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

3

4 Katrin Mischkale^a, Ilona Reimann^b, J. Zemke^a, P. König^a and Martin Beer^{a*}

5

6 ^aInstitute of Diagnostic Virology, Friedrich-Loeffler-Institut, D-17493 Greifswald-Insel

7 Riems, Germany

8 ^bInstitute of Molecular Biology, Friedrich-Loeffler-Institut, D-17493 Greifswald-Insel Riems,

9 Germany

10

11

12

13

14

15

16 *Corresponding author:

17 Dr. Martin Beer

18 Institute of Diagnostic Virology

19 FRIEDRICH-LOEFFLER-INSTITUT

20 Südufer 10

21 17493 Greifswald-Insel Riems

22 Phone +49 383517200

23 Fax +49 38351 7151

24 e-mail: Martin.Beer@fli.bund.de

25

1

26 **Abstract**

27 Based on their genomic sequences, two genotypes of Bovine viral diarrhea virus (BVDV) can
28 be differentiated, BVDV type 1 (BVDV-1) and BVDV type 2 (BVDV-2). The complete
29 genomic sequence of the highly virulent BVDV-2 strain 890 was cloned as cDNA to establish
30 the infectious cDNA clone p890FL. In *vitro*-synthesised full-length RNA of p890FL was
31 transfected into bovine cells and infectious virus could be recovered (v890FL). In *vitro*,
32 recombinant v890FL showed similar growth characteristics as wild type virus 890WT.
33 However, infection experiments in calves revealed an attenuation of recombinant v890FL in
34 comparison to the parental isolate. Both leukocytopenia and fever were less pronounced in
35 v890FL-infected calves. Nevertheless, viremia and virus shedding were comparable between
36 recombinant and parental BVDV 890. Furthermore, mutants with partial deletions of the
37 genomic region encoding for the autoprotease N^{pro} (p890ΔN^{pro}) or the capsid protein
38 (p890ΔC) were constructed and characterised. In order to generate pseudovirions, replicon
39 v890ΔC was efficiently *trans*-complemented on a helper cell line. In summary, the newly
40 developed construct p890FL represents the first infectious full-length cDNA clone for the
41 BVDV-2 strain 890 and offers a useful tool for further studies on the pathogenesis of
42 BVDV-2 and the development of novel recombinant BVDV-2 specific vaccine candidates.

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

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50 1. Introduction

51 The Bovine viral diarrhea virus (BVDV) belongs to the genus *Pestivirus* within the family
52 *Flaviviridae*. BVDV is closely related to the classical swine fever virus (CSFV) and the ovine
53 border disease virus (BDV) (Fauquet et al., 2005). The pestiviral genome consists of a single
54 stranded positive-sense RNA with a length of about 12.3 kb. It contains one large open
55 reading frame (ORF), which is flanked by non-translated regions (NTR) on both genome
56 termini. The single ORF is translated into one polyprotein, which is co- and post-
57 translationally processed into the mature proteins N^{pro}, C, E^{ms}, E1, E2, p7, NS2/3, NS4a,
58 NS4b, NS5a and NS5b by viral and cellular proteases (Collett et al., 1988; Lackner et al.,
59 2004; Meyers et al., 1989). In cell culture, two BVDV biotypes have been described:
60 cytopathogenic (cp) and non-cytopathogenic (ncp). While the cp biotype induces apoptosis
61 and cell death (Zhang et al., 1996), the ncp biotype leads to a persistent infection of cell
62 cultures (Donis and Dubovi, 1987). Since the late 1980s, a new type of BVDV infections with
63 severe thrombocytopenia associated with hemorrhagic syndrome in cattle has been described
64 in Northern America (Pellerin et al., 1994; Rebhun et al., 1989; Ridpath et al., 1994). In
65 Europe, first observations of hemorrhagic syndrome associated with BVDV were reported in
66 the early 1990s (Broes et al., 1992; Lecomte et al., 1996; Thiel, 1993). Analysis of different
67 isolates resulted in classification of BVDV into genotype 1 and 2. Because of their genetic,
68 antigenetic and phylogenetic marked differences, the isolates mentioned above were classified
69 as BVDV genotype 2. The highly virulent strain 890 was isolated by Ridpath et al., 1994.
70 Furthermore, vaccination against BVDV-1 provided only partial protection from BVDV-2
71 infections and most monoclonal antibodies against BVDV-1 failed to detect BVDV-2 (Bolin
72 et al., 1991; Ridpath et al., 1994). The ncp BVDV-2 strain 890 was the first BVDV-2 to be
73 completely sequenced (GenBank accession no. U18059). In comparison to other ncp
74 pestiviruses the ORF is elongated due to an insertion of 228 nucleotides in the genome
3

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

79

80 **2. Materials and Methods**

81 2.1. Cells and virus

82 Bovine oesophageal cells (KOP-R, RIE244), European bison thymus cells (WT-R, RIE758)
83 and interferon incompetent Madin-Darby bovine kidney cells (MDBK, RIE728) were
84 obtained from the collection of cell lines in veterinary medicine at the Federal Institute of
85 Animal Health, Insel Riems (CCLV). KOP-R cells were used for transfection of in *vitro*-
86 transcribed RNA and growth kinetics analysis. WT-R cells were used to establish the WT-R2
87 cell line, which in turn, was used in *trans*-complementation experiments. The interferon
88 incompetent MDBK cells were used for transfection of in *vitro*-transcribed RNA and
89 propagation of the Npro-deleted virus mutant. Cells were grown in Dulbecco's Modified
90 Eagle Medium (DMEM) supplemented with 10 % BVDV-free fetal calf serum (FCS).
91 BVDV-2 wild type strain 890 (v890WT) was kindly provided by H. Hehnen (Bayer AG,
92 Monheim, Germany).

93

94 2.2. Monoclonal antibodies

95 For the detection of BVDV proteins, monoclonal antibodies (mab) *WB 433* (anti-E^{tns}, CVL,
96 Weybridge), *WB210* (IgG1, anti-E^{tns}, CVL, Weybridge), *CA1/2* (anti-E2, Institute for
97 Virology, TiHo Hannover), *CA34/1/2* (anti-E2, Institute for Virology, TiHo Hannover), and
98 mab-mix *WB103/105* (anti-NS3, CVL, Weybridge) were used (Edwards et al., 1988).

99 Secondary antibody *anti-mouse IgG ALEXA⁴⁸⁸* (Molecular Probes) was used for

4

100 immunofluorescence (IF) staining.

101

102 2.3. Construction of the full-length cDNA clone and the deletion mutants

103 Plasmids were amplified in *Escherichia coli* DH10BTM cells (Invitrogen) and *Escherichia coli*
104 MDS42 (kindly provided by G.M. Keil, FLI) (Pósfei et al., 2006), respectively. Plasmid DNA
105 was purified by using Qiagen Plasmid Mini or Midi Kit or with the GFXTM Micro Plasmid
106 Prep Kit (Amersham). Primers used for plasmid construction are presented in table 1, primers
107 for mutagenesis of the plasmid constructs are listed in table 2 (synthesised by MWG-Biotech,
108 Ebersberg, Germany; or OPERON Biotechnologies, Berlin, Germany). Restriction enzyme
109 digestion and cloning procedures were performed according to standard protocols.
110 Construction of the infectious cDNA clone p890FL is schematically illustrated in figure 1.
111 Organisation of the capsid protein deletion mutant (p890ΔC) and of the N^{pro} deletion mutant
112 (p890ΔN^{pro}) is shown in figure 2.

113

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

122 The four PCR fragments (figure 1) were generated by RT-PCR using the appropriate primers
123 (table 1) and subsequently ligated into the plasmid vector pA (kindly provided by Gregor

124 Meyers, FLI Tübingen). *SmaI* sites at the 5' end of the subcloned fragment 1 and within
125 fragment 2 (nucleotide position 1978) were mutated by site-directed mutagenesis using the
126 QuickChangeII XL Site-Directed Mutagenesis Kit (Stratagene) and the respective primers
127 listed in table 2. Subsequently, by sequencing of the complete p890FL plasmid with the
128 Genome Sequencer (GS20, Roche/454), in all 8 mismatches compared to the parental virus
129 were detected. Two defects were eliminated by site-directed mutagenesis (1) a frame shift due
130 to the deletion of two nucleotides in the p7 encoding region and (2) a substitution of one
131 amino acid (aa) at the 3' end of the region encoding for the NS5a protein by using the primers
132 890_ORF and 890_ORF_r, and the primer pair 890_NS5 and 890_NS5_r, respectively.

133 For partial deletion of the N^{pro} encoding sequence, the plasmid p890FL and a PCR fragment
134 amplified with the primers 890_SalI and 890_Npro_r were cleaved with *SalI* and *SnaBI* and
135 ligated to generate the construct p890ΔN^{pro}-E2. A second PCR fragment was generated by
136 using the primer pair 890_Npro and 890_SnaBI, cleaved with *NotI* / *SnaBI* and cloned into
137 the *NotI* and *SnaBI* digested plasmid p890ΔN^{pro}-E2 to obtain the deletion mutant p890ΔN^{pro}.
138 The deletion encompasses nt 422-889 (aa 13-168) excepting the first 12 aa which overlap
139 with the internal ribosomal entry site (IRES) region.

140 The capsid-deleted replicon p890ΔC contained a deletion of aa 201-243 (nt 986-1114)
141 compared to the parental p890FL. The 32 N-terminal amino acids and the 27 C-terminal aa of
142 the capsid protein, which constitute an essential signalase recognition site and which direct
143 translocation of the envelope proteins into the endoplasmic reticulum (ER) for further
144 processing of the E^{ms}-E1-E2 polyprotein (Rümenapf et al., 1991), were retained. For
145 construction of p890ΔC, a PCR fragment using the primers 890_SalI and 890_Capsid_r was
146 amplified. p890FL and the PCR fragment were cleaved with *SalI* and *SnaBI* and ligated to
147 generate the construct p890ΔC-E2. In a second step, a PCR fragment was amplified by using

148 the primer pair 890_Capsid and 890_SnaBI_r. The resulting amplicon and p890 Δ C-E2 were
149 digested with *NotI* / *SnaBI* and both ligated to establish the deletion mutant p890 Δ C.

150

151 2.4. In *vitro* transcription and RNA transfection

152 In *vitro* transcription of the deletion mutants p890 Δ N^{pro}, p890 Δ C and the full-length construct
153 p890FL was performed using the T7 RiboMax Large-Scale RNA Production System
154 (Promega) according to the manufacturer's instructions after linearising the plasmids with *SmaI*.
155 The amount of RNA was estimated by ethidium bromide staining after agarose gel
156 electrophoresis. For RNA transfection, bovine cells were detached using a trypsin solution,
157 washed twice with phosphate buffered saline without Ca⁺⁺/Mg⁺⁺ (PBS-) and mixed with 1-5
158 μ g of in *vitro* synthesised RNA. Electroporation was done by using the GenePulser transfection
159 unit (Biorad) (two pulses at 850 V, 25 μ F and 156 ω).

160

161 2.5. Immunofluorescence staining

162 Cell cultures were fixed with 4% paraformaldehyde (PFA) and permeabilised with 0.01 %
163 digitonin (IF staining of NS3) or fixed/permeabilised with 80 % acetone (E^{ms}, E2), and
164 incubated with the appropriate working dilution of the respective antibodies for 30 min. After
165 one washing step with PBS⁻, cells were incubated with the Alexa⁴⁸⁸-conjugated secondary
166 antibody for 30 min and finally washed. IF was analysed by using a fluorescence microscope
167 (Olympus).

168

169 2.6. *Trans*-complementation of the replicon p890 Δ C

170 2.6.1. Establishment of C-E^{ms}-E1-E2 expressing WT-R2 cells

171 The genomic region encoding the structural proteins (C-E^{ms}-E1-E2) of ncp BVDV-1 strain

172 PT810 (Wolfmeyer et al., 1997) was cloned as a chemically synthesised synthetic open
173 reading frame (Syn-ORF, constructed by GeneArt, Regensburg, Germany). It consisted of
174 2694 nucleotides extending from nucleotide 890 to 3584 of the nucleotide sequence of BVDV
175 strain NADL (Collett et al., 1988), and was inserted into the pcDNA3.1 expression plasmid
176 (Invitrogen) using *KpnI* and *NotI* restriction sites. The nucleotide sequence of Syn-ORF had
177 been changed to remove splice sites (Schmitt et al., 1999), but retained the original amino
178 acid sequence of ncp BVDV strain PT810 (GenBank accession no. AY078406). Additionally,
179 the first codon of Syn-ORF was changed to a methionine to allow expression of the
180 polyprotein under the control of the *HCMV* immediate-early promoter present in pcDNA3.1,
181 and a stop codon was inserted behind the last codon. The resulting construct pcDNA_C-E2 (1
182 _g) was used to transfect WT-R cells with the SUPERFECT reagent (Qiagen). At 2 days post
183 transfection (p.t.), cell culture medium was changed to DMEM supplemented with 10 %
184 bovine serum and 0.5 mg of geneticin G418 per ml. G418-resistant colonies were isolated,
185 replated several times, and stained for E^{ms} and E2 expression using mab WB210, respectively
186 E2-mix (CA 1/2 and CA34/1/2).

187

188 2.6.2. *Trans*-complementation

189 *In vitro*-transcribed RNA of p890ΔC was transfected into WT-R2 cells and at 72 h p.t. RNA
190 replication was analysed by IF staining with NS3 specific mabs. Supernatants of transfected
191 cells were harvested and the titre of the pseudovirion progeny v890ΔC_{trans} was determined.
192 Serial passages of v890ΔC_{trans} were performed on complementing WT-R2 cells as well as
193 on non-complementing KOP-R cells.

194

195 2.7. Virus titration

196 Infectious titres were determined for virus stocks as well as for growth kinetics analyses, and
8

197 after *trans*-complementation of p890 Δ C. Cell culture supernatants of v890FL-, v890WT- and
198 890 Δ N^{pro}-infected cells were harvested, and supernatants containing the *trans*-complemented
199 pseudovirions (v890 Δ C_trans) were collected. After freezing, supernatants were titrated in
200 log₁₀-dilutions on KOP-R cells, and titres were determined as median tissue culture infective
201 dose per ml (TCID₅₀/ml).

202

203 2.8. Growth kinetics

204 For *in vitro* growth kinetics, KOP-R cells were infected with the recombinant virus v890FL,
205 v890 Δ N^{pro} and with the parental virus v890WT, respectively, at a multiplicity of infection
206 (MOI) of 1. Supernatants were collected at 0, 8, 12, 24, 48, 72 and 96 h post infection (p.i.)
207 and virus titres (TCID₅₀/ml) were determined.

208

209 2.9. Real-time RT-PCR analyses

210 In order to determine the viral RNA replication levels of v890FL, v890WT, and v890 Δ N^{pro},
211 KOP-R cells were infected at an MOI of 1 of the appropriate viruses. At 48 h p.i.,
212 supernatants and cells were separately collected and RNA was isolated by using the QIAamp
213 Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Uninfected
214 KOP-R cells were included as test control. In order to minimize the risk of cross
215 contamination, a one step RT-PCR was performed using the QuantiTect™ Probe RT-PCR Kit
216 (Qiagen). According to Hoffmann et al. (2005, 2006), 5 μ l RNA template were added to a
217 total volume of 25 μ l, containing 3.5 μ l RNase-free water, 12.5 μ l 2 \times QuantiTect Probe RT
218 reaction-buffer, 2.0 μ l panpesti-specific FAM-labeled primer/probe mix and 0.25 μ l RT-
219 enzyme mix. For quantification of the copy numbers, serially diluted BVDV-DI9-RNA
220 (Behrens et. al., 1998) was used as standard RNA. The following temperature profile was

221 used: 30 min at 50 °C (reverse transcription), 14 min at 95 °C (inactivation reverse
222 transcriptase/activation *Taq* polymerase), followed by 40 cycles of 30 sec at 95 °C
223 (denaturation), 30 sec at 57 °C (annealing) and 60 sec at 62 °C (elongation). Identical
224 temperature profiles were used for all real-time RT-PCR runs and fluorescence values were
225 recorded during the annealing steps.

226

227 2.10. Animal experiment

228 Ten Simmentaler breed calves, aged between 6 and 8 month, were shown to be free of
229 BVDV-antibodies and -antigen. Calves were randomly allocated into two groups of five
230 animals each, and inoculated with the recombinant v890FL and the parental 890WT virus,
231 respectively. Inoculation was done intranasally with 2×10^6 TCID₅₀ in a volume of 2 ml (1ml
232 per nostril). To confirm infectious titres, both viral suspensions were backtitrated on KOP-R
233 cells after inoculation. The animals were housed under identical conditions in two different
234 units and were monitored daily for clinical signs and rectal body temperatures. Blood samples
235 were collected to monitor viremia as well as to evaluate leukocyte and thrombocytes counts.
236 Nasal swabs were investigated for virus shedding throughout the experiment.

237

238 3. Results

239 3.1. Construction and characterisation of the infectious BVDV-2 cDNA clone p890FL

240 The full-length clone p890FL was constituted from four PCR fragments assembled in the low
241 copy vector pA (Meyers et al., 1996b). At the 5' end, the sequence of the T7 promoter was
242 added to enable *in vitro* transcription and at the 3' end a *SmaI* restriction site was introduced
243 for plasmid linearisation (figure 1). First sequence analyses revealed introduction of several
244 mutations into the full-length clone. In comparison to the parental virus a frame shift due to
245 the deletion of two nucleotides in the p7 encoding region and 7 aa substitutions allocated over
10

246 the ORF were detected, one in Npro (aa 147), two in E^{ms} (aa 392, aa 489), one in E2 (aa 851),
247 one in NS3 (aa 2057) and two in NS5a (aa 2905, aa 3221). Two of the mutations, the frame
248 shift and the aa substitution at position 3221 were eliminated by using site directed
249 mutagenesis. Subsequently, the p890FL cDNA clone was again completely sequenced,
250 resulting in detection of a bacterial insertion at aa position 648, accompanied by duplication
251 of the aa sequence GLR. Sequence analysis of the bacterial insertion showed similarities to
252 the bacterial IS10 element, which can be found in E. coli K-12 strain (data not shown).
253 Thereupon, we analysed the sequence of the RNA re-isolated from cells infected with
254 v890FL. The sequences of the bacterial insertion into the cDNA clone p890FL were not
255 present in the viral RNA of v890FL. As a consequence, we used the E. coli strain MDS42
256 (Pósfai et al., 2006) instead of E. coli strain DH10B for transformation of the new plasmid
257 constructs, due to the engineered genome of E. coli strain MDS42 without any sequences
258 encoding mobile bacterial genetic elements.

259 In order to generate infectious virus progeny, *in vitro*-transcribed RNA of p890FL was
260 transfected into KOP-R cells. 72 h p.t., RNA replication could be detected in nearly 100 % of
261 the cells by IF staining using NS3 specific mabs (figure 3). By passaging the transfection
262 supernatant, infectious virus v890FL could be recovered. A stock of the second passage was
263 used for *in vitro* and *in vivo* characterisation. The *in vitro* growth analyses indicated a very
264 similar growth of the recombinant virus v890FL compared to parental virus 890WT at earlier
265 time points, with a reduced final virus titre of v890FL (figure 4). In order to analyse RNA
266 replication levels, we performed real-time RT-PCR analyses, which showed similar RNA
267 replication levels of v890FL and v890WT. In supernatants of infected cells, 10⁸ to 10⁹ RNA
268 copies per ml were detected, and intracellular levels of viral RNA revealed around 10² RNA
269 copies per cell (table 3).

270 Furthermore, the animal experiment with v890FL and v890WT demonstrated an attenuated
11

271 phenotype of recombinant v890FL if compared to wild type virus 890WT. However, both
272 animal groups showed clinical signs of a severe BVDV infection like depression, reduced
273 feed intake, mild diarrhea and respiratory symptoms. In addition, all animals exhibited
274 pyrexia with a biphasic elevated body temperature curve, with mean maximum body
275 temperatures of nearly 41 °C for the wild type infected group and 39.7 °C for the group
276 infected with the recombinant virus v890FL (figure 5). Interestingly, in both groups no
277 thrombocytopenia could be observed. A marked leukocytopenia was present in both groups
278 with earlier recovery to pre-infection levels in the v890FL infected animals (figure 6). For
279 each animal, viremia could be detected at days 3 to 7 for the group infected with v890FL, and
280 at days 2 to 10 for the group infected with the parental virus v890WT (figure 7). In addition,
281 nasal virus shedding could be observed from day 2 to 10 p.i. (data not shown). Taken
282 together, we observed a milder clinical course after infection with v890FL, and without
283 ‘haemorrhagic syndrome’, compared to the BVDV-2 strain 890 (Bolin and Ridpath 1992).

284

285 3.2. Construction and characterisation of BVDV-2 deletion mutant p890 Δ N^{pro}

286 An N^{pro} autoprotease deletion mutant, p890 Δ N^{pro} (figure 2), was constructed on basis of the
287 infectious BVDV-2 clone p890FL by partial deletion of the genomic sequence encoding most
288 of N^{pro} (the first 36 nt overlapping with the BVDV IRES were retained). In order to detect
289 viral replication, in *vitro*-transcribed RNA of p890 Δ N^{pro} was transfected into interferon
290 negative MDBK cells. At 72 h p.t., expression of NS3 could be detected by IF staining in
291 nearly 100 % of the transfected cells (figure 3). Transfection supernatant was passaged and
292 infectious virus progeny v890 Δ N^{pro} could be recovered. Growth kinetics on interferon
293 competent KOP-R cells showed approximately 100-fold reduced growth of the deletion
294 mutant (figure 8). Nevertheless, real-time RT-PCR analyses indicated a similar RNA

295 replication level of v890 Δ N^{pro} in comparison to v890FL and v890WT (table 3).

296

297 3.3. Construction, *trans*-complementation and characterisation of BVDV-2 replicon p890 Δ C

298 Replicon p890 Δ C is characterised by a partial deletion of 43 aa within the encoding region for
299 the capsid protein (figure 2). 48 h post transfection of in *vitro*-transcribed RNA into non-
300 complementing KOP-R cells, autonomous replication of viral proteins could be detected in
301 nearly 100 % of the transfected cells by IF staining (figure 3), but no infectious virus progeny
302 could be recovered. For packaging of the replicon p890 Δ C, we established the new helper cell
303 line WT-R2 derived from the European bison. Like the first available helper cell line PT805
304 (Reimann et al., 2003), WT-R2 cells stably express a synthetic ORF encoding the BVDV-1
305 structural genes C-E^{ms}-E1-E2. For *trans*-complementation, in *vitro*-transcribed RNA of the
306 replicon p890 Δ C was transfected into WT-R2 cells, and 72 h p.t. autonomous virus
307 replication was detected by IF staining (figure 9). Infectious pseudovirions v890 Δ C_{trans}
308 could be recovered from transfection supernatants and were serially passaged on WT-R2 cells
309 (figure 9). However, no passaging was possible on non-complementing KOP-R cells, and no
310 replication competent revertants or pseudo-revertants could be detected.

311

312 4. Discussion

313 Several pestiviral infectious cDNA clones, including CSFV (Meyers et al., 1996a; Ruggli et
314 al., 1996) and BVDV-1 (Mendez et al., 1998; Meyers et al., 1996b; Vassilev et al., 1997),
315 have been described. However, only a single infectious BVDV-2 cDNA clone (strain
316 NY'93C) is published (Meyer et al., 2002). In addition, an infectious transcript of the
317 BVDV-2 strain 890 was established by Dehan et al. (2005). However, the construction of an
318 infectious cDNA clone of strain 890 failed. This study describes construction and

319 characterisation of the first infectious full-length cDNA clone of BVDV-2 strain 890
320 (p890FL) and its application for the development of further mutants (p890 Δ N^{pro} and
321 p890 Δ C). Although PCR amplification of the whole genome represents a simplification of the
322 cloning strategy, and has been described for pestiviruses (Rasmussen et al., 2008), the
323 generation of a full-length PCR fragment for the strain 890 failed. Therefore, p890FL was
324 constructed on the basis of four PCR fragments, which were assembled into the vector pA
325 (Meyers et al., 1996b). In *in vitro*-transcribed RNA of p890FL was transfected into bovine cells,
326 and replication could be demonstrated in nearly 100 % of the cells. Subsequently, infectious
327 virus progeny could be recovered (v890FL) from supernatants of transfected cells (figure 3).
328 In *in vitro*-characterisation of v890FL showed similar growth kinetics with only slightly reduced
329 virus titres, and a similar RNA replication level compared to the parental virus 890WT (figure
330 4). In infected animals however, we observed an attenuated phenotype of v890FL compared
331 to v890WT with lower mean body temperatures and a shorter viremia (figure 5, 6 and 7). Up
332 to now, the reason for in *vivo* attenuation of v890FL is not definitely resolved. In addition, our
333 experiments revealed for the parental virus 890WT altogether milder clinical symptoms
334 compared to BVDV-2 strain US890 (Bolin and Ridpath, 1992), which may be e.g. age or
335 breed related.

336 Attenuation of RNA-viruses recovered from cDNA clones reflects their genetic variability,
337 and has been also described for BVDV-2 before (Dehan et al., 2005; Meyer et al., 2002).
338 However, there are some amino acid substitutions in the full-length ORF of p890FL which
339 could possibly account for the in *vivo* attenuation: two in the E^{ns}, one in the E2, and one in
340 the NS5a encoding sequences. One of the E^{ns} point mutations is located at the C-terminus,
341 and is identical to a mutation in an infectious transcript of BVDV-2 890 described by Dehan
342 et al. (2005), which also showed an attenuated phenotype. The second aa substitution could be
343 found in the middle part of E^{ns} near the RNase motif. RNase activity is important for
14

344 virulence and pathogenicity of BVDV (Magkouras et al., 2008; Meyer et al., 2002; Meyers et
345 al., 2007), and therefore further studies will predominantly focus on the reconstitution of the
346 E^{ms} mutations. In contrast, the aa substitution within E2 and NS5a are not located in
347 previously defined functional regions (Johnson et al., 2001; Reed et al., 1998; Sapay et al.,
348 2006).

349 Furthermore, the cDNA clone p890FL was used for the construction of the deletion mutant
350 p890ΔN^{pro} by partial deletion of the genomic sequence encoding a predominant part of N^{pro}.

351 In contrast to CSFV and HCV, the extension of the IRES into the ORF of BVDV is not
352 defined in detail. In order to ensure full activity of the IRES, the first 12 codons were retained.

353 However, the minimum coding region essential for full efficacy of the IRES region is still
354 discussed. Recent reports describe for BVDV-1 the preservation of nine to 25 codons

355 downstream of the initial start codon to ensure full IRES activity (Moes and Wirth, 2007), and
356 for BVDV-2 Meyers et al. (2007) reported four residual codons as sufficient for acceptable

357 growth *in vitro*. Furthermore, alignment of BVDV-1 and BVDV-2 protein sequences resulted
358 in 13 out of the first 16 codons which are conserved in the BVDV polyprotein (Moes and

359 Wirth, 2007). For CSFV a similar conservation scheme is described (Moers and Wirth 2007).
360 17 codons of the N-terminus are required for full activity of the CSFV-IRES (Fletcher et al.,

361 2002). However, it has to be mentioned that preservation of the first 12 codons of the N^{pro}-
362 gene of BVDV-2 strain 890 were sufficient to maintain viral replication, but also resulted in a

363 capsid protein with an amino-terminal extension. The deletion mutant p890ΔN^{pro} was able to
364 replicate *in vitro*, and from supernatants of transfected interferon negative MDBK cells

365 infectious virus progeny v890ΔN^{pro} could be recovered. The v890ΔN^{pro} virus titres detected in
366 MDBK cells were comparable to the titres of v890FL detected in KOP-R cells (data not

367 shown), indicating that there is no marked influence of the amino-terminal extension of the
368 capsid protein on viral viability and growth in cell culture. Comparison of the *in vitro* growth

369 kinetics of v890 Δ N^{pro}, v890FL and v890WT on interferon-competent KOP-R cells revealed
370 an approximately 100-fold reduced growth of the deletion mutant v890 Δ N^{pro} due to the loss of
371 N^{pro} as an interferon antagonist (Gil et al., 2006). However, our results are in contrast to the
372 non-reduced *in vitro* growth of a BVDV-2 N^{pro} deletion mutant described by Meyers et al.
373 (2007). Furthermore, N^{pro} deletion mutants are useful candidates for efficient modified live
374 vaccines against BVDV-1 and BVDV-2 with the potency to induce sterile immunity without
375 the risk of establishing persistent infections (P. König unpublished data; Meyers et al., 2007;
376 Zemke et al., 2008).

377 In addition, the BVDV-2 replicon p890 Δ C, with a partial deletion of the genomic region
378 encoding the capsid protein, was constructed. The N-terminal 32 aa and the 27 C-terminal aa
379 of the capsid protein, which are essential for signalase recognition, translocation of the
380 envelope proteins into the ER, and further processing of the E^{ms}-E1-E2 polyprotein
381 (Rümenapf et al., 1991) were retained. *In vitro*-transcribed RNA of p890 Δ C was able to
382 replicate autonomously in non-complementing bovine cells, since the structural proteins are
383 not essential for pestiviral RNA replication (Behrens et al., 1998). Virus progeny could not be
384 recovered from the supernatant of transfected non-complementing bovine cells. However,
385 infectious virus could be generated by packaging the defective genomes by using a helper
386 virus (Kupfermann et al., 1996) or a helper cell line (Reimann et al., 2003). For *trans*-
387 complementation and packaging of the replicon p890 Δ C we constructed the new helper cell
388 line WT-R2 essentially as described for the helper cells PT805 (Reimann et al., 2003). The
389 replicon p890 Δ C was efficiently *trans*-complemented and packaged into pseudovirions by
390 using WT-R2 cells. Recombination or reversion during generation of the pseudovirions was
391 not observed, and in contrast to experiments with BVDV-1 Δ C replicons and PT805 cells
392 (Reimann et al., 2003, 2007), the recombinant WT-R2 cells even allowed the passaging of

393 BVDV-2 Δ C pseudovirions.

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

399

400 **Acknowledgements**

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403

404 **Conflict of interest statement**

405 None

406

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597 **Tables**

598 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	ATATCCCGGGCCTATTATCTTGGTGTTCCTTGG	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	ATATGAATTCCCCGGGGGGCCGTTAGAGGCATC CTCTAGTC	12486-12512 (-)
890_Npro	ATATGCGGCCGCATCCGATGAAGGGAGTAAGG GTGCT	890-913 (+)
890_Npro_r	ATATTACGTATGCGGCCGCTGTTTTGTATAAAA GTTCAATTTGAAAACAACCTCCATGTGCC	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 (-)

599 ^a genomic region of BVDV-2 strain 890 (GenBank accession no. U18059), symbols in

600 brackets show the polarity

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

Primer	Sequence (5'-3') ^a	Genomic region ^b
MutI	AGAACTAGTGGATCCC <u>GCG</u> CGTAATACGACTCAC TA	- (+)
MutI_r	TAGTGAGTCGTATTAC <u>GCG</u> CGGGATCCACTAGTTC T	- (-)
MutII	ACCAAGATAATAGGCC <u>A</u> GG <u>A</u> AGTTTGACACCA ACGCC	1961-1999 (+)
MutII_r	GGCGTTGGTGTCAA <u>CT</u> CTGGGCCTATTATCT TGGT	1961-1999 (-)
890_ORF	GCTGACACACAGTG <u>AT</u> ATTGAGGTTGTGGTC	3619-3649 (+)
890_ORF_r	GACCACAACCTCA <u>TA</u> TCACTGTGTGTCAGC	3619-3649 (-)
890_NS5	GGCTGACTTATATCACCTAATT <u>G</u> GCAGTGTGATA GTATAAAAAG	10024-10068 (+)
890_NS5_r	CTTTTTATACTATCAACACTG <u>C</u> CAATTAGGTGATA TAAGTCAGCC	10024-10068 (-)

606 ^a mutated nucleotides are underlined and in bold607 ^b genomic region of BVDV-2 strain 890 (GenBank accession no. U18059), symbols in
608 brackets show the polarity

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

622

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}

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640 **Figures**

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

651

652 Figure 2: Schematic depiction of the deletion mutants p890ΔN^{pro} and p890ΔC based on the
653 infectious cDNA clone p890FL. Filled boxes represent the regions encoding the BVDV
654 structural proteins. Horizontal dotted lines show the deleted regions and numbers indicate the
655 nucleotide (nt) or amino acid (aa) position in the BVDV full-length RNA. Lines at the left and
656 the right ends indicate non-translated regions. N^{pro}, autoprotease; C, capsid protein; E^{ms}, E1,
657 E2, envelope proteins; p7, non-structural protein; NS2 to NS5, non-structural proteins; 3'NTR
658 and 5'NTR, non-coding regions. The size-scale is given in kb.

659

660 Figure 3: IF analysis of bovine cells transfected with *in vitro* transcribed RNA of p890FL,
661 p890ΔN^{pro} or p890ΔC. In addition, supernatants of transfected cells were passaged on bovine
662 cells. At 72 h p.t. and 72 h p.i. NS3 expression was analyzed by IF staining using the mab WB
663 103/105. Untransfected/uninfected bovine cells were used as controls. A) p890FL RNA

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664 transfected into KOP-R cells and passage of the supernatants on KOP-R cells. B) p890 Δ N^{pro}
665 RNA transfected into interferon-incompetent MDBK cells and passage of the supernatants. C)
666 RNA of p890 Δ C transfected into KOP-R cells and passage of the supernatants on KOP-R
667 cells.

668

669 Figure 4: Growth kinetics of the recombinant virus v890FL (broken line) and the parental
670 virus v890WT (solid line). KOP-R cells were infected at an MOI of 1. Supernatants were
671 harvested at the indicated time points. After freezing and thawing, virus titres (TCID₅₀/ml)
672 were determined by titration on KOP-R cells. Standard deviations are shown as error bars.

673

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

677

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

681

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

687

688 Figure 8: Growth kinetics of the deletion mutant v890 Δ N^{pro} (dotted line) compared with the
689 recombinant virus v890FL (broken line) and the parental virus v890WT (solid line). KOP-R
690 cells were infected with the respective viruses at an MOI of 1. Supernatants were harvested at
691 the indicated time points. After a freezing and thawing procedure, virus titres (TCID₅₀/ml)
692 were determined by titration on KOP-R cells. Standard deviations are shown as error bars.

693

694 Figure 9: *Trans*-complementation studies with replicon p890 Δ C and WT-R2 helper cells. A)
695 The WT-R2 cell line stably expresses the synthetic structural genes C-E2 of BVDV-1, and E2
696 expression is shown by IF staining using an E2-mab mix (CA 1/2 and CA34/1/2). NS3 as a
697 marker for viral replication could not be detected by IF staining using the mab WB 103/105 in
698 non-transfected cells. B) Transfection of *in vitro*-transcribed RNA of p890 Δ C into WT-R2
699 cells. 72 h p.t. NS3 expression could be detected by IF staining using the mab WB 103/105.
700 C) Pseudovirions v890 Δ C_{trans} could be recovered from supernatants of transfected WT-R2
701 cells and were further passaged on WT-R2 cells. Replication of the pseudovirions was
702 detected by IF staining: 72 h p.i. NS3 expression could be detected.

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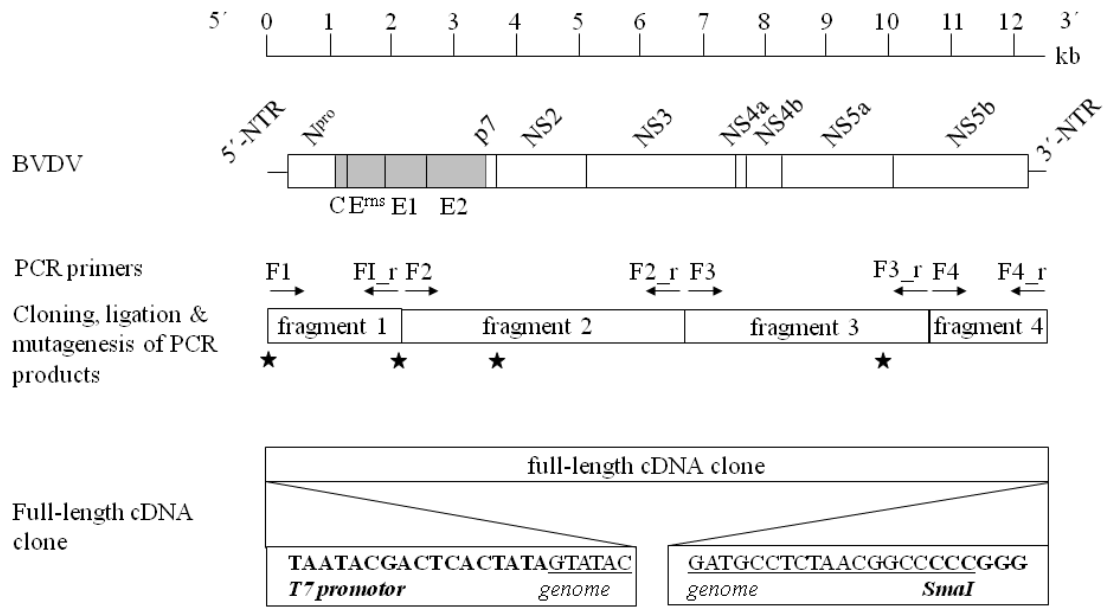
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712 Figure 1:



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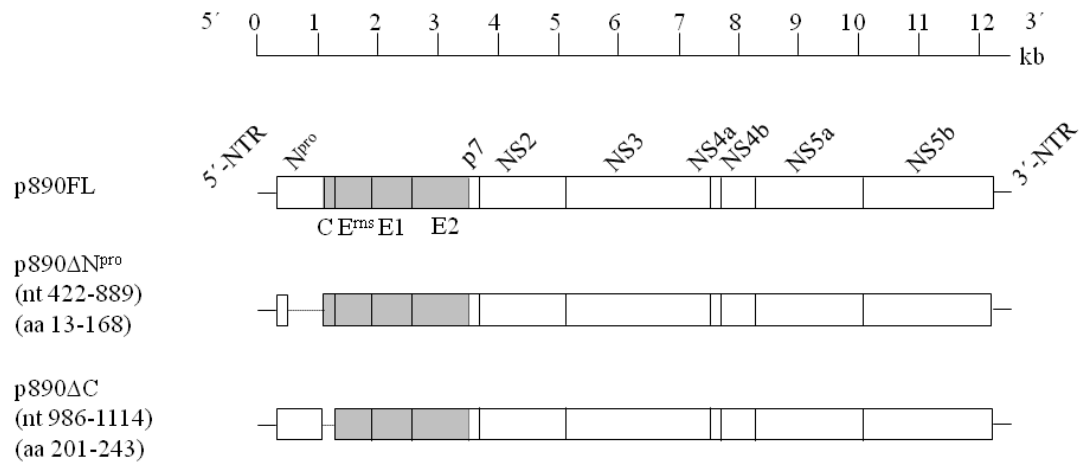
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725 Figure 2:



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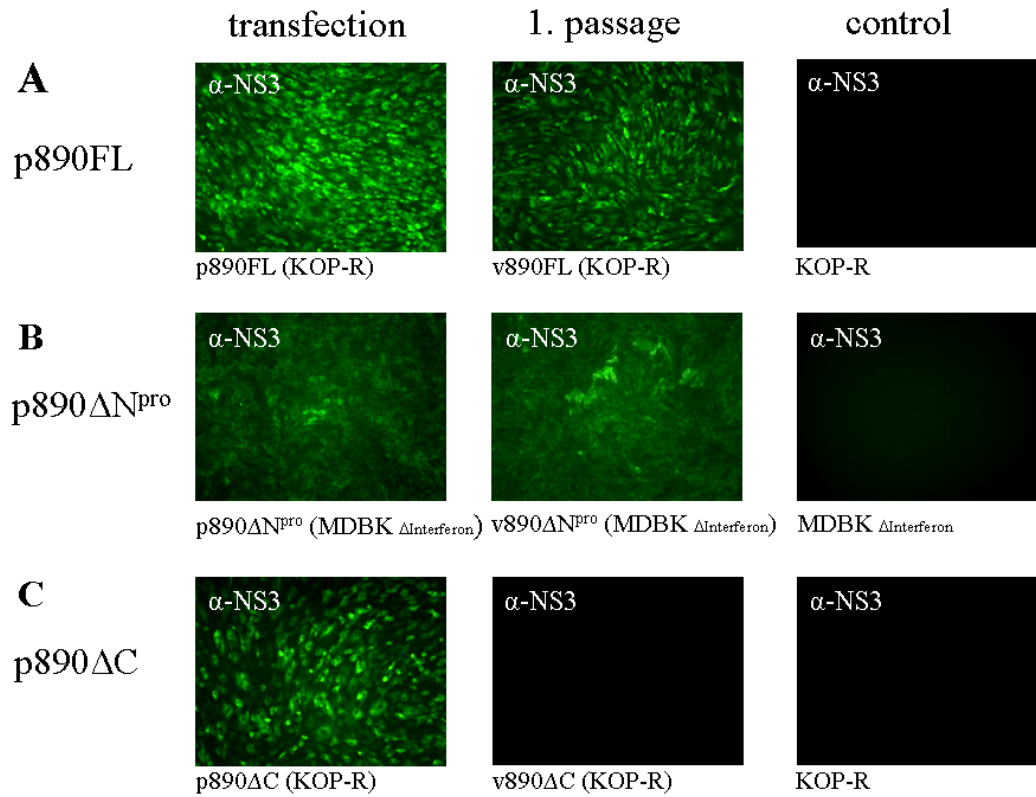
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738 Figure 3:

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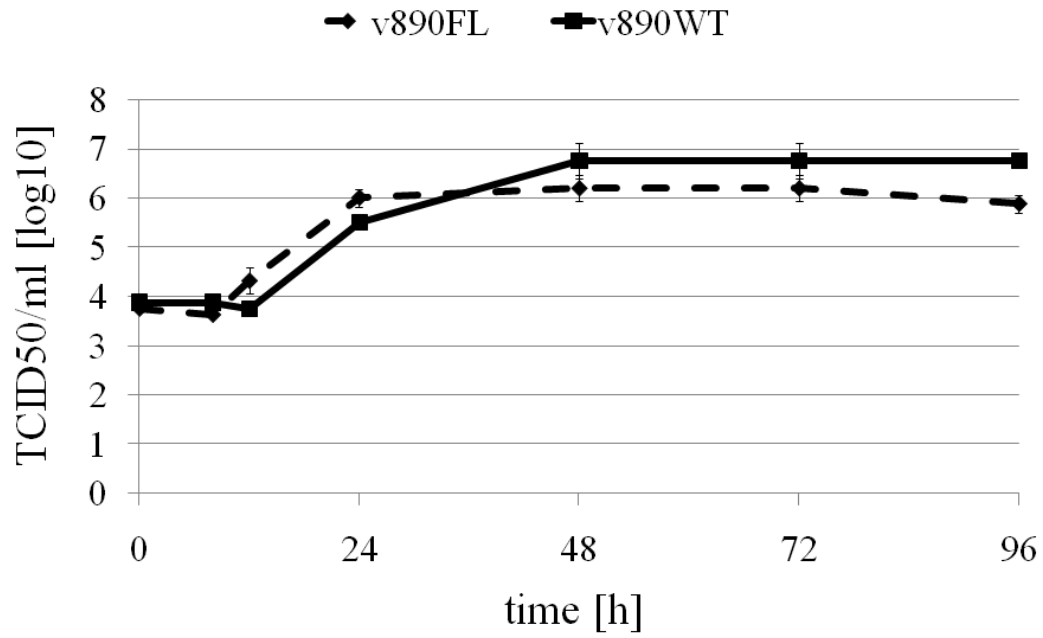
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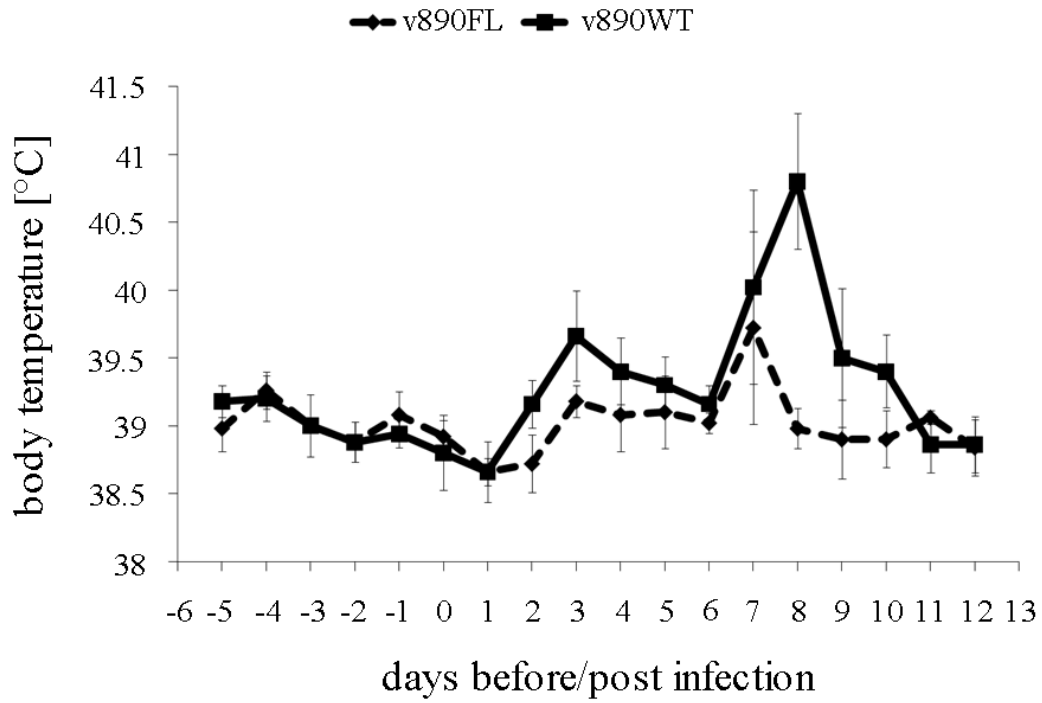
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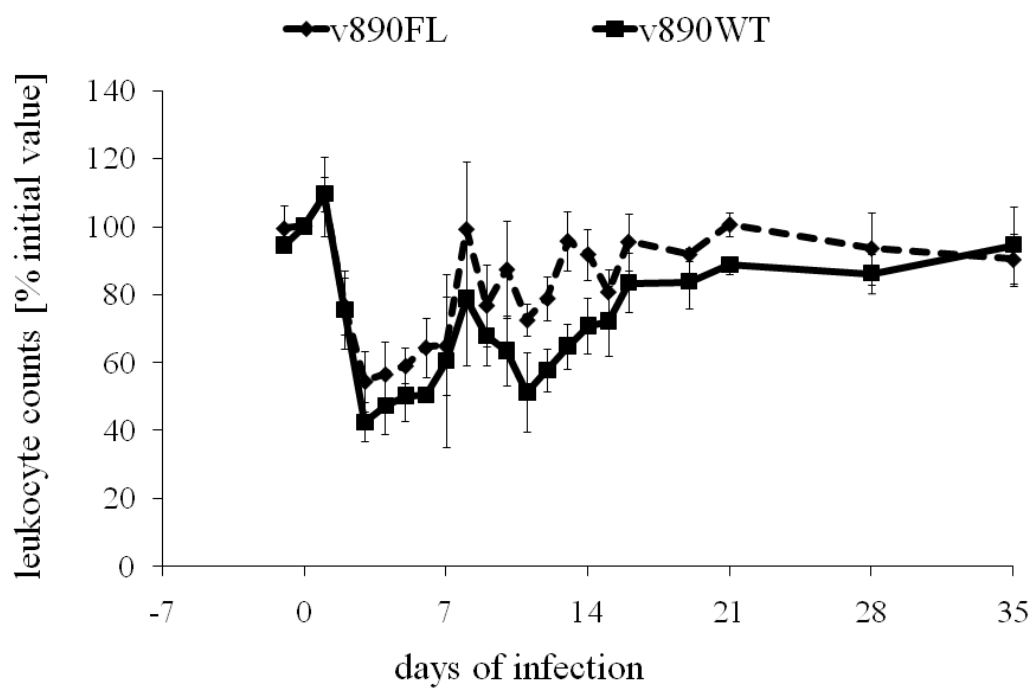
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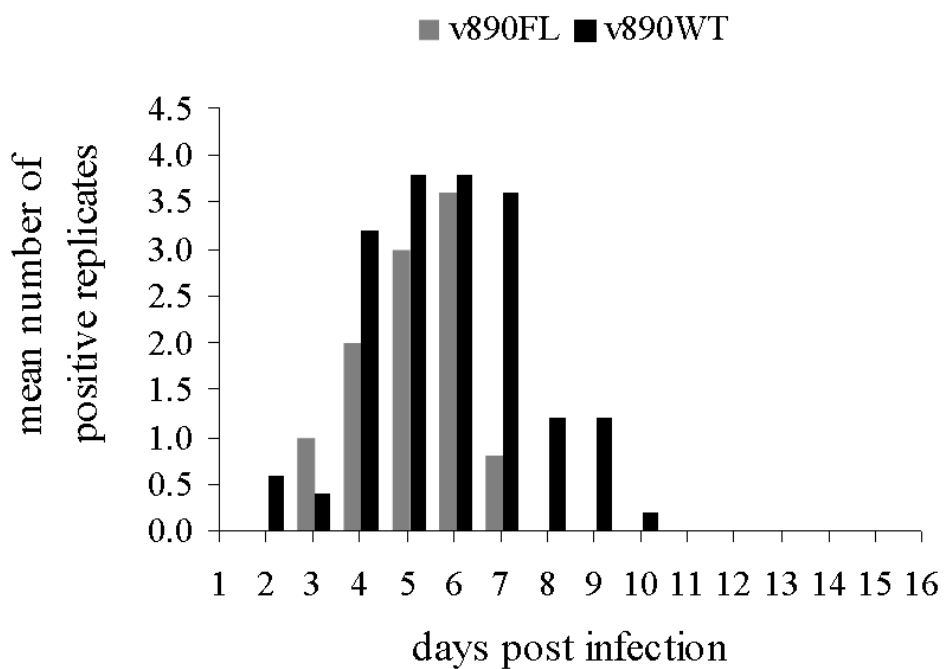
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790 Figure 7:



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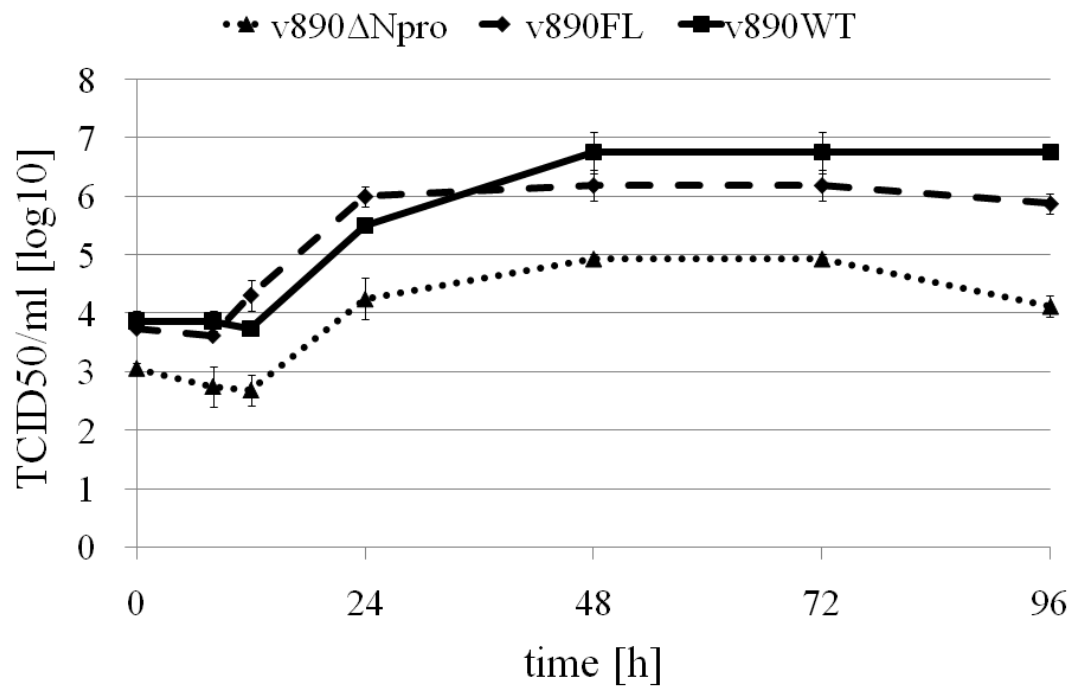
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803 Figure 8:



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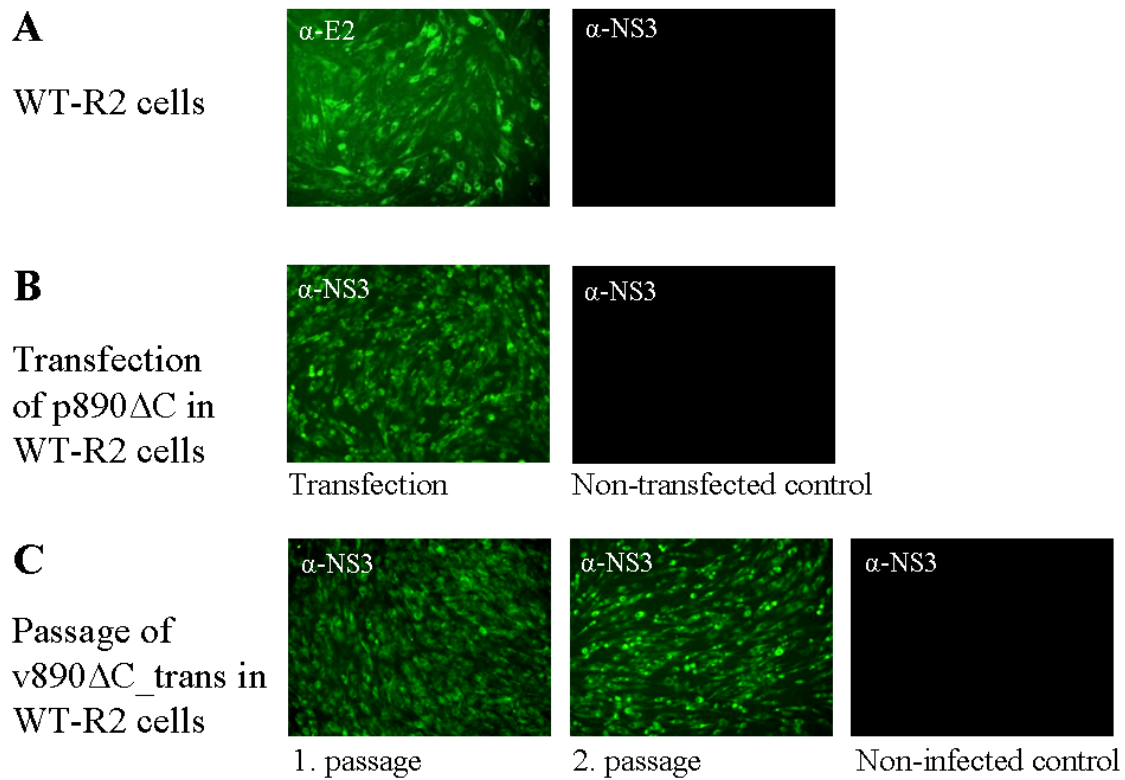
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816 Figure 9:



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