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

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28 **Abstract**

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

47

48

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

51

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53

54 **Introduction**

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

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

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

79 2005), but was also described in Europe (Wolfmeyer et al., 1997; Letellier et al., 1999,
80 Falcone et al., 1999) and other countries (Canal et al., 1998; Nagai et al., 1998).

81 Different approaches are adopted for disease eradication and various comprehensive control
82 programs tackling the reduction of losses in different countries are pursued. In this context,
83 especially in countries with a high prevalence, high cattle density and very active and quick
84 trading, BVDV vaccination is a valuable tool of BVDV control. However, after vaccination
85 with commercially available BVDV-1 vaccines, protection against BVDV-2 infection is often
86 limited due to a marked genetic and antigenic heterogeneity between BVDV-1 and BVDV-2
87 (Becher et al., 1999a; Beer et al., 2000). In some cases clinical disease can be prevented,
88 while viremia still occurs. But prevention of viremia is paramount for circumventing trans-
89 placental infection of the fetus and thus emergence of PI calves. Taking the heterogeneity and
90 the prevalence data into account, the demand for vaccines that provide a reliable prophylaxis
91 for both, BVDV-1 and BVDV-2, is strengthened (Beer et al., 2000; Fulton et al., 2003). In
92 current vaccination programs modified-live vaccines are used as well as inactivated ones, but
93 there are concerns about their safety and/or efficacy (Becher et al., 2001; Bolin and Ridpath,
94 1995). Modified-live vaccines are considered to be efficacious as immunogenic proteins are
95 amplified through viral replication, and offer a long lasting protection, but their safety is
96 questionable especially when being used during pregnancy (Moennig and Liess, 1995) or in
97 animals having contact to pregnant ones. The available inactivated vaccines are safe, but they
98 have disadvantages concerning fetal protection, duration of protection and production costs,
99 as high amounts of antigen and formulation with adjuvants are required (Beer et al., 2000;
100 Beer and Wolf, 2003; Bolin and Ridpath, 1995).

101 New developments aim at combining the immunogenicity of live attenuated vaccines with the
102 safety of inactivated ones by the use of genetically engineered constructs like vector vaccines,
103 DNA vaccines, subunit vaccines, and marker or deletion mutants (Bruschke et al., 1997;

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

115 In this study, selected genetically engineered BVDV deletion mutants derived from infectious
116 full-length cDNA clones were characterized *in vivo* in a vaccination/challenge trial. A BVDV-
117 1 and a BVDV-2 deletion mutant, each lacking a major part of the N^{pro} gene (BVDV-1ΔN^{pro} /
118 BVDV-2ΔN^{pro}), as well as pseudovirions derived from a BVDV-2 core protein deletion
119 mutant (replicon) were tested as modified live candidates against a heterologous, virulent
120 BVDV-2 isolate for challenge infection.

121

122 **Materials and Methods**

123

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

125 The cytopathogenic BVDV-1b strain CP7 was isolated from a case of fatal Mucosal Disease
126 as described previously (Corapi et al., 1988). The CP7 cDNA full-length clone is based on the
127 plasmid pA/BVDV and was kindly provided by G. Meyers (FLI Tübingen; Meyers et al.,

128 1996). The CP7 N^{pro} deletion mutant (BVDV-1ΔN^{pro}) is lacking 156 aminoacids (aa) of the
129 non-structural N^{pro} gene (nucleotide (nt) 422-889; Reimann et al., unpublished data).

130 The BVDV-2 strain 890 (Bolin and Ridpath, 1992) is an ncp strain belonging to the subgroup
131 BVDV-2a and was isolated in the USA from a heifer that died of acute BVDV infection. The
132 890 full-length cDNA clone (p890FL) was constructed and assembled by K. Mischkale et al.
133 as were the BVDV-2 mutants used in this study (Mischkale et al., 2008). For receiving
134 v890FLΔN^{pro} (BVDV-2ΔN^{pro}) a deletion of 156 aa was set as described above for BVDV-
135 1ΔN^{pro}. The v890FLΔC replicon (BVDV-2ΔC) has a partial deletion of 43 aa (nt 986-1114) in
136 the core protein encoding region.

137 The challenge strain, ncp BVDV-2a HI916, was isolated in Germany during an acute severe
138 outbreak of BVD (kindly provided by G. Wolf, LMU München; Martin et al., 2005). BVDV-
139 1b strain SE5508 was used in neutralisation assays as a heterologous BVDV-1 prototype
140 virus.

141 Cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10%
142 BVDV free foetal bovine serum and incubated at 37°C in a humidified atmosphere containing
143 5% CO₂.

144 A diploid bovine esophageal cell line, KOP-R (RIE244, Collection of cell lines in veterinary
145 medicine, CCLV, FLI Insel Riems) that is highly susceptible to BVDV infection was used for
146 virus isolation and neutralisation assays. The challenge virus stock was produced on Madin
147 Darby bovine kidney (MDBK) cells (RIE261; CCLV, FLI Insel Riems). Virus stocks of the
148 BVDV-1 and BVDV-2 N^{pro} deletion mutants were propagated on interferon-incompetent
149 MDBK cells (kindly provided by G. Keil, FLI; RIE728; CCLV, FLI Insel Riems). Generation
150 of v890ΔC_trans (BVDV-2ΔC pseudovirions) was carried out using a new wisent helper cell
151 line (WT-2; P. König, unpublished data). Cells and cell culture media were routinely screened
152 for the absence of BVDV and BVDV-specific antibodies (CCLV, FLI Insel Riems). *In vitro*

153 transcribed RNA of the BVDV-2 Δ C replicon was transfected by electroporation into the
154 *trans*-complementing helper cells. Infected, resp. transfected cell cultures were freeze/thawed
155 once. All virus stocks were subsequently cleared from cell debris through low speed
156 centrifugation, titrated on KOP-R cells and tested for sterility and absence of mycoplasma. All
157 virus preparations were stored at -70°C until use.

158

159 *Animals and experimental design*

160 25 conventionally reared female Holstein-Frisian calves were obtained from local farms after
161 being tested negative for BVDV and BHV-1 (antibodies, antigen and genome). Their age
162 ranged from 6 to 14 months. For the trial, animals were allocated into 5 different groups
163 (n = 5 per group) and were housed in the Biosafety Level - 3 facility of the FLI. For
164 vaccination, virus stocks were diluted in cell culture medium to a final concentration of 5 x
165 10⁵ tissue culture infective doses 50% (TCID₅₀) per ml. All calves were vaccinated
166 intramuscularly with 2-5 ml containing approximately 1 x 10⁶ TCID₅₀ per animal (see values
167 of the backtitrations enclosed in brackets behind the assigned mutant below). One group
168 received the BVDV-2 Δ N^{pro} (9.3 x 10⁵ TCID₅₀), one the BVDV-1 Δ N^{pro} (9.3 x 10⁵ TCID₅₀),
169 and one group received a mixture of both N^{pro} deletion mutants in a single application
170 (BVDV-2 Δ N^{pro} & BVDV-1 Δ N^{pro}; 1.3 x 10⁶ TCID₅₀; approximately 6.5 x 10⁵ TCID₅₀ of each
171 mutant). An additional group was vaccinated with the BVDV-2 Δ C-pseudovirions (first shot:
172 1.1 x 10⁶ TCID₅₀ / second shot: 6.3 x 10⁵ TCID₅₀) and the animals of the fifth group served as
173 non-vaccinated controls.

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

177 Intranasal challenge infection with the BVDV-2 strain HI916 ensued 35 days after the last
178 vaccination for all 25 animals with 2.3×10^6 TCID₅₀ in a volume of 2 ml (1ml per nostril)
179 with the help of a nebulizer. All viral suspensions were backtitrated on KOP-R cells after
180 inoculation to confirm infectious titres of the respective inoculum.

181

182 ***Clinical evaluation***

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

194

195 ***Samples***

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

200

201

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

203 Blood samples were taken by jugular venipuncture and collected in sterile blood collection
204 tubes (Monovette) with potassium EDTA as anticoagulant (Sarstedt, Nuembrecht, Germany).

205 Total white blood cell counts as well as thrombocyte counts were determined by size
206 distribution analysis with an Abbott CellDyn 3700 analyzer.

207

208 ***Virus isolation***

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

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

222 5mL of EDTA blood per animal and day were treated with an ammonium chloride blood
223 lysisbuffer (155 mM NH₄Cl, 10 mM KHCO₃, 1 mM EDTA/ pH 7.4). After haemolysis and
224 centrifugation at 3200 rpm, pelleted leukocytes were washed twice with phosphate buffered
225 saline without Ca⁺⁺/Mg⁺⁺ (PBS-) and were re-suspended in 1 ml PBS-. KOP-R cell cultures
226 were inoculated with 4 replicates per sample, containing approximately 3 x 10⁶

227 leukocytes/100 μ l each, and tested for BVDV using immunofluorescence analysis as
228 described above. Purified leukocytes were stored at -70°C until further use.

229

230 ***Serology***

231 Sterile blood collection tubes (Monovette) with a clot activator (Sarstedt, Nuembrecht,
232 Germany) were applied for the weekly serum sampling. After centrifugation at 3000 rpm,
233 serum aliquots were stored at -20°C . For serological investigations all sera were inactivated at
234 56°C for 45 minutes.

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

240 Furthermore, sera from all animals were tested in a standard neutralisation assay (NA) against
241 selected BVDV-1 and -2 strains (BVDV-1 SE5508, BVDV-2 890 and HI916). For all three
242 virus strains, inactivated serum samples were serially diluted (\log_2 steps) in triplicates of 50
243 μ l in 96-well plates using cell culture medium. 50 μ l of a virus dilution (1000 TCID₅₀/ml) was
244 added per well and the plates were incubated at 37°C . After 2 h of incubation, 100 μ l of a
245 KOP-R cell suspension (2×10^4 cells/well) were added and the plates were incubated for 4
246 days. BVDV antibody-positive and antibody-negative sera were used as test controls. The
247 virus titre was confirmed by backtitrations (\log_{10} dilutions, 8 parallels). Neutralisation, i.e.
248 absence of virus infection and replication, was detected by indirect immunofluorescence (see
249 above). Titres were expressed as reciprocal of the highest dilution that caused 50%
250 neutralisation (\log_2 ND₅₀).

251

252 *Sequence analysis*

253 Total RNA from cells transfected with the full-length RNA of the BVDV-2 Δ C mutant was
254 extracted with the QIAamp Viral RNA Mini kit (Qiagen, Hilden, Germany) according to the
255 manufacturer's instructions. E2-encoding sequences were amplified using the Superscript III
256 One-Step RT-PCR system (Invitrogen, Karlsruhe, Germany). After agarose gel
257 electrophoresis, the E2-PCR product was further purified with the QIAex II Gel Extraction kit
258 (Qiagen, Hilden, Germany). Sequencing of the E2 encoding region was carried out using the
259 Big Dye® Terminator v1.1 Cycle sequencing Kit (Applied Biosystems). Nucleotide
260 sequences were read with an automatic sequencer (3130 Genetic Analyzer, Applied
261 Biosystems, Foster City, USA) and analyzed using the Genetics Computer Group (GCG)
262 software version 11.1 (Accelrys Inc., San Diego, USA). Custom primers were used for
263 sequencing (MWG Biotech, Ebersberg, Germany).

264

265 **Results**

266 *Vaccination*

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

276 antibody titres against all three strains tested were not detectable after the first vaccination
277 (Fig. 3).

278 After vaccination of all groups at day 25 (2nd immunisation of the BVDV-2ΔC group), all
279 animals stayed within the physiological temperature range (Fig. 1). One animal from the
280 BVDV-1ΔN^{pro} and one out of the BVDV-2ΔN^{pro} group showed a small elevation in
281 temperature for one (day 28) to two days (day 31, day 32) respectively, but did not develop
282 fever. The BVDV-1ΔN^{pro} group showed some clinical reaction post vaccination (p. vacc.).
283 They had slightly elevated mean clinical score values for 2 days (day 28 and day 35) due to
284 mild respiratory symptoms (data not shown). None of the other groups had raised scores in
285 this period of monitoring. Calves in the groups which received the different ΔN^{pro}-mutants
286 had a short and monophasic decline in their leukocyte counts, which were reduced up to 28%
287 (mean group value BVDV-1ΔN^{pro} & BVDV-2ΔN^{pro}, day 29) p. vacc. (Fig. 4). In both, the
288 BVDV-1ΔN^{pro} and the BVDV-2ΔN^{pro} group, up to 20% reduction of mean values was
289 observed (days 29-30 and days 30-32). Thrombocyte counts also slightly decreased in all
290 three sampled groups paralleling leukocyte counts (data not shown).

291 Vaccine virus was detected by isolation on cell culture in the nasal swab sample from one
292 BVDV-2ΔN^{pro}-immunised animal on one single day (day 32, 1 replicate after blind passage;
293 Table 1). No nasal virus shedding could be observed in the other groups. In addition, animals
294 from all three ΔN^{pro}-groups groups had a very limited vaccine virus viremia (Table 2). Virus
295 could be isolated from purified leukocytes from at least two animals per group for at least one
296 day between day 29 and day 33 of the trial (BVDV-1ΔN^{pro}: 3 animals, 2 days; BVDV-1ΔN^{pro}
297 & BVDV-2ΔN^{pro}: 3 animals, 1 to 2 days; BVDV-2ΔN^{pro}: 2 animals, 3 to 4 days). All animals
298 vaccinated on day 25 with the N^{pro} deletion mutants were scored positive in a NS3 blocking
299 ELISA (Fig. 2) from day 14 after vaccination. As mentioned above, animals in the
300 BVDV-2ΔC group stayed negative after first immunisation but showed a clear boost in

301 antibody development reacting ELISA positive as soon as 7 days after their second
302 vaccination. Mean blocking values between 91 % and 95 % were reached in the different
303 vaccination groups prior to challenge infection. Slightly lower mean inhibition values (81 %)
304 were found in the BVDV-2 Δ N^{pro} group (Fig. 2). All control animals stayed seronegative
305 during the vaccination period. Neutralising antibody titres (Fig. 3) were found in all
306 immunised groups as soon as 14 days after vaccination except for the BVDV-2 Δ C-
307 pseudovirion group. Testing sera against the heterologous BVDV-1 strain SE5508, highest
308 pre-challenge titres with a mean value of up to 891 ND₅₀ were reached in the groups receiving
309 BVDV-1 Δ N^{pro} (alone or in the mixed application). All other groups stayed basal in their titres
310 (mean values \leq 2 ND₅₀) prior to challenge (Fig. 3). Using the BVDV -2 challenge strain
311 HI916 in a neutralisation assay, slightly different results were obtained. Highest titres after
312 immunisation were reached in the group receiving the BVDV-2 Δ N^{pro} mutant (mean value 97
313 ND₅₀) followed by the BVDV-1 Δ N^{pro} & BVDV-2 Δ N^{pro} and the BVDV-1 Δ N^{pro} group (28
314 ND₅₀/11 ND₅₀). The group vaccinated with the pseudovirion preparation (BVDV-2 Δ C),
315 which was clearly positive in the ELISA by day 7 after booster vaccination, however stayed at
316 basal to non detectable neutralising titre levels ($<$ 2 ND₅₀) until challenge infection. Similar
317 values and trends were seen against BVDV-2 strain 890. Mean titres of 79 ND₅₀ were found
318 in the BVDV-2 Δ N^{pro} vaccinated animals at the day of challenge. The graduation between the
319 groups was similar to the one obtained against BVDV-1 HI916 (BVDV-1 Δ N^{pro} & BVDV-
320 2 Δ N^{pro} 18 ND₅₀; BVDV-1 Δ N^{pro} 11 ND₅₀). In the BVDV-2 Δ C group, neutralising antibodies
321 ranged from very low to undetectable (mean values \leq 2 ND₅₀) even against the parental
322 BVDV-2 890 strain.

323

324

325

326 ***Challenge infection***

327 After challenge infection at day 60 of the trial, all control animals showed typical and clear
328 signs of clinical disease. They had a biphasic rise in their body temperatures, a slight one at
329 day 3, and a pronounced one at days 8 and 9 p. chall. with maximum mean group values of up
330 to 41°C (Fig. 1). Simultaneously, a marked rise in clinical scores could be found peaking at
331 days 8 to 10 (data not shown). Besides fever, all calves had marked respiratory symptoms
332 (coughing and mucopurulent nasal discharge), depression with reduced appetite, and 2
333 animals showed watery diarrhea for 2 to 3 days. In contrast, clinical effects of the challenge
334 infection were clearly reduced - or even absent - in the vaccinated groups. Vaccinated animals
335 had an elevation in body temperature at day 7 p. chall. to different degrees (Fig. 1). Highest
336 mean values were reached in the BVDV-1 Δ N^{pro} (40°C) and in the BVDV-1 Δ N^{pro} & BVDV-
337 2 Δ N^{pro} (39.8°C) groups. The BVDV-2 Δ C group stayed in the physiological temperature
338 range, peaking at 39.4°C. Temperature reaction went along with moderated respiratory
339 symptoms in the BVDV-1 Δ N^{pro} group (data not shown). The only animals that stayed
340 completely unaffected regarding their body temperature and clinical signs were those
341 vaccinated with BVDV-2 Δ N^{pro}.

342 After challenge infection, all control animals developed a severe leukopenia (Fig. 4). They
343 had a bi- to triphasic decrease (days 3, 7 and 13 p. chall.) in leukocyte counts with maximum
344 levels of 48 % reduction at day 7 after challenge. The vaccinated animals showed no or only a
345 monophasic decline of the leukocyte numbers. A maximum decrease of about 12 % in the
346 BVDV-1 Δ N^{pro} and the BVDV-2 Δ C group was found at day 4. In addition, those animals
347 quickly recovered to pre-infection counts (day 7 p. chall.). The group that received the mixed
348 application of Δ N^{pro} mutants (BVDV-1 Δ N^{pro} & BVDV-2 Δ N^{pro}) had a mean drop of 20 % at
349 day 5 p. chall. and of 24 % at day 10 p. chall., and these reduced leukocyte counts persisted
350 till the end of the trial (day 89/day28 p.chall.) with a mean reduction of 20 % (Fig. 4). BVDV-

351 $2\Delta N^{\text{pro}}$ vaccinated animals showed no decrease in leukocyte blood counts after challenge
352 infection. Thrombocyte counts were not as heavily affected by BVDV-2 infection as
353 expected. The control animals had a mean reduction to a maximum of 35 % at day 3 after test
354 infection. All other groups showed no or only a monophasic less marked decrease (data not
355 shown). No clinical effects like bloody diarrhoea, petechia or hematomas on injection/injury
356 sites, which were described for the challenge virus strain, were observed. Thereafter counts
357 notably increased in the controls (to mean values of 195 %), the BVDV- $1\Delta N^{\text{pro}}$ group (175
358 %) and slightly in the BVDV- $1\Delta N^{\text{pro}}$ & BVDV- $2\Delta N^{\text{pro}}$ one (125 %) corresponding to severity
359 of infection and disease (data not shown).

360 Performing virus isolation, we found a long and pronounced challenge virus viremia in the
361 control animals for up to 11 days (day 62 – day 73; Table 2) while there was a clear reduction
362 in duration (day 63 – day 68) and amount in all vaccinated groups (BVDV- $2\Delta C$: 4 animals, 1
363 day; BVDV- $1\Delta N^{\text{pro}}$: all animals, 1 to 5 days; BVDV- $1\Delta N^{\text{pro}}$ & BVDV- $2\Delta N^{\text{pro}}$: 2 animals, 1 to
364 3 days). In contrast, no challenge virus could be isolated from the leukocytes of the
365 BVDV- $2\Delta N^{\text{pro}}$ group.

366 Furthermore, challenge virus was detectable in the nasal swab samples of all control animals
367 from day 61 till day 71 (Table 1). Duration (day 62 – day 68) and levels of nasal virus
368 shedding were again markedly reduced in the vaccinated animals (BVDV- $2\Delta C$: all animals, 1
369 to 4 days; BVDV- $1\Delta N^{\text{pro}}$: 4 animals, 1 to 3 days; BVDV- $1\Delta N^{\text{pro}}$ & BVDV- $2\Delta N^{\text{pro}}$: 2 animals,
370 1 to 2 days). No challenge virus could be recovered from the nasal swab samples of the
371 BVDV- $2\Delta N^{\text{pro}}$ -vaccinated animals.

372 From day 14 p. chall., all control animals scored positive in the NS3 blocking ELISA, while
373 NS3 antibodies in all other groups were slightly boosted. Mean blocking values of 100 %
374 were reached in the five groups at day 89 (Fig. 2). Infection with BVDV-2 HI916 also
375 induced a boost in neutralising antibodies titres in the immunised groups detected by

376 neutralisation assays peaking at day 14 to day 28 p. chall. (Fig. 3). The controls developed
377 detectable neutralising titres by day 14. Against BVDV-1 SE5508 maximum titres were
378 reached in the BVDV-1 Δ N^{pro} and BVDV-1 Δ N^{pro} & BVDV-2 Δ N^{pro} group with 3821 ND₅₀ and
379 1552 ND₅₀ respectively. The titres in the other three groups also increased, but mean values
380 peaked at a markedly lower level (97 ND₅₀ and 16 ND₅₀), leaving a distinct gap between the
381 two groups vaccinated with the BVDV-1 mutant and those receiving solely BVDV-2 (mutants
382 or challenge strain). Tested against the BVDV-2 strain 890, sera of the BVDV-1 Δ N^{pro} animals
383 featured maximum titres of 1552 ND₅₀. Values of all other groups followed closely and at the
384 term of the study end titres were very similar in all groups (588 ND₅₀). Highest neutralising
385 titres against the BVDV-2 strain HI916 remained lower (274 ND₅₀; BVDV-1 Δ N^{pro} group),
386 but all mean group values were again similar at the end of the trial (181 ND₅₀) with slightly
387 lower values for the BVDV-2 Δ C-immunised group (69 ND₅₀). In general, the conducted
388 neutralising assays in this study showed that titres of BVDV-2 exposed animals against
389 BVDV-2 strains were lower than those of the BVDV-1 vaccinated animals against the applied
390 BVDV-1 strain.

391

392 *Sequencing results of the E2 region of BVDV-2 Δ C*

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

399

400

401 **Discussion**

402 In contrast to vaccines currently licensed in Europe, commercially available vaccines in the
403 United States, where BVDV-2 is highly prevalent, include BVDV-2 strains (Ridpath, 2005).
404 Although data show a markedly lower prevalence in Europe (Wolfmeyer et al., 1997; Beer
405 and Wolf, 1999), outbreaks of severe acute disease associated with hemorrhagic syndrome,
406 reproductive losses and high lethality rates are reported. Thus, future vaccines will prove
407 advantageous to also mediate a stable protection against BVDV-2 strains (Becher et al., 2000;
408 Beer et al., 2000). In order to test and compare new potential BVDV-2 vaccine candidates *in*
409 *vivo* we experimentally vaccinated cattle and subsequently challenged them with a recent,
410 virulent German BVDV-2 field strain (HI916), which allowed graduated classification
411 between the different vaccinated groups. The results of this study show that clinical
412 symptoms, leukopenia, viremia and nasal virus shedding after experimental infection of
413 calves could be clearly reduced or even prevented with all three tested BVDV mutants
414 compared to the markedly affected control animals. However, thrombocytopenia and
415 hemorrhagic syndrome described after field-infection with BVDV-2 HI916 (Martin et al.;
416 2005), could not be observed.

417 In this study, a BVDV-2 replicon construct (Mischkale et al., 2008) with a deletion in the core
418 protein region could be successfully complemented *in trans* with the help of a newly
419 established helper cell line, expressing the BVDV-1-PT810 structural proteins C to E2
420 (König, unpublished data). Infectious particles, so called pseudovirions, could be generated in
421 sufficient amounts to characterise BVDV-2 Δ C *in vivo*. Following immunisation with
422 BVDV-2 Δ C-pseudovirions neither nasal virus shedding nor pseudovirion viremia was
423 detectable as it was previously described for a BVDV-1 Δ C mutant (Reimann et al., 2007).
424 These *in vivo* results clearly indicate that pseudovirions are defective in second cycle (DISC)
425 and therefore neither horizontally nor vertically transmissible after vaccination. Serological

426 responses after immunisation with pseudovirions resembled those of inactivated vaccines
427 (Beer and Wolf, 2003), showing low NS3 antibody levels after the first application and a clear
428 and quick boost effect after the second one 25 days later. Unexpectedly, these antibodies had
429 very low to non-existent neutralising abilities against any of the tested BVDV-1 and -2
430 strains, which is most likely the reason for the reduced protective effect in comparison to the
431 previously published data from immunisation trials using BVDV-1 Δ C (Reimann et al., 2007).
432 Therefore, we sequenced the E2 encoding region of the replicon cDNA and found a single
433 nucleotide change at nucleotide position 2736 leading to an amino acid change in a highly
434 immunogenic region (leucine to histidine; aa 109 of the p890FL E2). For CSFV it was
435 demonstrated by van Rijn (2007) that the local variability by one or more aminoacids in the
436 E2 region may lead to differences in affinity, avidity and in cross-neutralisation. Therefore,
437 we speculate that the altered E2 of BVDV-2 Δ C could entail a lack of neutralising E2
438 antibodies and thus less efficient protective qualities. Possible functional defects of the
439 mutated BVDV-2 Δ C E2 protein could be masked and functionally complemented in the
440 pseudovirion particles by the structural proteins of a BVDV-1 strain provided by the helper
441 cell line. In the animal, the non-replicative BVDV-1 E2 protein from the putative hybrid-
442 envelopes did not induce detectable levels of neutralising antibodies against the tested
443 BVDV-1 strain. However, BVDV-2 Δ C had clear protective effects, which underlines the
444 importance of cell-mediated responses to Pestivirus infection as it was described previously
445 (Beer et al., 1997; Larsson and Fossum, 1992; Kimman et al., 1993; Pauly et al., 1995).
446 As previously described, the non-structural N^{pro} protein interferes with the host's innate
447 immune response by interacting with interferon regulatory factor-3 (IRF3) and targeting it for
448 proteasomal degradation (Hilton et al., 2006). Knocking out this immunosuppressive function
449 through deletion of an essential part of the genome aims at attenuating BVDV strains making
450 them future vaccine candidates with enhanced safety. CSFV mutants with a deletion in the

451 N^{pro} gene already showed to be attenuated (Mayer et al., 2003). BVDV-1ΔN^{pro} mutants also
452 proved to be highly attenuated even in pregnant animals and to mediate complete protection
453 against a heterologous BVDV-1 challenge (Meyers et al., 2007; König et al., unpublished
454 data). In our study, we could show that *in vivo* both, the BVDV-1 and the BVDV-2 N^{pro}
455 deletion mutant provided clinical protection against challenge infection. Interestingly, lower
456 homologous titres of neutralising antibodies were necessary to gain a “sterile immunity” than
457 it was described for previous studies (Bolin and Ridpath, 1995; Beer et al., 2000). It turned
458 out that a neutralising titre of 64 ND₅₀ to 128 ND₅₀ against the challenge strain prior to
459 infection was sufficient to prevent systemic challenge virus infection, irrespective to the titres
460 against the reference BVDV-1 strain SE5508. In all three groups NS3-specific antibodies
461 could be detected as soon as 14 days after vaccination indicating effective replication of all
462 mutants. The group immunised with BVDV-1ΔN^{pro} had high neutralising antibodies against
463 the BVDV-1 strain, which were apparently less effective in neutralising BVDV-2 strains *in*
464 *vitro*, and this group was less protected against challenge infection than were the others. In
465 contrast to the results of Beer et al. (2000), there was a marked difference in the titres
466 developed against BVDV-1 and BVDV-2 when both N^{pro} deletion mutants were
467 simultaneously applied. A possible explanation could be the more efficient infection and
468 higher replication levels of the cp BVDV-1ΔN^{pro}, subsequently leading to interference with
469 BVDV-2ΔN^{pro} infection and replication. This assumption is supported by our findings that re-
470 isolated virus from leukocyte samples was characterized as BVDV-1 by selective
471 immunofluorescence staining. Nevertheless, only the BVDV-2ΔN^{pro} group developed a
472 complete protective immune response inducing a “sterile immunity” against heterologous
473 BVDV-2-challenge infection.

474 The BVDV-2 strain 890 served as basis for the first approach generating this kind of BVDV-2
475 deletion mutants as it offers many options for virulence studies. This advantage was used in

476 the presented proof-of-principle study to investigate in detail how effectively the two applied
477 mutation strategies, deleting a structural protein or a protein acting as an immunosuppressing
478 modulator, attenuate such an isolate *in vivo*. Obtained results could then be more reliably
479 transferred to less virulent strains that would be used for further developments. As infectious
480 virus from the constructed BVDV-2 full-length cDNA clone (p890FL) is still moderately
481 virulent in cattle (Mischkale et al., 2008), it could be concluded from this trial that all tested
482 BVDV-2 deletion mutants are further attenuated. It should be additionally beneficial to the
483 safety of future BVDV-2 deletion mutants to originate from a cytopathogenic full-length
484 cDNA clone, since cp viruses are the standard for attenuated BVDV-1 live vaccine
485 preparations (Fulton et al., 2003; van Oirschot et al., 1999; Beer and Wolf, 2003). If
486 cytopathogenic vaccine viruses would be able to reach the fetus, abortion could be induced in
487 sporadic cases, but the possibility of induction of a persistent infection would be excluded, as
488 there are no data that indicate reversion of a cp strain to an ncp one *in vivo* (Ridpath et al.,
489 2005). Meyers et al. (2007) have shown that prevention of transplacental infection with
490 BVDV-1 and -2 ΔN^{pro} mutants requires a further elimination of the RNase activity of E^{RNS}.
491 To investigate this safety aspect, vaccination studies with BVDV-2 N^{pro} deletion mutants in
492 pregnant animals have to be carried out. Reversions or recombinations of the assessed ncp
493 BVDV-2 ΔN^{pro} as well as for prospective cp BVDV-2 ΔN^{pro} mutants with a second strain, as
494 described previously (Becher et al., 1999b; Meyers et al., 1992), cannot be excluded by our
495 present results. At least for BVDV-2 ΔC these events are highly unlikely as discussed for
496 BVDV-1 replicons that were thoroughly analysed *in vitro* (Reimann et al., 2003, 2007). Both
497 issues, that would require extensive double infection studies *in vitro* as well as *in vivo*, were
498 not addressed in the setup of this first trial, which served as promising basis for future
499 developments.

500

501

502 Conclusions

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

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

514

515

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523

524 Conflict of interest statement

525 None

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699 **Tables**

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

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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	0	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	0	0	0	0
	762	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	775	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	777	0	0	0	0	0	0	0	0	0	0	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	0
	486									0	0	0	0	0	0	0	0	0	0	0
	472									0	0	0	0	0	0	0	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 & BVDV-1 ΔNpro	494									0	0	0	0	0	0	0	0	0	0	0
	502									0	0	0	0	0	0	0	0	0	0	0
	503									0	0	0	0	0	0	0	0	0	0	0
	764									0	0	0	0	0	0	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	0	0	0	0	0	0
	499									0	0	0	0	0	1	0	0	0	0	0
	509									0	0	0	0	0	0	0	0	0	0	0
	767									0	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	0	0	0
	759	0	0	0	0	0	1	0	0	0	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	0	0	0
	775	0	0	3	0	4	4	1	0	0	0	0	0	0	0	0	0	0	0
	777	0	0	0	0	0	2	0	0	0	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	0
	497	0	0	0	0	1	2	4	4	4	1	4	1	0	0	0	0	0	0
	500	0	1	0	1	2	1	2	3	4	3	4	1	0	0	0	0	0	0
	753	0	0	0	0	0	0	3	2	2	1	0	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	0
BVDV-1ΔNpro	505	0	0	0	0	1	0	1	2	0	0	0	0	0	0	0	0	0	0
	486	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0
	472	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	814	0	0	0	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	0	0	0
BVDV-2ΔNpro & BVDV-1 ΔNpro	494	0	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0
	502	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0
	503	0	0	0	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	0	0	0
	779	0	0	0	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	0	0	0
	480	0	0	0	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	0	0	0
	509	0	0	0	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	0	0	0

702 Nasal virus shedding of vaccine (A) /challenge (B) virus: Highly susceptible KOP-R cell
703 cultures were inoculated with 4 replicates of nasal swab fluids (100 µl / replicate) and after 3-
704 4 days virus replication was verified by immunofluorescence staining. Results were scored
705 according to the number of positive inoculations (grey underlay) out of the 4 replicates (0 =
706 no BVDV isolation → 4 = all inoculations BVDV positive). A first result was confirmed after
707 one blind passage of the supernatants. Samples that were only detected positive after
708 passaging are highlighted in light grey.

709

710 **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	0	0	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	0	0	0	0	0
	762	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	775	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	777	0	0	0	0	0	0	0	0	0	0	0	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 & BVDV-1 ΔNpro	494										0	0	0	0	0	2	1	0	0	0	0
	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	0	0	0	0
	759	0	0	0	0	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	0	0	0	0
	775	0	0	0	0	0	3	0	0	0	0	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	0	0	0	0
control	496	0	0	1	2	4	4	4	4	4	4	4	1	0	1	0	0	0	0	
	497	0	0	2	1	3	4	4	4	4	4	2	2	2	0	0	0	0	0	
	500	0	0	2	4	4	4	4	4	4	4	1	1	0	0	0	0	0	0	
	753	0	0	1	1	1	4	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	0	0	0	
	486	0	0	0	1	1	3	4	1	0	0	0	0	0	0	0	0	0	0	
	472	0	0	0	0	2	3	2	1	0	0	0	0	0	0	0	0	0	0	
	814	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	
	819	0	0	0	0	2	0	0	0	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	0	0	0	0	0	0	0	0	
	502	0	0	0	2	3	4	0	0	0	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	0	0	0	
	764	0	0	0	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	0	0	0	
BVDV-2ΔNpro	468	0	0	0	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	0	0	0	
	499	0	0	0	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	0	0	0	
	767	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	

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

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737 **Figures**

738

739 **Figure 1:** Mean group values of the rectal body temperatures throughout the course of the
740 study. (☉) marks day of vaccination and (☛) the day of challenge infection. Temperatures of
741 the groups were recorded daily until 3 weeks after challenge infection. $< 39.5^{\circ}\text{C}$ =
742 physiological temperature; $>40^{\circ}\text{C}$ = fever. Dotted lines border the raised temperature range
743 ($39.5 - 40^{\circ}\text{C}$). One animal of the BVDV-1 $\Delta\text{N}^{\text{pro}}$ group could not be measured after day 38 due
744 to its behavior. The mean values were calculated from the other 4 animals from that day on.
745 Standard deviations (error bars) are not depicted for the individual groups for clarity.

746

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

753

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

759

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

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

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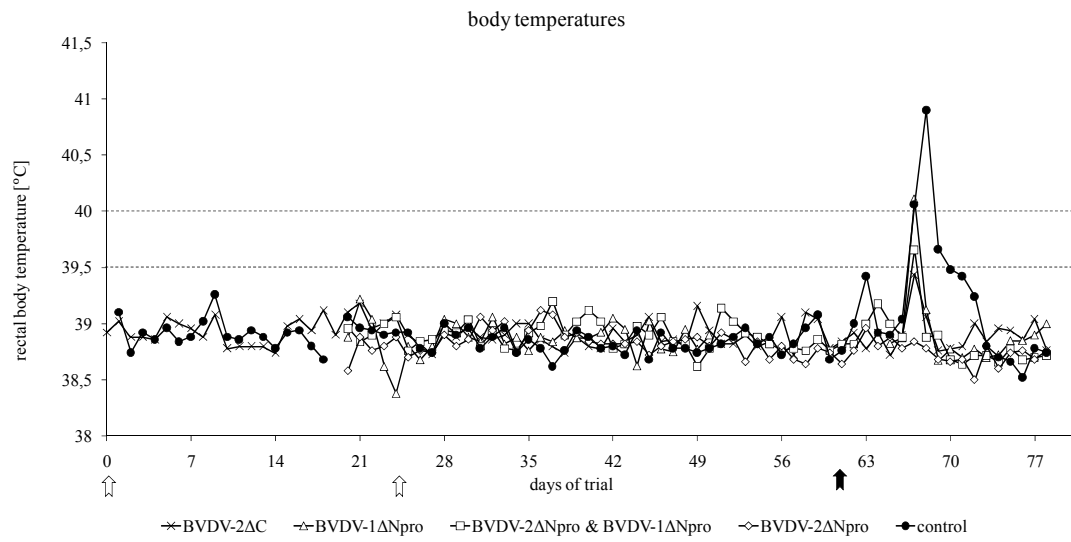
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791 **Figure 1**

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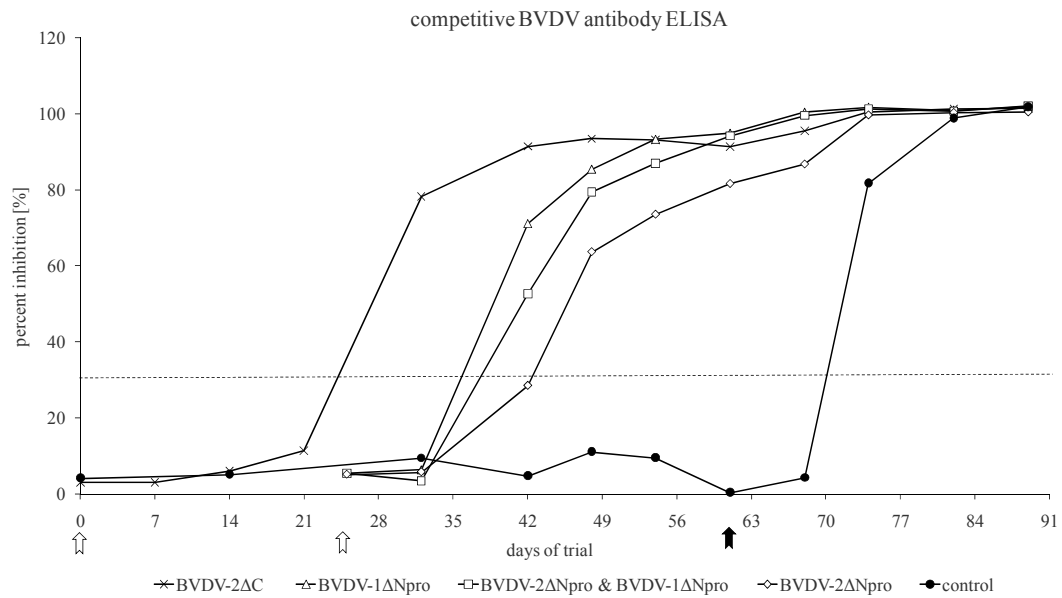
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809 **Figure 2**

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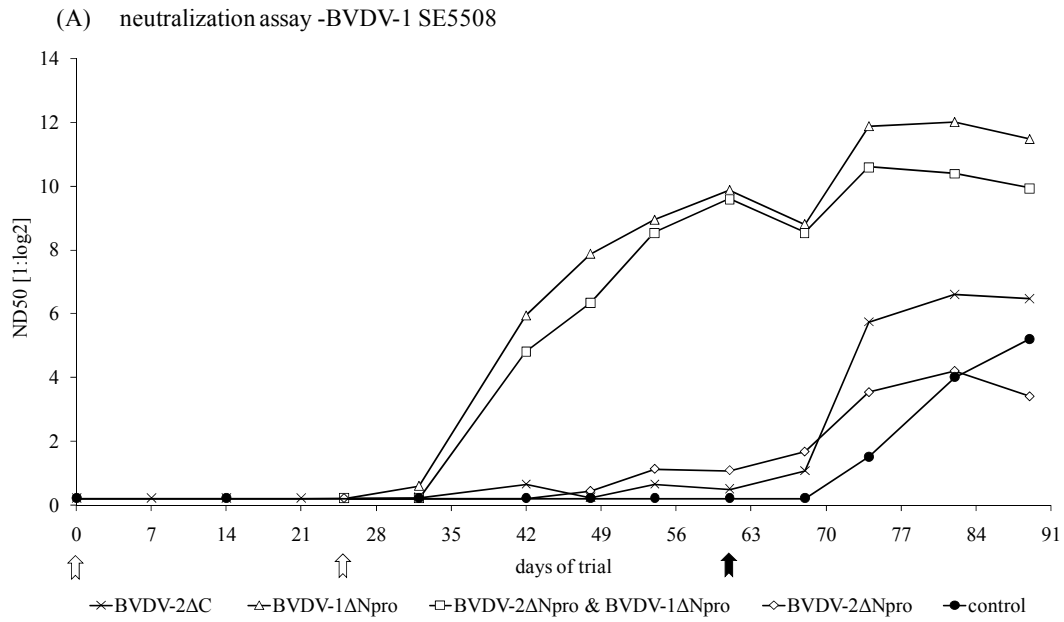
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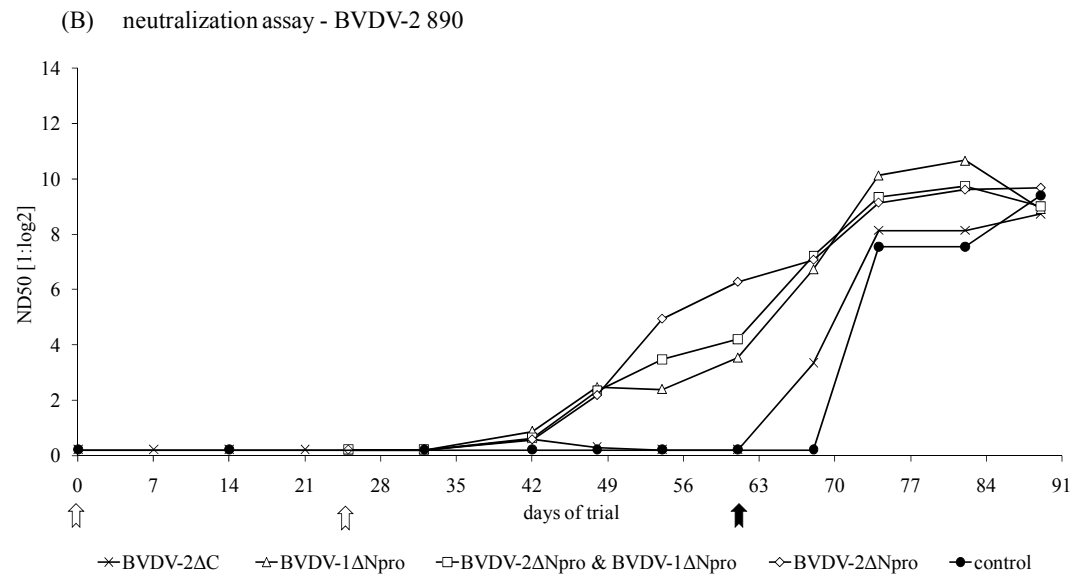
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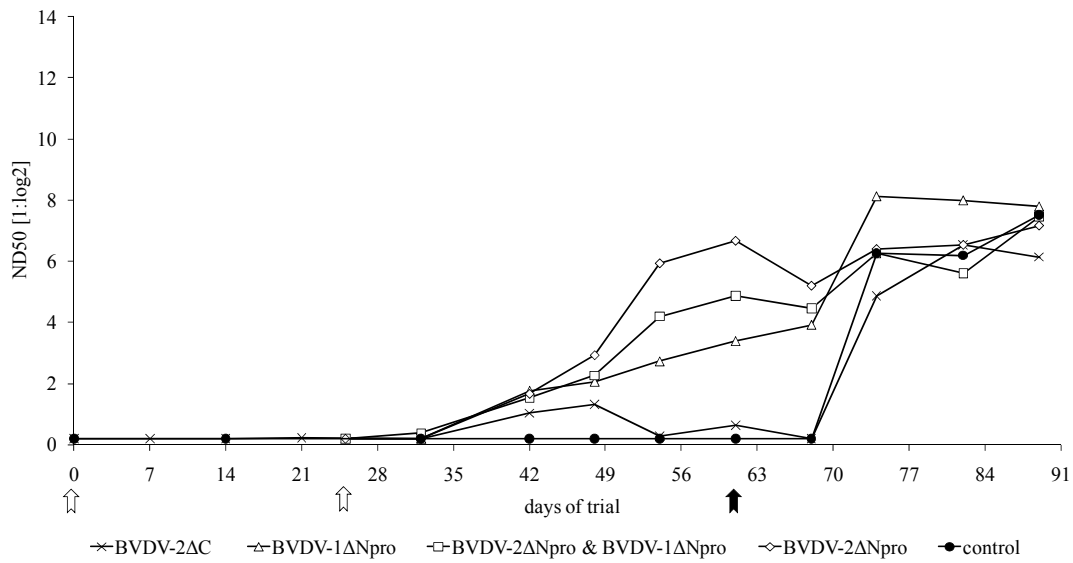
826 **Figure 3**

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(C) neutralization assay -BVDV-2 HI916



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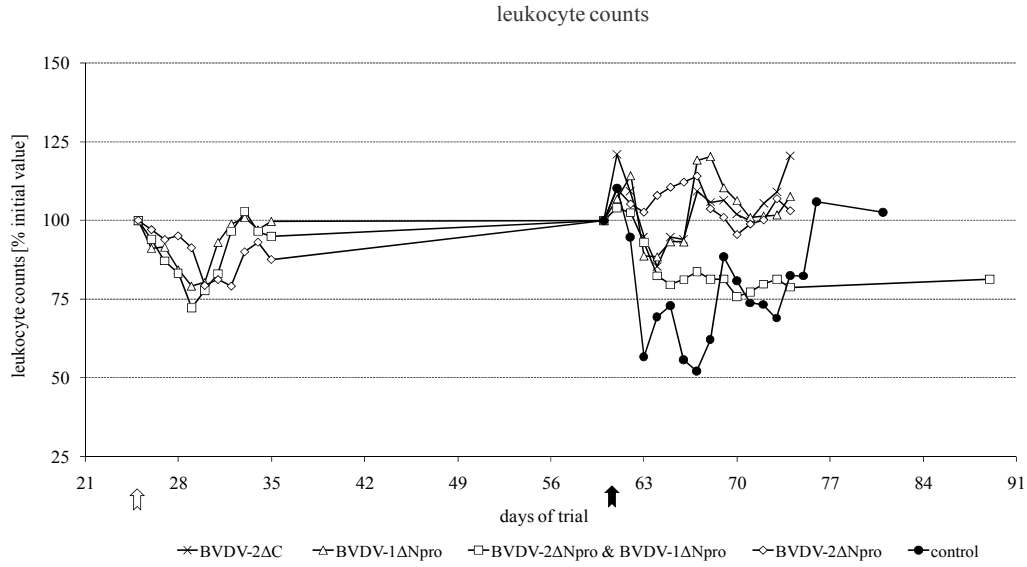
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846 **Figure 4**

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