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1 Special Issue Veterinary Microbiology: 7th Pestivirus Meeting

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Short communication

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5 **Characterisation of a new chimeric marker vaccine candidate with a mutated**
6 **antigenic E2-epitope**

7

8 Ilona Reimann^a, Klaus Depner^b, Katrin Utke^b, Immanuel Leifer^b, Elke Lange^c,
9 and Martin Beer^{b*}

10

11 ^aInstitute of Molecular Biology, ^bInstitute of Diagnostic Virology, ^cInstitute of Infectology,
12 Friedrich-Loeffler-Institut, D-17493 Greifswald-Insel Riems, Germany

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20 *Corresponding author:

21 Dr. Martin Beer

22 Institute of Diagnostic Virology

23 Friedrich-Loeffler-Institut

24 Suedufer 10

25 17493 Greifswald-Insel Riems

26 Phone: Martin.Beer@fli.bund.de

27 **Abstract**

28 A new chimeric pestivirus “CP7_E1E2alf_TLA”, based on the infectious cDNA of
29 bovine viral diarrhoea virus (BVDV) strain CP7, was constructed. The substitution of BVDV
30 E1 and E2 with the respective proteins of classical swine fever (CSF) strain Alfort 187 allows
31 an optimal heterodimerization of E1 and E2 in the chimeric virus, which is beneficial for
32 efficient and authentic virus assembly and growth. In addition, for implementation of E2-
33 based marker diagnostics, the previously described antigenic CSFV-specific TAVSPTTLR-
34 epitope was exchanged with the corresponding E2-epitope of BVDV strain CP7.
35 Recombinant virus CP7_E1E2alf_TLA displayed a growth defect, and was not reacting with
36 monoclonal antibodies used in commercial E2 antibody blocking ELISAs. Therefore, efficacy
37 as well as marker properties of CP7_E1E2alf_TLA were investigated in an animal experiment
38 with both a high dose and a low dose vaccine preparation. All CP7_E1E2alf_TLA-vaccinated
39 animals seroconverted until day 14 post challenge infection with neutralizing antibodies.
40 Furthermore, at the day of challenge infection CP7_E1E2alf_TLA-immunised animals
41 showed distinct lower ELISA-values in a commercial CSFV-E2-antibody test in comparison
42 to the C-strain vaccinated controls. However, E2-ELISA reactivity as well as neutralizing
43 titres were directly connected to the dosage used for vaccination, and only the low dose group
44 had E2-ELISA values below threshold until challenge infection. Following challenge
45 infection with highly virulent CSFV-strain Koslov, all vaccinees were protected, however,
46 short-term fever episodes and very limited CSFV-genome detection with very low copy
47 numbers could be observed. In conclusion, manipulation of the TAVSPTTLR-epitope within
48 the tested chimeric virus resulted in a slightly reduced efficacy, but the E2-marker properties
49 unexpectedly did not allow a clear differentiation of infected from vaccinated animals in some
50 cases.

51

52 **Keywords:** Pestivirus, classical swine fever, marker vaccine, DIVA, chimeric construct

53

54 **1. Introduction**

55 Classical swine fever (CSF) is one of the most important infectious diseases of pigs.
56 Large-scale CSF outbreaks have a disastrous economic effect in countries with an industrial
57 pig production and are associated with mass culling of animals. In addition, CSF outbreaks in
58 domestic pigs implicate trade restrictions of pigs and pig products (Artois et al., 2002;
59 Edwards et al., 2000). Currently a non-vaccination policy is implemented in the EU. But due
60 to ethical, welfare and economical aspects the development of emergency vaccines, allowing
61 the differentiation of vaccinated from infected animals, is indispensable. Furthermore, due to
62 the risk of CSFV transmission from wild boar to domestic pigs (Artois et al., 2002), a
63 modified live vaccine should be available which also could be used for oral vaccination of
64 wild boar. At the moment, no licensed CSFV live vaccine features “marker properties”,
65 allowing a differentiation of vaccinated and infected animals (*Differentiating Infected from*
66 *Vaccinated Animals “DIVA”*) (Beer et al., 2007). Until now, only CSF subunit marker
67 vaccines based on recombinant, baculovirus-expressed envelope protein E2 are registered
68 (Bouma et al., 1999). However, the immune response after vaccination with the E2-subunit
69 vaccine is delayed, and pigs have to be vaccinated twice for a sufficient protection (Depner et
70 al., 2001; de Smit et al., 2001a). Facilitated by the establishment of infectious cDNA clones of
71 classical swine fever virus (CSFV) and bovine viral diarrhoea virus (BVDV) (Meyers, et al.,
72 1996; Moorman et al., 1996; Rüggli et al., 1996; Vassilev et al., 1997) trans-complemented
73 deletion mutants (Wjdjoatmodjo et al., 2000; Maurer et al., 2005; Reimann et al., 2007) and
74 chimeric pestiviruses (van Gennip et al., 2000; Reimann et al., 2004, König et al., 2007a,
75 Wehrle et al., 2007, Rasmussen et al., 2007) are the most promising candidates for the
76 development of new, attenuated DIVA vaccines.

77 Serological diagnostics of pestivirus infections are often based on the detection of antibodies
78 directed against the non-structural protein NS3 or the envelope proteins E2 and E^{ms}. Since

79 NS3-specific antibodies are pan-pestivirus specific, only E2 and E^{ms} specific antibodies can
80 be used for serological differentiation of different pestivirus species (Wensvoort et al., 1989,
81 Paton, 1995, Beer et al., 2007). Due to the high sensitivity and specificity, CSFV E2-ELISAs
82 would be the best choice for CSF DIVA diagnostics. However, the available vaccines (E2-
83 Subunit, C-strain) are based on unchanged CSFV E2 proteins which do not enable E2-based
84 DIVA diagnostics. Therefore, chimeric pestiviruses had been generated on the basis of CSFV
85 and BVDV backbones, respectively. CSFV backbone chimeras are based on vaccine strain C.
86 Substitution of CSFV E2 and E^{ms} with the analogous proteins of a BVDV type 2 strain,
87 respectively, resulted in viable chimeric viruses, which protected pigs from a lethal CSFV
88 infection and allowed either an E2 or E^{ms} based marker diagnostics (van Gennip et al., 2000,
89 de Smit et al., 2001b). The BVDV-based chimeric pestivirus CP7_E2alf had been constructed
90 on the basis of BVDV strain CP7 by replacing the original E2 protein with the E2 protein of
91 CSFV Alfort 187 (Reimann et al., 2004). CP7_E2alf virus was proven to be completely
92 avirulent and efficacious after intramuscular (i.m.) and oral immunisation of domestic pigs
93 and wild boar (Reimann et al., 2004; König et al., 2007a, 2007b). The BVDV background of
94 CP7_E2alf allowed an E^{ms} based serological marker diagnostics as well as a genetic DIVA
95 diagnostics (Reimann et al., 2004; Hoffmann et al., 2005, 2006).

96 In this study, we describe a newly developed, BVDV-based chimera, expressing both
97 CSFV E1 and E2. In order to allow E2-based in addition to E^{ms}-based serological marker
98 diagnostics, CSFV E2 was modified by substitution of the antigenic TAVSPTTLR-epitope
99 (Lin et al., 2000; Liu et al., 2006) with the corresponding epitope of BVDV strain CP7. The
100 chimeric virus was characterized *in vitro*, and vaccine efficacy and marker properties were
101 investigated in an immunization and challenge experiment.

102

103 **2. Material and Methods**

104 *2.1. Cell cultures and viruses*

105 SK-6 swine kidney cells (SK-6; CCLV RIE262) were obtained from the Collection of Cell
106 Lines in Veterinary Medicine, FLI, Insel Riems, Germany (CCLV). Cells were grown in
107 Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% BVDV free fetal calf
108 serum at 37°C in a humidified atmosphere containing 5% CO₂.

109 CSFV strains Alfort 187 and Koslov were obtained from the National Reference
110 Laboratory of CSF (Friedrich-Loeffler-Institut Insel Riems, Germany). Virus titers were
111 determined by end point titrations. Cells seeded in microtitration plates were infected with 10-
112 fold serial dilutions of clarified supernatants. The titres expressed in TCID₅₀ per milliliter
113 were obtained by immunofluorescence (IF) staining of the SK-6 cells with the monoclonal
114 antibody (mab) C16 directed against the pestiviral protein NS3 (Institute of Virology, TiHo
115 Hannover, Germany) and an Alexa Fluor®488-conjugated F(ab')₂ fragment of goat anti-
116 mouse IgG (Molecular Probes). For detection of the chimeric E2-protein, the monoclonal
117 antibodies CA34, HC34 and HC/TC16 were used (kindly provided by Irene Greiser-Wilke;
118 Institute of Virology, TiHo Hannover, Germany). Standard immunofluorescence analyses
119 using a fluorescence microscope (IX51, Olympus) were performed as previously described
120 (Beer et al., 1997).

121

122 *2.3. Polymerase chain reaction and sequencing*

123 For PCR, a PTC-200 thermal cycler (MJ Research, Inc.) was used. DNA based
124 amplification was done with the Expand High Fidelity PCR System (Roche Molecular
125 Biochemicals) according to the supplier's protocol. For RT-PCR, total RNA of virus-infected
126 cells was extracted using the TRIZOL reagent (Invitrogen). cDNA was produced from RNA
127 by using the OneStep RT-PCR Kit (Qiagen) with sequence-specific primers. The PCR
128 products were directly sequenced using appropriate primers. Sequencing was carried out
129 using a Big Dye® Terminator v1.1 Cycle sequencing Kit (Applied Biosystems). Nucleotide
130 sequences were read with an automatic sequencer (3130 Genetic Analyzer, Applied

131 Biosystems) and analyzed using the Genetics Computer Group software version 11.1
132 (Accelrys Inc., San Diego, USA).

133

134 2.4. Plasmid construction

135 The chimeric pestivirus clones pA/CP7_E1E2alf and pA/CP7_E1E2alf_TLA, depicted
136 in Fig. 1, were constructed by using the full-length cDNA clone pA/BVDV (Meyers et al.,
137 1996). For the generation of pA/CP7_E1E2alf, genomic regions encoding BVDV E1 and E2
138 were deleted (nucleotides 1860-3566) and a PCR fragment comprising CSFV E1 and E2 was
139 inserted. In addition, by site-directed mutagenesis amino acid (aa) 479 within BVDV E^{ms} was
140 mutated from glycine to arginine, and restriction sites created during the cloning procedure
141 were deleted resulting in plasmid pA/CP7_E1E2alf. The plasmid with a chimeric E2 gene,
142 pA/CP7_E1E2alf_TLA, was constructed by site-directed mutagenesis of the antigenic CSFV-
143 specific TAVSPTTLR epitope within E2 to TLANKDTLA, the corresponding BVDV CP7
144 epitope. The specified nucleotide positions (nt) correspond to the BVDV CP7 genome
145 (Accession No. U63479), and the CSFV Alfort 187 sequence (Accession No. X87939).
146 Detailed information concerning the cloning steps is available on request.

147

148 2.5 Rescue of recombinant virus

149 For virus recovery, *in vitro*-transcribed RNA of plasmids pA/CP7_E1E2alf and
150 pA/CP7_E1E2alf_TLA (1 to 5 µg) were transfected into SK6 cells by electroporation. *In vitro*
151 transcription, transfection of SK-6 cells, virus rescue and multi-step growth kinetics were
152 performed as described previously (Reimann et al., 2004). Virus stocks were prepared by
153 passaging the virus three times on SK-6 cells, containing a titre of 10^5 - 10^7 TCID₅₀/ml. The
154 identity of the recombinant viruses was confirmed by RT-PCR and subsequent sequencing of
155 the PCR products.

156

157 2.6. *Immunization and challenge experiment*

158 In two animal experiments, 5- to 7-week old crossbred weanling pigs, free of
159 pestiviruses and neutralizing pestivirus antibodies, were randomly divided into vaccine
160 groups of 5 (chimeric constructs) or 3 (C-strain) pigs. The animals of the vaccine group were
161 immunized with CP7_E1E2alf_TLA derived from infectious chimeric cDNA clone
162 pA/CP7_E1E2alf_TLA. All animals were inoculated intramuscularly (i.m.) with 2 ml of cell
163 culture supernatant containing $10^{4.75}$ TCID₅₀ (low dose [ld] group; n=5) and $10^{6.5}$ TCID₅₀,
164 (high dose [hd] group; n=5), respectively. The third group (n=3) was vaccinated with 2 ml
165 [$>10^2$ 50% protective doses (PD₅₀) corresponding to $10^{4.1}$ 50% tissue culture-infective doses
166 (TCID₅₀)] of a commercial available C-strain vaccine (Riemser Arzneimittel AG) according
167 to the manufacturer's instructions. All vaccinated animals and naïve controls were challenged
168 28 days later oronasally with highly virulent CSFV strain Koslov ($2 \times 10^{6.0}$ TCID₅₀/animal).
169 During the course of the experiment, pigs were monitored daily for clinical signs of CSF.
170 Nasal and oral swabs as well as blood samples were collected for virological and serological
171 examinations in intervals of 2 to 7 days. Blood samples were also analysed for differential
172 leukocyte counts by using an automatic cell counter (Cell-Dyn^R3700, Abbott).

173 Virus was isolated from blood samples by using co-cultivation of 1×10^6 blood
174 leucocytes with SK-6 cells in duplicate. After an incubation period of 5 to 6 days, the cells
175 were analyzed for NS3-expression by immunofluorescence. Viral RNA was detected by real-
176 time RT-PCR with a real-time PCR-Cycler MX3005pro (Stratagene) in serum, nasal swabs
177 and leucocytes as described by Hoffmann et al. (2005).

178 Neutralizing titres were determined as neutralizing dosage 50% using the CSF virus
179 strain Alfort 187 according to the EU Diagnostic Manual. In addition, serum or plasma
180 samples were tested for the presence of antibodies against CSFV E2 and E^{ms} with the
181 commercialized ELISA assays HerdCheck CSFV Ab, (IDEXX) and Ceditest CSFV-E^{RNS} Ab
182 (Cedi-Diagnostics/Prionics).

183

184 **3. Results and discussion**185 *3.1. Construction and in vitro-characterization of the recovered BVDV/CSFV chimeras*

186 Aim of this study was to generate a BVDV-based chimeric pestivirus as a potential
187 CSFV vaccine candidate allowing additionally to the E^{RNS}-based marker diagnostics an E2-
188 based differentiation of infected from vaccinated animals. Possible E2 markers can rely on the
189 modification of the antigenic regions “BC” or “A” within CSFV E2 (van Rijn et al., 1994).
190 However, CSF E2 antibody ELISAs commercially available in Europe are using monoclonal
191 antibodies directed against the antigenic “TAVSPTTLR-epitope” within the antigenic region
192 A. Therefore, one favourite option for E2-DIVA diagnostics could be the use of vaccine
193 viruses with a modified TAVSPTTLR-epitope in combination with the commercial E2
194 antibody ELISAs as accompanying test systems. Here, in a first step a chimeric BVDV/CSFV
195 full-length clone, pA/CP7_E1E2alf was constructed, in which the E1 and E2 encoding regions
196 of cytopathogenic BVDV strain CP7 were replaced by the corresponding regions of CSFV
197 strain Alfort 187, and amino acid G⁴⁷⁹ within the genomic region encoding for BVDV E^{rns}
198 was mutated to R for an optimized virus growth (Hulst et al., 2000; Reimann et al., 2004).
199 Furthermore, based on plasmid pA/CP7_E1E2alf, the cDNA construct
200 pA/CP7_E1E2alf_TLA was generated by substitution of the antigenic TAVSPTTLR-epitope
201 (Lin et al., 2000) within the CSFV E2 with the corresponding BVDV CP7 epitope
202 (TLANKDTLA) resulting in a total of six exchanged amino acids (Fig. 1).

203 In vitro-transcribed RNA of both cDNA constructs pA/CP7_E1E2alf and
204 pA/CP7_E1E2alf_TLA was able to replicate after transfection into SK-6 cells (data not
205 shown). At 24 h after transfection, recombinant virus progeny CP7_E1E2alf and
206 CP7_E1E2alf_TLA could be isolated from transfection supernatants by inoculation of SK-6
207 cells. Cells infected with the recombinant viruses could be stained (Fig. 2A) by using a
208 panpesti-specific mab (anti-Pesti, NS3-specific, C16). The modified chimeric

209 CP7_E1E2alf_TLA virus could be differentiated from parental CP7_E1E2alf virus by IF
210 staining with CSFV E2-specific mabs directed against the TAVSPTTLR-epitope (e.g. anti-
211 CSFV E2, TAV, HC34). In contrast to a non-TAVSPTTLR-specific mab (anti-CSFV E2,
212 none TAV, HC/TC16), the TAVSPTTLR-specific mab detected only CP7_E1E2alf-infected
213 SK-6 cells. In addition, due to the substitution of BVDV E2 with CSFV E2, SK-6 cells
214 infected with either CP7_E1E2alf or CP7_E1E2alf_TLA could not be detected with a BVDV
215 E2-specific mab (anti-BVDV E2, CA34).

216 Growth kinetics of the chimeric viruses CP7_E1E2alf and CP7_E1E2alf_TLA were
217 performed in porcine SK-6 cells. Surprisingly, the multistep growth curve of
218 CP7_E1E2alf_TLA showed a decreased replication efficiency resulting in about tenfold
219 reduced virus titers in comparison to parental CP7_E1E2alf (Fig. 2B). A possible explanation
220 could be that the modified CSFV E2 protein has an altered structure inducing a reduced virus
221 replication due to changes in the efficacy of virus assembly, release and/or attachment.
222 However, the role of a change of tropism should be low since virus growth of
223 CP7_E1E2alf_TLA was similarly decreased in bovine KOP-R cells as it was for
224 CP7_E1E2alf or CP7_E2alf (data not shown; Reimann et al., 2004).

225

226 3.2. Immunization and challenge infection of pigs

227 The marker and vaccine properties of CP7_E1E2alf_TLA were investigated in
228 immunization and challenge experiments in comparison with the C-strain vaccine (Riemser
229 Arzneimittel AG) as “gold standard”. After immunization of pigs with the C-strain vaccine
230 and CP7_E1E2alf_TLA, respectively, the general condition of the animals was good and no
231 clinical symptoms could be observed. CP7_E1E2alf_TLA was completely avirulent, and no
232 vaccine virus transmission was observed (data not shown).

233 After oro-nasal challenge infection with the highly virulent CSFV strain Koslov at day
234 28 post vaccination (dpv; day 0 post challenge infection [dpc]), all pigs vaccinated with

235 CP7_E1E2alf_TLA showed a short increase of body temperatures ($>40^{\circ}\text{C}$) between 3 and 5
236 dpc (Table 1). At the day of challenge infection, mean homologous antibody titers of 1:15
237 (low dosis group = ld group) and 1:74 (high dosis group = hd group) could be detected in the
238 CP7_E1E2alf_TLA-immunized animals with a CSFV Alfort 187 specific neutralization
239 assay. In comparison, a mean titer of CSFV Alfort 187 neutralizing antibodies of 1:33 was
240 determined for the C-strain-immunized group. No challenge virus could be re-isolated from
241 blood leucocytes of any of the immunized animals between 0 and 28 dpc, suggesting that no
242 virus shedding occurred. However, low levels of CSFV genome could be detected in serum
243 samples of animals of both groups immunized with CP7_E1E2alf_TLA between 3 to 6 dpc
244 with cycle of threshold (ct) values of about 35 (less than 100 genome copies). In detail, CSFV
245 genome was detected in 5/5 vaccinated pigs (low dose) at 4 dpc, and in 1/5 pigs at 6 dpc
246 (Table 1). In serum samples of animals, which were immunized with a high dose of
247 CP7_E1E2alf_TLA, CSFV genomes were detected at 3 dpc (4/5 animals), and 5 dpc (2/5
248 animals, Table 1). In summary, CP7_E1E2alf_TLA showed a slightly reduced protective
249 efficacy in comparison to the C-strain vaccine. In addition, the newly constructed mutants
250 were also less efficacious than the prototype BVDV-based chimera CP7_E2alf (Reimann et
251 al., 2004, König et al., 2007b).

252

253 3.3. *Differentiating infected from immunized animals*

254 Following immunization and challenge infection, “marker or DIVA serology” was
255 determined using selected CSFV-specific E^{RNS} and E2 antibody blocking ELISAs. Pigs
256 immunized with CP7_E1E2alf_TLA scored negative until day 28 p.v. by using the E^{RNS} -
257 antibody ELISA (data not shown), a result which is comparable to the data obtained with the
258 chimeric vaccine candidate CP7_E2alf (Reimann et al., 2004), and which allowed a
259 differentiation of vaccinated from infected animals. Furthermore, in the more sensitive CSFV
260 E2 antibody ELISA, CP7_E1E2alf_TLA-immunized animals showed distinct lower titers in

261 comparison to the C-strain-vaccinated animals before challenge infection, and animals of the
262 low dose group scored negative until 28 dpv. Nevertheless, 3 out of 5 animals of the high
263 dose group reached ELISA titers above the threshold, and scored positive before challenge
264 infection (Fig. 3). Following challenge infection with CSFV Koslov, all vaccinated animals
265 scored clearly positive in all CSFV-specific antibody ELISAs. Therefore, despite a marked
266 difference in the E2-specific antibody reactivity of the E2-manipulated recombinants, a clear
267 differentiation of vaccinated from infected pigs was not possible with the commercial E2-
268 antibody ELISAs in some cases, especially when a high titer preparation induced higher
269 immune responses. Obviously, the exchange of several amino acids within the TAVSPTTLR-
270 epitope could not completely block the induction of E2-antibodies with a certain blocking
271 efficacy against the A-epitope specific mabs of the commercial ELISAs. This is in contrast to
272 previously published data using a C-strain-based chimera with “BC” and “A” epitopes
273 exchanged with the corresponding sequences of a BVDV type 2 strain (van Gennip et al.,
274 2000). However, further studies are needed to evaluate the data of the C-strain chimera using
275 high titer preparations or multiple vaccinations. In addition, further studies have to test the
276 suitability of changed cut-off values of the E2-ELISAs allowing e.g. the differentiation with
277 an acceptable loss of sensitivity. Nevertheless, DIVA diagnostics was possible on the basis of
278 the tested E^{ms}-antibody ELISA, but the sensitivity of this assay for the detection of wild type
279 CSFV infections is lower than that of the widely used CSFV E2-antibody ELISAs.

280 We conclude from the data, that the TAVSPTTLR-mutant CP7_E1E2alf_TLA
281 induced an only slightly reduced protection compared to the C-strain vaccine, but did not
282 achieve the expected additional DIVA properties concerning E2-based serology.
283 Nevertheless, the new marker vaccine candidate allowed E^{ms}-based serological marker
284 diagnostics as well as genetic DIVA, and could be a good basis for further adaptations to
285 optimize the DIVA properties. However, “fine tuning” of BVDV backbone constructs for
286 improved DIVA properties includes the risk of reduced efficacy due to e.g. E2 manipulation.

287 Therefore, the ideal marker vaccine will be a balance between efficacy and safety on one site,
288 and DIVA properties on the other site. Further studies will focus on the definition of antigenic
289 regions/epitopes with low influence on the protective efficacy and a potential for DIVA
290 diagnostics.

291

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298

299 **Conflict of interest statement**

300 None

301

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388 **Tables:**389 **Table 1:**

390 Results of the immunization and challenge experiments

| | C-strain vaccine | CP7_E1E2alf_TLA Group “low dose” (10 ^{4.75} TCID ₅₀) | CP7_E1E2alf_TLA Group “high dose” (10 ^{6.5} TCID ₅₀) |
|--|-----------------------------|---|---|
| Clinical symptoms | No | fever (at days 4 to 5 p.c.) | fever (at days 3 to 5 p.c.) |
| Virus shedding | No | No | No |
| Mean VNT-Titer (CSFV Alfort 187, 28 d p.v.) | 1:33 | 1:15 | 1:74 |
| CSFV genome (real time RT-PCR) | No | Yes 5/5 animals at day 4 p.c. 1/5 animals at day 6 p.c. | Yes 4/5 animals at day 3 p.c. 2/5 animals at day 5 p.c. |
| CSFV E ^{ms} Ab blocking ELISA | 14 to 21 d p.v. positive | 14 to 21 d p.c. positive | 14 to 21 d p.c. positive |
| CSFV E2 Ab blocking ELISA | 21 d p.v. positive | 2 to 6 d p.c. Positive | 2 to 6 d p.c. positive |

391 d p.v., days post vaccination; d p.c., days post challenge

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397 **Figure legends:**

398 Fig 1: Schematic representation of the parental BVDV full-length cDNA pA/BVDV and the
 399 engineered constructs pA/CP7_E1E2alf and pA/CP7_E1E2alf_TLA. Filled boxes represent
 400 the BVDV structural protein region and filled boxes with horizontal lines demonstrate the
 401 inserted CSFV envelope proteins E1 and E2. The vertical dotted line indicate the modified
 402 CSFV TAVSPTTLR epitope, which had been substituted with the corresponding BVDV
 403 TLANKDTLA epitope. Lines at the left and right ends indicate untranslated regions. N^{pro},
 404 autoprotease; C, capsid protein; E^{ms}, E1, E2, envelope proteins; p7, NS2 to NS5, nonstructural
 405 proteins; 3'-NTR and 5'-NTR, non-coding regions. The scale is given in kb.

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407 Figure 2: (A) IF analysis of SK6 cells infected with CP7_E1E2alf and CP7_E1E2alf_TLA,
 408 respectively. The cells were stained using panpesti-specific mab (C16, NS3), BVDV E2-
 409 specific mab (CA34) and CSFV E2-specific mabs (HC34, TAV; HC/TC16, none TAV). The
 410 panpesti-specific mab C16 stained cells infected with both chimeras CP7_E1E2alf and
 411 CP7_E1E2alf_TLA, respectively. Both CSFV E2-specific mabs (HC34, A-TAV; HC/TC16,
 412 A-none TAV) detected cells infected with CP7_E1E2alf. E2 protein expressed by
 413 CP7_E1E2alf_TLA infected cells could only be stained by using the CSFV E2 none TAV-
 414 specific mab HC/TC16. Because of the substitution of the parental BVDV E1 and E2 infected
 415 cells remained IF-negative by using the BVDV E2-specific mab. (B) Multistep growth curves
 416 of chimeric viruses CP7_E1E2alf and CP7_E1E2alf_TLA in SK-6 cells. Confluent
 417 monolayers of SK6 cells were infected at a multiplicity of infection (m.o.i.) of 1 and clarified
 418 supernatants were titrated in 10-fold dilutions at 0, 8, 24, 48, and 72 h after inoculation. Virus
 419 titers are given as TCID₅₀/ml.

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421 Figure 3: CSFV E2-specific ELISA antibodies after immunization with the C-strain vaccine
 422 and CP7_E1E2alf_TLA with a low dose (ld) or a high dose (hd) detected by using the

423 IDEXX HerdCheck CSFV Ab ELISA. ELISA values are given in blocking percentages.
424 Samples with more than 40 % blocking are scored positive and samples with 30 % to 40 %
425 are doubtful. The mean values of the C-strain vaccine group (n=3) and the
426 CP7_E1E2alf_TLA groups (n=5) are given, and standard deviations are shown as error bars.
427 The mean value of the C-strain group scored positive with about 50 % inhibition at day 21
428 p.v. and more than 60 % at the day of the challenge infection. The mean values of the
429 CP7_E1E2alf_TLA low dose group (l.d.) were negative until challenge infection, by using a
430 higher dose of CP7_E1E2alf_TLA (h.d.) positive mean ELISA values were observed before
431 challenge infection.

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449 **Figures**

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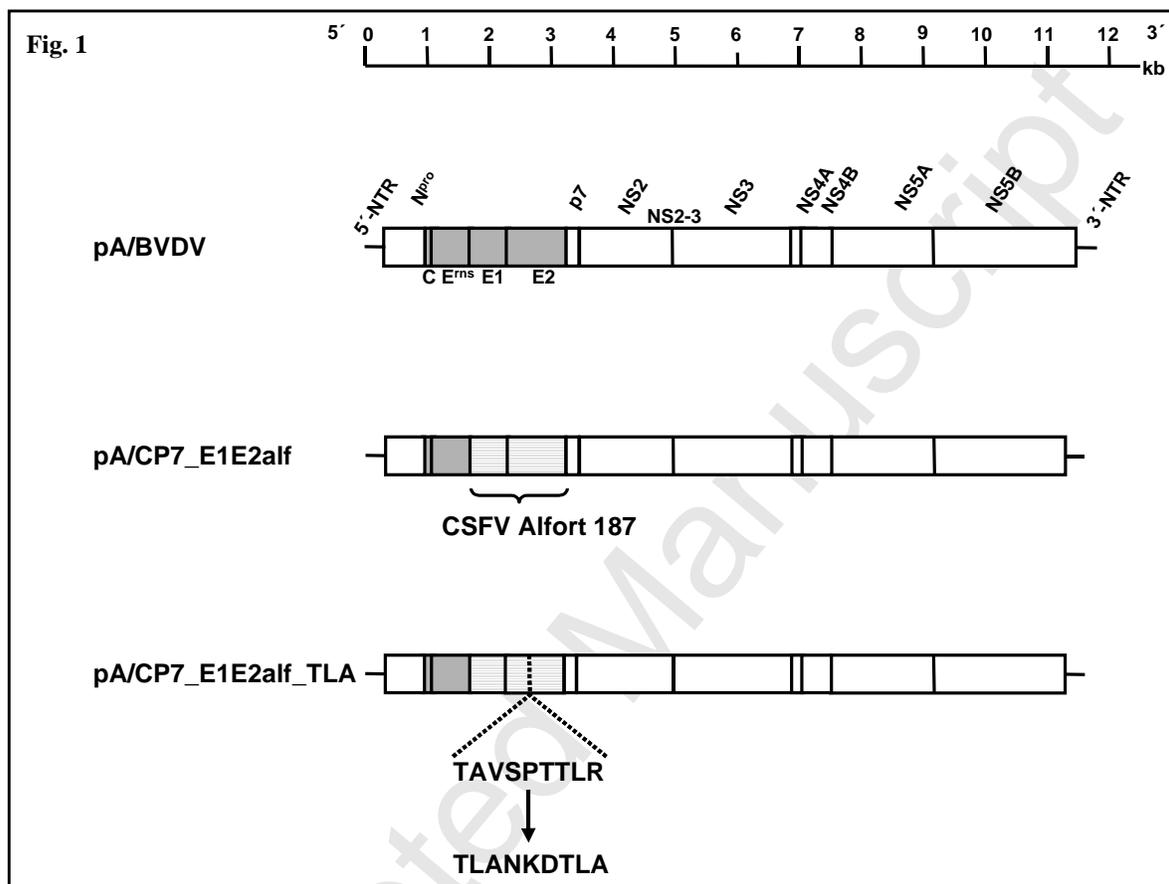
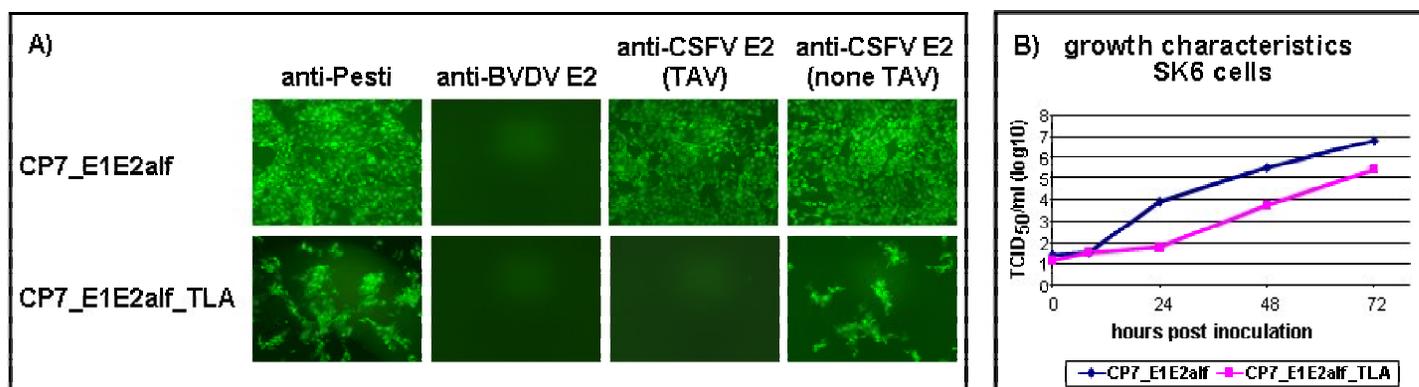


Fig. 2



475 **Fig. 3**

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