenesis, replication and immunoprophylaxis.

## 2. Materials and Methods

### 2.1. Cells and virus

Bovine oesophageal cells (KOP-R, RIE244, CCLV), European bison thymus cells (WT-R, RIE758, CCLV) and interferon incompetent Madin-Darby bovine kidney cells (MDBK, RIE728, CCLV) were obtained from the collection of cell lines in veterinary medicine at the Federal Research Institute of Animal Health, Insel Riems (CCLV). Cells were grown in Dulbecco´s Modified Eagle Medium (DMEM) supplemented with 10 % BVDV-free fetal calf serum (FCS). BVDV-2 wild type strain 890 (v890WT) was kindly provided by H. Hehnen (Bayer AG, Monheim, Germany).

### 2.2. Monoclonal antibodies

For the detection of BVDV proteins, monoclonal antibodies (mab) *WB 433* (anti-$E^{rns}$, CVL, Weybridge), *WB210* (IgG1, anti-$E^{rns}$, CVL, Weybridge), *CA1/2* (anti-E2, Institute for Virology, TiHo Hannover), *CA34/1/2* (anti-E2, Institute for Virology, TiHo Hannover), and mab-mix *WB103/105* (anti-NS3, CVL, Weybridge) were used (Edwards et al., 1988). Secondary antibody *anti*-mouse IgG $ALEXA^{488}$ (Molecular Probes) was used for immunofluorescence (IF) staining.

### 2.3. Construction of the full-length cDNA clone and the deletion mutants

Plasmids were amplified in Escherichia coli DH10B™ cells (Invitrogen) and Escherichia coli MDS42 (kindly provided by G.M. Keil, FLI) (Pósfei et al., 2006), respectively. Plasmid DNA

was purified by using Qiagen Plasmid Mini or Midi Kit or with the GFX™Micro Plasmid Prep Kit (Amersham). Primers used for plasmid construction are presented in table 1, primers for mutagenesis of the plasmid constructs are listed in table 2 (synthesised by MWG-Biotech, Ebersberg, Germany; or OPERON Biotechnologies, Berlin, Germany). Restriction enzyme digestion and cloning procedures were performed according to standard protocols. Construction of the infectious cDNA clone p890FL is schematically illustrated in figure 1. Organisation of the capsid protein deletion mutant (p890ΔC) and of the $N^{pro}$ deletion mutant (p890Δ$N^{pro}$) is shown in figure 2.

The full-length cDNA clone p890FL was constituted from four PCR fragments. RNA for RT-PCR was extracted from bovine cells infected with the parental virus 890WT using TRIZOL reagent (Gibco-Life Technologies) or RNeasy Mini Kit (Qiagen). Copy DNA was generated by using the SuperScript™III Reverse Transcriptase (Invitrogen) according to the instructions of the manufacturer. RT–PCR was performed by using the One-step RT–PCR Kit (Qiagen) or the SuperScript™III One-Step RT-PCR System with Platinum®TaqDNAPolymerase (Invitrogen) according to the supplier´s protocol. DNA based amplification was done using the Expand High Fidelity PCR System (Roche Molecular Biochemicals).

The four PCR fragments (figure 1) were generated by RT-PCR using the appropriate primers (table 1) and subsequently ligated into the plasmid vector pA (kindly provided by Gregor Meyers, FLI Tübingen). *SmaI* sites at the 5´end of the subcloned fragment 1 and within fragment 2 (nucleotide position 1978) were mutated by site-directed mutagenesis using the QuickChangeII XL Site-Directed Mutagenesis Kit (Stratagene) and the respective primers listed in table 2. Subsequently, by sequencing of the complete p890FL plasmid with the Genome Sequencer (GS20, Roche/454) several mismatches compared to the parental virus were detected. Two defects were eliminated by site-directed mutagenesis (1) a frame shift due to the deletion of two nucleotides in the E2 encoding region and (2) a substitution of one

amino acid (aa) at the 3´end of the region encoding for the NS5a protein by using the primers 890_ORF and 890_ORF_r, and the primer pair 890_NS5 and 890_NS5_r, respectively.

For partial deletion of the $N^{pro}$ encoding sequence, the plasmid p890FL and a PCR fragment amplified with the primers 890_SalI and 890_Npro_r were cleaved with *SalI* and *SnaBI* and ligated to generate the construct p890Δ$N^{pro}$-E2. A second PCR fragment was generated by using the primer pair 890_Npro and 890_SnaBI, cleaved with *NotI* / *SnaBI* and cloned into the *NotI* and *SnaBI* digested plasmid p890Δ$N^{pro}$-E2 to obtain the deletion mutant p890Δ$N^{pro}$. The deletion encompasses nt 422-889 (aa 13-168) excepting the first 12 aa which overlap with the internal ribosomal entry site (IRES) region.

The capsid-deleted replicon p890ΔC contained a deletion of aa 201-243 (nt 986-1114) compared to the parental p890FL. The 32 N-terminal amino acids and the 27 C-terminal aa of the capsid protein, which constitute an essential signalase recognition site and which direct translocation of the envelope proteins into the endoplasmatic reticulum (ER) for further processing of the $E^{rns}$-E1-E2 polyprotein (Rümenapf et al., 1991), were retained. For construction of p890ΔC, a PCR fragment using the primers 890_SalI and 890_Capsid_r was amplified. p890FL and the PCR fragment were cleaved with *SalI* and *SnaBI* and ligated to generate the construct p890ΔC-E2. In a second step, a PCR fragment was amplified by using the primer pair 890_Capsid and 890_SnaBI_r. The resulting amplicon and p890ΔC-E2 were digested with *NotI* / *SnaBI* and both ligated to establish the deletion mutant p890ΔC.

2.4. In *vitro* transcription and RNA transfection

In *vitro* transcription of the deletion mutants p890Δ$N^{pro}$, p890ΔC and the full-length construct p890FL was performed using the T7 RiboMax Large-Scale RNA Production System (Promega) according to the manufactur´s instructions after linearising the plasmids with *SmaI*. The amount of RNA was estimated by ethidiumbromid staining after agarose gel electrophoresis. For RNA transfection, bovine cells were detached using a trypsin solution,

washed twice with phosphate buffered saline without Ca++/Mg++ (PBS-) and mixed with 1-5 µg of in *vitro* sythesised RNA. Electroporation was done by using the GenePulser transfection unit (Biorad) (two pulses at 850 V, 25 µF and 156 ω).

2.5. Immunofluorescence staining

Cell cultures were fixed with 4% paraformaldehyde (PFA) and permeabilised with 0.01 % digitonin (IF staining of NS3) or fixed/permeabilised with 80 % acetone ($E^{rns}$, E2), and incubated with the appropriate working dilution of the respective antibodies for 30 min. After one washing step with $PBS^{-}$, cells were incubated with the $Alexa^{488}$-conjugated secondary antibody for 30 min and finally washed. IF was analysed by using a fluorescence microscope (Olympus).

2.6. *Trans*-complementation of the replicon p890ΔC

2.6.1. Establishment of C-$E^{rns}$-E1-E2 expressing WT-R2 cells

The genomic region encoding the structural proteins (C-$E^{rns}$-E1-E2) of ncp BVDV-1 strain PT810 (Wolfmeyer et al., 1997) was cloned as a chemically synthesised synthetic open reading frame (Syn-ORF, constructed by GeneArt, Regensburg, Germany). It consisted of 2694 nucleotides extending from nucleotide 890 to 3584 of the nucleotide sequence of BVDV strain NADL (Collett et al., 1988), and was inserted into the pcDNA3.1 expression plasmid (Invitrogen) using *Kpn*I and *NotI* restriction sites. The nucleotide sequence of Syn-ORF had been changed to remove splice sites (Schmitt et al., 1999), but retained the original amino acid sequence of ncp BVDV strain PT810 (GenBank accession no. AY078406). Additionally, the first codon of Syn-ORF was changed to a methionine to allow expression of the polyprotein under the control of the *HCMV* immediate-early promoter present in pcDNA3.1, and a stop codon was inserted behind the last codon. The resulting construct pcDNA_C-E2 (1 _g) was used to transfect WT-R cells with the SUPERFECT reagent (Qiagen). At 2 days post

transfection (p.t.), cell culture medium was changed to DMEM supplemented with 10 % bovine serum and 0.5 mg of geneticin G418 per ml. G418-resistant colonies were isolated, replated several times, and stained for $E^{rns}$ and E2 expression using mab WB210, respectively E2-mix (CA 1/2 and CA34/1/2).

2.6.2. *Trans*-complementation

In *vitro*-transcribed RNA of p890ΔC was transfected into WT-R2 cells and at 72 h p.t. RNA replication was analysed by IF staining with NS3 specific mabs. Supernatants of transfected cells were harvested and the titre of the pseudovirion progeny v890ΔC_trans was determined. Serial passages of v890ΔC_trans were performed on complementing WT-R2 cells as well as on non-complementing KOP-R cells.

2.7. Virus titration

Infectious titres were determined for virus stocks as well as for growth kinetics analyses, and after *trans*-complementation of p890ΔC. Cell culture supernatants of v890FL-, v890WT- and 890Δ$N^{pro}$-infected cells were harvested, and supernatants containing the *trans*-complemented pseudovirions (v890ΔC_trans) were collected. After freezing, supernatants were titrated in log10-dilutions on KOP-R cells, and titres were determined as median tissue culture infective dose per ml ($TCID_{50}$/ml).

2.8. Growth kinetics

For in *vitro* growth kinetics, KOP-R cells were infected with the recombinant virus v890FL, v890Δ$N^{pro}$ and with the parental virus v890WT, respectively, at a multiplicity of infection (MOI) of 1. Supernatants were collected at 0, 8, 12, 24, 48, 72 and 96 h post infection (p.i.) and virus titres ($TCID_{50}$/ml) were determined.

2.9. Real-time RT-PCR analyses

In order to determine the viral RNA replication levels of v890FL, v890WT, and v890ΔN$^{pro}$, KOP-R cells were infected at an MOI of 1 of the appropriate viruses. At 48 h p.i., supernatants and cells were separately collected and RNA was isolated by using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer´s instructions. Uninfected KOP-R cells were included as test control. In order to minimize the risk of cross contamination, a one step RT-PCR was performed using the QuantiTect™ Probe RT-PCR Kit (Qiagen). According to Hoffmann et al. (2005, 2006), 5 µl RNA template were added to a total volume of 25 µl, containing 3.5 µl RNase-free water, 12.5 µl 2×QuantiTect Probe RT reaction-buffer, 2.0 µl panpesti-specific FAM-labeled primer/probe mix and 0.25 µl RT-enzyme mix. For quantification of the copy numbers, serially diluted BVDV-DI9-RNA (Behrens et. al., 1998) was used as standard RNA. The following temperature profile was used: 30 min at 50 °C (reverse transcription), 14 min at 95 °C (inactivation reverse transcriptase/activation *Taq* polymerase), followed by 40 cycles of 30 sec at 95 °C (denaturation), 30 sec at 57 °C (annealing) and 60 sec at 62 °C (elongation). Identical temperature profiles were used for all real-time RT-PCR runs and fluorescence values were recorded during the annealing steps.

2.10. Animal experiment

Ten Simmentaler breed calves, aged between 6 and 8 month, were shown to be free of BVDV-antibodies and -antigen. Calves were randomly allocated into two groups of five animals each, and inoculated with the recombinant v890FL and the parental 890WT virus, respectively. Inoculation was done intranasally with $2\times10^6$ $TCID_{50}$ in a volume of 2 ml (1ml per nostril). To confirm infectious titres, both viral suspensions were backtitrated on KOP-R cells after inoculation. The animals were housed under identical conditions in two different units and were monitored daily for clinical signs and rectal body temperatures. Blood samples

were collected to monitor viremia as well as to evaluate leukocyte and thrombocytes counts. Nasal swabs were investigated for virus shedding throughout the experiment.

## 3. Results

### 3.1. Construction and characterisation of the infectious BVDV-2 cDNA clone p890FL

The full-length clone p890FL was constituted from four PCR fragments assembled in the low copy vector pA (Meyers et al., 1996b). At the 5´end, the sequence of the T7 promoter was added to enable in *vitro* transcription and at the 3´end a *SmaI* restriction site was introduced for plasmid linearisation (figure 1). First sequence analyses revealed introduction of several mutations into the full-length clone. Two of the mutations, a frame shift due to the deletion of two nucleotides in the E2 encoding region, and a substitution of one aa at the 3´end of the region encoding for the NS5a protein were eliminated by using site directed mutagenesis. Subsequently, the p890FL cDNA clone was again completely sequenced, resulting in detection of a bacterial insertion at aa position 648, accompanied by duplication of the aa sequence GLR. Sequence analysis of the bacterial insertion showed similarities to the bacterial IS10 element, which can be found in E. coli K-12 strain (data not shown). Thereupon, we analysed the sequence of the RNA re-isolated from cells infected with v890FL. The sequences of the bacterial insertion into the cDNA clone p890FL were not present in the viral RNA of v890FL. As a consequence, we used the E. coli strain MDS42 (Pósfai et al., 2006) instead of E. coli strain DH10B for transformation of the new plasmid constructs, due to the engineered genome of E. coli strain MDS42 without any sequences encoding mobile bacterial genetic elements.

In order to generate infectious virus progeny, in *vitro*-transcribed RNA of p890FL was transfected into KOP-R cells. 72 h p.t., RNA replication could be detected in nearly 100 % of the cells by IF staining using NS3 specific mabs (figure 3). By passaging the transfection supernatant, infectious virus v890FL could be recovered. A stock of the second passage was

used for in *vitro* and in *vivo* characterisation. The in *vitro* growth analyses indicated a very similar growth of the recombinant virus v890FL compared to parental virus 890WT, with only slightly reduced final virus titres (figure 4). In order to analyse RNA replication levels, we performed real-time RT-PCR analyses, which showed similar RNA replication levels of v890FL and v890WT. In supernatants of infected cells, $10^8$ to $10^9$ RNA copies per ml were detected, and intracellular levels of viral RNA revealed around $10^2$ RNA copies per cell (table 3).

Furthermore, the animal experiment with v890FL and v890WT demonstrated an attenuated phenotype of recombinant v890FL if compared to wild type virus 890WT. However, both animal groups showed clinical signs of a severe BVDV infection with a biphasic elevated body temperature curve, with a mean maximum body temperatures of nearly 41 °C for the wild type infected group and 39.7 °C for the group infected with the recombinant virus v890FL (figure 5). Interestingly, in both groups no thrombocytopenia could be observed. A marked leukocytopenia was present in both groups, but at lower levels for the v890FL-infected animals (figure 6). For each animal, viremia could be detected at days 3 to 7 for the group infected with v890FL, and at days 2 to 10 for the group infected with the parental virus v890WT (figure 7). In addition, nasal virus shedding could be observed from day 2 to 10 p.i. (data not shown).

3.2. Construction and characterisation of BVDV-2 deletion mutant p890Δ$N^{pro}$

An $N^{pro}$ autoprotease deletion mutant, p890Δ$N^{pro}$ (figure 2), was constructed on basis of the infectious BVDV-2 clone p890FL by partial deletion of the genomic sequence encoding most of $N^{pro}$ (the first 36 nt overlapping with the BVDV IRES were retained). In order to detect viral replication, in *vitro*-transcribed RNA of p890Δ$N^{pro}$ was transfected into interferon negative MDBK cells. At 72 h p.t., expression of NS3 could be detected by IF staining in nearly 100 % of the transfected cells (figure 3). Transfection supernatant was passaged and

infectious virus progeny v890ΔN$^{pro}$ could be recovered. Growth kinetics on interferon competent KOP-R cells showed approximately 100-fold reduced growth of the deletion mutant (figure 8). Nevertheless, real-time RT-PCR analyses indicated a similar RNA replication level of v890ΔN$^{pro}$ in comparison to v890FL and v890WT (table 3).

3.3. Construction, *trans*-complementation and characterisation of BVDV-2 replicon p890ΔC

Replicon p890ΔC is characterised by a partial deletion of 43 aa within the encoding region for the capsid protein (figure 2). 48 h post transfection of in *vitro*-transcribed RNA into non-complementing KOP-R cells, autonomous replication of viral proteins could be detected in nearly 100 % of the transfected cells by IF staining (figure 3), but no infectious virus progeny could be recovered. For packaging of the replicon p890ΔC, we established the new helper cell line WT-R2 derived from the European bison. Like the first available helper cell line PT805 (Reimann et al., 2003), WT-R2 cells stably express a synthetic ORF encoding the BVDV-1 structural genes C-E$^{rns}$-E1-E2. For *trans*-complementation, in *vitro*-transcribed RNA of the replicon p890ΔC was transfected into WT-R2 cells, and 72 h p.t. autonomous virus replication was detected by IF staining (figure 9). Infectious pseudovirions v890ΔC_trans could be recovered from transfection supernatants and were serially passaged on WT-R2 cells (figure 9). However, no passaging was possible on non-complementing KOP-R cells, and no replication competent revertants or pseudo-revertants could be detected.

**4. Discussion**

Several pestiviral infectious cDNA clones, including CSFV (Meyer et al., 2003; Ruggli et al., 1996) and BVDV-1 (Mendez et al., 1998; Meyers et al., 1996; Vassilev et al., 1997), have been described. However, only a single infectious BVDV-2 cDNA clone (strain NY´93C) is published (Meyer et al., 2002). In addition, an infectious transcript of the BVDV-2 strain 890

was established by Dehan et al. (2005). However, the construction of an infectious cDNA clone of strain 890 failed. This study describes construction and characterisation of the first infectious full-length cDNA clone of BVDV-2 strain 890 (p890FL) and its application for the development of further mutants (p890ΔN$^{pro}$ and p890ΔC). Although PCR amplification of the whole genome represents a simplification of the cloning strategy, and has been described for pestiviruses (Rasmussen et al., 2008), the generation of a full-length PCR fragment for the strain 890 failed. Therefore, p890FL was constructed on the basis of four PCR fragments, which were assembled into the vector pA (Meyers et al., 1996). In *vitro*-transcribed RNA of p890FL was transfected into bovine cells, and replication could be demonstrated in nearly 100 % of the cells. Subsequently, infectious virus progeny could be recovered (v890FL) from supernatants of transfected cells (figure 3). In *vitro*-characterisation of v890FL showed similar growth kinetics with only slightly reduced virus titres, and a similar RNA replication level compared to the parental virus 890WT (figure 4). In infected animals however, we observed an attenuated phenotype of v890FL compared to v890WT with lower mean body temperatures, leukocytopenia at a lower levels and a shorter viremia (figure 5, 6 and 7). Attenuation of RNA-viruses recovered from cDNA clones reflects their genetic variability, and has been also described for BVDV-2 before (Dehan et al., 2005; Meyer et al., 2002). Up to now, the reason for in *vivo* attenuation of v890FL is not definitely resolved. However, there are some amino acid substitutions in the full-length ORF of p890FL which could possibly account for the in *vivo* attenuation: two in the E$^{rns}$, one in the E2, and one in the NS5a encoding sequences. One of the E$^{rns}$ point mutations is located at the C-terminus, and is identical to a mutation in an infectious transcript of BVDV-2 890 described by Dehan et al. (2005), which also showed an attenuated phenotype. The second aa substitution could be found in the middle part of E$^{rns}$ near the RNase motif. RNase activity is important for virulence and pathogenicity of BVDV (Magkouras et al., 2008; Meyer et al., 2002; Meyers et al., 2007), and therefore further studies will predominantly focus on the E$^{rns}$ mutations. In

contrast, the aa substitution within E2 and NS5a are not located in previously defined functional regions (Johnson et al., 2001; Reed et al., 1998; Sapay et al., 2006).

Furthermore, the cDNA clone p890FL was used for the construction of the deletion mutant p890Δ$N^{pro}$ by partial deletion of the genomic sequence encoding a predominant part of $N^{pro}$. In contrast to CSFV and HCV, the extension of the IRES into the ORF of BVDV is not defined in detail. In order to ensure full activity of the IRES, the first 12 codons were retained. However, the minimum coding region essential for full efficacy of the IRES region is still discussed. Recent reports describe for BVDV-1 the preservation of nine to 25 codons downstream of the initial start codon to ensure full IRES activity (Moes and Wirth, 2007), and for BVDV-2 Meyers et al. (2007) reported four residual codons as sufficient for acceptable growth in *vitro*. Furthermore, alignment of BVDV-1 and BVDV-2 protein sequences, resulted in 13 out of the first 16 codons which are conserved in the BVDV polyprotein (Moes and Wirth, 2007). For CSFV a similar conservation scheme is described (Moers and Wirth 2007). 17 codons of the N-terminus are required for full activity of the CSFV-IRES (Fletcher et al., 2002). However, it has to be mentioned that preservation of the first 12 codons of the $N^{pro}$-gene of BVDV-2 strain 890 were sufficient to maintain viral replication, but also resulted in a capsid protein with an amino-terminal extension. The deletion mutant p890Δ$N^{pro}$ was able to replicate in *vitro,* and from supernatants of transfected interferon negative MDBK cells infectious virus progeny v890Δ$N^{pro}$ could be recovered. The v890Δ$N^{pro}$ virus titres detected in MBDK cells were comparable to the titres of v890FL detected in KOP-R cells (data not shown), indicating that there is no marked influence of the amino-terminal extension of the capsid protein on viral viability and growth in cell culture. Comparison of the in *vitro* growth kinetics of v890Δ$N^{pro}$, v890FL and v890WT on interferon-competent KOP-R cells revealed an approximately 100-fold reduced growth of the deletion mutant v890Δ$N^{pro}$ due to the loss of $N^{pro}$ as an interferon antagonist (Gil et al., 2006). However, our results are in contrast to the non-reduced in *vitro* growth of a BVDV-2 $N^{pro}$ deletion mutant described by Meyers et al.

(2007). Furthermore, $N^{pro}$ deletion mutants are useful candidates for efficient modified live vaccines against BVDV-1 and BVDV-2 with the potency to induce sterile immunity without the risk of establishing persistent infections (P. König unpublished data; Meyers et al., 2007; Zemke et al., 2008).

In addition, the BVDV-2 replicon p890ΔC, with a partial deletion of the genomic region encoding the capsid protein, was constructed. The N-terminal 32 aa and the 27 C-terminal aa of the capsid protein, which are essential for signalase recognition, translocation of the envelope proteins into the ER, and further processing of the $E^{rns}$-E1-E2 polyprotein (Rümenapf et al., 1991) were retained. In *vitro*-transcribed RNA of p890ΔC was able to replicate autonomously in non-complementing bovine cells, since the structural proteins are not essential for pestiviral RNA replication (Behrens et al., 1998). From supernatants of transfected non-complementing bovine cells no infectious virus progeny could be recovered. However, infectious virus could be generated by packaging the defective genomes by using a helper virus (Kupfermann et al., 1996) or a helper cell line (Reimann et al., 2003). For *trans*-complementation and packaging of the replicon p890ΔC we constructed the new helper cell line WT-R2 essentially as described for the helper cells PT805 (Reimann et al., 2003). The replicon p890ΔC was efficiently *trans*-complemented and packaged into pseudovirions by using WT-R2 cells. Recombination or reversion during generation of the pseudovirions was not observed, and in contrast to experiments with BVDV-1ΔC replicons and PT805 cells (Reimann et al., 2003, 2007), the recombinant WT-R2 cells even allowed the passaging of BVDV-2ΔC pseudovirions.

In conclusion, the established infectious full-length cDNA clone of BVDV-2 strain 890 could enable new insights in viral biology, especially studies of the 228 nt insertion into the NS2 encoding region of the ncp strain 890, and pathogenesis of BVDV-2. Furthermore, the generated viral mutants can be the basis for the generation of novel safe and efficacious BVDV-2 vaccines.

**Acknowledgements**

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## Tables

Table 1: nucleotide sequence of PCR primers used for plasmid constructions

| Primer | Sequence (5´to 3´) | Genomic region [a] |
|---|---|---|
| F1 | TTAACCCGGGTAATACGACTCACTATAGTATAC GAGATTAGCTAAAGT | 1-21 (+) |
| F1_r | ATATCCCGGGGCCTATTATCTTGGTGTTTCTTGG | 1950-1982 (-) |
| F2 | ATATCCCGGGAAGTTTGACACCAACGCCGAAG ATGGC | 1976-2007 (+) |
| F2_r | ATATCCCGGGACGCGTTGGCACGAACACGAGC ATGTTGCC | 6569-6598 (-) |
| F3 | CGATACGCGTAACATGGCAGTAGAAACAGC | 6593-6618 (+) |
| F3_r | GTTCTTACTCTCTAGATAACCGGCTGCTCCC | 10804-10834 (-) |
| F4 | GGGAGCAGCCGGTTATCTAGAGAGTAAGAAC | 10804-10834 (+) |
| F4_r | ATATGAATTCCCCGGGGGCCGTTAGAGGCATC CTCTAGTC | 12486-12512 (-) |
| 890_Npro | ATATGCGGCCGCATCCGATGAAGGGAGTAAGG GTGCT | 890-913 (+) |
| 890_Npro_r | ATATTACGTATGCGGCCGCTGTTTTGTATAAAA GTTCATTTGAAAACAACTCCATGTGCC | 381-421 (-) |
| 890_Capsid | GGATGCGGCCGCACCTGAATCAAGAAAGAAAT TGG | 1115-1136(+) |
| 890_Capsid_r | ATATTACGTATGCGGCCGCTTCTGACTCTTTTGG GGC | 968-985 (-) |
| 890_SalI | GGACGTCGACAAACTTTGAATTGG | 37-60 (+) |
| 890_SnaBI_r | CCACAGTACGTATTTACCACCCAAC | 3508-3532 (-) |

[a] genomic region of BVDV-2 strain 890 (GenBank accession no. U18059), symbols in brackets show the polarity

Table 2: PCR primers used for site directed mutagenesis of plasmid constructs

| Primer | Sequence (5´-3´)[a] | Genomic region[b] |
|---|---|---|
| MutI | AGAACTAGTGGATCCC**G**CG**C**GTAATACGACTCACTA | - (+) |
| MutI_r | TAGTGAGTCGTATTAC**G**CG**C**GGGATCCACTAGTTCT | - (-) |
| MutII | ACCAAGATAATAGGCCC**A**GG**A**AAGTTTGACACCAACGCC | 1961-1999 (+) |
| MutII_r | GGCGTTGGTGTCAAACTT**T**CC**T**GGGCCTATTATCTTGGT | 1961-1999 (-) |
| 890_ORF | GCTGACACACAGTG**AT**ATTGAGGTTGTGGTC | 3619-3649 (+) |
| 890_ORF_r | GACCACAACCTCAA**TA**TCACTGTGTGTCAGC | 3619-3649 (-) |
| 890_NS5 | GGCTGACTTATATCACCTAATT**G**GCAGTGTTGATAGTATAAAAG | 10024-10068 (+) |
| 890_NS5_r | CTTTTTATACTATCAACACTGC**C**AATTAGGTGATATAAGTCAGCC | 10024-10068 (-) |

[a] mutated nucleotides are underlined and in bold

[b] genomic region of BVDV-2 strain 890 (GenBank accession no. U18059), symbols in brackets show the polarity

Table 3: Results of the real-time RT-PCR analyses of the recombinant viruses v890ΔN$^{pro}$, v890FL, and the wild type virus v890WT. KOP-R cells were infected at an MOI of 1. 48 h p.i. supernatants and cells were harvest, respectively.

| Virus | Supernatants (RNA copies/ml) | Cells (RNA copies/cell) |
|---|---|---|
| v890ΔN$^{pro}$ | $10^{8.02}$ | $10^{1.79}$ |
| v890FL | $10^{8.27}$ | $10^{2.08}$ |
| v890WT | $10^{8.80}$ | $10^{1.79}$ |

**Figures**

Figure 1: Schematic representation of the construction of the infectious cDNA clone p890FL. The viral genome was amplified in 4 PCR fragments with 4 separate PCR reactions. The PCR products were cloned into the vector pA (G. Meyers et al., 1996b). At the 5´NTR the sequence of the T7 promoter was added to enable in *vitro* transcription. For plasmid linearisation a *SmaI* restriction site was introduced at the 3´NTR. Mutagenesis steps during construction of the cDNA clone are indicated by stars. Filled boxes represent the BVDV structural protein region. Lines at the left and the right ends indicate non-translated regions. $N^{pro}$, autoprotease; C, capsid protein; $E^{rns}$, E1, E2, envelope proteins; p7, non-structural protein; NS2 to NS5, non-stuctural proteins; 3'NTR and 5'NTR, non-coding regions. The size-scale is given in kb.

Figure 2: Schematic depiction of the deletion mutants p890$\Delta N^{pro}$ and p890ΔC based on the infectious cDNA clone p890FL. Filled boxes represent the regions encoding the BVDV structural proteins. Horizontal dotted lines show the deleted regions and numbers indicate the nucleotide (nt) or amino acid (aa) position in the BVDV full-length RNA. Lines at the left and the right ends indicate non-translated regions. $N^{pro}$, autoprotease; C, capsid protein; $E^{rns}$, E1, E2, envelope proteins; p7, non-structural protein; NS2 to NS5, non-structural proteins; 3'NTR and 5'NTR, non-coding regions. The size-scale is given in kb.

Figure 3: IF analysis of bovine cells transfected with in *vitro* transcribed RNA of p890FL, p890$\Delta N^{pro}$ or p890ΔC. In addition, supernatants of transfected cells were passaged on bovine cells. At 72 h p.t. and 72 h p.i. NS3 expression was analyzed by IF staining using the mab WB 103/105. Untransfected/uninfected bovine cells were used as controls. A) p890FL RNA transfected into KOP-R cells and passage of the supernatants on KOP-R cells. B) p890$\Delta N^{pro}$

RNA transfected into interferon-incompetent MDBK cells and passage of the supernatants. C) RNA of p890ΔC transfected into KOP-R cells and passage of the supernatants on KOP-R cells.

Figure 4: Growth kinetics of the recombinant virus v890FL (broken line) and the parental virus v890WT (solid line). KOP-R cells were infected at an MOI of 1. Supernatants were harvested at the indicated time points. After freezing and thawing, virus titres ($TCID_{50}$/ml) were determined by titration on KOP-R cells. Standard deviations are shown as error bars.

Figure 5: Mean body temperatures of calves (n=5) after intranasal infection with the recombinant v890FL (broken lines) and the wild type virus v890WT (solid lines), respectively. Standard deviations are shown as error bars.

Figure 6: Mean leukocyte counts of calves (n=5) following intranasal infection with the recombinant v890FL (broken lines) and the wild type virus v890WT (solid lines), respectively. The initial values were set to 100%. Standard deviations are shown as error bars.

Figure 7: Course of viremia in calves infected with the recombinant v890FL (dark grey bars) and the wild type virus v890WT (black bars), respectively. Viremia was determined by co-culture of purified leukocytes on highly susceptible KOP-R cells (4 replicates per animal/day). Virus replication was detected by immunofluorescence staining. Mean values are calculated from positive replicates of 5 animals each.

Figure 8: Growth kinetics of the deletion mutant v890Δ$N^{pro}$ (dotted line) compared with the recombinant virus v890FL (broken line) and the parental virus v890WT (solid line). KOP-R

cells were infected with the respective viruses at an MOI of 1. Supernatants were harvested at the indicated time points. After a freezing and thawing procedure, virus titres ($TCID_{50}$/ml) were determined by titration on KOP-R cells. Standard deviations are shown as error bars.

Figure 9: *Trans*-complementation studies with replicon p890ΔC and WT-R2 helper cells. A) The WT-R2 cell line stably expresses the synthetic structural genes C-E2 of BVDV-1, and E2 expression is shown by IF staining using an E2-mab mix (CA 1/2 and CA34/1/2). NS3 as a marker for viral replication could not be detected by IF staining using the mab WB 103/105 in non-transfected cells. B) Transfection of in *vitro*-transcribed RNA of p890ΔC into WT-R2 cells. 72 h p.t. NS3 expression could be detected by IF staining using the mab WB 103/105. C) Pseudovirions v890ΔC_trans could be recovered from supernatants of transfected WT-R2 cells and were further passaged on WT-R2 cells. Replication of the pseudovirions was detected by IF staining: 72 h p.i. NS3 expression could be detected.

Figure 1:

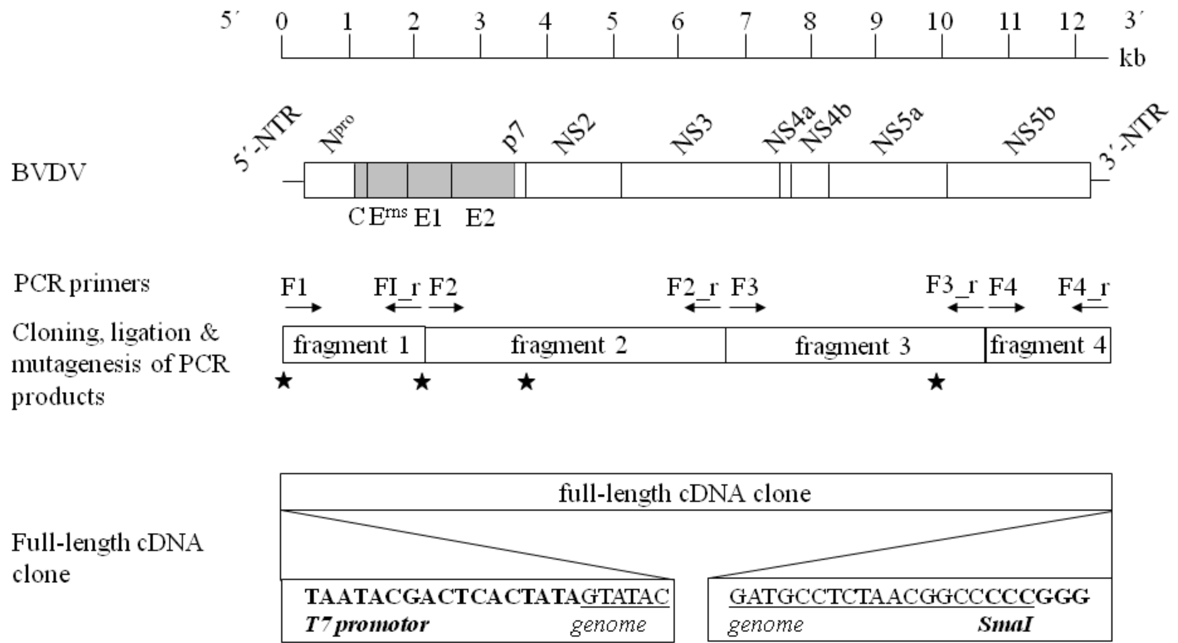


Figure 2:

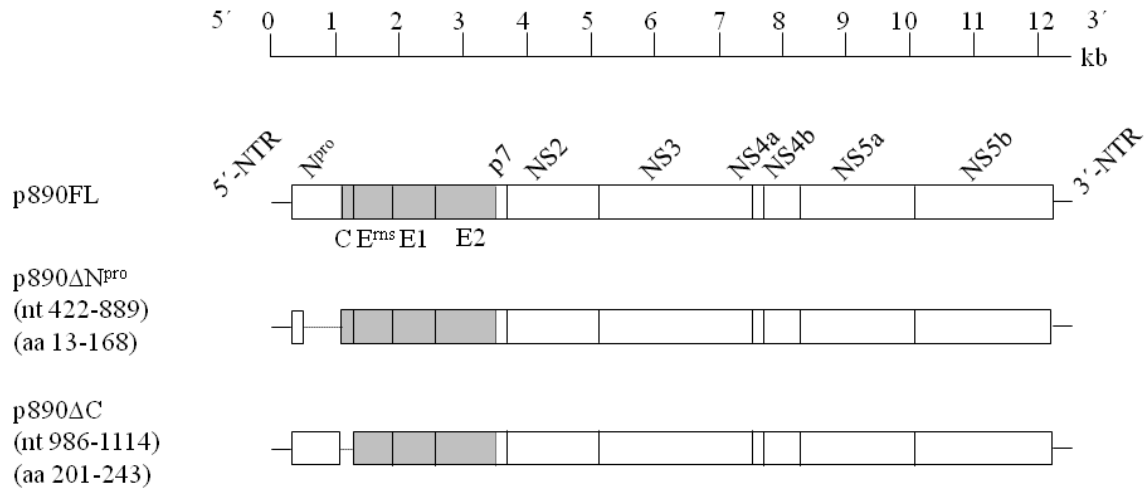
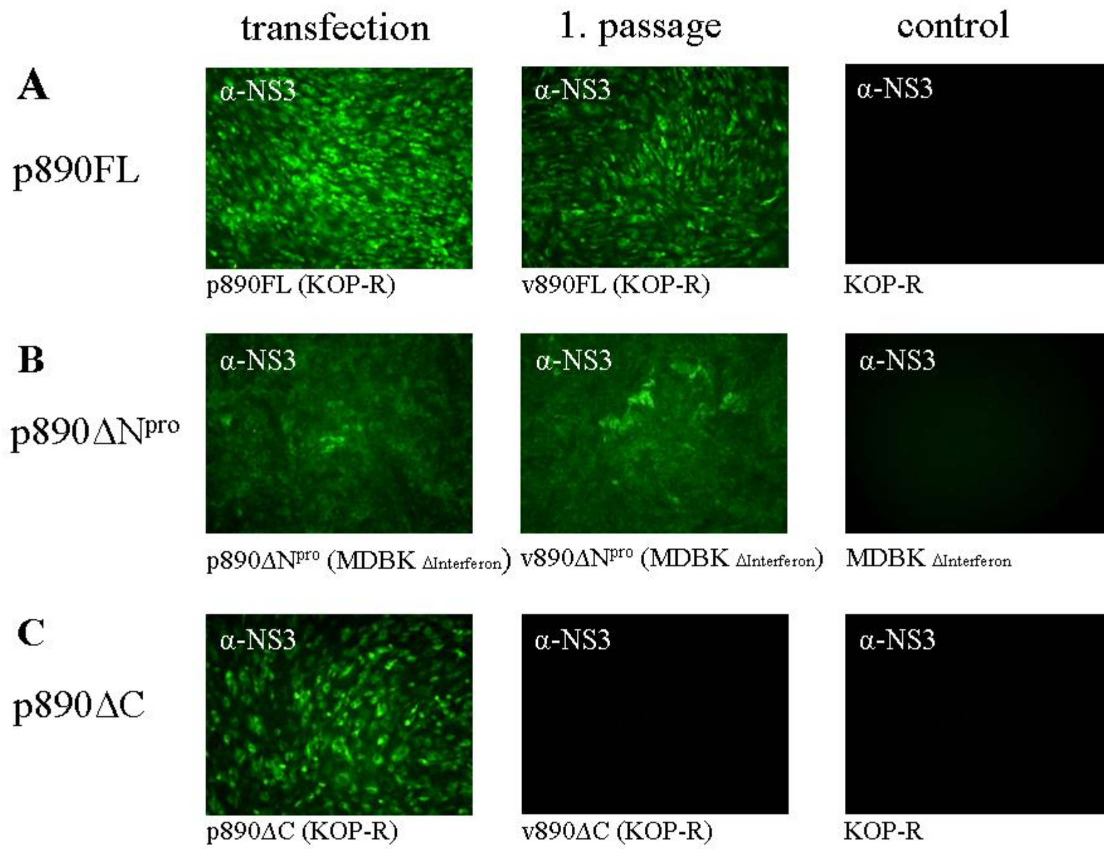


Figure 3:



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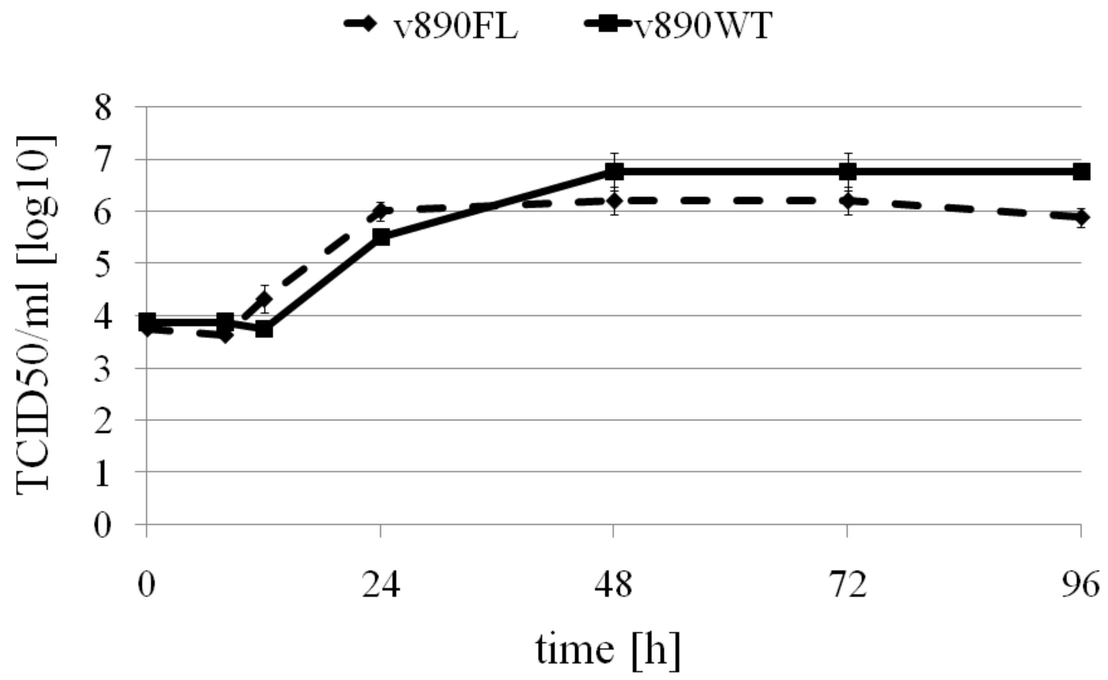

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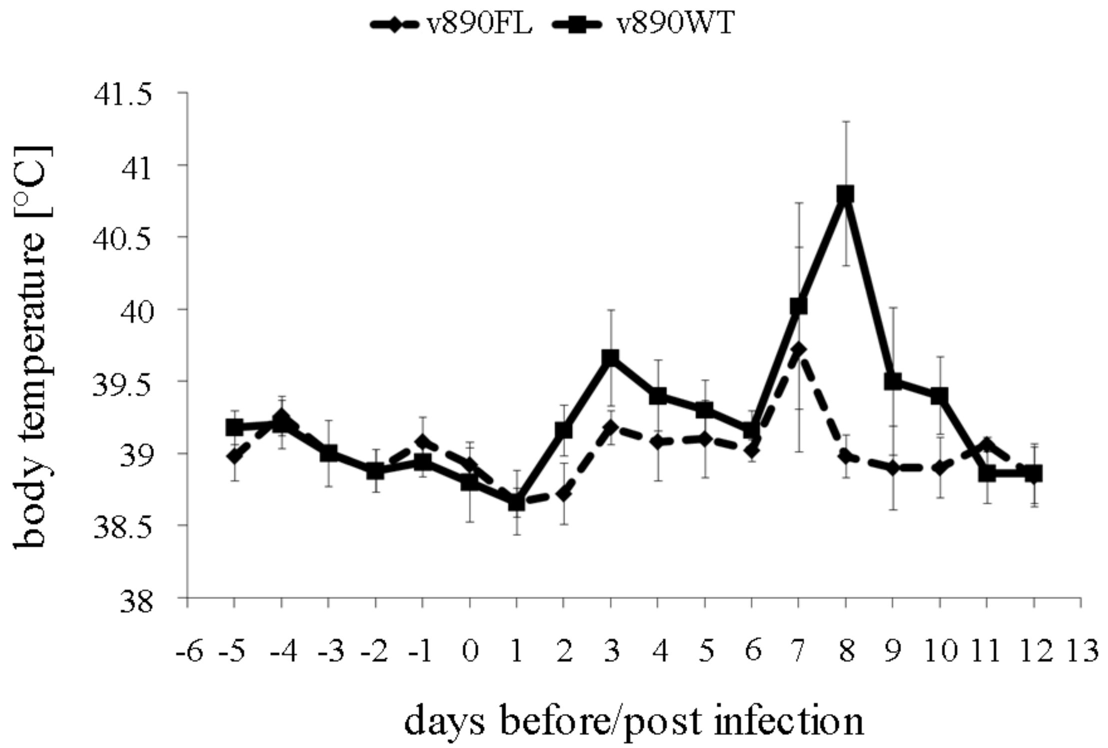

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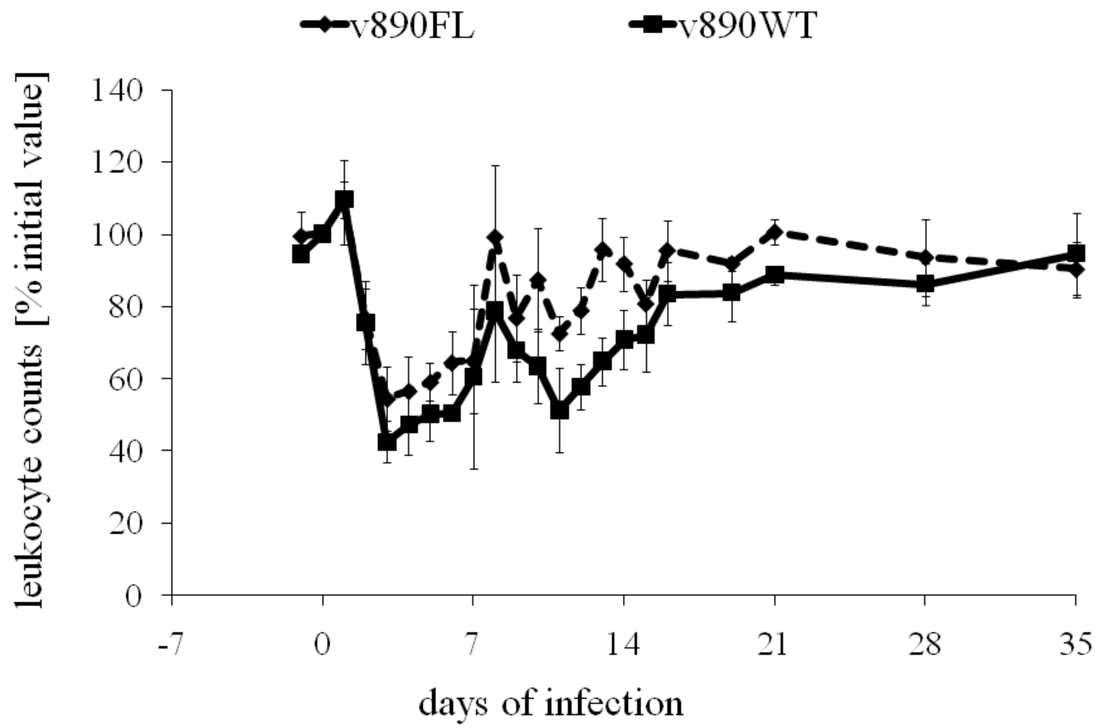

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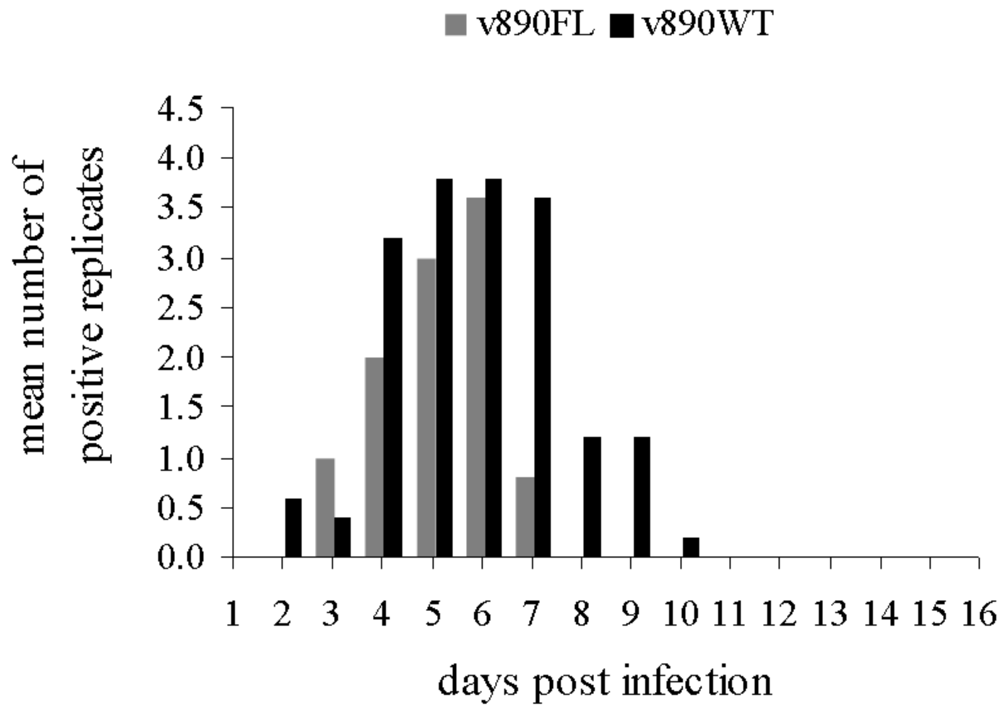


Figure 8:

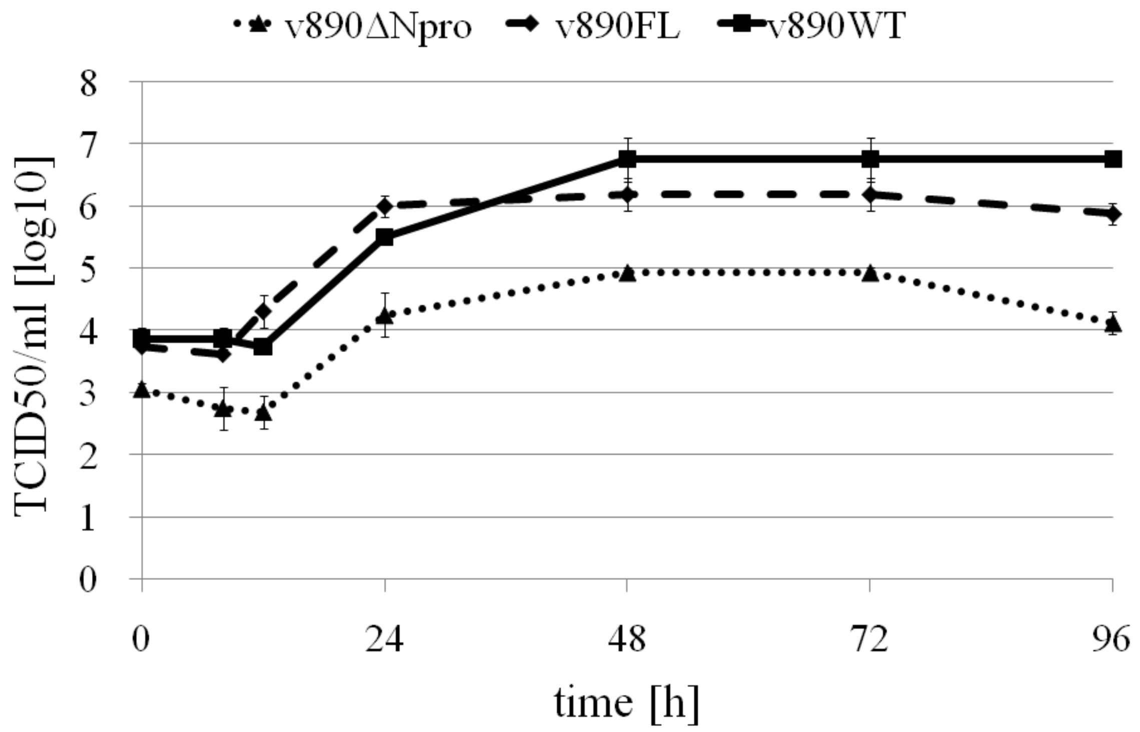
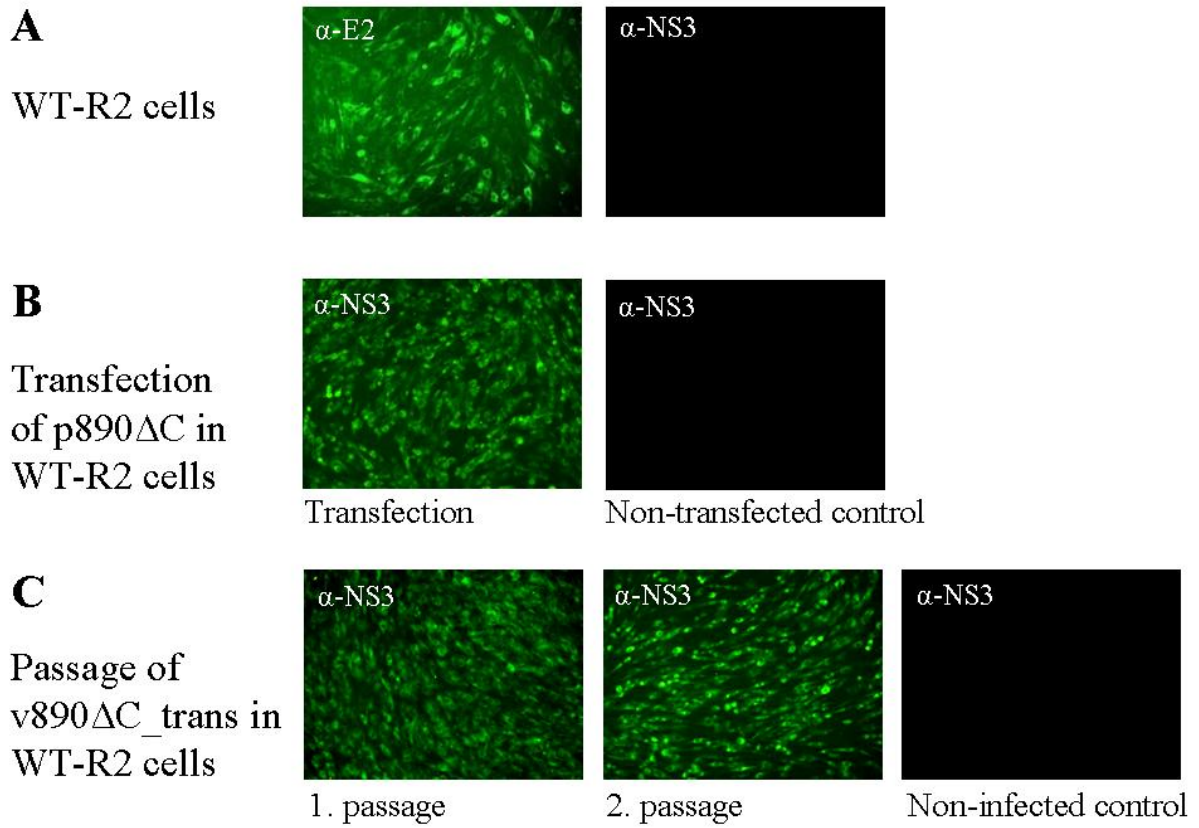


Figure 9:



## 3.2. Publication 2

J. Zemke, P. König, K. Mischkale, I. Reimann, M. Beer

**Novel BVDV-2 mutants as new candidates for modified live vaccines**

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# Novel BVDV-2 mutants as new candidates for modified live vaccines

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## Abstract

Protection against Bovine viral diarrhea virus (BVDV) type 2 infection of commercially available vaccines is often limited due to marked genetic and antigenic differences between BVDV type 1 (BVDV-1) and 2 (BVDV-2). Therefore, the immunogenicity of selected BVDV-1 and -2 mutants derived from infectious full-length cDNA clones and their use as modified-live vaccine candidates against challenge infection with a virulent heterologous BVDV-2 field isolate were investigated. Deletion mutants of BVDV-1 and BVDV-2 lacking a part of the $N^{pro}$ gene (BVDV-1Δ$N^{pro}$ / BVDV-2Δ$N^{pro}$) were used as well as a packaged replicon with a deletion in the structural core protein encoding region (BVDV-2ΔC-pseudovirions). The 25 calves used in this vaccination/challenge trial were allocated in five groups (n=5/group). One group received BVDV-1Δ$N^{pro}$ (1 shot), one group BVDV-2Δ$N^{pro}$ (1 shot), one group received both, BVDV-1Δ$N^{pro}$ and BVDV-2Δ$N^{pro}$ (1 shot), and one group was immunised with the BVDV-2ΔC pseudovirions (2 shots). The fifth group served as non-vaccinated control group. All groups were challenged intranasally with the BVDV-2 strain HI916 and monitored for signs of clinical disease, virus shedding and viremia. All tested BVDV vaccine candidates markedly reduced the outcome of the heterologous virulent BVDV-2 challenge infection showing graduated protective effects. The BVDV-2Δ$N^{pro}$ mutant was able to induce complete protection and a "sterile immunity" upon challenge. Thus it represents a promising candidate for an efficacious future live vaccine.


**Keywords**: Bovine viral diarrhea virus type 2; pestivirus; vaccination; infectious pestivirus clone; pseudovirions; modified-live vaccine;

## Introduction

The two species of Bovine viral diarrhea virus, BVDV-1 and BVDV-2, are grouped in the genus *Pestivirus* within the family of *Flaviviridae* (Mayo, 2002) together with other important animal pathogens such as Classical swine fever virus (CSFV) and Border disease virus (BDV). The genome of these enveloped viruses consists of a positive-sense single stranded RNA of about 12.3 kb in size. Analysis revealed at least eleven genetic groups within BVDV-1 (BVDV-1 a-k; Vilcek et al., 2001) and two within BVDV-2 (BVDV-2 a-b; Becher et al., 1999a). Furthermore, a cytopathogenic (cp) and a non-cytopathogenic (ncp) biotype can be differentiated in both species with respect to their effects on cells *in vitro*.

BVD is spread worldwide and goes along with high economic losses in the cattle industry (Brownlie et al., 1984; Houe, 1995). Most postnatal infections with both BVDV species take a subclinical course or cause only mild disease which can go along with fever, respiratory symptoms and reproductive disorders such as reduced fertility, abortions, congenital defects or stillbirth. In contrast to the infection of immunocompetent animals, which normally leads to an immune response and as a result to elimination of the virus, infection of pregnant animals in the first trimester with an ncp strain of one of both BVDV species may lead to transplacental infection of the fetus and to the development and birth of persistently infected calves (PIs) (Moennig and Liess, 1995).

In the 1980ies, a new acute severe form of BVD was observed in North America (Carman et al., 1998; Corapi et al., 1989, 1990). The course of disease was characterized by a hemorrhagic syndrome, associated with pronounced thrombocytopenia and a significant lethality rate (Pellerin et al., 1994; Ridpath et al., 1994). Subsequently, the causative strains were found to be genetic distinct from previous BVDV isolates and were typed as BVDV-2 (Pellerin et al., 1994; Ridpath et al., 1994; Harpin et al., 1995). Up to now, the prevalence of BVDV-2 increased especially in North America (up to 50 % of BVDV isolates; Ridpath,

2005), but was also described in Europe (Wolfmeyer et al., 1997; Letellier et al., 1999, Falcone et al., 1999) and other countries (Canal et al., 1998; Nagai et al., 1998).

Different approaches are adopted for disease eradication and various comprehensive control programs tackling the reduction of losses in different countries are pursued. In this context, especially in countries with a high prevalence, high cattle density and very active and quick trading, BVDV vaccination is a valuable tool of BVDV control. However, after vaccination with commercially available BVDV-1 vaccines, protection against BVDV-2 infection is often limited due to a marked genetic and antigenic heterogeneity between BVDV-1 and BVDV-2 (Becher et al., 1999a; Beer et al., 2000). In some cases clinical disease can be prevented, while viremia still occurs. But prevention of viremia is paramount for circumventing trans-placental infection of the fetus and thus emergence of PI calves. Taking the heterogeneity and the prevalence data into account, the demand for vaccines that provide a reliable prophylaxis for both, BVDV-1 and BVDV-2, is strengthened (Beer et al., 2000; Fulton et al., 2003). In current vaccination programs modified-live vaccines are used as well as inactivated ones, but there are concerns about their safety and/or efficacy (Becher et al., 2001; Bolin and Ridpath, 1995). Modified-live vaccines are considered to be efficacious as immunogenic proteins are amplified through viral replication, and offer a long lasting protection, but their safety is questionable especially when being used during pregnancy (Moennig and Liess, 1995) or in animals having contact to pregnant ones. The available inactivated vaccines are safe, but they have disadvantages concerning fetal protection, duration of protection and production costs, as high amounts of antigen and formulation with adjuvants are required (Beer et al., 2000; Beer and Wolf, 2003; Bolin and Ridpath, 1995).

New developments aim at combining the immunogenicity of live attenuated vaccines with the safety of inactivated ones by the use of genetically engineered constructs like vector vaccines, DNA vaccines, subunit vaccines, and marker or deletion mutants (Bruschke et al., 1997; Reimann et al., 2007). Vaccination with pseudovirions e.g. already proved to be successful for

BVDV-1. These pseudovirions are infectious virus particles derived from engineered mutants with a deletion in the structural protein region (replicons), and are produced through *in trans*-complementation by a helper cell line. They are safe through their inability to assemble new infectious virions when replicating in non-complementing cells so they are “defective in second cycle” (DISC; Reimann et al., 2007; Widjojoatmodjo et al., 2000). Another approach aims at attenuation through the deletion of the non-structural protein $N^{pro}$, which is involved in the suppression of the host’s innate immune system. Mutants were tested safe and efficacious for BVDV-1 (Meyers et al., 2007; P. König, unpublished data) and CSFV (Tratschin et al., 1998; Mayer et al., 2003). Those mutants can be propagated on conventional cell lines as the autoprotease $N^{pro}$ is dispensable for viral growth *in vitro*.

In this study, selected genetically engineered BVDV deletion mutants derived from infectious full-length cDNA clones were characterized *in vivo* in a vaccination/challenge trial. A BVDV-1 and a BVDV-2 deletion mutant, each lacking a major part of the $N^{pro}$ gene (BVDV-1$\Delta N^{pro}$ / BVDV-2$\Delta N^{pro}$), as well as pseudovirions derived from a BVDV-2 core protein deletion mutant (replicon) were tested as modified live candidates against a heterologous, virulent BVDV-2 isolate for challenge infection.

## Materials and Methods

### *Virus strains, deletion mutants and cells*

The cytopathogenic BVDV-1b strain CP7 was isolated from a case of fatal Mucosal Disease as described previously (Corapi et al., 1988). The CP7 cDNA full-length clone is based on the plasmid pA/BVDV and was kindly provided by G. Meyers (FLI Tübingen; Meyers et al., 1996). The CP7 $N^{pro}$ deletion mutant (BVDV-1$\Delta N^{pro}$) is lacking 156 aminoacids (aa) of the non-structural $N^{pro}$ gene (nucleotide (nt) 422-889; Reimann et al., unpublished data).

The BVDV-2 strain 890 (Bolin and Ridpath, 1992) is an ncp strain belonging to the subgroup BVDV-2a and was isolated in the USA from a heifer that died of acute BVDV infection. The 890 full-length cDNA clone (p890FL) was constructed and assembled by K. Mischkale et al. as were the BVDV-2 mutants used in this study (Mischkale et al., 2008). For receiving v890FLΔ$N^{pro}$ (BVDV-2Δ$N^{pro}$) a deletion of 156 aa was set as described above for BVDV-1Δ$N^{pro}$. The v890FLΔC replicon (BVDV-2ΔC) has a partial deletion of 43 aa (nt 986-1114) in the core protein encoding region.

The challenge strain, ncp BVDV-2a HI916, was isolated in Germany during an acute severe outbreak of BVD (kindly provided by G. Wolf, LMU München; Martin et al., 2005). BVDV-1b strain SE5508 was used in neutralisation assays as a heterologous BVDV-1 prototype virus.

Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% BVDV free foetal bovine serum and incubated at 37°C in a humidified atmosphere containing 5% $CO_2$.

A diploid bovine esophageal cell line, KOP-R (RIE244, Collection of cell lines in veterinary medicine, CCLV, FLI Insel Riems) that is highly susceptible to BVDV infection was used for virus isolation and neutralisation assays. The challenge virus stock was produced on Madin Darby bovine kidney (MDBK) cells (RIE261; CCLV, FLI Insel Riems). Virus stocks of the BVDV-1 and BVDV-2 $N^{pro}$ deletion mutants were propagated on interferon-incompetent MDBK cells (kindly provided by G. Keil, FLI; RIE728; CCLV, FLI Insel Riems). Generation of v890ΔC_trans (BVDV-2ΔC pseudovirions) was carried out using a new wisent helper cell line (WT-2; P. König, unpublished data). Cells and cell culture media were routinely screened for the absence of BVDV and BVDV-specific antibodies (CCLV, FLI Insel Riems). *In vitro* transcribed RNA of the BVDV-2ΔC replicon was transfected by electroporation into the *trans*-complementing helper cells. Infected, resp. transfected cell cultures were freeze/thawed once. All virus stocks were subsequently cleared from cell debris through low speed

centrifugation, titrated on KOP-R cells and tested for sterility and absence of mycoplasma. All virus preparations were stored at -70°C until use.

***Animals and experimental design***

25 conventionally reared female Holstein-Frisian calves were obtained from local farms after being tested negative for BVDV and BHV-1 (antibodies, antigen and genome). Their age ranged from 6 to 14 months. For the trial, animals were allocated into 5 different groups (n = 5 per group) and were housed in the Biosafety Level - 3 facility of the FLI. For vaccination, virus stocks were diluted in cell culture medium to a final concentration of 5 x $10^5$ tissue culture infective doses 50% ($TCID_{50}$) per ml. All calves were vaccinated intramuscularly with 2-5 ml containing approximately 1 x $10^6$ $TCID_{50}$ per animal (see values of the backtitrations enclosed in brackets behind the assigned mutant below). One group received the BVDV-2Δ$N^{pro}$ (9.3 x $10^5$ $TCID_{50}$), one the BVDV-1Δ$N^{pro}$ (9.3 x $10^5$ $TCID_{50}$), and one group received a mixture of both $N^{pro}$ deletion mutants in a single application (BVDV-2Δ$N^{pro}$ & BVDV-1Δ$N^{pro}$; 1.3 x $10^6$ $TCID_{50}$; approximately 6.5 x $10^5$ $TCID_{50}$ of each mutant). An additional group was vaccinated with the BVDV-2ΔC-pseudovirions (first shot: 1.1 x $10^6$ $TCID_{50}$ / second shot: 6.3 x $10^5$ $TCID_{50}$) and the animals of the fifth group served as non-vaccinated controls.

Two different vaccination schemes were used: animals that received the BVDV-2ΔC-pseudovirions were immunised twice, the shots given 25 days apart (day 0 and day 25).All other groups were immunised only once at day 25.

Intranasal challenge infection with the BVDV-2 strain HI916 ensued 35 days after the last vaccination for all 25 animals with 2.3 x $10^6$ $TCID_{50}$ in a volume of 2 ml (1ml per nostril) with the help of a nebulizer. All viral suspensions were backtitrated on KOP-R cells after inoculation to confirm infectious titres of the respective inoculum.

***Clinical evaluation***

Body temperatures were measured daily during the whole trial. Mean values of the BVDV-1ΔN$^{pro}$ group had to be calculated from four animals from day 39 on, as the behavior of one animal did not allow further measurement. The calves were examined for adverse reactions immediately after vaccination and challenge infection. Further clinical examinations were carried out during the sampling periods and comprised noting signs of clinical disease, focusing on respiratory and digestive disorders, and controlling general health status (depression, feed intake and behavior). Cumulative clinical scores were obtained using a defined scoring system, ranking signs from 0 (inconspicuous) to 4 (markedly abnormal). The clinical score was normalized to a cutoff line for mean group values at 2 points, above which we stated the score as raised. The cut-off was calculated from pre-vaccination signs and permanent basal symptoms like mild nasal discharge and sporadic coughing.

***Samples***

Nasal swabs and blood samples were taken daily over a period of 10 days after vaccination (after the first for the BVDV-2ΔC group) and for 14 days after challenge infection (16 days in the control group). Serum samples were obtained weekly throughout the course of the study. Specimens were subjected to virological, hematological and serological investigations.

***Hematological investigations (differential blood cell counts)***

Blood samples were taken by jugular venipuncture and collected in sterile blood collection tubes (Monovette) with potassium EDTA as anticoagulant (Sarstedt, Nuembrecht, Germany). Total white blood cell counts as well as thrombocyte counts were determined by size distribution analysis with an Abbott CellDyn 3700 analyzer.

***Virus isolation***

Virus isolation was done in cell culture, inoculating monolayers of KOP-R cells with 4 replicates per animal and specimen. After 3 to 4 days of incubation viral infection and replication was detected by indirect immunofluorescence staining of NS3 protein using mab mix WB103/105 (anti-NS3, CCpro, Germany). Binding was detected using ALEXA488 goat-anti-mouse-IgG conjugate (Molecular Probes). Evaluation was carried out using an IX51 fluorescence microscope (Olympus, Hamburg, Germany). Furthermore, one blind passage of supernatants was performed after 3 to 4 days of inoculation.

For determination of nasal virus shedding, swab sticks containing a sterile cotton plug (Medical wire and equipment MW&E, Corsham, England) were used. Swabs were submerged in 1 ml of cell culture medium supplemented with 200 U/ml penicillin, 200 µg/ml streptomycin and 5 µg/ml amphotericin B (Sigma–Aldrich, Deisenhofen, Germany). Four replicates per sample (100 µl/replicate) were co-cultivated on KOP-R cells and analyzed as described above. Samples were stored at -70°C until further use.

5mL of EDTA blood per animal and day were treated with an ammonium chloride blood lysisbuffer (155 mM $NH_4Cl$, 10 mM $KHCO_3$, 1 mM EDTA/ pH 7.4). After haemolysis and centrifugation at 3200 rpm, pelleted leukocytes were washed twice with phosphate buffered saline without Ca++/Mg++ (PBS-) and were re-suspended in 1 ml PBS-. KOP-R cell cultures were inoculated with 4 replicates per sample, containing approximately $3 \times 10^6$ leukocytes/100 µl each, and tested for BVDV using immunofluorescence analysis as described above. Purified leukocytes were stored at -70°C until further use.

***Serology***

Sterile blood collection tubes (Monovette) with a clot activator (Sarstedt, Nuembrecht, Germany) were applied for the weekly serum sampling. After centrifugation at 3000 rpm,

serum aliquots were stored at -20°C. For serological investigations all sera were inactivated at 56°C for 45 minutes.

A commercially available competitive NS3 antibody ELISA (PrioCHECK® BVDV Ab) was used and samples were processed following the manufacturer's instructions (Prionics AG, Switzerland). In order to confirm results, we additionally tested all samples with a second antibody ELISA, primarily detecting E2 antibodies (HerdChek® BVDV Ab, IDEXX Europe B.V.), according to the manufacturer's instructions.

Furthermore, sera from all animals were tested in a standard neutralisation assay (NA) against selected BVDV-1 and -2 strains (BVDV-1 SE5508, BVDV-2 890 and HI916). For all three virus strains, inactivated serum samples were serially diluted (log2 steps) in triplicates of 50 µl in 96-well plates using cell culture medium. 50 µl of a virus dilution (1000 $TCID_{50}$/ml) was added per well and the plates were incubated at 37°C. After 2 h of incubation, 100 µl of a KOP-R cell suspension (2 x $10^4$ cells/well) were added and the plates were incubated for 4 days. BVDV antibody-positive and antibody-negative sera were used as test controls. The virus titre was confirmed by backtitrations (log10 dilutions, 8 parallels). Neutralisation, i.e. absence of virus infection and replication, was detected by indirect immunofluorescence (see above). Titres were expressed as reciprocal of the highest dilution that caused 50% neutralisation ($log_2$ $ND_{50}$).

***Sequence analysis***

Total RNA from cells transfected with the full-length RNA of the BVDV-2ΔC mutant was extracted with the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. E2-encoding sequences were amplified using the Superscript III One-Step RT-PCR system (Invitrogen, Karlsruhe, Germany). After agarose gel electrophoresis, the E2-PCR product was further purified with the QIAex II Gel Extraction kit (Qiagen, Hilden, Germany). Sequencing of the E2 encoding region was carried out using the

Big Dye® Terminator v1.1 Cycle sequencing Kit (Applied Biosystems). Nucleotide sequences were read with an automatic sequencer (3130 Genetic Analyzer, Applied Biosystems, Foster City, USA) and analyzed using the Genetics Computer Group (GCG) software version 11.1 (Accelrys Inc., San Diego, USA). Custom primers were used for sequencing (MWG Biotech, Ebersberg, Germany).

## Results

### *Vaccination*

Following first vaccination with the pseudovirions (day 0), the general condition of the animals in the BVDV-2ΔC group remained unaffected. The animals showed neither adverse reactions nor a temperature rise (Fig. 1) or clinical signs of disease. No leukopenia could be observed (data not shown). To the contrary, there was even a slight increase in mean leukocyte counts up to a maximum of 147 % on day 7. Neither shedding via nasal excretions nor pseudovirion viremia was detectable by virus isolation in cell culture (Table 1 and Table 2). Therefore, the group was not sampled following second vaccination. In the NS3-specific blocking ELISA, the pseudovirion-immunised animals developed only a marginal rise in the inhibition levels and remained negative until booster vaccination (Fig. 2). Neutralising antibody titres against all three strains tested were not detectable after the first vaccination (Fig. 3).

After vaccination of all groups at day 25 ($2^{nd}$ immunisation of the BVDV-2ΔC group), all animals stayed within the physiological temperature range (Fig. 1). One animal from the BVDV-1Δ$N^{pro}$ and one out of the BVDV-2Δ$N^{pro}$ group showed a small elevation in temperature for one (day 28) to two days (day 31, day 32) respectively, but did not develop fever. The BVDV-1Δ$N^{pro}$ group showed some clinical reaction post vaccination (p. vacc.). They had slightly elevated mean clinical score values for 2 days (day 28 and day 35) due to mild respiratory symptoms (data not shown). None of the other groups had raised scores in

this period of monitoring. Calves in the groups which received the different $\Delta N^{pro}$-mutants had a short and monophasic decline in their leukocyte counts, which were reduced up to 28% (mean group value BVDV-1$\Delta N^{pro}$ & BVDV-2$\Delta N^{pro}$, day 29) p. vacc. (Fig. 4). In both, the BVDV-1$\Delta N^{pro}$ and the BVDV-2$\Delta N^{pro}$ group, up to 20% reduction of mean values was observed (days 29-30 and days 30-32). Thrombocyte counts also slightly decreased in all three sampled groups paralleling leukocyte counts (data not shown).

Vaccine virus was detected by isolation on cell culture in the nasal swab sample from one BVDV-2$\Delta N^{pro}$-immunised animal on one single day (day 32, 1 replicate after blind passage; Table 1). No nasal virus shedding could be observed in the other groups. In addition, animals from all three $\Delta N^{pro}$-groups groups had a very limited vaccine virus viremia (Table 2). Virus could be isolated from purified leukocytes from at least two animals per group for at least one day between day 29 and day 33 of the trial (BVDV-1$\Delta N^{pro}$: 3 animals, 2 days; BVDV-1$\Delta N^{pro}$ & BVDV-2$\Delta N^{pro}$: 3 animals, 1 to 2 days; BVDV-2$\Delta N^{pro}$: 2 animals, 3 to 4 days). All animals vaccinated on day 25 with the $N^{pro}$ deletion mutants were scored positive in a NS3 blocking ELISA (Fig. 2) from day 14 after vaccination. As mentioned above, animals in the BVDV-2ΔC group stayed negative after first immunisation but showed a clear boost in antibody development reacting ELISA positive as soon as 7 days after their second vaccination. Mean blocking values between 91 % and 95 % were reached in the different vaccination groups prior to challenge infection. Slightly lower mean inhibition values (81 %) were found in the BVDV-2$\Delta N^{pro}$ group (Fig. 2). All control animals stayed seronegative during the vaccination period. Neutralising antibody titres (Fig. 3) were found in all immunised groups as soon as 14 days after vaccination except for the BVDV-2ΔC-pseudovirion group. Testing sera against the heterologous BVDV-1 strain SE5508, highest pre-challenge titres with a mean value of up to 891 $ND_{50}$ were reached in the groups receiving BVDV-1$\Delta N^{pro}$ (alone or in the mixed application). All other groups stayed basal in their titres (mean values $\leq$ 2 $ND_{50}$) prior to challenge (Fig. 3). Using the BVDV -2 challenge strain

HI916 in a neutralisation assay, slightly different results were obtained. Highest titres after immunisation were reached in the group receiving the BVDV-2Δ$N^{pro}$ mutant (mean value 97 $ND_{50}$) followed by the BVDV-1Δ$N^{pro}$ & BVDV-2Δ$N^{pro}$ and the BVDV-1Δ$N^{pro}$ group (28 $ND_{50}$/11 $ND_{50}$). The group vaccinated with the pseudovirion preparation (BVDV-2ΔC), which was clearly positive in the ELISA by day 7 after booster vaccination, however stayed at basal to non detectable neutralising titre levels (< 2 $ND_{50}$) until challenge infection. Similar values and trends were seen against BVDV-2 strain 890. Mean titres of 79 $ND_{50}$ were found in the BVDV-2Δ$N^{pro}$ vaccinated animals at the day of challenge. The graduation between the groups was similar to the one obtained against BVDV-1 HI916 (BVDV-1Δ$N^{pro}$ & BVDV-2Δ$N^{pro}$ 18 $ND_{50}$; BVDV-1Δ$N^{pro}$ 11 $ND_{50}$). In the BVDV-2ΔC group, neutralising antibodies ranged from very low to undetectable (mean values ≤ 2 $ND_{50}$) even against the parental BVDV-2 890 strain.

***Challenge infection***

After challenge infection at day 60 of the trial, all control animals showed typical and clear signs of clinical disease. They had a biphasic rise in their body temperatures, a slight one at day 3, and a pronounced one at days 8 and 9 p. chall. with maximum mean group values of up to 41°C (Fig. 1). Simultaneously, a marked rise in clinical scores could be found peaking at days 8 to 10 (data not shown). Besides fever, all calves had marked respiratory symptoms (coughing and mucopurulent nasal discharge), depression with reduced appetite, and 2 animals showed watery diarrhea for 2 to 3 days. In contrast, clinical effects of the challenge infection were clearly reduced - or even absent - in the vaccinated groups. Vaccinated animals had an elevation in body temperature at day 7 p. chall. to different degrees (Fig. 1). Highest mean values were reached in the BVDV-1Δ$N^{pro}$ (40°C) and in the BVDV-1Δ$N^{pro}$ & BVDV-

2ΔN$^{pro}$ (39.8°C) groups. The BVDV-2ΔC group stayed in the physiological temperature range, peaking at 39.4°C. Temperature reaction went along with moderated respiratory symptoms in the BVDV-1ΔN$^{pro}$ group (data not shown). The only animals that stayed completely unaffected regarding their body temperature and clinical signs were those vaccinated with BVDV-2ΔN$^{pro}$.

After challenge infection, all control animals developed a severe leukopenia (Fig. 4). They had a bi- to triphasic decrease (days 3, 7 and 13 p. chall.) in leukocyte counts with maximum levels of 48 % reduction at day 7 after challenge. The vaccinated animals showed no or only a monophasic decline of the leukocyte numbers. A maximum decrease of about 12 % in the BVDV-1ΔN$^{pro}$ and the BVDV-2ΔC group was found at day 4. In addition, those animals quickly recovered to pre-infection counts (day 7 p. chall.). The group that received the mixed application of ΔN$^{pro}$ mutants (BVDV-1ΔN$^{pro}$ & BVDV-2ΔN$^{pro}$) had a mean drop of 20 % at day 5 p. chall. and of 24 % at day 10 p. chall., and these reduced leukocyte counts persisted till the end of the trial (day 89/day28 p.chall.) with a mean reduction of 20 % (Fig. 4). BVDV-2ΔN$^{pro}$ vaccinated animals showed no decrease in leukocyte blood counts after challenge infection. Thrombocyte counts were not as heavily affected by BVDV-2 infection as expected. The control animals had a mean reduction to a maximum of 35 % at day 3 after test infection. All other groups showed no or only a monophasic less marked decrease (data not shown). No clinical effects like bloody diarrhoea, petechia or hematomas on injection/injury sites, which were described for the challenge virus strain, were observed. Thereafter counts notably increased in the controls (to mean values of 195 %), the BVDV-1ΔN$^{pro}$ group (175 %) and slightly in the BVDV-1ΔN$^{pro}$ & BVDV-2ΔN$^{pro}$ one (125 %) corresponding to severity of infection and disease (data not shown).

Performing virus isolation, we found a long and pronounced challenge virus viremia in the control animals for up to 11 days (day 62 – day 73; Table 2) while there was a clear reduction in duration (day 63 – day 68) and amount in all vaccinated groups (BVDV-2ΔC: 4 animals, 1

day; BVDV-1ΔN$^{pro}$: all animals, 1 to 5 days; BVDV-1ΔN$^{pro}$ & BVDV-2ΔN$^{pro}$: 2 animals, 1 to 3 days). In contrast, no challenge virus could be isolated from the leukocytes of the BVDV-2ΔN$^{pro}$ group.

Furthermore, challenge virus was detectable in the nasal swab samples of all control animals from day 61 till day 71 (Table 1). Duration (day 62 – day 68) and levels of nasal virus shedding were again markedly reduced in the vaccinated animals (BVDV-2ΔC: all animals, 1 to 4 days; BVDV-1ΔN$^{pro}$: 4 animals, 1 to 3 days; BVDV-1ΔN$^{pro}$ & BVDV-2ΔN$^{pro}$: 2 animals, 1 to 2 days). No challenge virus could be recovered from the nasal swab samples of the BVDV-2ΔN$^{pro}$-vaccinated animals.

From day 14 p. chall., all control animals scored positive in the NS3 blocking ELISA, while NS3 antibodies in all other groups were slightly boostered. Mean blocking values of 100 % were reached in the five groups at day 89 (Fig. 2). Infection with BVDV-2 HI916 also induced a boost in neutralising antibodies titres in the immunised groups detected by neutralisation assays peaking at day 14 to day 28 p. chall. (Fig. 3). The controls developed detectable neutralising titres by day 14. Against BVDV-1 SE5508 maximum titres were reached in the BVDV-1ΔN$^{pro}$ and BVDV-1ΔN$^{pro}$ & BVDV$^{-2}$ΔN$^{pro}$ group with 3821 $ND_{50}$ and 1552 $ND_{50}$ respectively. The titres in the other three groups also increased, but mean values peaked at a markedly lower level (97 $ND_{50}$ and 16 $ND_{50}$), leaving a distinct gap between the two groups vaccinated with the BVDV-1 mutant and those receiving solely BVDV-2 (mutants or challenge strain). Tested against the BVDV-2 strain 890, sera of the BVDV-1ΔN$^{pro}$ animals featured maximum titres of 1552 $ND_{50}$. Values of all other groups followed closely and at the term of the study end titres were very similar in all groups (588 $ND_{50}$). Highest neutralising titres against the BVDV-2 strain HI916 remained lower (274 $ND_{50}$; BVDV-1ΔN$^{pro}$ group), but all mean group values were again similar at the end of the trial (181 $ND_{50}$) with slightly lower values for the BVDV-2ΔC-immunised group (69 $ND_{50}$). In general, the conducted neutralising assays in this study showed that titres of BVDV-2 exposed animals against

BVDV-2 strains were lower than those of the BVDV-1 vaccinated animals against the applied BVDV-1 strain.

***Sequencing results of the E2 region of BVDV-2 ΔC***

Due to widely differing results of the NS3-specific ELISA and the neutralisation assay for the BVDV-2ΔC-immunised group, we sequenced the region of the replicon encoding the E2 protein. The E2 protein is the major immunogen of BVDV and the predominant inducer of neutralising antibodies. We found one nucleotide change compared to the corresponding sequence of the parental full-length cDNA clone. It was located at nucleotide position 2736 referred to the full-length cDNA and leads to an amino acid change from leucine to histidine.

## Discussion

In contrast to vaccines currently licensed in Europe, commercially available vaccines in the United States, where BVDV-2 is highly prevalent, include BVDV-2 strains (Ridpath, 2005). Although data show a markedly lower prevalence in Europe (Wolfmeyer et al., 1997; Beer and Wolf, 1999), outbreaks of severe acute disease associated with hemorrhagic syndrome, reproductive losses and high lethality rates are reported. Thus, future vaccines will prove advantageous to also mediate a stable protection against BVDV-2 strains (Becher et al., 2000; Beer et al., 2000). In order to test and compare new potential BVDV-2 vaccine candidates *in vivo* we experimentally vaccinated cattle and subsequently challenged them with a recent, virulent German BVDV-2 field strain (HI916), which allowed graduated classification between the different vaccinated groups. The results of this study show that clinical symptoms, leukopenia, viremia and nasal virus shedding after experimental infection of calves could be clearly reduced or even prevented with all three tested BVDV mutants compared to the markedly affected control animals. However, thrombocytopenia and

hemorrhagic syndrome described after field-infection with BVDV-2 HI916 (Martin et al.; 2005), could not be observed.

In this study, a BVDV-2 replicon construct (Mischkale et al., 2008) with a deletion in the core protein region could be successfully complemented *in trans* with the help of a newly established helper cell line, expressing the BVDV-1-PT810 structural proteins C to E2 (König, unpublished data). Infectious particles, so called pseudovirions, could be generated in sufficient amounts to characterise BVDV-2ΔC *in vivo*. Following immunisation with BVDV-2ΔC-pseudovirions neither nasal virus shedding nor pseudovirion viremia was detectable as it was previously described for a BVDV-1ΔC mutant (Reimann et al., 2007). These *in vivo* results clearly indicate that pseudovirions are defective in second cycle (DISC) and therefore neither horizontally nor vertically transmissible after vaccination. Serological responses after immunisation with pseudovirions resembled those of inactivated vaccines (Beer and Wolf, 2003), showing low NS3 antibody levels after the first application and a clear and quick boost effect after the second one 25 days later. Unexpectedly, these antibodies had very low to non-existent neutralising abilities against any of the tested BVDV-1 and -2 strains, which is most likely the reason for the reduced protective effect in comparison to the previously published data from immunisation trials using BVDV-1ΔC (Reimann et al., 2007). Therefore, we sequenced the E2 encoding region of the replicon cDNA and found a single nucleotide change at nucleotide position 2736 leading to an amino acid change in a highly immunogenic region (leucine to histidine; aa 109 of the p890FL E2). For CSFV it was demonstrated by van Rijn (2007) that the local variability by one or more aminoacids in the E2 region may lead to differences in affinity, avidity and in cross-neutralisation. Therefore, we speculate that the altered E2 of BVDV-2ΔC could entail a lack of neutralising E2 antibodies and thus less efficient protective qualities. Possible functional defects of the mutated BVDV-2ΔC E2 protein could be masked and functionally complemented in the pseudovirion particles by the structural proteins of a BVDV-1 strain provided by the helper

cell line. In the animal, the non-replicative BVDV-1 E2 protein from the putative hybrid-envelopes did not induce detectable levels of neutralising antibodies against the tested BVDV-1 strain. However, BVDV-2ΔC had clear protective effects, which underlines the importance of cell-mediated responses to Pestivirus infection as it was described previously (Beer et al., 1997; Larsson and Fossum, 1992; Kimman et al., 1993; Pauly et al., 1995).

As previously described, the non-structural $N^{pro}$ protein interferes with the host's innate immune response by interacting with interferon regulatory factor-3 (IRF3) and targeting it for proteasomal degradation (Hilton et al., 2006). Knocking out this immunosuppressive function through deletion of an essential part of the genome aims at attenuating BVDV strains making them future vaccine candidates with enhanced safety. CSFV mutants with a deletion in the $N^{pro}$ gene already showed to be attenuated (Mayer et al., 2003). BVDV-1Δ$N^{pro}$ mutants also proved to be highly attenuated even in pregnant animals and to mediate complete protection against a heterologous BVDV-1 challenge (Meyers et al., 2007; König et al., unpublished data). In our study, we could show that *in vivo* both, the BVDV-1 and the BVDV-2 $N^{pro}$ deletion mutant provided clinical protection against challenge infection. Interestingly, lower homologous titres of neutralising antibodies were necessary to gain a "sterile immunity" than it was described for previous studies (Bolin and Ridpath, 1995; Beer et al., 2000). It turned out that a neutralising titre of 64 $ND_{50}$ to 128 $ND_{50}$ against the challenge strain prior to infection was sufficient to prevent systemic challenge virus infection, irrespective to the titres against the reference BVDV-1 strain SE5508. In all three groups NS3-specific antibodies could be detected as soon as 14 days after vaccination indicating effective replication of all mutants. The group immunised with BVDV-1Δ$N^{pro}$ had high neutralising antibodies against the BVDV-1 strain, which were apparently less effective in neutralising BVDV-2 strains *in vitro*, and this group was less protected against challenge infection than were the others. In contrast to the results of Beer et al. (2000), there was a marked difference in the titres developed against BVDV-1 and BVDV-2 when both $N^{pro}$ deletion mutants were

simultaneously applied. A possible explanation could be the more efficient infection and higher replication levels of the cp BVDV-1Δ$N^{pro}$, subsequently leading to interference with BVDV-2Δ$N^{pro}$ infection and replication. This assumption is supported by our findings that re-isolated virus from leukocyte samples was characterized as BVDV-1 by selective immunofluorescence staining. Nevertheless, only the BVDV-2Δ$N^{pro}$ group developed a complete protective immune response inducing a "sterile immunity" against heterologous BVDV-2-challenge infection.

The BVDV-2 strain 890 served as basis for the first approach generating this kind of BVDV-2 deletion mutants as it offers many options for virulence studies. This advantage was used in the presented proof-of-principle study to investigate in detail how effectively the two applied mutation strategies, deleting a structural protein or a protein acing as an immunosuppressing modulator, attenuate such an isolate *in vivo*. Obtained results could then be more reliably transferred to less virulent strains that would be used for further developments. As infectious virus from the constructed BVDV-2 full-length cDNA clone (p890FL) is still moderately virulent in cattle (Mischkale et al., 2008), it could be concluded from this trial that all tested BVDV-2 deletion mutants are further attenuated. It should be additionally beneficial to the safety of future BVDV-2 deletion mutants to originate from a cytopathogenic full-length cDNA clone, since cp viruses are the standard for attenuated BVDV-1 live vaccine preparations (Fulton et al., 2003; van Oirschot et al., 1999; Beer and Wolf, 2003). If cytopathogenic vaccine viruses would be able to reach the fetus, abortion could be induced in sporadic cases, but the possibility of induction of a persistent infection would be excluded, as there are no data that indicate reversion of a cp strain to an ncp one *in vivo* (Ridpath et al., 2005). Meyers et al. (2007) have shown that prevention of transplacental infection with BVDV-1 and -2 Δ$N^{pro}$ mutants requires a further elimination of the RNase activity of $E^{RNS}$. To investigate this safety aspect, vaccination studies with BVDV-2 $N^{pro}$ deletion mutants in pregnant animals have to be carried out. Reversions or recombinations of the assessed ncp

BVDV-2ΔN$^{pro}$ as well as for prospective cp BVDV-2 ΔN$^{pro}$ mutants with a second strain, as described previously (Becher et al., 1999b; Meyers et al., 1992), cannot be excluded by our present results. At least for BVDV-2ΔC these events are highly unlikely as discussed for BVDV-1 replicons that were thoroughly analysed *in vitro* (Reimann et al., 2003, 2007). Both issues, that would require extensive double infection studies *in vitro* as well as *in vivo,* were not addressed in the setup of this first trial, which served as promising basis for future developments.

## Conclusions

All BVDV vaccine candidates tested for safety and efficacy markedly reduced the outcome of the heterologous BVDV-2 challenge infection in cattle while showing graduated protective effects with regards to clinical symptoms, nasal virus shedding and viremia. The BVDV-2ΔNpro mutant provided complete protection leading to a “sterile immunity” against the highly virulent BVDV-2 challenge infection, facilitating its possible use as a future efficacious vaccine candidate.

Furthermore, the results of this study implicate further investigations, such as (i) consecutive vaccination with BVDV-1ΔNpro and BVDV-2ΔNpro to test for enhanced protection, (ii) construction and immunogenicity testing of a BVDV-2ΔC mutant without the observed amino acid exchange in the E2 region and (iii) development of a cp BVDV-2 full-length cDNA clone including corresponding deletion mutants.

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## Tables

**Table 1**: Virus isolation from nasal swab samples

**Virus isolation from nasal swabs**

**(A) Virus isolation from nasal swabs following vaccination**

| group | ear tag no | days of trial | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
| BVDV-2ΔC | 758 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | |
| | 759 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | |
| | 762 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | |
| | 775 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | |
| | 777 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | |
| control | 496 | | | | | | | | | | | | | | | | | | | |
| | 497 | | | | | | | | | | | | | | | | | | | |
| | 500 | | | | | | | | | | | | | | | | | | | |
| | 753 | | | | | | | | | | | | | | | | | | | |
| | 773 | | | | | | | | | | | | | | | | | | | |
| BVDV-1ΔNpro | 505 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 486 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 472 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 814 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 819 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BVDV-2ΔNpro & BVDV-1 ΔNpro | 494 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 502 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 503 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 764 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 779 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BVDV-2ΔNpro | 468 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 480 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 499 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 |
| | 509 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 767 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

**(B) Virus isolation from nasal swabs following challenge infection**

| group | ear tag no | days of trial | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 |
| BVDV-2ΔC | 758 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 759 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 762 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 775 | 0 | 0 | 3 | 0 | 4 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 777 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| control | 496 | 0 | 0 | 0 | 0 | 3 | 4 | 4 | 4 | 4 | 1 | 1 | 2 | 0 | 0 | 0 | 0 | 0 |
| | 497 | 0 | 0 | 0 | 0 | 1 | 2 | 4 | 4 | 4 | 1 | 4 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 500 | 0 | 1 | 0 | 1 | 2 | 1 | 2 | 3 | 4 | 3 | 4 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 753 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 773 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| BVDV-1ΔNpro | 505 | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 486 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 472 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 814 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 819 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| BVDV-2ΔNpro & BVDV-1 ΔNpro | 494 | 0 | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 502 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 503 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 764 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 779 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| BVDV-2ΔNpro | 468 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 480 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 499 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 509 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 767 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |

Nasal virus shedding of vaccine (A) /challenge (B) virus: Highly susceptible KOP-R cell cultures were inoculated with 4 replicates of nasal swab fluids (100 µl / replicate) and after 3-4 days virus replication was verified by immunofluorescence staining. Results were scored according to the number of positive inoculations (grey underlay) out of the 4 replicates (0 = no BVDV isolation → 4 = all inoculations BVDV positive). A first result was confirmed after one blind passage of the supernatants. Samples that were only detected positive after passaging are highlighted in light grey.

**Table 2:** Virus isolation from purified leukocytes

**Virus isolation from purified leukocytes**

**(A) Virus isolation from leukocytes following vaccination**

| group | ear tag no | days of trial 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BVDV-2ΔC | 758 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | |
| | 759 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | |
| | 762 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | |
| | 775 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | |
| | 777 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | | | | | | | | | | |
| control | 496 | | | | | | | | | | | | | | | | | | | | |
| | 497 | | | | | | | | | | | | | | | | | | | | |
| | 500 | | | | | | | | | | | | | | | | | | | | |
| | 753 | | | | | | | | | | | | | | | | | | | | |
| | 773 | | | | | | | | | | | | | | | | | | | | |
| BVDV-1ΔNpro | 505 | | | | | | | | | | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 486 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 0 | 0 | 0 |
| | 472 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 |
| | 814 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 819 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BVDV-2ΔNpro & | 494 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 0 | 0 | 0 | 0 |
| BVDV-1 ΔNpro | 502 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 503 | | | | | | | | | | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 764 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 779 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| BVDV-2ΔNpro | 468 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 480 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 1 | 2 | 1 | 0 | 0 | 0 |
| | 499 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 509 | | | | | | | | | | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 767 | | | | | | | | | | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 0 | 0 | 0 |

**(B) Virus isolation from leukocytes following challenge infection**

| group | ear tag no | days of trial 60 | 61 | 62 | 63 | 64 | 65 | 66 | 67 | 68 | 69 | 70 | 71 | 72 | 73 | 74 | 75 | 76 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BVDV-2ΔC | 758 | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 759 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 762 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 775 | 0 | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 777 | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| control | 496 | 0 | 0 | 1 | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 1 | 0 | 1 | 0 | 0 | 0 |
| | 497 | 0 | 0 | 2 | 1 | 3 | 4 | 4 | 4 | 4 | 4 | 2 | 2 | 2 | 0 | 0 | 0 | 0 |
| | 500 | 0 | 0 | 0 | 2 | 4 | 4 | 4 | 4 | 4 | 4 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 753 | 0 | 0 | 1 | 1 | 1 | 4 | 4 | 4 | 4 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | 773 | 0 | 0 | 2 | 0 | 4 | 4 | 4 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | | | | | | | | | | | | 0 | | | |
| BVDV-1ΔNpro | 505 | 0 | 0 | 0 | 4 | 4 | 4 | 4 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 486 | 0 | 0 | 0 | 1 | 1 | 3 | 4 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 472 | 0 | 0 | 0 | 0 | 0 | 2 | 3 | 2 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 814 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 819 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| BVDV-2ΔNpro & | 494 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| BVDV-1 ΔNpro | 502 | 0 | 0 | 0 | 2 | 3 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 503 | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 764 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 779 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| BVDV-2ΔNpro | 468 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 480 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 499 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 509 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |
| | 767 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | | |

Viremia of vaccine (A) / challenge (B) virus: Highly susceptible KOP-R cell cultures were inoculated with 4 replicates of purified leukocytes (3 x $10^6$ leukocytes / replicate) and after 3 to 4 days virus replication was verified by immunofluorescence staining. Results were scored according to the number of positive inoculations (grey underlay) out of the 4 replicates (0 = no BVDV isolation → 4 = all inoculations BVDV positive). A first result was confirmed after one blind passage of the supernatants. Samples that were only detected positive after passaging are highlighted in light grey.

## Figures

**Figure 1**: Mean group values of the rectal body temperatures throughout the course of the study. (⇧) marks day of vaccination and (⬆) the day of challenge infection. Temperatures of the groups were recorded daily until 3 weeks after challenge infection. < 39.5°C = physiological temperature; >40°C = fever. Dotted lines border the raised temperature range (39.5 - 40°C). One animal of the BVDV-1$\Delta N^{pro}$ group could not be measured after day 38 due to its behavior. The mean values were calculated from the other 4 animals from that day on. Standard deviations (error bars) are not depicted for the individual groups for clarity.

**Figure 2**: Development of NS3-specific antibodies after vaccination with the indicated mutants (⇧) and challenge infection with BVDV-2 strain HI916 (⬆). Serological responses were monitored employing a competitive NS3 antibody ELISA (Ceditest® BVDV, Cedi Diagnostics B.V. now: PrioCHECK® BVDV Ab, Prionics AG). Relative blocking values are indicated as mean group values. The dotted line is marking the threshold value of the test. Standard deviations (error bars) are not depicted for the individual groups for clarity.

**Figure 3:** Neutralising antibody titres against BVDV-1 SE5508 (A), the BVDV-2 890 wild type (B) and the BVDV-2 challenge strain HI916 (C) after vaccination and challenge infection were determined. Animals were vaccinated at day 0 (BVDV-2ΔC) and day 25 (all groups) (⇧). Challenge infection followed at day 60 (⬆). All values are given as mean group values. Standard deviations (error bars) are not shown for clarity of the curves.

**Figure 4**: Blood leukocytes were counted after vaccination(s) (day 0 and day 25) (⇧) and after challenge infection (day 60) (⬆) with a CELL-DYN® 3700 haematology analyser (Abbott, Chicago, USA) using EDTA-blood. Mean values of the different groups are shown in percent

of the initial values, which were set to 100 % prior to vaccination/challenge. Results of the BVDV-2ΔC group after vaccination (day 0) are not shown for clarity of the figure. No decline could be observed in this group following first immunisation and they were not sampled after the second one. Controls were measured starting at time of challenge infection. Standard deviations (error bars) are not depicted for the individual groups for clarity.

**Figure 1**

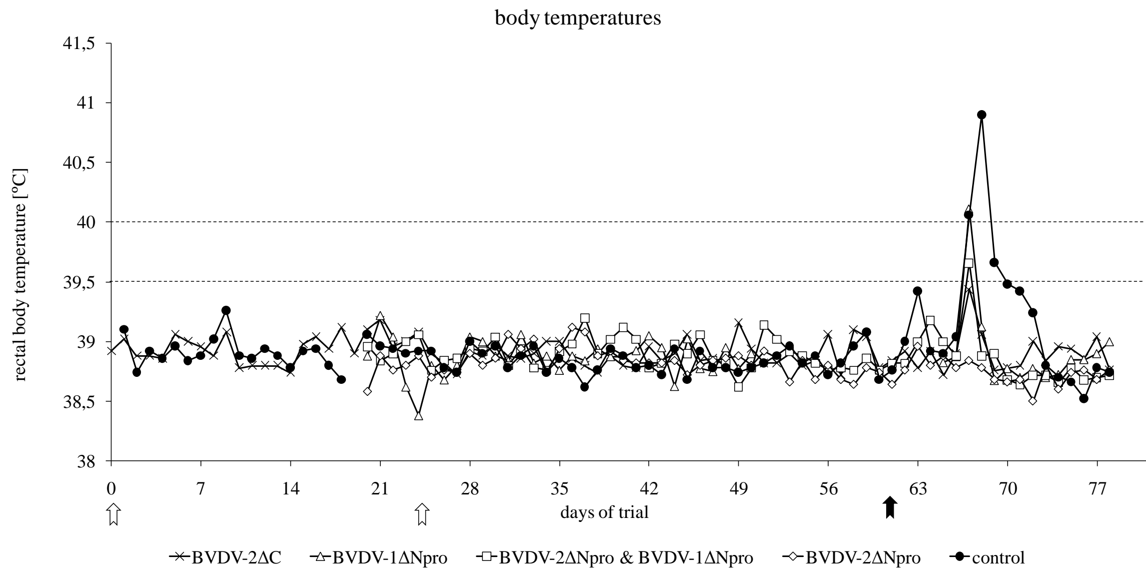


**Figure 2**

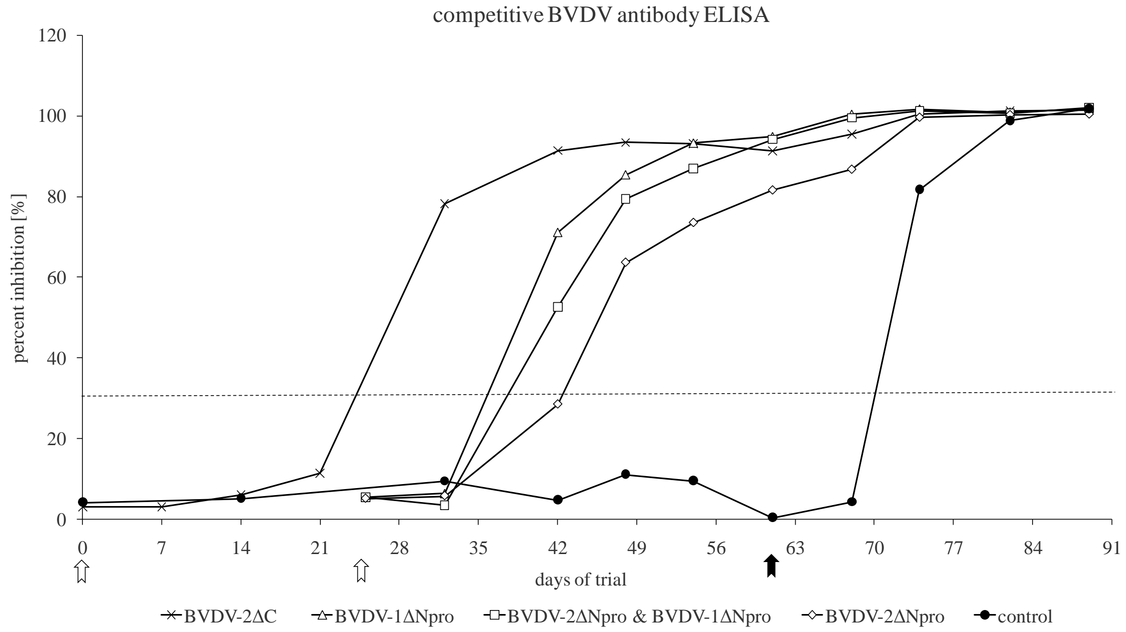
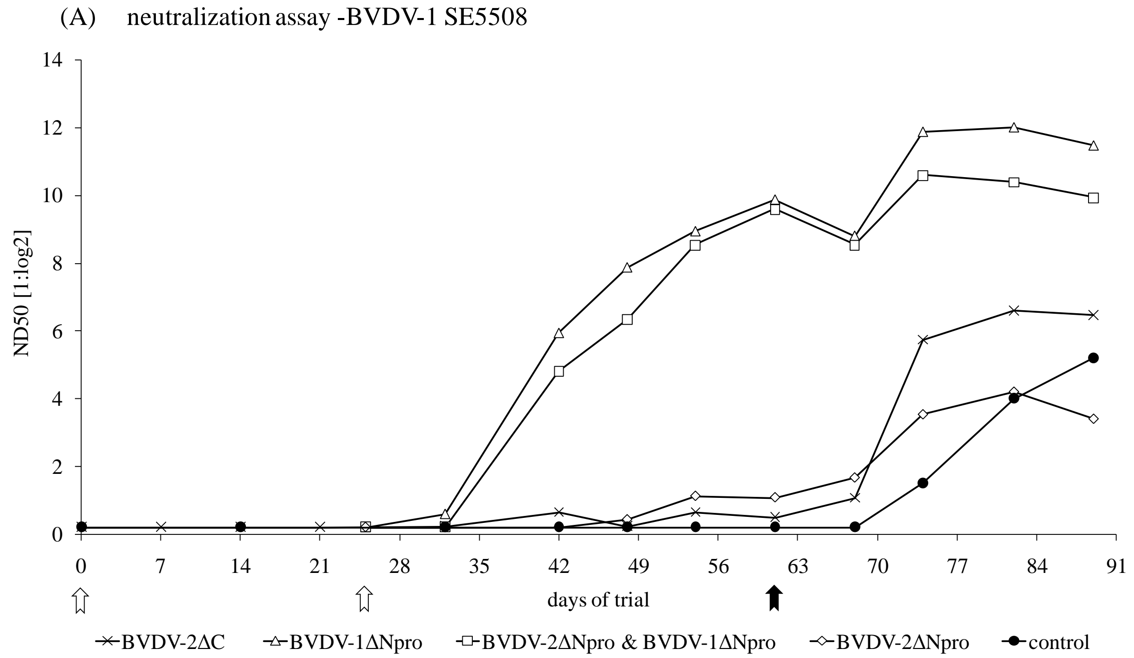
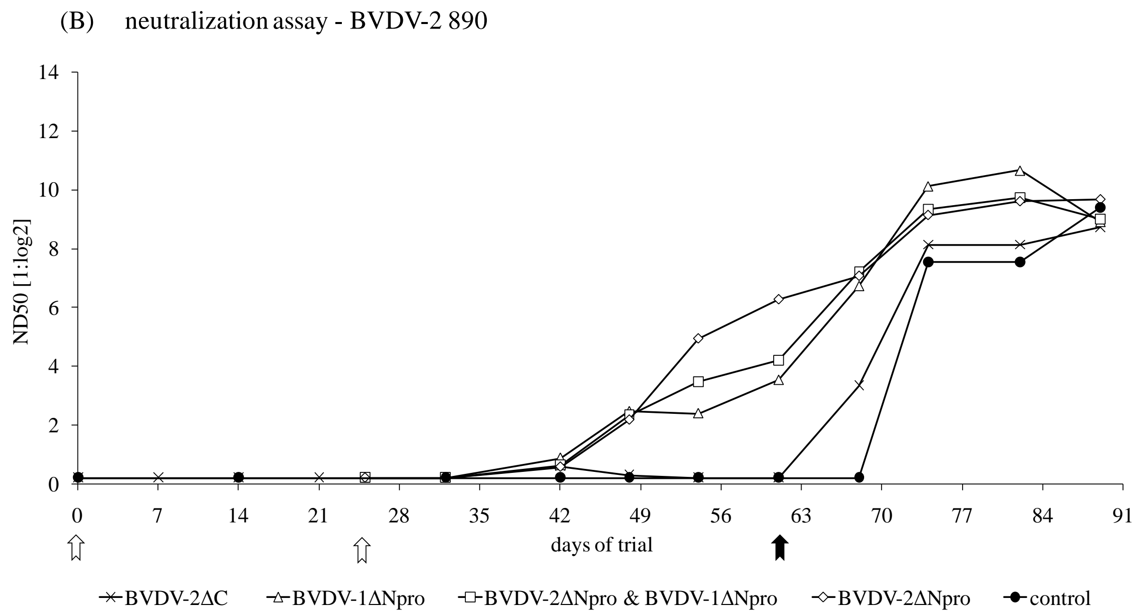


**Figure 3**



(C) neutralization assay -BVDV-2 HI916

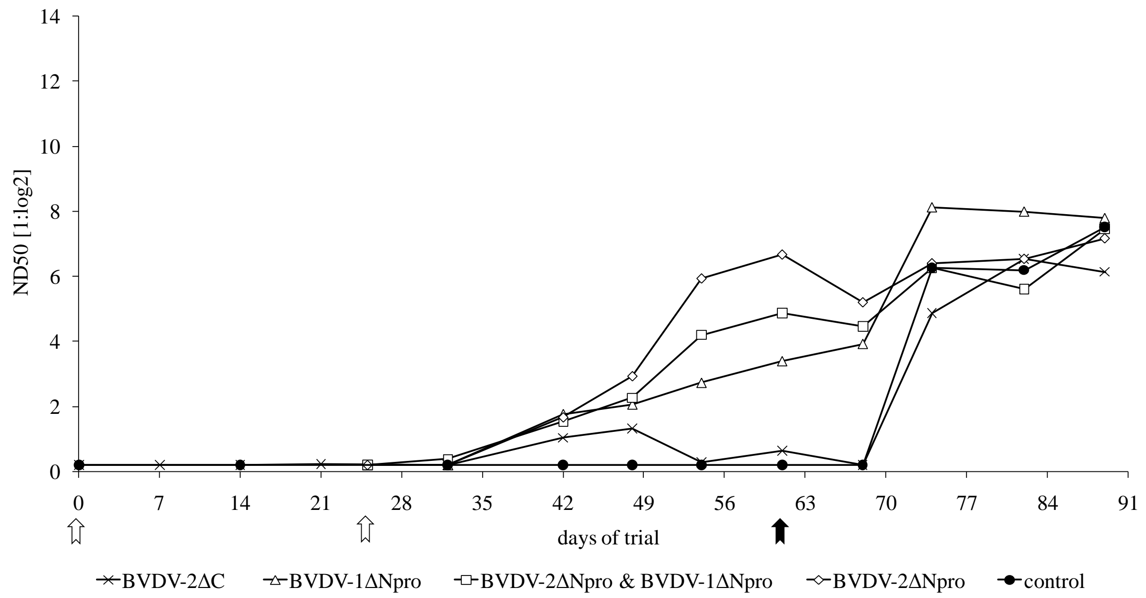
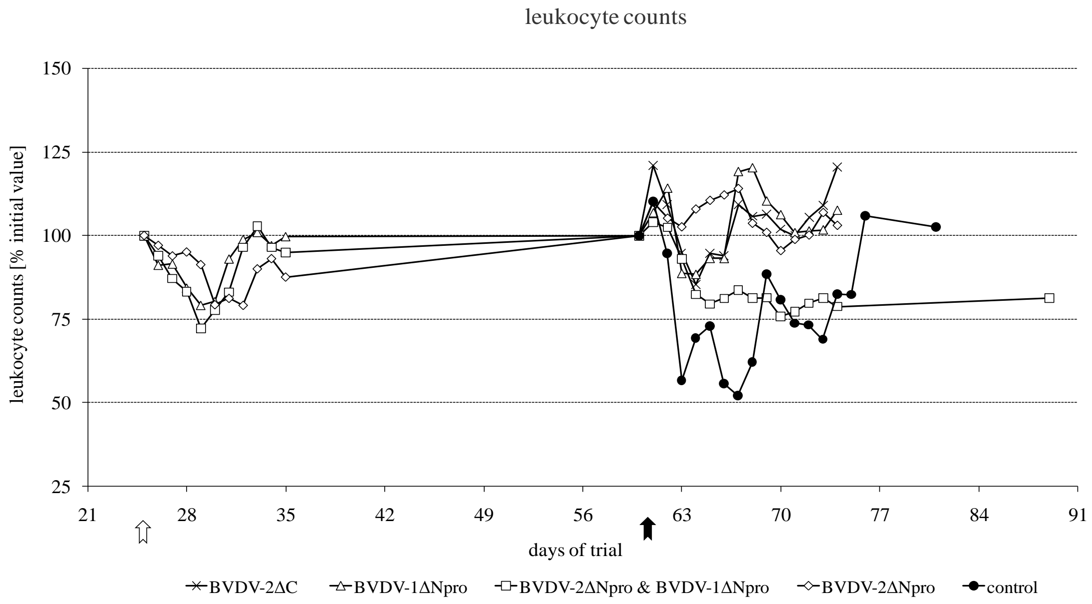


**Figure 4**



# 4. Extended discussion

Both types of vaccines against Bovine viral diarrhea virus licensed in Europe today, inactivated vaccines and modified-live virus (MLV) vaccines (Beer and Wolf, 2003), have clear disadvantages. Several new approaches using molecular techniques seek to combine the advantages of both systems while counterbalancing their drawbacks. The safety of an inactivated formulation and the immunogenicity of a modified-live virus vaccine (preventing viremia and fetal infection) (Greiser-Wilke et al., 2003; Beer and Wolf, 2003) are benchmarks for new candidates. Future vaccines will be held to a high standard, especially in the context of state-run control and eradication schemes like the compulsory program adopted in Germany in 2008 (Verordnung zum Schutz der Rinder vor einer Infektion mit dem Bovinen Virusdiarrhoe-Virus [BVDV-Verordnung]; Anonymous, 2008). Implementing European guidelines, the "Tierimpfstoff-Verordnung" in its 2006 revision is the legal basis for the licensing of veterinary vaccines in Germany.
BVDV-2 strains are commonly included in licensed vaccines in the U.S. due to the high prevalence of BVDV-2. In spite of the lower prevalence in Europe, it would also be advantageous for vaccines licensed in Germany if reliable protection against both species could be mediated by a single vaccine (Becher and Thiel, 2000; Beer et al., 2000). Relevant prototypes have been described (Beer et al., 2000). Progress in vaccine developments meeting the standards mentioned above only seems possible by further refining molecular approaches. Similar to the designs presented in this work, several attempts to attenuate modified-live virus vaccines have been published, among them deletions in the 5' UTR (Makoschey et al., 2004), a knock-out of the RNase function of $E^{RNS}$ (von Freyburg et al., 2004) or replicon systems (Reimann et al., 2003, 2007).

In the studies presented here, v890FL virus generated from a recently constructed ncp BVDV-2 full-length cDNA clone (p890FL) was characterised *in vivo* and compared to the parental strain by infection of cattle. Further, attenuated mutants of v890FL were examined in a subsequent vaccination and challenge trial assessing their safety and efficacy against a virulent heterologous BVDV-2 field isolate. The level of cross-protection of different vaccines or vaccine candidates against BVDV-1 and -2 has been examined in a number of studies. Makoschey et al. (2001) observed clinical protection against BVDV-2 when animals were vaccinated with an inactivated vaccine, in another experiment a BVDV-1 MLV vaccine even protected against viremia after challenge with BVDV-2 (Dean and Leyh, 1999). A

BVDV-1 mutant that had already proved to be safe and efficacious against BVDV-1 challenge (König et al., unpublished) was included in this trial to investigate its cross-neutralizing properties. The BVDV-1 mutant was administered either individually or in combination with the BVDV-2 mutant, since a combined application would be favourable for field use.

### 4.1. Full-length cDNA clone of ncp BVDV-2 strain 890 (p890FL): *in vivo* characterisation of generated virus (v890FL)

Mischkale et al. (2009) established a full-length cDNA clone (p890FL) based on the ncp BVDV-2a prototype strain 890. Virus (v890FL) was generated by transfection of bovine cells (KOP-R; RIE244; CCLV) with p890FL RNA and subsequent passaging. The parental strain 890 and v890FL were compared by intranasally infecting cattle and monitoring them daily for clinical and virological parameters over a period of 21 days. In the trial, v890FL displayed an attenuated phenotype, in particular by reduced clinical signs. A shorter duration of shedding over nasal mucosa and shorter viremia were observed. Antibody development (NS3 and neutralizing antibodies) was slightly delayed, but levels at day 28 were similar. Since the dose used for infection was the same for both strains, this suggests a lower *in vivo* replication efficiency of v890FL, in contrast to *in vitro* results of Mischkale et al. (2009). Causes for this attenuation have not yet been determined. Mischkale et al. (2009) describe four amino acid substitutions, two in the $E^{RNS}$ coding region, a third in the E2 gene and the fourth in the NS5a region. The latter two are not in previously defined functional regions (Johnson et al., 2001; Sapay et al., 2006). The first substitution in the $E^{RNS}$ region is similar to a mutation described by Dehan et al. (2005) in an attenuated infectious transcript of BVDV-2 890 while the second substitution is close to the RNase motif, an important virulence factor of BVDV (Meyer et al., 2002; Meyers et al., 2007). This could affect the virulence of v890FL and lead to the slight attenuation observed *in vivo*.

Full-length clones constructed for CSFV (C strain: van Gennip et al., 1999 / Eystrup: Mayer et al., 2003), BVDV-1 (pa/BVDV/Ins-: Meyers et al., 1996; NADL: Mendez et al., 1998) and BVDV-2 (NY93: Meyer et al., 2002) proved to be useful for investigating pestiviral functions and mechanisms (Ruggli and Rice, 1999). Despite its suitability as a basis for vaccine developments, the full-length infectious cDNA clone of BVDV-2 reference strain 890 lends itself to modifying its genomic structure (Meyers et al., 1996; Vassilev et al., 1997). This facilitates investigations on mechanisms of its ncp biotype, the function of the insertion in the

NS2 region of the parental BVDV-2 strain 890 and the replication cycle, as well as pathogenicity and interference features of BVDV-2. Due to its good replication efficiency and remaining virulence, v890FL is very suitable for testing attenuation options *in vivo*, as effects in less virulent strains are not as obvious. Mutants with a deletion either in the structural Capsid protein gene (replicons) or in the genome region of the non-structural protein $N^{pro}$ were constructed and tested in a vaccination-challenge trial.

### 4.2. Vaccination-challenge trial

Protection against field strains in the intended area of application is of utmost importance, and the selection of vaccine strains must be epidemiologically justified. Accordingly, HI916, a recent German BVDV-2 field isolate (Martin et al., 2005), was picked as a challenge strain. In a preceding animal trial (not included in the present work), it was shown that this isolate allows infection via the natural route (intranasally) and induces clear reproducible signs of disease, thus permitting comparisons between the different vaccinated groups. Thrombocytopenia and signs of hemorrhagic syndrome that had been observed in the outbreak where this strain was isolated (Martin et al., 2005), could never be reproduced in our trials with this isolate.

#### 4.2.1. Replicon p890ΔC: trans-complementation and vaccination of cattle with pseudovirions (v890ΔC)

Replicons are capable of effective replication without generation of infectious progeny (Harada et al., 2000; Reimann et al., 2003). The assembly of new infectious virions is inhibited by the deletion of the genes of one or more (structural) proteins, which are dispensable for virus replication (Behrens et al., 1998). *In vitro*, infectious progeny can be subsequently obtained by *trans*-complementation through a helper cell line expressing the missing proteins or by co-infection with a helper virus capable of *in trans* complementing the replicon proteins (Harada et al., 2000; Grassmann et al., 2001; Reimann et al., 2003, 2007). Based on the infectious full-length clone of BVDV-2 strain 890, a replicon lacking a major central part of the capsid protein gene was constructed (Mischkale et al., 2009). Amino acids (aa) 201-243 (nucleotides [nt] 986-1114) of p890FL were deleted (p890ΔC) while 32 aa at the N-terminal end and 27 aa at the C-terminal end remained. These are essential signalase recognition sequences and needed for correct further processing (Rümenapf et al., 1991) and

replication. Results of van Gennip et al. (2002) indicated difficulties in *trans*-complementation by a helper cell line system. Effective *trans*-complementation of CSFV E2 deletion mutants could be observed in only one of three E2 expressing cell lines after several serial passages. In the presented work effective *trans*-complementation could be demonstrated in a newly established helper cell line (WT-R2; RIE758; CCLV) (König et al., unpublished) constitutively expressing the structural proteins C to E2 of a BVDV-1 strain. This kind of *trans*-complementation was described previously for another helper cell line (PT_805) by Reimann et al. (2003, 2007), but WT-R2 cells provide a higher percentage of expression and better complementing efficiency (data not shown). Pseudovirions could be generated in sufficient amounts for the vaccination trials. *In vitro* infectivity was confirmed on complementing and non-complementing cell lines, while only the new cell line WT-R2 enabled serial passaging of v890ΔC. Signs of interference and markedly reduced susceptibility to BVDV infection that were described for the PT_805 cells (Reimann et al., 2003, 2007) and other cell lines expressing E2 (Hulst and Moormann, 1997; van Gennip et al., 2002) were not observed in the WT-R2 cell line. Passages on non-complementing cell lines showed no infection or replication, so recombination events with the BVDV structural protein cassette in the cell line could be excluded throughout our studies. In replicon systems for other families, these occurred frequently (Weiss and Schlesinger, 1991; Bredenbeek et al., 1993). Infectious revertants could not be detected even after several serial passages on non-complementing and complementing cells. These events were described as highly unlikely for BVDV-1 replicons, and the used *trans*-complementing system (Reimann et al., 2003, 2007). One possible reason is the use of BVDV-1 proteins for *trans*-complementing a BVDV-2 replicon, as homologous recombination may be complicated by genetic differences between strains and species. RNA-dependent RNA polymerase can switch strains/matrices in the genes of the non-structural proteins (Becher et al., 2001), but a switch in the structural protein region or a double template switch that would be necessary for a recombination event in the described *trans*-complementing system with the synthetic open reading frame (ORF) plasmid (Reimann et al., 2003) has not yet been reported and is obviously very improbable.

Dual vaccination of cattle with these pseudovirions within the presented work led to a detectable immune answer without leukopenia, viremia or nasal virus shedding. No negative reactions were observed after immunisation. No BVDV-specific clinical symptoms could be observed post-vaccination, thus animals were not sampled after the second vaccination. The developed level of NS3 antibody resembles that after use of an attenuated vaccine (Beer and Wolf; 2003). NS3 antibody levels were low after first vaccination, but a quick, clear boost

was observed after the second vaccination 25 days later. NS3-positive reactions were also described for killed vaccines but only as a weak signal after several booster shots (Beer et al., 2000). Unexpectedly and in contrast to the NS3 response, antibodies with neutralizing abilities (nab) against homologous or heterologous strains were not detected and titres stayed minimal throughout the vaccination period. Sequence data analysis of the virus mutant revealed a single nucleotide exchange leading to an amino acid substitution (leucine to histidine at nt position 2736 of p890FL) in a highly immunogenic region of the E2 protein, the major immunogen of BVDV. For CSFV van Rijn (2007) demonstrated differences in affinity, avidity and cross-neutralization due to the E2 variability of one or more amino acids. This could explain the absence of detectable neutralising antibodies and as a consequence the reduced protective effect of v890 ΔC after challenge infection compared to previous studies with similar BVDV-1 replicons (Reimann et al., 2007). BVDV-1 proteins expressed by WT-R2 cells can mask defects in the structural protein region so that infectivity is not affected. Proteins of the non-replicative hybrid envelope did not lead to a humoral response *in vivo*, as no increase in nab levels against BVDV-1 strains could be found. Despite this lack of neutralising antibodies, the clinical outcome of the challenge, viremia and shedding were clearly reduced in animals of the v890 ΔC vaccinated group, clearly emphazising the importance of cell-mediated immunity in BVDV infections (Larsson and Fossum, 1992; Beer et al., 1997).

Taking into account the results of previous experiments using BVDV-1 mutants with a similar deletion in the capsid protein region (Reimann et al., 2007), it is concluded that due to their limited one-time infectiousness the *trans*-complemented pseudovirions are as safe *in vivo* as they are *in vitro*. They replicate efficiently leading to protein expression appropriately answered by the animal immune system. After a first application they are able to initialize the activation of memory cells, which leads to a quicker and better response after booster immunization (prime-boost effect). These pseudovirions were defective in second cycle (DISC), no infectious progeny was produced, which would lead to infection of other cells and spread throughout the susceptive tissues of the host and subsequent transmission to other animals. Infection is restricted to cells at the site of application. When considering the use of pseudovirions as vaccines, it is essential to administer amounts sufficient for evoking an immune response as the infectivity is limited in contrast to the replication competence. The replicon system has the advantages of replication-competent MLV vaccine, but similar to an inactivated vaccine higher amounts of virions (pseudovirions) and at least one booster shot are required. Improvement of the immune answer when administering replicons could be

achieved by addition of classical or genetic adjuvants like immune stimulating factors IL-12 or GM-CSF as was already suggested by van Gennip et al. (2002) for CSFV replicons. As inactivated vaccines are described to mediate a shorter duration of immunity (Hofmann, 1998) with a decline as soon as 5 month after booster immunization, the stability of immunity induced after vaccination with pseudovirions needs to be determined, but was not an issue in this study.

It was shown for the first time that a BVDV-2 replicon (p890ΔC) can be successfully complemented *in trans* by a new helper cell line. The generated pseudovirions (v890ΔC) were capable of inducing an immune answer *in vivo* leading to a partial protection with a clearly reduced outcome of a virulent heterologous BVDV-2 field strain challenge. Recombinations and reversions restoring the ability to produce infectious progeny, which could lead to systemic spread and transmission, were not observed. The safety advantage of the replicon approach was demonstrated, confirming the results of previous studies (Reimann et al., 2007). In this regard pseudovirions are comparable to inactivated vaccines as transmission is not possible (Thierauf, 1993; Wolf et al., 1996). On the other hand, v890ΔC did not display the efficacy of a MLV, most likely because of an acquired mutation in a highly immunogenic region of the E2 gene. Its impact on the immunogenicity of v890ΔC can be tested by site-directed mutagenesis of p890ΔC, substituting the histidinde with a leucine, followed by *in vivo* application of both variants and monitoring neutralizing antibody titres.

Nevertheless, the obvious protective effect v890ΔC pseudovirions had even without detectable nab titers could be due to the developed cell-mediated immunity, which plays an important role in the hosts defense against BVDV infections (Beer et al., 1997).

### 4.2.2. Attenuation by deleting $N^{pro}$ (p890Δ$N^{pro}$): vaccination of cattle with v890Δ$N^{pro}$

Another approach to generate attenuated future vaccine candidates with enhanced safety is the deletion of an essential part of the genome region coding for the non-structural protein $N^{pro}$ unique to pestiviruses. $N^{pro}$ is the first protein encoded in the single pestivirus ORF and dispensable for virus replication and the generation of infectious progeny (Tratschin et al., 1998). This protein was described previously as an important, but not the only, virulence factor for pestiviruses. It interferes with mechanisms of the innate immune system, leading to inhibition of apoptosis and interferon-α/β production (Ruggli et al., 2005). BVDV achieves this effect by interaction with interferon regulatory factor 3 (IRF-3), a cellular transcription factor controlling interferon-α/β genes, and targeting it for proteasomal degradation (Hilton et

al., 2006). This function has mainly been observed in ncp strains and was supposed to enable the establishment of persistent infections in the fetus. Chen et al. (2007) described this way of interference likewise for a cp strain. The IRF-3 interaction and the autoprotease activity are independent but structurally overlapping functions. For CSFV, the inhibition of transcription of the IRF-3 gene has been shown by La Rocca et al. (2005), so CSFV seems to interfere at a different level in the same pathway. Recently a specific zinc-binding TRASH motif has been shown to be essential for virus mediated targeting of IRF-3 (Szymanski et al., 2009). *In vitro*, high interferon production and a lower replication efficiency of dendritic cells were described by Bauhofer et al. (2005) for CSFV$\Delta N^{pro}$. Attenuation *in vivo* has also been shown for CSFV (Mayer et al., 2004) and BVDV-1 deletion mutants (König et al., unpublished). Complete protection against a BVDV-1 challenge after BVDV-1$\Delta N^{pro}$ vaccination was demonstrated by König et al. (unpublished).

The virus mutant p890$\Delta N^{pro}$ investigated in this study lacks a major part of the genomic region coding for the non-structural autoproteinase $N^{pro}$, namely aa 13 to 168 of p890FL. The first 12 amino acids were retained to ensure IRES functionality vital for translation. Subsequently the capsid protein showed an N-terminal elongation. 13 out of the first 16 codons from the initial start codon seem to be conserved in the polyprotein of BVDV (Moes and Wirth, 2007). Different numbers (nine to 25) were described as essential for BVDV-1 (Moes and Wirth, 2007) while Meyers et al. (2007) described 4 residual codons sufficient for IRES function in a BVDV-2 full-length cDNA clone. Generation of v890 $\Delta N^{pro}$ vaccine stock was conducted by transfection and subsequent passaging on an interferon-incompetent bovine cell line (RIE728; CCLV). Replication was effective as was recovery of sufficient amounts of infectious virus. Titres were lower than for v890FL and 890 wildtype in a standard diploid bovine oesophageal cell line (KOP-R; RIE244; CCLV). This was also demonstrated by Mischkale et al. (2009) in growth kinetics on KOP-R cells and is most likely due to the loss of the interferon-antagonistic function of $N^{pro}$ (Gil et al., 2006). The virus stock v890 $\Delta N^{pro}$ was tested for correct deletion by a selective PCR and sequence analysis spanning the region of the deletion.

In this trial cattle were intramuscularly vaccinated with the already described and tested BVDV-1$\Delta N^{pro}$ (König et al., unpublished) and the newly generated v890$\Delta N^{pro}$. One group received BVDV-1$\Delta N^{pro}$, one v890$\Delta N^{pro}$ and one group received a mixed application of BVDV-1$\Delta N^{pro}$ and v890$\Delta N^{pro}$. Both mutants showed a clearly attenuated phenotype as no clinical symptoms were observed after vaccination. In the group receiving v890$\Delta N^{pro}$, virus shedding was observed in one animal on one day at a very low level. Limited vaccine virus

viremia was observed in all groups. Mutants provided clinical protection against the heterologous challenge infection but to different degrees. Only vaccination with v890ΔN$^{pro}$ led to sterile immunity completely preventing challenge virus viremia and shedding. Mixed application was more efficient than BVDV-1ΔN$^{pro}$, but unexpectedly inferior to v890ΔN$^{pro}$. Higher infection and replication efficiencies of the cytopathic BVDV-1ΔN$^{pro}$ could be a reason together with mechanisms of interference described for BVDV (Harada et al.; 2000). Only BVDV-1 could be reisolated after vaccination from blood leukocytes identified by selective indirect immunofluorescence.

This graduated protection was also reflected by the developed antibody titres. In general, it can be concluded that lower neutralizing titres against BVDV-2, evolved after vaccination, were needed to mediate complete challenge protection than described in other studies (Bolin and Ridpath, 1996; Beer et al., 2000). A titre of 64 to 128 ND50 at the day of challenge seemed sufficient for inducing a “sterile immunity” as it was observed in the v890ΔN$^{pro}$ group. High neutralizing titres detected in the BVDV-1ΔN$^{pro}$ group against a BVDV-1 strain were obviously less effective in neutralizing BVDV-2 strains. Mixed application led to different titres against BVDV-1 and -2 strains, as titres against BVDV-1 were clearly higher which promotes the assumption of better replication and/or higher immunogenicity of the BVDV-1 mutant. Effective replication could be demonstrated in all groups referring to the NS3 antibodies developed.

The presented data clearly demonstrate complete protection against a heterologous BVDV-2 challenge after vaccination with v890ΔN$^{pro}$. This mutant was as effective as other MLV vaccines in inducing a sterile immunity against challenge when administered individually. This was shown before for BVDV-1 mutants (ncp/cp) lacking the same region of the N$^{pro}$ gene. They proofed to be attenuated, safe and efficacious when used against a heterologous BVDV-1 challenge (König et al., unpublished). Cross-protection after vaccination with BVDV-1ΔN$^{pro}$ was incomplete as challenge virus viremia and shedding were observed, underlining the need for vaccines containing both species for reliable prevention of BVDV field infection. The mixed application was more efficacious than BVDV-1ΔN$^{pro}$, but did not offer complete protection either. Future studies with similar mutants based on cp BVDV-2 strains with similar replication efficiency have to be conducted to investigate this issue, as testing of sequential vaccination (due to prevalences in Germany: 1$^{st}$ shot BVDV-1 ΔN$^{pro}$, 2$^{nd}$ shot v890 ΔN$^{pro}$) did not lead to better protection (data not shown). The most promising candidate v890ΔN$^{pro}$ is still posing a minimal risk of vaccine virus transmission due to (very limited) viremia and shedding. The risk of transmission to other animals in contact could not

be excluded as has been shown for other MLV vaccines (Thierauf, 1993). Meyers et al. (2007) found that abolishing the $N^{pro}$ function in BVDV-2 was not sufficient for prevention of fetal infection. An additional deletion of the RNase function of $E^{RNS}$ protein was required for reliable attenuation. Other studies revealed that $N^{pro}$ deletion is sufficient for BVDV-1 and mutants seemed not capable of transplacental transmission when pregnant animals were infected (König et al., unpublished). But safety and efficacy studies with pregnant animals were not part of this proof-of-principle trial and neither were possibilities of recombination and reversion. These events cannot be ruled out based on the presented results, although they are very unlikely and were not observed *in vitro*. Extensive co-infection studies would be required to investigate this important issue *in vivo*.

### 4.3. Conclusions and outlook

Virus generated from a newly established full-length cDNA clone of BVDV-2 reference strain 890 was shown to be virulent, but slightly attenuated *in vivo* compared to the wild type parental strain. The attenuation of a highly virulent BVDV-2 strain by two completely different approaches was demonstrated in the second proof-of-principle study. A replicon construct missing part of the capsid protein coding region and an $N^{pro}$ gene knock-out construct where an important immunosuppressive function of pestiviruses was disabled were tested. Both were clearly attenuated *in vivo* and mediated graduated protection against a virulent heterologous BVDV-2 challenge, as did a BVDV-1$\Delta N^{pro}$ mutant. All vaccine candidates clearly reduced or in case of v890$\Delta N^{pro}$ completely prevented clinical symptoms, complete blood count deviations and viremia after challenge. The results of this study are relevant to future developments in BVDV vaccination. They could be beneficial for designing new chimeric pestiviruses, of which some have been constructed (van Gennip et al., 2002) and tested successfully (Reimann et al., 2003; Koenig et al., 2007; Leifer et al., 2009a). Some constructs like CP7_E2alf feature beneficial marker properties which can be used in standard or newly developed diagnostic methods (Koenig et al., 2007; Leifer et al., 2009b) and would be advantageous for BVDV vaccines too. However, future vaccine candidates will derive their origin from less virulent BVDV strains for further attenuation. Additionally, a cytopathic BVDV strain, standard in most vaccine preparations (Fulton et al., 2003; Beer and Wolf, 2003), would offer additional advantages in safety as induction of persistently infected offspring would be excluded both by the biotype and for example the deletion of $N^{pro}$.

## 5. Summary

Vaccination against Bovine Viral Diarrhea Virus plays a major role in the obligatory German control program decided in 2008. Both kinds of vaccines licensed today for the use in cattle have disadvantages: MLV vaccines concerning their safety and killed vaccines concerning efficacy, especially in terms of cross-neutralization.

In the presented work, virus (v890FL) generated by transfection of conventional bovine cell lines with RNA derived from a BVDV-2 full-length clone (p890FL; Mischkale et al., 2009) was used to infect cattle. Its effects compared to the wild type strain 890 were investigated. It could be shown that it was still virulent but slightly attenuated. This functional full-length clone offers many possibilities to investigate further virulence mechanisms and genetic features of the BVDV-2 strain 890.

Further, two mutants derived from this BVDV-2 full-length cDNA clone were tested in a vaccination-challenge trial in cattle randomly allocated in groups of five heads each following two approaches of attenuation. Their safety and efficacy as vaccine candidates were investigated.

1$^{st}$ approach: Replicon (p890ΔC) and pseudovirions (v890ΔC)

Replication competent BVDV-2 genomes with a deletion in the coding region for the structural Capsid protein were transfected into a recently established *trans*-complementing helper cell line, constitutively expressing BVDV-1 structural proteins C to E2, and were *trans*-complemented effectively. Pseudovirions in sufficient amounts for vaccination purposes were produced. The group of cattle receiving v890ΔC was vaccinated twice.

2$^{nd}$ approach: Deletion of $N^{pro}$ (v890Δ$N^{pro}$ and BVDV-1Δ$N^{pro}$)

A BVDV-2 mutant lacking the major part of the nonstructural $N^{pro}$ protein (v890Δ$N^{pro}$) was propagated on a cell line deficient of Interferon production, as was an already described BVDV-1Δ$N^{pro}$ mutant. One group of animals was vaccinated with v890Δ$N^{pro}$, one with BVDV-1Δ$N^{pro}$ and one with a combination of both administered in a single application. Animals vaccinated with $N^{pro}$ deletion mutants received only one shot.

There was no vaccine virus shedding or viremia in the pseudovirion group. For the first time, the safety described for the replicon system could be verified for BVDV-2. A short viremia was observed in the groups vaccinated with $N^{pro}$ mutants. A very limited shed of v890Δ$N^{pro}$ (one animal on one day) was detected.

All animals including a naïve control group were challenged with a virulent heterologous German BVDV-2 field strain. The tested BVDV vaccine candidates markedly reduced the outcome of the heterologous virulent BVDV-2 challenge infection showing graduated protective effects in terms of reduced time and amount of shedding and viremia and milder clinical symptoms.
Unlike previous studies, the protection after vaccination with replicons was not complete. This could be explained by nominal (undetectable) neutralizing antibody titers due to a mutation in the E2 gene.
The v890ΔN$^{pro}$ mutant was able to induce complete protection and a "sterile immunity" upon heterologous challenge, still bearing the risk of a very limited vaccine virus shedding. The deletion of N$^{pro}$ was shown to be an additional clear attenuation factor in BVDV-2, as it was confirmed for the BVDV-1ΔN$^{pro}$ mutant in this trial. This will be beneficial for future developments.

Besides, the results of this study implicate further investigations, such as construction and immunogenicity testing of a p890ΔC mutant without the observed amino acid exchange in the E2 region and development of a cp BVDV-2 full-length cDNA clone including corresponding deletion mutants.

# 6. Zusammenfassung

Immunisierung gegen die Bovine Virusdiarrhoe spielt eine entscheidende Rolle im 2008 verabschiedeten, verpflichtenden BVDV Bekämpfungsprogramm. Die zwei Arten von Vakzinen, welche derzeit in Deutschland zur Immunisierung von Rindern zugelassen sind, haben jeweils klare Nachteile: Lebendvakzinen sind nicht vollständig sicher in ihrer Anwendung, während Totvakzinen einen oft nur unzulänglichen Schutz bieten, vor allem vor Infektionen mit heterologen Stämmen.

In der vorliegenden Arbeit wurden Viren (v890FL), ausgehend von einem neu konstruierten BVDV-2 Volllängen cDNA Klon (p890FL; Mischkale et al., 2009), nach RNA Transfektion und Anzucht auf konventionellen Rinderzelllinien, *in vivo* charakterisiert und mit dem Wildtyp Stamm verglichen. v890FL verhielt sich *in vivo* virulent, jedoch schwach attenuiert im Vergleich zum Wildtyp. Der vorliegende Volllängenklon bietet vielfältige Möglichkeiten um weitere Virulenzmechanismen sowie genetische Eigenschaften des BVDV-2 Stammes 890 im Detail zu untersuchen.

Zwei verschiedene Ansätze wurden verfolgt um attenuierte Mutanten vom BVDV-2 Klon p890FL zu generieren. Diese wurden als Vakzinekandidaten gegen einen heterologen, virulenten BVDV-2 Challenge eingesetzt. Rinder in Gruppen von jeweils 5 Tieren wurden geimpft und die Sicherheit und Wirksamkeit der Mutanten untersucht.
1. Ansatz: Replikon (p890ΔC) basierte Pseudovirionen (v890ΔC)
Replikationskompetente BVDV-2 Genome mit einer Deletion im Bereich des Capsid kodierenden Gens wurden in eine neu etablierte *trans*-komplementierende Helferzelllinie transfiziert, welche permanent die Strukturproteine C bis E2 eines BVDV-1 Stammes exprimiert. Replikons wurden effizient *in trans*-komplementiert und Pseudovirionen konnten in ausreichenden Mengen gewonnen werden. Die Gruppe, der v890ΔC Pseudovirionen appliziert wurden, wurde zweifach immunisiert.
2.Ansatz: Deletion des $N^{pro}$ Gens (v890Δ$N^{pro}$ und BVDV-1Δ$N^{pro}$)
Für die Anzucht von BVDV-1 und BVDV-2 Mutanten, denen der größte Teil des Nichtstrukturproteins $N^{pro}$ fehlt, wurde eine Interferon-defiziente Zelllinie verwendet. Eine Tiergruppe wurde mit v890Δ$N^{pro}$ geimpft, eine erhielt BVDV-1Δ$N^{pro}$ und eine weitere beide Mutanten gemischt in einer Applikation. Alle drei Gruppen wurden nur einmal geimpft.

Weder Vakzinevirusausscheidung noch Virämie konnten in der Gruppe, die mit Pseudovirionen geimpft wurde, nachgewiesen werden. Somit wurde die schon beschriebene Sicherheit des Replikonansatzes *in vivo* durch diese Studie erstmals auch für BVDV-2 bestätigt.In den anderen immunisierten Gruppen wurde eine kurze Virämie post vaccinationem beobachtet, bei einem Tier aus der $\Delta N^{pro}$ Gruppe sogar eine geringgradige Ausscheidung an einem Tag.
Alle geimpften Tiere inklusive naïver Kontrollen wurden mit einem virulenten heterologen Deutschen BVDV-2 Feldisolat infiziert. Die Immunisierung der Tiere zeigte einen deutlichen protektiven Effekt gegen die Challengeinfektion, wenngleich sehr abgestuft zwischen den einzelnen Gruppen. Die Tiere zeigten eine verminderte und verkürzte Ausscheidung und Virämie und deutlich geringer ausgeprägte klinische Symptome.
Im Gegensatz zu vorhergehenden Studien wurde kein vollständiger Schutz durch die zweifache Immunisierung mit Pseudovirionen erreicht. Dies könnte vermutlich durch eine einzelne Mutation im E2 Protein und die damit verbundenen extrem niedrigen Titer neutralisierender Antikörper gegen die getesteten Stämme begründet sein.
Eine einmalige Vakzinierung mit v890$\Delta N^{pro}$ führte zu einer sterilen Immunität und somit vollständigem Schutz vor einer Challenge Infektion mit einem heterologen BVDV-2 Isolat, birgt aber ein Restrisiko aufgrund der minimalen Ausscheidung der Vakzineviren.
Eine deutliche Attenuierung durch Deletion des $N^{pro}$ Proteins konnte in diesem Versuch für BVDV-2 ebenfalls gezeigt, sowie für BVDV-1 bestätigt werden und für zukünftige Weiterentwicklungen eine wichtige Grundlage darstellen.

Die vorliegenden Ergebnisse können durch weitere Untersuchungen ergänzt werden z.B. durch die Konstruktion eines p890ΔC ohne Mutation im E2 und einen vergleichenden Test der immunogenen Wirkung *in vivo*. Weiterhin wäre die Entwicklung einer zytopathogenen Variante des Volllängenklons p890FL erstrebenswert zur zusätzlichen Sicherheit generierter Mutanten *in vivo*.

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## 8. Abbreviations

| | |
|---|---|
| aa | amino acid/amino acids |
| BDV | Border Disease Virus |
| BVDV | Bovine Viral Diarrhea Virus |
| C-terminal | Carboxy-terminal end (3’end) |
| CCLV | Collection of Cell Lines in Veterinary medicine (FLI-Insel Riems) |
| cDNA | complementary DNA |
| cp | cytopathic |
| CSFV | Classical Swine Fever Virus |
| DISC | Defective In Second Cycle |
| DIVA | Differentiating Infected from Vaccinated Animals |
| DNA | Deoxyribonucleic Acid |
| ELISA | Enzyme-Linked Immunosorbent Assay |
| et al. | et alii/et aliae |
| FCS | Fetal Calf Serum |
| Fig. | Figure |
| fl | full-length |
| GM-CSF | Granulocyte Macrophage Colony-Stimulating Factor |
| IFN | Interferon |
| IL-12 | Interleukin-12 |
| IRES | Internal Ribosomal Entry Site |
| IRF-3 | Interferon Regulatory Factor-3 |
| kb | kilobase |
| LDLR | Low Density Lipoprotein Receptor |
| LMU | Ludwig-Maximilians-Universität, Munich |
| mab | monoclonal antibody/antibodies |
| MLV | Modified Live Virus |
| N-terminal | Amino-terminal (5’end) |
| nab | neutralizing antibody/antibodies |
| ncp | noncytopathic |
| ND50 | 50% Neutralizing Dose |

| | |
|---|---|
| nm | nanometer |
| nt | nucleotide |
| ORF | Open Reading Frame |
| PCR | Polymerase Chain Reaction |
| % | percent |
| PI | persistently infected |
| RNA | Ribonucleic Acid |
| RNase | Ribonuclease |
| RT-PCR | Reverse Transcription Polymerase Chain Reaction |
| TNF-α | Tumor Necrosis Factor - α |
| U.S. | United States of America |
| UTR | Untranslated Region |
| VRP | Virus Replicon Particles |

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