



HAL
open science

Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by vaccination and challenge in cattle

G. Savini, C. Hamers, A. Conte, P. Migliaccio, B. Bonfini, L. Teodori, M. Di Ventura, P. Hudelet, C. Schumacher, V. Caporale

► **To cite this version:**

G. Savini, C. Hamers, A. Conte, P. Migliaccio, B. Bonfini, et al.. Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by vaccination and challenge in cattle. *Veterinary Microbiology*, 2008, 133 (1-2), pp.1. 10.1016/j.vetmic.2008.05.032 . hal-00532439

HAL Id: hal-00532439

<https://hal.science/hal-00532439>

Submitted on 4 Nov 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Title: Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by vaccination and challenge in cattle

Authors: G. Savini, C. Hamers, A. Conte, P. Migliaccio, B. Bonfini, L. Teodori, M. Di Ventura, P. Hudelet, C. Schumacher, V. Caporale



PII: S0378-1135(08)00214-9
DOI: doi:10.1016/j.vetmic.2008.05.032
Reference: VETMIC 4062

To appear in: *VETMIC*

Received date: 30-1-2008
Revised date: 20-5-2008
Accepted date: 26-5-2008

Please cite this article as: Savini, G., Hamers, C., Conte, A., Migliaccio, P., Bonfini, B., Teodori, L., Di Ventura, M., Hudelet, P., Schumacher, C., Caporale, V., Assessment of efficacy of a bivalent BTV-2 and BTV-4 inactivated vaccine by vaccination and challenge in cattle, *Veterinary Microbiology* (2007), doi:10.1016/j.vetmic.2008.05.032

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1 **ASSESSMENT OF EFFICACY OF A BIVALENT BTV-2 AND BTV-4 INACTIVATED VACCINE**
2 **BY VACCINATION AND CHALLENGE IN CATTLE**

3

4 Savini G^{1*}, Hamers C², Conte A¹, Migliaccio P¹, Bonfini B¹, Teodori L¹, Di Ventura M¹.
5 Hudelet P², Schumacher C². Caporale V¹.

6 ¹ :Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale", Via Campo Boario,
7 64100, Teramo, Italy

8 ² : MERIAL S.A.S. 254 rue M.Mérieux 69007 Lyon France

9

10 **Corresponding author:** Giovanni Savini

11 Dept. of Virology

12 OIE Reference laboratory for Bluetongue

13 Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale"

14 Via Campo Boario, 64100 - Teramo - Italy

15 Telephone: +390861 332440

16 Fax No. +390861 332251

17 E-mail: g.savini@izs.it

18

19 **ABSTRACT**

20 The efficacy of a bivalent inactivated vaccine against bluetongue virus (BTV) serotypes 2 (BTV-
21 2) and 4 (BTV-4) was evaluated in cattle by general and local examination, serological follow-
22 up, and challenge.

23 Thirty-two 4 month-old calves were randomly allocated into 2 groups of 16 animals each. One
24 group was vaccinated subcutaneously (s/c) with two injections of bivalent inactivated vaccine
25 at a 28-day interval, and the second group was left unvaccinated and used as control. Sixty-
26 five days after first vaccination, 8 vaccinated and 8 unvaccinated calves were s/c challenged
27 with 1 mL of 6.2 Log₁₀ TCID₅₀/mL of an Italian field isolate of BTV serotype 2, while the
28 remaining 8 vaccinated and 8 unvaccinated animals were challenged by 1 mL of 6.2 Log₁₀

29 TCID₅₀/mL of an Italian field isolate of BTV serotype 4. Three additional calves were included in
30 the study and used as sentinels to confirm that no BTV was circulating locally.

31 At the time of the challenge, only one vaccinated animal did not have neutralizing antibodies
32 against BTV-4, while the remaining 15 showed titres of at least 1:10 for either BTV-2 or BTV-4.
33 However, the BTV-2 component of the inactivated vaccine elicited a stronger immune response
34 in terms of both the number of virus neutralization (VN) positive animals and antibody titres.
35 After challenge, no animal showed signs of disease. Similarly, none of the vaccinated animals
36 developed detectable viraemia while bluetongue virus serotype 2 and 4 titres were detected in
37 the circulating blood of all unvaccinated animals, commencing on day 3 post challenge and
38 lasting 16 days. It is concluded that administration of the bivalent BTV-2 and 4 inactivated
39 vaccine resulted in a complete prevention of detectable viraemia in all calves when challenged
40 with high doses of BTV-2 or BTV-4.

41

42 **KEY WORDS:** Bluetongue virus serotype 2, Bluetongue virus serotype 4, Cattle, Inactivated
43 vaccine

44

45 **INTRODUCTION**

46 Bluetongue is an infectious, non-contagious disease of wild and domestic ruminants caused by
47 an RNA virus belonging to the family *Reoviridae*, genus *Orbivirus*. Biting midges of the
48 *Culicoides* genus are the biological vector of the virus, and their distribution affects the
49 spreading of the infection in the temperate and tropical regions of the world (Gibbs et al.,
50 1994; Tabachnick, 2004). The disease is typically evident in sheep and only recently clinical
51 cases have unexpectedly been observed in cattle (Verwoerd and Erasmus, 2004; Toussaint et
52 al., 2006). Since 1998, BTV infection has spread progressively all over the Mediterranean
53 Basin, Balkan areas and more recently in Northern Europe. To date, 7 serotypes have been
54 detected in the Mediterranean Basin: BTV-1, BTV-2, BTV-4, BTV-8, BTV-9, BTV-15 and BTV-16
55 (Mellor and Wittmann, 2002; OIE, 2006a,b,c,d,e,f,g,h; Saegerman et al., 2008). In Italy, the
56 first evidence of BTV infection was recorded in Sardinia in August 2000, and since then,
57 numerous outbreaks of BTV serotypes 1, 2, 4, 9 and 16 have been reported impacting most of

58 the central and southern regions of Italy (Calistri et al., 2004, OIE, 2006f). The incursion of
59 BTV into Europe is having a considerable negative economic impact, partly due to direct losses
60 from death and reduced production in affected livestock but, more importantly, because of the
61 total ban of ruminant trade between BTV-infected and non-infected areas (Calistri et al.,
62 2004). Despite the low occurrence of clinical cases in bovines, BTV is one of the 16 diseases
63 formerly ranked as list 'A' by the Office International des Epizooties (OIE). As a consequence,
64 Bluetongue affected countries are banned from trading livestock and livestock products. To
65 reduce direct losses due to disease and indirect losses due to the trade embargo caused by
66 virus circulation, European authorities have been undertaking vaccination campaigns according
67 to their individual national policies, the geographic distribution of the incurring BTV
68 serotype(s), and the availability of appropriate vaccines. Prior to 2003, only live vaccines were
69 used. They were the only product available in the market. They are inexpensive and have
70 proven highly effective in preventing bluetongue disease in the areas where they have been
71 used (Savini et al. 2007). However, they could be inadequately attenuated, depress milk
72 production in lactating sheep; and be teratogenic if used in pregnant animals (Savini et al.
73 2004; MacLachlan et al. 1985). Because of their potential to replicate in the organism attaining
74 titres capable of infecting vectors, vaccine viruses have been demonstrated to spread into the
75 environment (Ferrari et al. 2005, Savini et al. 2007) with the potential for reversion to
76 virulence and re-assortment of their genes with those of wild type BTV (Murray et al 1996,
77 Venter et al. 2004, Ferrari et al. 2005, Monaco et al. 2006).

78 With this respect, whole inactivated virus vaccines would represent a safer alternative. Since
79 2005 BTV inactivated vaccines have been in the market and used in vaccination campaigns in
80 Italy, France, Spain and Portugal. Even though most of the animal movements are associated
81 to cattle, the inactivated vaccine launched in the market was for sheep use only.

82 This paper describes the clinical evaluation of a commercial inactivated vaccine containing
83 purified BTV serotypes 2 and 4 in cattle. An experiment was conducted to determine the level
84 of protection induced by the vaccine against a challenge with homologous serotypes.

85

86 **MATERIALS AND METHODS**

87 **1. Vaccine**

88 A bivalent BTV-2/BTV-4 inactivated vaccine produced at an industrial scale by Merial (France)
89 was used in this study. The vaccine is inactivated and adjuvanted with Saponin/Aluminium
90 hydroxide.

91 Both BTV-2 and BTV-4 vaccine strains originated from France (Corsica).

92 Sixteen cattle were vaccinated with 1 mL of the vaccine on day 0 and on day 28. The vaccine
93 was administered s/c on the lateral face of the neck, height centred on the left (day 0) or right
94 (day 28) side. Rectal temperatures of all animals (including controls) were recorded prior to
95 vaccinations.

96

97 **2. Challenge strain**

98 Field isolates recovered from Sardinian infected animals during the BTV outbreaks of 2000-
99 2003 were used as challenge strains: the 8341/00 strain for BTV-2 and 10353/03 for BTV-4.
100 Before challenging, each strain was amplified by one passage in VERO (African green monkey
101 kidney) cell cultures. The strains were titrated after cell passage and then used as challenge
102 strain. Viral suspensions were diluted in phosphate buffer saline (PBS, pH 7,2) to adjust the
103 viral titre to $10^{6.2}$ TCID₅₀/mL. Each animal was challenged s/c on the left face of the neck on
104 day 65 with 1 mL of its respective viral suspension.

105

106 **3. Animals**

107 This study was conducted between November 2005 and April 2006 in the province of Teramo
108 at an altitude of 1000 m in an insect-proof facility.

109 Thirty-five 4 month-old calves that were free of respiratory, digestive, umbilical and osteo-
110 articular disease were included in the study. All animals originated from BTV-free herd, located
111 in a BTV-free area (France) and tested negative for BTV-antibodies (c-ELISA) before entering
112 the study.

113 Thirty-two calves were randomly allocated into 2 groups of 16 animals each. One group was
114 vaccinated and the second group was left unvaccinated and used as control.

115 Sixty-five days after first vaccination, 8 vaccinated and 8 unvaccinated calves were challenged
116 by BTV serotype 2, while the remaining 8 vaccinated and 8 unvaccinated animals were
117 challenged by BTV serotype 4.

118 Thus, animals were allocated to four groups : (1) "Controls BTV-2", (2) "Controls BTV-4", (3)
119 "Vaccinates BTV-2", (4) "Vaccinates BTV-4".

120 The three remaining animals were kept as environmental controls (not vaccinated nor
121 challenged), and used as sentinels to confirm that no BTV was circulating locally.

122 On days 2, 14, 28 (before vaccination), 42, 65 (before challenge), and 86 all cattle were blood
123 sampled by jugular puncture with plain tubes; serum samples were tested by c-ELISA and
124 titrated for antibodies against BTV-2, BTV-4, BTV-9 and BTV-16 by virus neutralisation test
125 (VN).

126 Prior to challenge, all bovines were blood sampled for detection of viraemia by jugular
127 puncture with ethylene-diaminetetra-acetic acid (EDTA) tubes. After challenge, blood samples
128 were taken 3 times a week for 42 days (day 65 -> day 107).

129 To exclude viral circulation in the stable, the environmental control animals were bled once a
130 week for the entire trial length. Serum samples were tested by ELISA. In case of positive
131 reaction they were subsequently typed by VN.

132

133 **4. Virological and serological tests**

134 The presence and titre of the virus in the blood were assessed by titration on VERO cells,
135 according to the methods described by Savini et al., 2004 and OIE Manual of diagnostic tests
136 and vaccines for terrestrial animals (2004). The presence of BTV in EDTA-blood samples was
137 also assessed by using the RT-PCR which amplifies a portion of S5 as described by Katz et al.
138 (1993). Its sensitivity for BTV-2 and BTV-4 of either field or vaccine origin was evaluated by
139 testing viral suspensions whose titer was previously determined.

140 On day 7, in the absence of cytopathic effect (CPE), cultures were scraped, centrifuged, and
141 the supernatant was re-passaged into a 24 microplate flat bottomed wells containing a
142 confluent monolayer of VERO cells and the plates handled as described before. In the presence
143 of CPE as well as at the end of the second passage, cells were scraped and collected from each

144 well. Cells and medium were then centrifuged at 812 g for 10 min. and the pellet was re-
145 suspended in approximately 1 mL of PBS. Ten μL of the cell suspension were subsequently put
146 onto a well of a multiwell slide, fixed for 20 min. in acetone at -20°C and checked for the
147 presence of BTV by immunofluorescence (IF) using BTV monoclonal antibodies. Virus
148 characterised as BTV was consequently typed by virus microneutralisation assays using type-
149 specific antisera. The virus titer was determined using the Reed and Munch formula applied on
150 the four replicates after the first passage. Those samples which showed CPE after the second
151 passage or were positive to IF were considered positive for BT with a titer of less than $10^{2.3}$
152 $\text{TCID}_{50}/\text{mL}$. (Savini et al. 2007)

153 As for the virus neutralisation assays, the method described by Savini et al. 2007 was applied
154 to both challenge inocula to demonstrate their serotype. The positive and negative controls as
155 well as OIE standard reference BTV serotypes 2, 4, 9 and 16 were kindly provided by the OIE
156 reference laboratory, Onderstepoort Veterinary Institute (OVI) in South Africa.

157 The antibody response was monitored using both the c-ELISA (Lelli et al., 2003) and VN test
158 (Savini et al., 2004).

159

160 **5. Statistical analysis**

161 Differences between the neutralising titres against BTV-2 and 4 per sampling day in the
162 vaccinated group after immunisation, were analysed using the non-parametric Wilcoxon test
163 for paired groups (Siegel and Castellani, 1998), while differences between BTV-2 and 4
164 viraemic titres per sampling day in the control groups were analysed using the non-parametric
165 Mann-Whitney test for independent groups. The probability of the various possible sensitivity
166 values of the C-ELISA in the vaccinated animals were estimated through a Bayesian approach
167 using the Beta ($s+1$, $n-s+1$) distribution (Sivia, 1996) where s is the total number of positives
168 and n is the total number of tested animals. Different Beta distributions were calculated on the
169 basis of different periods from vaccination. The probability distribution of the percentage of
170 positive animals shows not only the most probable value of sensitivity, but also the level of
171 uncertainty due to sample size. From an epidemiological point of view, it is far more interesting
172 to know the percentage of vaccinated animals that are protected after being challenged with

173 homologous BTV serotypes. The probability that more than a certain percentage of animals
174 would be protected after challenge was calculated by using 1-Beta (s+1, n-s+1).

175

176 **RESULTS**

177 **1. Virological results**

178 After challenge, none of the vaccinated animals developed detectable viraemia by either cell
179 culture or RT-PCR. Conversely bluetongue virus nucleic acid and serotype 2 and 4 titres were
180 detected in the circulating blood of all unvaccinated animals. In these animals the RT-PCR
181 detected BTV starting on day 5 post infection (pi) and remained positive up to the end of the
182 experimental period (42 days pi). The cell culture virus isolation detected BTV-2 and BTV-4
183 titers commencing on day 3 pi and lasting 16 days. In both infections, maximum viral titre was
184 detected on day 10 pi with average titres of $10^{4.02}$ TCID₅₀/mL and $10^{3.54}$ TCID₅₀/mL for BTV-2
185 and BTV-4, respectively (Fig. 1). The difference between BTV-2 and BTV-4 viraemic titres was
186 statistically significant ($p < 0.05$), with BTV-2 titres much higher than those with BTV-4. Figure
187 2 shows the curve of the probability that vaccinated animals are protected, not showing any
188 detectable viraemia after challenge infection with homologous BTV.

189

190 **2. Serological results**

191 None of the vaccinated animals showed detectable c-ELISA antibodies after the first vaccine
192 injection. Conversely, commencing on day 42 (14 days after the second shot), the immuno-
193 enzymatic assay detected antibodies in all vaccinated calves. Figure 3 shows the probability
194 distributions of the percentage of calves showing c-ELISA antibodies after being vaccinated
195 with the inactivated bivalent BTV-2 and 4 vaccine. Apart from those used as environmental
196 controls, all animals were positive to c-ELISA on day 86.

197 For BTV-2, 28 days after first vaccination, 13 had sero-converted, but at low titres. By day 42,
198 all had BTV-2 neutralizing antibodies. For BTV-4, none of the calves had detectable BTV-4
199 neutralizing antibodies 28 days after the first vaccination. By day 42, fifteen out of sixteen had
200 BTV-4 neutralizing antibodies. On day 28, the number of BTV-2 positive calves was
201 significantly higher ($p < 0.05$) than that of BTV-4. In one animal BTV-4 antibodies were not

202 detected. The difference between BTV-2 and BTV-4 neutralising titres was statistically
203 significant ($p < 0.05$) at all time points between day 14 and day 65, with BTV-2 titres being
204 much higher than those of BTV-4. However, for both serotypes, the highest peak of antibody
205 response was observed on day 42 (Fig. 4). No animals developed BTV-9 and BTV-16 antibody
206 titres. On day 86, BTV-2 and BTV-4 challenged groups developed homologous neutralising
207 antibodies while no BTV antibody titres were observed in the animals used as environmental
208 controls.

209 Neither BTV nor antibodies were detected in the blood samples taken from the environmental
210 control group.

211

212 **DISCUSSION**

213 Vaccination against BTV is a very important tool, not only for the control of the disease but
214 more importantly, for 'safe' trade of live ruminants in accordance to OIE standards and EU
215 legislation. To prevent BTV infection of ruminants, different types of vaccines, including
216 inactivated and modified live virus (MLV) vaccines, Virus-like particles (VLP) produced from
217 recombinant baculoviruses, and recombinant vaccinia virus vectored vaccines have been
218 manufactured (Roy and Erasmus, 1992, Boone et al., 2007).

219 Of these, the inactivated and MLV vaccines only are now available in the market and used in
220 the official vaccination campaigns. Although VLPs are safe and neat, their inconsistent efficacy
221 when used in field trials (Roy et al., 1990, 1992, 1994; Roy, 2004) and difficulties with
222 commercial production, cost, and long-term stability make them ineligible for field use (Savini
223 et al., 2007). Similarly, recombinant vector vaccines expressing both VP2 and VP5, even
224 though revealing some potential in terms of safety and protection, still require further
225 development before being ready for field use (Lobato et al., 1997). Conversely, MLV are
226 cheap, easy to produce in large quantities, able to elicit protective immunity after a single
227 inoculation, and have been proven effective in preventing clinical bluetongue (BT) disease in
228 the areas where they have been used (Patta et al., 2004; Dungu et al., 2004). Nevertheless
229 BTV MLV vaccines suffer from a variety of documented potential drawbacks including side
230 effects due to under-attenuation of the modified strains and their capacity of passing the

231 placental barrier, and the spread of vaccine strain in the environment with the potential for
232 reversion to virulence and re-assortment with field isolates.

233 Inactivated vaccines have been recently developed and marketed. The efficacy of an
234 inactivated vaccine is fully dependent on the dose of virus, resulting in significantly higher
235 virus mass than that of MLV. Two doses, in the presence of adjuvant, may often be required
236 for inactivated vaccines considerably increasing the cost of vaccination. Inactivated vaccines
237 for BTV-2 and/or BTV-4 have been developed, commercialised and successfully employed in
238 the 2005-2006 BTV vaccination campaigns. Despite this success, the real costs of bluetongue
239 infection came from the ban on trading live animals in general and cattle in particular. The
240 inactivated vaccines available in the market are primarily registered for sheep and very few
241 information were available on the use of these products in cattle, at the time of this study. In
242 this study, the administration of the bivalent BTV-2 and BTV-4 inactivated vaccine was safe
243 and resulted in a complete prevention of detectable viraemia in all calves when infected with
244 high doses of virulent BTV-2 or BTV-4. It occurred when using either the cell culture method or
245 the RT-PCR assay. It has to be said however that, amongst the two assays, the cell culture
246 isolation is the method capable of giving information on the presence of infectious virus. Two
247 doses of the immunological product completely prevented vaccinated animals from developing
248 viraemia, which statistically signify a virological protection of at least 83.8% (95% confidence
249 interval) of vaccinated animals. It means that the product is not only safe but also effective in
250 protecting all vaccinated animals from viraemia, including the calf which did not develop
251 neutralising antibodies against BTV-4. At the time of the challenge, that calf was the only
252 vaccinated animal which did not develop neutralizing antibodies, while the remaining 15
253 showed titres of at least 1:10 for either BTV-2 or BTV-4. The study showed that the BTV-2
254 component of the inactivated vaccine elicited a stronger immune response in terms of both the
255 number of VN positive animals and antibody titres. However, the differences observed might
256 also relate to the VN technique and further studies are required to demonstrate higher
257 immunological stimulation of certain serotypes as compared to others. Concerning the
258 diagnostic tests, it was surprising to note that 13 animals, even if at low titres, had sero-
259 converted for BTV-2 28 days after first vaccination, whereas no c-ELISA antibodies were

260 detected in vaccinated animals following the first injection. These data were unexpected as c-
261 ELISA normally detects antibodies earlier than VN. It is known that neutralising antibodies are
262 stimulated by the structural proteins of the outer capsid, VP2 and partially VP5, while
263 antibodies to the inside capsid, VP7, are detected by the c-ELISA (Huismans and Erasmus, 1981,
264 Jeggo et al., 1991). An ineffective antigen stimulation by the VP7 following vaccination could be
265 an explanation of this discrepancy, or alternatively, it could be due to a poor performance of
266 the c-ELISA in detecting antibodies in vaccinated animals.

267 It is concluded that the BTV-2 & BTV-4 bivalent inactivated vaccine tested in the experiment
268 safely and effectively induces protective immunity in cattle, making it suitable for BTV
269 vaccination campaigns and particularly ideal for use in BTV-free countries where emergency
270 ring vaccination may be necessary. Moreover, because it is an inactivated vaccine, its use does
271 not prevent the possibility of using DIVA strategy based on the detection of antibodies versus
272 BTV non structural proteins to distinguish vaccinated from infected animals.

273

274 REFERENCES

- 275 Boone, J.D., Balasuriya, U.B., Karaca, K., Audonnet, J.C., Yao, J., He, L., Nordgren, R.,
276 Monaco, F., Savini, G., Gardner, I.A., MacLachlan, N.J., 2007. Recombinant canarypox virus
277 vaccine co-expressing genes encoding the VP2 and VP5 outer capsid proteins of bluetongue
278 virus induces high level protection in sheep. *Vaccine* 25, 672-678.
- 279 Calistri, P., Giovannini, A., Conte, A., Nannini, D., Santucci, U., Patta, C., Rolesu, S. and
280 Caporale, V., 2004. Bluetongue in Italy: Part I. *Veterinaria Italiana* 40, 243-251.
- 281 Dungu, B., Gerdes, T., Smit, T., 2004. The use of vaccination in the control of bluetongue in
282 southern Africa. *Veterinaria Italiana* 40, 616-622.
- 283 Ferrari, G., De Liberato, C., Scavia, G., Loenzetti, R., Zini, M., Farina, M., et al., 2005. Active
284 circulation of bluetongue vaccine virus serotype-2 among unvaccinated cattle in central Italy.
285 *Prev. Vet. Med.* 68,103-113.
- 286 Flanagan, M., Johnson, S.J., 1996. The effects of vaccination of Merino ewes with an
287 attenuated Australian bluetongue virus serotype 23 at different stages of gestation. *Aust. Vet.*
288 *J.* 72, 455-457.

- 289 Gibbs, E.P., Greiner, E.C., 1994. The epidemiology of bluetongue. *Comparative Immunology,*
290 *Microbiology and Infectious Diseases* 17, 197-206.
- 291 Huismans, H. and Erasmus, B.J., 1981. Identification of the serotype specific and group
292 specific antigens of bluetongue virus. *Onderstepoort J. Vet. Res.* 48, 51-58.
- 293 Jeggo, M., Wright, P., Anderson, J., Eaton, B., Afshar, A., Pearson, J., Kirkland, P. and Ozawa,
294 Y., 1992. Review of the IAEA meeting in Vienna on standardization of the competitive ELISA
295 test and reagents for the diagnosis of bluetongue. *In Bluetongue, African horse sickness and*
296 *related orbiviruses* (T.E. Walton & B.I. Osburn, eds). *Proc. Second International Symposium,*
297 *Paris, 17-21 June 1991.* CRC Press, Boca Raton, 547-569.
- 298 Katz, J.B., Gustafson, G.A., Alstad, A.D., Adler, K.A., Moser, K.M., 1993. Colorimetric diagnosis
299 of prolonged bluetongue viremia in sheep, using an enzyme-linked oligonucleotide sorbent
300 assay of amplified viral nucleic acids. *Am. J. Vet. Res.*, 54, 2021-2026.
- 301 Lelli, R., Portanti, O., Langella, V., Luciani, M., Di Emidio, B. and Conte, A., 2003. Produzione
302 di un kit ELISA competitiva per la diagnosi sierologica della Bluetongue. *Veterinaria Italiana,*
303 47, 5-13.
- 304 Lobato, Z.I., Coupar, B.E., Gray, C.P., Lunt, R., Andrew, M.E., 1997. Antibody responses and
305 protective immunity to recombinant vaccinia expressed-expressed bluetongue virus antigens.
306 *Veterinary Immunology and Immunopathology* 59, 293-309.
- 307 MacLachlan, N.J., Osburn, B.I., Stott, J.L., Ghalib, H.W., 1985. Orbivirus infection of the bovine
308 fetus. *Prog. Clin. Biol. Res.* 178, 79-84.
- 309 Mellor, P.S., Wittmann, E.J., 2002. Bluetongue virus in the Mediterranean Basin 1998-2001.
310 *The Veterinary Journal* 164, 20-37.
- 311 Monaco, F., Cammà, C., Serini, S., Savini, G., 2006. Differentiation between field and vaccine
312 strain of bluetongue virus serotype 16. *Vet. Microbiol.* 116, 45-52.
- 313 Murray, P.K., and Eaton, B.T., 1996. Vaccine for bluetongue. *Aust. Vet. J.* 73, 207-210.
- 314 Office International Des Epizooties (OIE), *Manual of Diagnostic Tests and Vaccines for*
315 *Terrestrial Animals, OIE Standards Commission (Eds), 5th Edition, 2006.*
- 316 OIE. World Organisation for Animal Health, 2006a. Bluetongue in The Netherlands. *Disease*
317 *Information,* 24 August, 19, 612-613.

318 OIE. World Organisation for Animal Health, 2006b. Bluetongue in Belgium. Disease
319 Information, 24 August, 19, 616-617.

320 OIE. World Organisation for Animal Health, 2006c. Bluetongue in Germany. Disease
321 Information, 24 August, 19, 618.

322 OIE. World Organisation for Animal Health, 2006d. Bluetongue in France. Disease Information,
323 31 August, 19, 636-637.

324 OIE. World Organisation for Animal Health, 2006e. Bluetongue in Bulgaria. Disease
325 Information, 19 October, 19, 736.

326 OIE. World Organisation for Animal Health, 2006f. Bluetongue in Italy. Disease Information, 9
327 November, 19, 775-776.

328 OIE. World Organisation for Animal Health, 2006g. Bluetongue in Israel. Disease Information,
329 30 November, 19, 842.

330 OIE. World Organisation for Animal Health, 2006h. Bluetongue in Luxembourg. Disease
331 Information, 7 December, 19, 852.

332 Patta, C., Giovannini, A., Rolesu, S., Nannini, D., Savini, G., Calistri, P., et al., 2004.
333 Bluetongue vaccination in Europe: the Italian experience. *Veterinaria Italiana* 40, 601-610.

334 Roy, P., Urakawa, T., Van Dijk, A.A., Erasmus, B.J., 1990. Recombinant virus vaccine for
335 bluetongue disease in sheep. *J. Virol.* 64, 1998-2003.

336 Roy, P., French, T., Erasmus, B.J., 1992. Protective efficacy of virus-like particles for
337 bluetongue disease. *Vaccine* 10, 28-32.

338 Roy, P., Bishop, D.H., LeBlois, H., Erasmus, B.J., 1994. Long-lasting protection of sheep
339 against bluetongue challenge after vaccination with virus-like particles: evidence for
340 homologous and partial heterologous protection. *Vaccine* 12, 805-811.

341 Roy, P., 2004. Genetically engineered structure-based vaccine for bluetongue disease.
342 *Veterinaria Italiana*, 40, 594-600.

343 Saegerman, C., Berkvens, D., Mellor, P.S., 2008. Bluetongue epidemiology in the European
344 Union. *Emerg. Infect. Dis.* [serial on the Internet]. 2008 Apr [date cited]. Available from
345 <http://www.cdc.gov/EID/content/14/4/539.htm>.

- 346 Savini, G., Monaco, F., Facchinei, A., Pinoni, C., Salucci, S., Cofini, F., et al., 2003. Field
347 vaccination of sheep with bivalent modified-live vaccine against bluetongue virus serotypes 2
348 and 9: effect on milk production. *Veterinaria Italiana* 40, 627-630.
- 349 Savini, G., Monaco, F., Citarella, R., Calzetta, G., Panichi, G., Ruiu, A. and Caporale, V., 2004.
350 Monovalent modified vaccine against bluetongue virus serotype 2: immunity studies in cows.
351 *In Bluetongue, Part II* (N.J. MacLachlan & J.E. Pearson, eds). Proc. Third International
352 Symposium, Taormina, 26-29 October. *Veterinaria Italiana* 40, 664-667.
- 353 Savini, G., Nicolussi, P., Pilo, G., Colorito, P., Fresi, S., Teodori, L., Leone, A., Bonfini, B. and
354 Patta, C., 2007. Study of the safety and efficacy of a recombinant vaccine for bluetongue virus
355 serotype 2. *Veterinaria Italiana* 43, 815-820.
- 356 Savini, G., MacLachlan, N.J., Calistri, P., Sanchez-Vizcaino, J.M., Zientara, S., 2007. Vaccines
357 against bluetongue in Europe. *Comparat. Immunol. Microbiol. Infect. Dis.*
358 doi:10.1016/j.cimid.2007.07.006
- 359 Siegel, S., Castellan Jr., N.J., 1988. *Nonparametric Statistic for Behavioral Sciences*. 2nd
360 edition. McGraw-Hill International Editions (Statistic series), New York, NY, USA.
- 361 Sivia, D.S., 1996. *Data analysis – a Bayesian tutorial*. Oxford University Press.
- 362 Tabachnick, W.J., 2004. *Culicoides* and the global epidemiology of bluetongue virus infection.
363 *Veterinaria Italiana* 40, 145-150.
- 364 Toussaint, J.F., Vandebussche, F., Mast, J., De Meester, L., Goris, N., Van Dessel, W.,
365 Vanopdenbosche, E., Kerkhofs, P., De Clercq, K., Zientara, S., Sailleau, C., Czaplicki, G.,
366 Depoorter, G., Dochy, J.M., 2006. Bluetongue in northern Europe. *The Veterinary Record* 159,
367 327.
- 368 Venter, G.J., Gerdes, G.H., Mellor, P.S., Paweska, J.T., 2004. Transmission potential of South
369 African *Culicoides* species for live-attenuated bluetongue virus. *Veterinaria Italiana* 40, 198-
370 202.
- 371 Veronesi, E., Hamblin, C., Mellor, P.S., 2005. Live attenuated bluetongue virus vaccine viruses
372 in Dorset poll sheep, before and after passage in vector midges (Diptera: Ceratopogonidae).
373 *Vaccine* 23, 5509-5516.

374 Verwoerd, D., Erasmus, B.J., 2004. Bluetongue. In: Coetzer J.A., Tustin R.C. (Eds). Infectious
375 Diseases of Livestock, 2nd ed. Oxford University Press, Cape Town, 1201-1220.

376

377

378

379

380

Accepted Manuscript

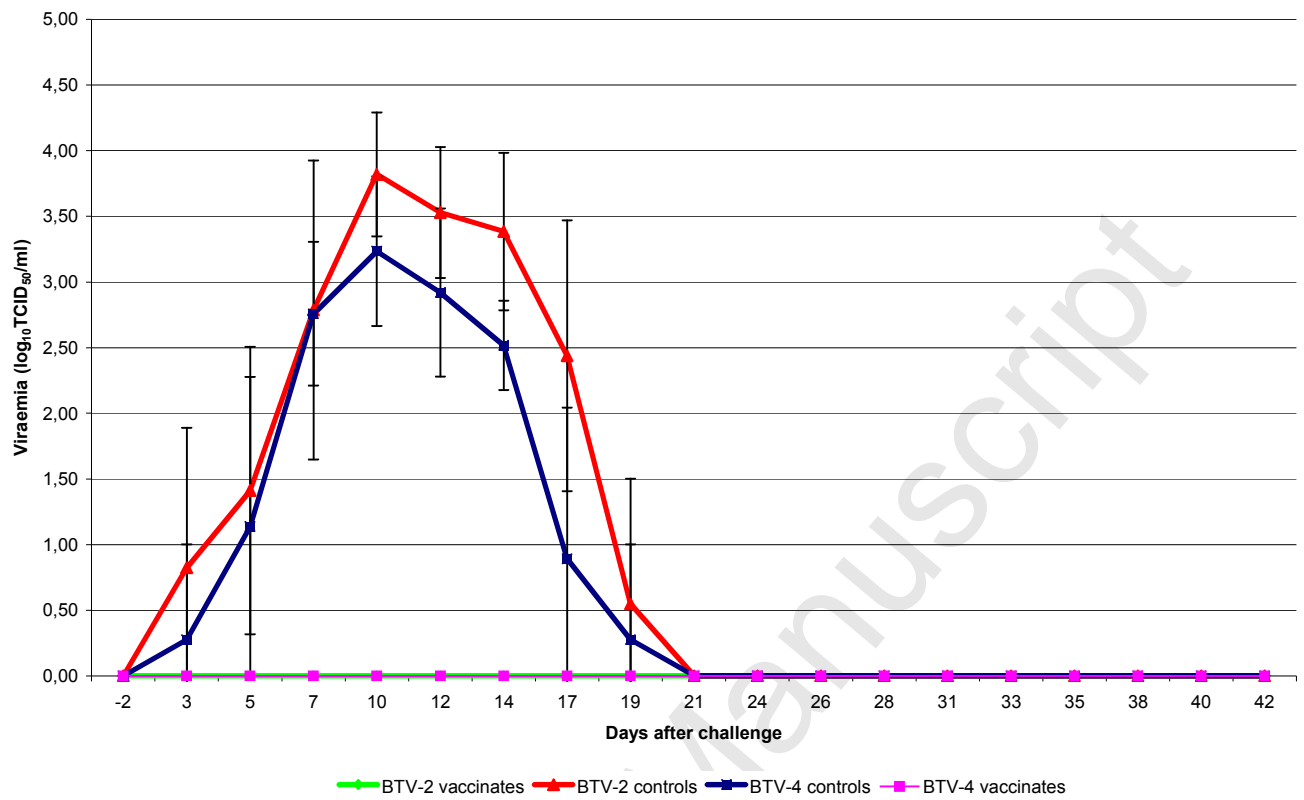


Figure 1: Titers of infectious BTV in blood samples of vaccinated and control groups following challenge infection with BTV-2 and BTV-4 field isolates

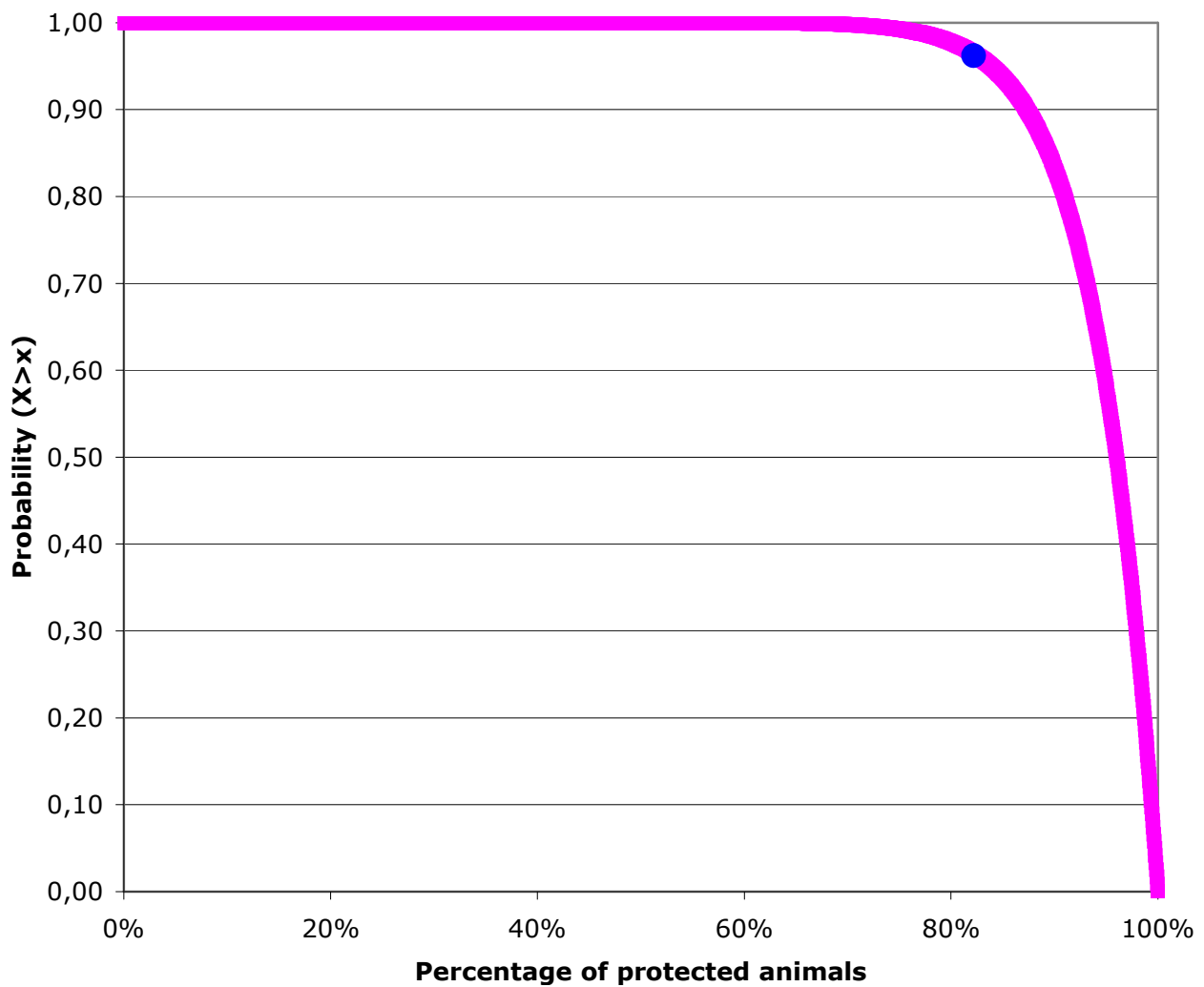


Figure 2: Probability that calves vaccinated with an inactivated bivalent BTV-2 and BTV-4 vaccine are protected against homologous challenge. The P value is equal or bigger to the x-axis percentages. According to the trial, at least 83.8% (blue circle) of vaccinated animals with 95% confidence level would be protected (no viraemia) when challenged with BTV-2 or BTV-4 field isolates.

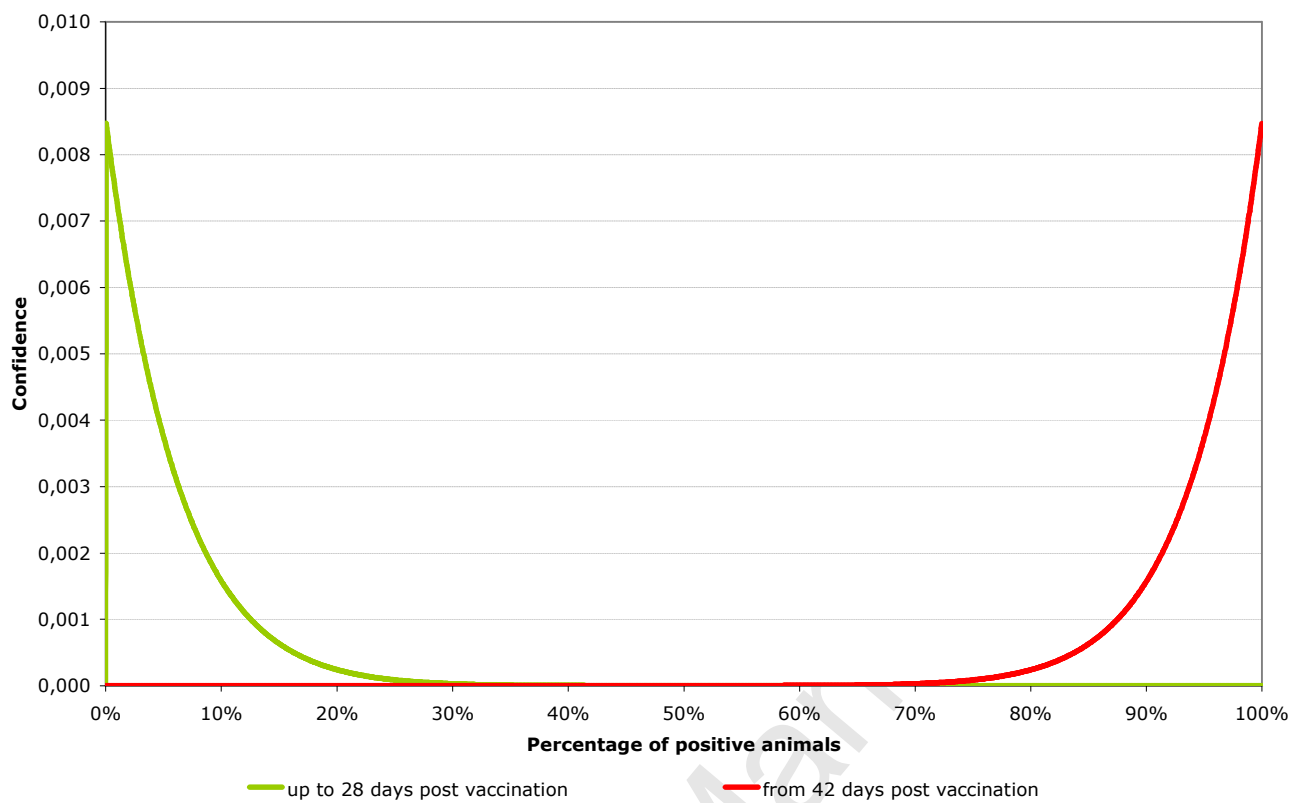


Figure 3: Probability distributions of the percentage of c-ELISA positive calves after vaccination with inactivated bivalent BTV-2 and BTV-4 vaccine

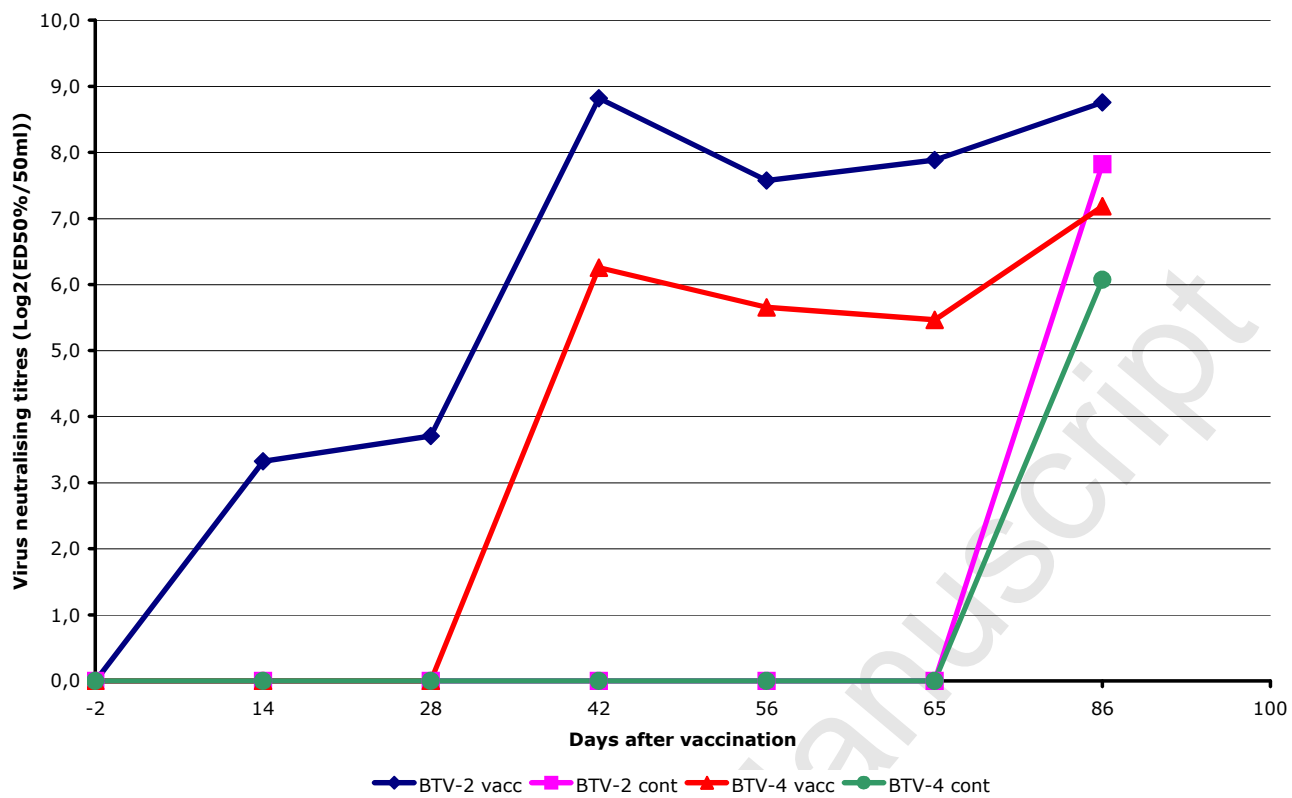


Figure 4: Average evolution of BTV-2 and BTV-4 serum neutralising titres in the vaccinated and unvaccinated groups