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1 Transplacental and oral transmission of wild-type bluetongue virus serotype 8 in  
2 cattle after experimental infection

3

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26

27 **Abstract**

28 Potential vertical transmission of wild-type bluetongue virus serotype 8 (BTV-8) in cattle was  
29 explored in this experiment. We demonstrated transplacental transmission of wild-type BTV-  
30 8 in one calf and oral infection with BTV-8 in another calf. Following the experimental BTV-  
31 8 infection of seven out of fifteen multi-parous cows eight months in gestation, each newborn  
32 calf was tested prior to colostrum intake for transplacental transmission of BTV by RRT-  
33 PCR. If transplacental transmission was not established the calves were fed colostrum from  
34 infected dams or colostrum from non-infected dams spiked with BTV-8 containing blood.  
35 One calf from an infected dam was born RRT-PCR positive and BTV-specific antibody (Abs)  
36 negative, BTV was isolated from its blood. It was born with clinical signs resembling  
37 bluetongue and lived for two days. Its post-mortem tissue suspensions were RRT-PCR  
38 positive. Of the seven calves fed colostrum from infected dams, none became infected. Of the  
39 six calves fed colostrum from non-infected dams spiked with infected blood, one calf became  
40 PCR-positive at day 8 post partum (dpp), seroconverted 27 days later, and remained RRT-  
41 PCR and Abs positive for the duration of the experiment (i.e. 70 dpp). This work  
42 demonstrates that transplacental transmission in late gestation and oral infection of the  
43 neonate with wild-type BTV-8 is possible in cattle under experimental conditions.

44

45 **bluetongue / transplacental and oral transmission / pregnant cattle / vertical**  
46 **transmission / late gestation**

47

## 48 1. Introduction

49       Bluetongue virus (BTV) (family *Reoviridae*, genus *Orbivirus*) is an arthropod-borne  
50 double-stranded RNA virus with 24 serotypes that can cause the non-contagious infectious  
51 disease bluetongue (BT) in ruminants. BT is a World Organization for Animal Health (OIE)  
52 reportable disease and is of considerable socioeconomic concern and of major importance in  
53 the international trade of animals and animal products. Since 1998 southern and central  
54 Europe have seen incursions of five different serotypes of BTV (1, 2, 4, 9 and 16) (Mellor and  
55 Wittmann, 2002; Saegerman et al., 2008; Schwartz-Cornil, 2008). A sixth serotype, BTV-8,  
56 was first identified in northern Europe in August 2006 (Elbers et al., 2008b; Saegerman et al.,  
57 2008). Its recrudescence in 2007 in the Netherlands, Germany, Belgium, Luxembourg and  
58 northern France, and its subsequent emergence in the United Kingdom, Denmark, Switzerland  
59 and the Czech Republic, has raised the question how BTV-8 survives between vector seasons.

60       Some of the hypotheses on this overwintering ability of BTV are prolonged or persistent  
61 infection in vertebrate hosts, the persistence of the virus within surviving adult vectors during  
62 the winter, and transovarial transmission through the vector (Purse et al., 2005; Sellers and  
63 Mellor, 1993; Takamatsu et al., 2003). The transmission of the virus between its ruminant  
64 hosts is almost exclusively by the bites of *Culicoides* midges. Of the indigenous European  
65 *Culicoides* species, principally *C. dewulfi*, the *Obsoletus* complex, and recently found *C.*  
66 *chiopterus*, can be infected with BTV-8 (Dijkstra et al., 2008; Mehlhorn et al., 2007;  
67 Meiswinkel et al., 2007) and might turn out to be competent vectors. The distinct feature that  
68 BTV-8 survives the winter by passing from pregnant infected ruminants to their offspring  
69 while *in utero*, is raised following observations in the Netherlands and Northern Ireland in  
70 March 2008 that PCR-positive ruminant offspring were born to dams that were infected with  
71 bluetongue virus the previous year (Menzies et al., 2008; van Wuijckhuise, 2008), and by a  
72 Belgian study of cattle and sheep in the field (De Clercq et al., 2008). Additionally the reports

73 of aborted and newborn calves from BTV-8 infected dams with severe developmental defects  
74 of the brain, i.e. hydranencephaly, proves natural vertical transmission of the European strain  
75 of BTV-8 (De Clercq et al., 2008; Vercauteren, 2008). Before this, it was generally believed  
76 that wild-type BTV lacks the capability for transplacental transmission, whereas cell adapted  
77 BTV can (Gibbs et al., 1979; Kirkland and Hawkes, 2004). Concerning other transmission  
78 routes of BTV, oral transmission, including oral transmission, was suggested recently for  
79 BTV-8 in ruminants (Menzies et al., 2008) and was shown only once by a repeated  
80 experiment for other serotypes in sheep (Jochim et al., 1965; Luedke and Jochim, 1968),  
81 while it was demonstrated in carnivores (Alexander et al., 1994; Jauniaux et al., 2008).

82 BTV-8 present in northern Europe is unusual in its transmission by novel vectors and the  
83 ability to cause disease and mortality in cattle (Elbers et al., 2008a; Schwartz-Cornil, 2008),  
84 also other transmission routes (De Clercq et al., 2008; Menzies et al., 2008; Vercauteren,  
85 2008; Wouda et al., 2008) seem to be part of the new characteristics, but experimental  
86 evidence applying serotype 8 in ruminants to support the field observations is missing.

87 The objective of this work was to explore the potential transplacental and oral  
88 transmission of wild-type BTV-8 after experimental infection in cattle. Therefore the calves  
89 born from infected multi-parous dams were tested immediately after birth for transplacental  
90 BTV-8 infection and subsequently allocated to a feed-group to test for possible oral BTV-8  
91 infection.

92

## 93 **2. Materials and Methods**

94

### 95 **2.1. Animals**

#### 96 ***2.1.1. Pregnant multi-parous cows***

97 This study was conducted by permission of the Animal Ethics Committee of the  
98 Central Veterinary Institute of Wageningen UR. Following pre-screening on clinical health,  
99 the absence of BTV by RRT-PCR and of BTV-specific antibodies (Abs), and on being late in  
100 gestation with the expected calving date around mid-February 2007, sixteen pregnant multi-  
101 parous Holstein-Friesian cows were commercially sourced from different Dutch holdings.  
102 They were housed together in a tie-stall a week before inoculation. During this week they  
103 were gradually adjusted from rough silage to grass pellets, observed for clinical signs, and  
104 screened for BTV Abs and virus in serum and blood and for Bovine Virus Diarrhoea Virus  
105 (BVDV) shedders by antigen ELISA. One cow calved before the onset of the experiment and  
106 was left out. Seven of the remaining fifteen cows were infected with BTV-8 as described  
107 below. The other eight cows served as non-infected dams. Nets, fully covering the udder,  
108 were attached to each cow to prevent newborn calves from drinking colostrum directly.

109

### 110 **2.1.2. Calves**

111 Newborn calves from the cows were housed in single calf boxes positioned separately  
112 in the housing of the dams. Blood and serum samples were taken from the calves straight after  
113 birth and before colostrum intake. The BTV status from these samples determined the  
114 allocation to a particular feed-group. After three days being fed with colostrum from the  
115 allocated feed-group the calves were gradually adjusted from colostrum to a commercial  
116 milk-replacer and milk pellets.

117

## 118 **2.2. Virus origin**

### 119 **2.2.1 BTV-8 inoculate to infect the cows**

120 The virus used to infect cows in this experiment was BTV-8 from field infected  
121 pregnant Dutch Holstein-Friesian heifers in 2006, estimated as at least day 40 to 50 post

122 infection (Thiry et al., 2006), passed once as full blood through BTV-8 negative Dutch  
123 Holstein-Friesian heifers. EDTA blood from the viremic heifers at days 7 and 21 post  
124 infection (dpi) was stored at -70°C, thawed before use, and pooled to obtain a sufficient  
125 quantity to inoculate the cows for this experiment.

126

### 127 **2.2.2. BTV-8 inoculate to spike colostrum from non-infected dams**

128 The virus used to spike colostrum from non-infected Dutch Holstein-Friesian cows  
129 was obtained by withdrawal of 500 ml EDTA blood from each of the seven BTV-8 infected  
130 multi-parous cows at 8 dpi, i.e. in the acute phase of infection before the detection of BTV-  
131 specific Abs. This blood was pooled, stored at -70°C and thawed before use. Per calf 100 ml  
132 was divided over the colostrum portions of the first three days, i.e. three liter colostrum per  
133 day with one to two volume percent of full blood.

134

## 135 **2.3. BTV Tests**

### 136 **2.3.1 BTV RRT-PCR**

137 EDTA blood was tested for the presence of BTV RNA by an in-house developed real-  
138 time reverse transcriptase PCR (RRT-PCR) detecting all 24 serotypes of BTV. Briefly, viral  
139 dsRNA from 200 µl diluted (1:1 with PBS) EDTA blood was isolated using the Total Nucleic  
140 Acid Isolation Kit (Roche Diagnostics Nederland b.v., Almere, Netherlands) in the isolation  
141 robot MagNa Pure (Roche Diagnostics Nederland b.v., Almere, Netherlands) according to  
142 manufacturer's instructions. Isolated dsRNA samples were tested using primers (Eurogentec  
143 Nederland b.v., Maastricht, Netherlands),  
144 the forward primer is 5'-AGTGTCGCTGCCATGCTATC-3'  
145 and the reverse primer is 5'-GCGTACGATGCGAATGCA-3',

146 and a Taqman probe 5'-6FAM-CGAACCTTTGGATCAGCCCGGA-XTMR-PH (Tib  
147 MolBiol, Berlin, Germany) targeting segment 10 of the BTV genome. RRT-PCR was  
148 performed by use of the LightCycler RNA Master Hybridization Probes kit (Kit, Roche  
149 Diagnostics Nederland b.v., Almere, Netherlands) with a LightCycler 2.0 (Roche Diagnostics  
150 Nederland b.v., Almere, Netherlands). Template RNA (5  $\mu$ l) was added to a reaction mixture  
151 containing 0.25  $\mu$ M of the forward and reverse primer, 0.25  $\mu$ M probe, 2.75 mM  $MnCl_2$ , 7.5  
152  $\mu$ l Kit and 0.2  $\mu$ l RNAsin (RNAsin, 40U/  $\mu$ l, Promega Benelux b.v., Leiden, Netherlands) in a  
153 final volume of 20  $\mu$ l. Thermocycling conditions of the RRT-PCR were: 20sec 98 °C, 20min  
154 61 °C, 30sec 95 °C, (1sec 95 °C, 10sec 61 °C, 15sec 72 °C)x45 followed by 30sec 40 °C and  
155 storage at 4 °C. Amplification was monitored real time by  $OD_{530}/OD_{640}$  using software  
156 version 4.05 (Roche Diagnostics Nederland b.v., Almere, Netherlands). Three positive  
157 controls were included containing different virus dilutions of BTV1 grown on BHK21 cells in  
158 DMEM with 5% fetal bovine serum and diluted in the same growth medium. The weak  
159 positive control, containing the highest virus dilution, must result in a visible curve therewith  
160 determining the cut-off. DNase free phosphate-buffered saline (PBS) solution was used as  
161 negative control. A run was successful when all negative controls were negative, and positive  
162 controls were positive. Results of test samples were considered positive if the software  
163 generated a crossing point (cp) value and a sigmoid curve with a signal ( $OD_{530}/OD_{640}$ ) at least  
164 partly above the cut-off value. Results were considered doubtful in case of a sigmoid-shaped  
165 curve completely below the cut-off value, or of all other not interpretable curves.

166

### 167 **2.3.2. BTV ELISA**

168 Sera were tested for BTV-specific Abs in a commercial ELISA (ID Screen®  
169 Bluetongue Competition ELISA kit, ID Vet, Montpellier, France) according to  
170 manufacturer's instructions.

171

172 **2.3.3. BTV Virus isolation**

173 Virus isolation (VI) was performed on embryonated chicken eggs (ECE) according to  
174 the OIE Manual (OIE, 2007). After one passage on ECE, homogenates were prepared and the  
175 supernatant was tested in the BTV RRT-PCR.

176

177 **2.4. BVDV Tests**178 **2.4.1. BVDV Antigen ELISA**

179 To detect possible BVDV shedders, EDTA blood was tested in a commercial BVDV  
180 antigen ELISA (IDEXX Europe b.v., Hoofddorp, Netherlands) according to manufacturer's  
181 instructions.

182

183 **2.4.2. BVDV Antibody ELISA**

184 Sera were tested to detect BVDV-specific Abs in a commercial ELISA (Prionics  
185 Lelystad, Lelystad, Netherlands) according to manufacturer's instructions.

186

187 **2.4.3. BVDV Virus isolation**

188 Virus isolation (VI) was performed, following the method described by Smith *et al.*  
189 (Smith *et al.*, 1988) modified by using an anti-BVDV hyper immune serum (HIS) of pigs  
190 instead of monoclonals and the use of DAKOPATTS conjugate labelled with anti-pig-HRPO  
191 (Wensvoort *et al.*, 1986). Serum samples were tested instead of the ideally used buffy coat of  
192 blood samples. Because BVDV VI was performed in retrospect haemolysis of the samples  
193 due to frozen storage did not allow for testing of the buffy coat.

194

195 **2.5. Study design**

196 **2.5.1. Dams: BTV infection**

197           Seven multi-parous Holstein-Friesian cows, eight months pregnant, were inoculated  
198 with BTV-8, 20 ml i.v. in the v.jugularis and 20 ml s.c. on the right side of the neck, i.e. the  
199 infected dams (I). The other eight pregnant cows were not inoculated, i.e. the non-infected  
200 dams (NI). EDTA blood and serum samples were collected daily in the week following the  
201 day of inoculation (d0), every other day in the second week, and once a week thereafter.  
202 These samples were tested using the RRT-PCR for the detection of BTV RNA and the ELISA  
203 for the detection of BTV-specific Abs. Body temperature was recorded daily, with fever  
204 defined above 39,5°C. The animals were observed and physically examined every day and  
205 any clinical signs were scored using a clinical reaction index (CRI) modified from that of  
206 Huismans and others (Huismans et al., 1987). The administration of analgesic and  
207 antimicrobial treatment was allowed. The cows were let to calve spontaneously. After calving  
208 the cows were milked at least two days to collect colostrum and than euthanized by  
209 intravenous injection of a combination of 200 mg/ml embutramide, 50 mg/ml  
210 mebezoniumjodide and 5 mg/ml tetracaine hydrochloride (T61; Intervet Nederland b.v.,  
211 Boxmeer, Netherlands).

212

213 **2.5.2. Calves: Transplacental BTV transmission**

214           Immediately after birth the calves were tested for possible transplacental transmission  
215 of BTV. The EDTA blood sample and serum sample drawn straight after birth and before the  
216 first ingestion of colostrum were tested for BTV by RRT-PCR and ELISA, respectively.  
217 According to the outcome of the tests, each calf was allocated to the appropriate feed-group in  
218 order to be able to study BTV infection by ingestion of colostrum. When the calves tested  
219 positive for BTV, they were allocated to a feed-group with colostrum from non-infected dams  
220 (col-NI).

221

222 **2.5.3. Calves: oral BTV infection**

223 Cows were let to calve naturally and spontaneously, randomizing the order of birth from  
224 infected or non-infected dams. Inducing the calving process in any way was not acceptable,  
225 also concerning the fact that the precise conception dates were unknown. The calves born  
226 uninfected were allocated to a feed-group, i.e. colostrum from infected dams (col-I) or  
227 colostrum from non-infected dams spiked with blood from infected dams (col-NI+bld-I).  
228 Each calf was fed 3 litre of the appointed colostrum per day for at least three days. The  
229 colostrum uptake was assured by visual inspection of the amount taken in by the calves  
230 together with the turbidimetric determination of the concentration of total Immunoglobulin G  
231 ([IgG]) in calf sera at 3 dpp by the Animal Health Services in The Netherlands.  
232 EDTA blood and serum samples were collected daily in the week of birth (d0), every other  
233 day in the second week, and once a week thereafter. These samples were tested using the  
234 RRT-PCR for the detection of BTV RNA and the ELISA for the detection of BTV-specific  
235 Abs. Body temperature was recorded daily, with fever defined above 39,5°C. The animals  
236 were observed and physically examined every day and any clinical signs were recorded. The  
237 administration of analgesic and antimicrobial treatment was allowed. If the clinical signs in  
238 the animals became so severe that their welfare was unacceptably compromised, they were  
239 euthanized. At the end of the experiment the calves were euthanized by intravenous injection  
240 of a combination of 200 mg/ml embutramide, 50 mg/ml mebezoniumjodide and 5 mg/ml  
241 tetracaine hydrochloride (T61; Intervet Nederland b.v., Boxmeer, Netherlands).

242

243 **3. Results**244 **3.1. Dams: BTV infection**

245 All of the seven BTV-8 infected dams were RRT-PCR positive at three dpi and BTV  
246 specific Abs positive at 12 dpi (Fig. 1). Five of the infected dams developed clinical signs.  
247 These signs started with the loss of appetite, anorexia, depression, salivation, nasal discharge  
248 and conjunctivitis. Three of these dams had very high CRI scores (Tab I). They developed  
249 severe lesions of face and feet, i.e. hyperaemic mucosa of nose and mouth, lesion of oral  
250 and/or nasal mucosa, painful feet, oedema of the legs, severe coronitis, recumbency due to  
251 laminitis or coronitis (Fig. 2). The clinical signs are conform the clinical signs caused by  
252 BTV-8 reported in cattle from the field in northern Europe (Elbers et al., 2008a; Elbers et al.,  
253 2008b).

254 The eight non-infected dams remained RRT-PCR and Abs negative, no clinical signs  
255 were observed.

256 All dams pre-tested negative for BVDV antigen in the antigen ELISA. Before  
257 inoculation with BTV-8, seven of the sixteen dams had BVDV-specific antibodies, i.e. 965,  
258 966, 969, 970, 973, 974 and 975. Of the BTV-8 inoculated dams that did not have BVDV  
259 antibodies before inoculation, 967, 977 and 978 sero-converted to BVDV-specific antibodies  
260 between 7 and 15 dpi (partus at 11 dpi), 7 and 22 dpi (partus 23 dpi), and 9 and 12 dpi (partus  
261 33 dpi), respectively. Non-cytopathogenic BVDV was isolated at 7 dpi for dam 967 and 977  
262 and at 12 dpi for dam 968 (partus at 22 dpi). The non-infected dams did not seroconvert to  
263 BVDV and no BVDV was isolated.

264 Six of the seven infected dams gave birth to six calves, the calf of the other infected  
265 dam, dam 977, died *in utero* and during the caesarean section the moment of death was  
266 antedated at least two days. Seven non-infected dams gave birth to eight calves. Non-infected  
267 dam 963 was diagnosed with a *torsio uteri*, and during the caesarean the calf was also found  
268 dead *in utero*.

269

### 270 **3.2. Calves: Transplacental BTV transmission**

271 Calf 987 from infected dam 978 was born with a hyperaemic nose and hyperaemic  
272 conjunctivae of the right eye (Fig. 3). The pre-colostrum tests resulted in a RRT-PCR Cp  
273 value of 21.6, in comparison, the average Cp value of the maximum positive controls in all  
274 the PCR runs was 27. The ELISA was negative for BTV-specific Abs. This calf died two days  
275 after it was born (see Table II). Post-mortem tissue suspensions of spleen, liver, kidney, lung,  
276 nose, mesenteric lymphnode, heart, brain were all RRT-PCR positive. Bluetongue virus was  
277 isolated from blood of the day of birth of this calf.

278 The calves from the other infected dams were RRT-PCR and Abs negative at birth.  
279 From the calf of dam 977 that died *in utero*, spleen and liver tissue was tested by RRT-PCR  
280 with a negative outcome. The calves from the non-infected dams were born with negative  
281 BTV pre-colostrum test results.

282 All calves, from infected and non-infected dams, were born without BVDV-specific  
283 antibodies. BVDV virus isolation was performed on the serum samples and on spleen and  
284 liver tissue suspensions from calf 987 and the calf from dam 977, which were all negative.

285

### 286 **3.3. Calves: oral BTV infection**

287 Allocation to a feed-group of each calf was according to its BTV status at birth, i.e.  
288 transplacental transmission, and by the BTV infection status of its respective dam (see Table  
289 II). Calf 987 was the only calf infected with BTV at birth and hence fed with col-NI. The  
290 other calves were given colostrum from infected dams (col-I) (N=7) or colostrum from non-  
291 infected dams spiked with BTV-8 (col-NI+bldI) (N=6). Maternal antibody intake per calf was  
292 detected by measuring the total [IgG] in serum at 3 dpp, excluding calf 986 (dam-I col-I) that  
293 died at 1 dpp. Eleven calves took in maternal antibodies by colostrum as indicated by [IgG] in  
294 serum at 3 dpp. Only marginal uptake of maternal antibodies was detected for calf 989,

295 although it did drink the total offered volume of colostrum. BTV-specific Abs were detected  
296 from day 1 in all calves fed col-I, except for earlier mentioned calf 989 (only at 12 dpp) and  
297 calf 983. The marginally low intake of maternal antibodies by colostrum intake of calf 989  
298 might explain the fact that BTV-specific antibodies could only be detected at one day. Calf  
299 983 remained negative for BTV Abs as it was fed colostrum from milkings of the day of birth  
300 from its own dam 967, who gave birth at 11 dpi, one day before she became Abs positive  
301 (from 12 dpi). All calves with BTV-specific Abs except for 988, dropped below detection  
302 level before the end of the experiment. All six calves who were fed with BTV-specific Abs  
303 free colostrum, i.e. col-NI+bld-I, remained negative for BTV-specific Abs, except for calf  
304 979. This calf 979 (dam-NI, col-NI+bld-I) was RRT-PCR positive from 8 dpp, with a Cp of  
305 24.6 (peak at 21 dpp of Cp 21.9), and BTV-specific Abs were detected from day 35 pp. This  
306 calf remained BTV and BTV-specific Abs positive until the end of the experiment, i.e. 70  
307 dpp.

308 All calves except for 989 had BVDV-specific antibodies at the day they left the  
309 experiment.

310

#### 311 4. Discussion

312 This work provides indications to support recent field observations that BTV-8 present in  
313 northern Europe can be transplacentally transmitted to offspring of infected dams. Moreover,  
314 this work provides evidence that oral transmission of wild-type BTV-8 might also occur. In  
315 short: Experimentally transplacental transmission with BTV-8 was detected in one calf (987)  
316 born from a dam infected late in gestation, and oral infection was detected in one calf (979)  
317 from a non-infected dam fed with colostrum from a non-infected dam spiked with blood from  
318 the infected dams.

319 Transplacental transmission of bluetongue virus has been described before for cattle (De  
320 la Concha-Bermejillo et al., 1993; Luedke et al., 1977a, b; MacLachlan et al., 2000;  
321 Waldvogel et al., 1992), sheep (Flanagan and Johnson, 1995; Richardson et al., 1985) and  
322 dogs (Brown et al., 1996; Osburn, 1994; Wilbur et al., 1994), but only with cell-attenuated  
323 virus strains. Experiments with wild-type bluetongue virus did not result in vertically  
324 transmitted bluetongue virus (Acree et al., 1991; Hubbert et al., 1972; Parsonson et al., 1994),  
325 except for a study with BTV11 in two elk cows in which a criticized method for virus  
326 detection has been used (Stott et al., 1982). Regarding the effect of the moment of infection of  
327 the dam in gestation, only infections early in gestation have been studied with cell attenuated  
328 virus. Late-term infection has only been studied by directly infecting bovine fetuses through  
329 the uterine wall (Jochim, 1974; Waldvogel et al., 1992).

330 The finding in this study that transplacental transmission of wild-type BTV-8 after  
331 experimental infection of a pregnant cow late in gestation is possible, provides support for the  
332 recent observations in the Netherlands and Northern Ireland (Menzies et al., 2008) and in  
333 Belgium (De Clercq et al., 2008) that PCR-positive ruminant offspring are being born to dams  
334 that were infected with BTV-8, and the increase in reports of aborted and newborn calves  
335 from BTV-8 infected dams with severe developmental defects of the brain, i.e.  
336 hydranencephaly (Vercauteren, 2008; Wouda et al., 2008). The importance of vertical and  
337 oral transmission in general, and in particular for the overwintering and spread of BTV  
338 remains unclear, but definitely depends on the significance of the natural BTV-spread in a  
339 respective season, area or country. At the same time it raises some immediate questions, as to  
340 whether BTV-8 present in northern Europe stems from an attenuated strain, and if there are  
341 conditions to facilitate the incidental BTV crossing over the placenta, like for instance  
342 interaction with co-infections, and whether the recent findings could explain a possible  
343 overwintering mechanism.

344           Regarding the potential role played by co-infections, interaction with BVDV as a  
345 known placenta crosser (Hewicker-Trautwein et al., 1994) could be a target for further  
346 discussion and research. After all, dam 978 of calf 987 did seroconvert to BVDV after  
347 inoculation with BTV-8, but so did other BTV infected dams without delivering a BTV  
348 positive calf. The only difference between these dams that seroconverted to BVDV after  
349 BTV-8 infection was that the dam 978 of calf 987 seroconverted to BVDV 11 days or more  
350 prior to delivery and the others just before or around the partus date. She also delivered her  
351 calf 10 days later than the others. Therefore she could have been infected 10 days earlier in  
352 her pregnancy than the other dams, whereby both BTV and BVDV could have had more time  
353 to cause vascular damage and hence interact to infect the calf *in utero*. Although no BVDV-  
354 specific antibodies could be detected in the calves at birth and no BVDV could be isolated  
355 from liver and spleen suspensions from calf 987, BVDV might have helped BTV to cross the  
356 barrier.

357           Oral transmission of BTV-8 was detected in one calf out of thirteen calves fed with  
358 potentially infecting colostrum. This calf, 979, born from a non-infected dam was fed with  
359 spiked colostrum. As oral transmission of BTV has not been described for ruminants before,  
360 except once for domestic sheep with BTV10 (Jochim et al., 1965; Luedke and Jochim, 1968)  
361 and for wild carnivores by eating infected prey (Alexander et al., 1994), this outcome might  
362 reveal a different incidental route of virus transmission for BTV-8.

363           The underlying hypothesis for oral BTV transmission was that virus could leak into  
364 the colostrum and that by drinking this infected colostrum calves could become infected. BTV  
365 is known to be associated with endothelial cells, to interact with the cell membrane of  
366 erythrocytes and therewith to be able to evade neutralizing antibodies, and to replicate in  
367 dividing lymphocytes (MacLachlan, 1994). Blood cells leaking into colostrum or milk in the  
368 mammary gland could therefore transport the virus. To study whether this happens under

369 natural conditions, seven calves born BTV negative were fed with colostrum from infected  
370 dams. Furthermore, to mimic virus-infected colostrum without BTV-specific antibodies as  
371 might be the case when a dam gets infected very late in gestation, the colostrum from non-  
372 infected dams was spiked with BTV-8 infected blood without BTV-specific antibodies. This  
373 was fed to six calves born BTV negative.

374       Regarding evidence of virus transmission by colostrum or milk, there are not many  
375 studies to be found. Maedi Visna virus (MVV) can be transmitted to lambs by feeding  
376 colostrum of infected ewes (Alvarez et al., 2005; Preziuso et al., 2004). Foot-and-Mouth  
377 disease virus (FMDV) is shed in milk, which can then be spread (Tomasula and Konstance,  
378 2004). For calf bovine leukosis virus (BLV), colostrum transmission of BLV to neonatal calves  
379 is possible if the dam is co-infected with bovine immunodeficiency virus (BIV) and BLV  
380 (Meas et al., 2002). However in the case of dams being infected with only BLV, colostrum  
381 antibodies are shown to be protective and thus to prevent colostrum BLV transmission (Nagy et  
382 al., 2007). In comparison, the colostrum from infected dams fed to seven calves, except 983,  
383 contained BTV-specific Abs. These antibodies might have neutralized the potential presence  
384 of BTV virus in the colostrum, thereby protecting the calves against BTV infection. Equally,  
385 the absence of BTV-specific Abs in the spiked colostrum fed to six calves might have  
386 provided an opportunity for the virus to infect a calf by ingestion. Still, to reach more  
387 conclusive results regarding the potential oral transmission of BTV in the presence of BTV-  
388 specific antibodies further study is needed. Ideally this is done by spiking colostrums that  
389 contain different titers of BTV-specific Abs with different amounts of BTV.

390

## 391 **5. Conclusion**

392       In conclusion, transplacental transmission in late gestation of wild-type BTV-8 was  
393 demonstrated by this study in one calf after experimental infection of the dam, as was oral

394 BTV-8 infection of the neonate by infected colostrum. Our findings support the possibility of  
395 alternative transmission routes for BTV-8 that may play a part in explaining its overwintering  
396 between vector seasons. To elucidate the unique characteristics of this BTV-8 strain further  
397 research is required, for instance to identify its ability to cross the placenta at a molecular  
398 level. Furthermore it may lead to clues about the origin of the BTV-8 strain present in  
399 northern Europe.

400

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405

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570 **Tables**

571

572 **Table I. Clinical scores\* of seven infected mothers: multi-parous Holstein-Friesian cows**573 **infected experimentally with wild-type BTV-8 approximately one month before calving**

Mother	Days scored DS	Fever		Anorexia		Face	Feet	Total score
		D	D/DS*4	D	D/DS*4	0-4	0-4	
1	22	1	<b>0.2</b>	8	<b>1.5</b>	<b>1</b>	<b>0</b>	<b>2.6</b>
2	22	2	<b>0.4</b>	8	<b>1.5</b>	<b>1</b>	<b>1</b>	<b>3.8</b>
3	33	3	<b>0.4</b>	5	<b>0.6</b>	<b>3</b>	<b>4</b>	<b>8.0</b>
4	29	1	<b>0.1</b>	0	<b>0.0</b>	<b>0</b>	<b>0</b>	<b>0.1</b>
5	42	3	<b>0.3</b>	0	<b>0.0</b>	<b>0</b>	<b>0</b>	<b>0.3</b>
6	23	11	<b>1.9</b>	17	<b>3.0</b>	<b>4</b>	<b>4</b>	<b>12.9</b>
7	33	3	<b>0.4</b>	12	<b>1.5</b>	<b>3</b>	<b>3</b>	<b>7.8</b>

574 \* Clinical scores were calculated for the clinical signs developed between day one after

575 inoculation and the moment of euthanasia as follows. Fever: the number of days (D) with a

576 temperature &gt; 39,5°C divided by the total number of days scored (DS) after inoculation times

577 4, rounded up to one decimal; Anorexia: calculated like Fever score for days with less or no

578 appetite; Lesion score: Face (rhinitis, conjunctivitis, salivation, hyperaemia, ulcers of mucosa,

579 facial oedema), Feet (lameness, coronitis, recumbency due to inflamed feet) each scored from

580 0-4 if present with 4 being the most severe. The range of the total score is 0 to 16.

581 **Table II. Wild-type BTV-8 (non)-infected dams and transplacental transmission and**  
 582 **oral infection with BTV-8 of their respective calves.**

Dams				Calves									
Group	ID <sup>a</sup>	BTV infection		† <sup>b</sup>	ID	Transplacental			Feedgroup	Colostrum			†
		BTV				Birth	BTV			[IgG] <sup>e</sup>	BTV		
		PCR	ELISA				PCR	ELISA			PCR	ELISA	
		(+ in dpi <sup>c</sup> )	(dpi)		(dpi dams)	(at 0 dpp <sup>d</sup> )		(g/L)	(+ in dpp)	(dpp)			
Infected	967 (1) <sup>k</sup>	3-†	12-†	22	<b>983</b>	11	-	-	col-I <sup>f</sup>	8	-	-	67
	968 (2)	3-†	12-†	22	<b>984</b>	13	-	-		7	-	1-16	65
	970 (4)	3-12	12-†	29	<b>986</b>	17	-	-		ND <sup>g</sup>	-	-	1
	969 (3)	3-†	12-†	33	<b>988</b>	25	-	-	col-NI+bld-I	4	-	1-53	53
	975 (5)	3-†	12-†	42	<b>991</b>	40	-	-		4	-	-	38
	978 (7)	3-†	9-†	33	<b>987</b>	23	+	-	col-NI	1	0-2	-	2
	977 (6)	2-†	12-†	23	X <sup>h</sup>								
Non-Infected	972	-	-	22	<b>985</b>	16	-	-	col-I	10	-	1-20	62
	974	-	-	36	<b>989</b>	30	-	-		1	-	12	48
	973	-	-	36	<b>990</b>	33	-	-		9	-	1-32	46
	971 <sup>l</sup>	-	-	9	<b>979</b>	8	-	-	col-NI+bld-I	5	8-70	35-70	70
	966	-	-	15	<b>980</b>	8	-	-		3	-	-	70
	964	-	-	22	<b>981</b>	11	-	-		3	-	-	67
	965	-	-	71	<b>982</b>	11	-	-		13	-	-	67
	963	-	-	15	<b>992</b>	58	-	-		12	-	-	10
X <sup>i</sup>													
Excluded	976	-	-	-3	X <sup>j</sup>	-3							

583

584 <sup>a</sup> ID = identification number585 <sup>b</sup> † = day of euthanization/death586 <sup>c</sup> dpi = days post infection587 <sup>d</sup> dpp = days post partum

588 <sup>e</sup> The concentration (in gram per liter) of total Immunoglobulin G was tested in serum from  
 589 the calves three days post partum, as an indicator for the intake of antibodies from colostrum.

590 <sup>f</sup> feedgroups: col-I: colostrum from infected dam; col-NI+bld-I: colostrum from non-infected  
 591 dam spiked with blood from infected dam; col-NI: colostrum from non-infected dam

592 <sup>g</sup> ND = not done, this calf lived for only 1 day.593 <sup>h</sup> caesarian section, calf found dead antedated at least 2 days594 <sup>i</sup> torsio uteri, calf found dead with caesarian595 <sup>j</sup> This calf was born before the onset of the experiment and therefore excluded596 <sup>k</sup> the numbers between brackets refer to clinical signs of the infected dams in table I and Fig.1597 <sup>l</sup> Dam 971 gave birth to a twin, calves 979 and 980.

598 **Figure captions**

599

600 **Figure 1. Onset and duration of positive test results for bluetongue virus (BTV) PRC,**  
601 **ELISA, fever ( $T > 39,5^{\circ}\text{C}$ ), and recorded clinical signs of bluetongue in days post**  
602 **inoculation (dpi) with wild-type bluetongue serotype 8 for seven multi-parous Dutch**  
603 **Holstein-Friesian cows infected at eight months gestation.**

604 Note that the scale is discontinuous after 7 dpi. The numbers above the bars mark the duration  
605 for the individual infected mothers. † indicates the death of the numbered animal.

606

607 **Figure 2. Severe clinical signs of bluetongue in wild-type BTV-8 infected Dutch Holstein-**  
608 **Friesian dams.**

609 **a)** Mucous nasal discharge at 19 dpi

610 **b)** Severe general illness, apathy, anorexia, at 20 dpi

611 **c)** Severe coronitis at 21 dpi (blue discoloration of coronary band)

612

613 **Figure 3. Hyperaemic nose of transplacentally BTV-8 infected calf.**

614

## Figures:

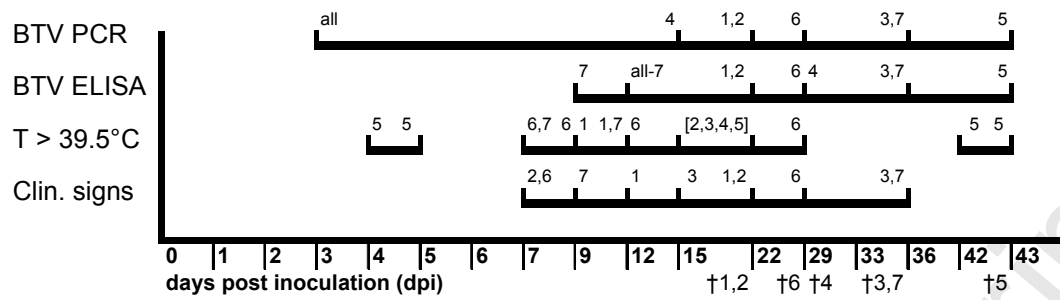


Figure 1.



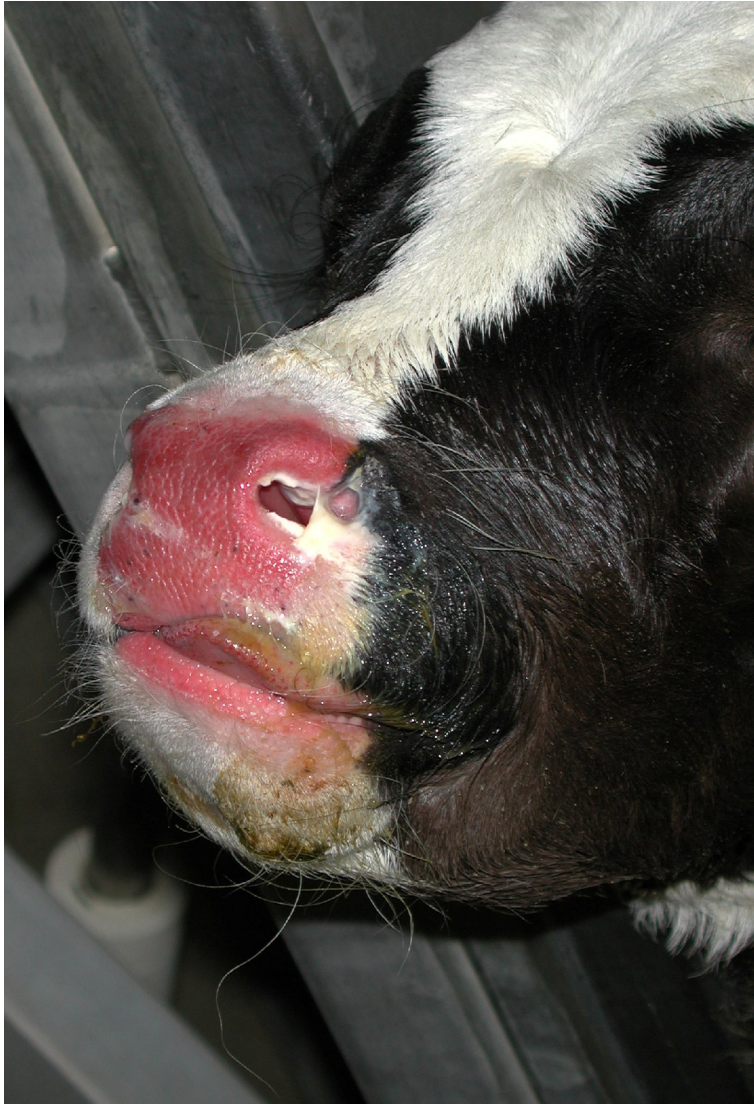
Figure 2a.



Figure 2b



Figure 2c



**Figure 3**

## Tables

**Table I. Clinical scores\* of seven infected mothers: multi-parous Holstein-Friesian cows infected experimentally with wild-type BTV-8 approximately one month before calving**

Mother	Days scored DS	Fever		Anorexia		Face	Feet	Total score
		D	D/DS*4	D	D/DS*4	0-4	0-4	
1	22	1	<b>0.2</b>	8	<b>1.5</b>	<b>1</b>	<b>0</b>	<b>2.6</b>
2	22	2	<b>0.4</b>	8	<b>1.5</b>	<b>1</b>	<b>1</b>	<b>3.8</b>
3	33	3	<b>0.4</b>	5	<b>0.6</b>	<b>3</b>	<b>4</b>	<b>8.0</b>
4	29	1	<b>0.1</b>	0	<b>0.0</b>	<b>0</b>	<b>0</b>	<b>0.1</b>
5	42	3	<b>0.3</b>	0	<b>0.0</b>	<b>0</b>	<b>0</b>	<b>0.3</b>
6	23	11	<b>1.9</b>	17	<b>3.0</b>	<b>4</b>	<b>4</b>	<b>12.9</b>
7	33	3	<b>0.4</b>	12	<b>1.5</b>	<b>3</b>	<b>3</b>	<b>7.8</b>

\* Clinical scores were calculated for the clinical signs developed between day one after

inoculation and the moment of euthanasia as follows. Fever: the number of days (D) with a temperature >39,5°C divided by the total number of days scored (DS) after inoculation times 4, rounded up to one decimal; Anorexia: calculated like Fever score for days with less or no appetite; Lesion score: Face (rhinitis, conjunctivitis, salivation, hyperaemia, ulcers of mucosa, facial oedema), Feet (lameness, coronitis, recumbency due to inflamed feet) each scored from 0-4 if present with 4 being the most severe. The range of the total score is 0 to 16.

**Table II. Wild-type BTV-8 (non)-infected dams and transplacental transmission and oral infection with BTV-8 of their respective calves.**

Dams				Calves									
Group	ID <sup>a</sup>	BTV infection		† <sup>b</sup>	ID	Transplacental			Colostrum			†	
		BTV				Birth	BTV		Feedgroup	[IgG] <sup>e</sup>	BTV		
		PCR	ELISA				PCR	ELISA			PCR		ELISA
		(+ in dpi <sup>c</sup> )	(dpi)		(dpi dams)	(at 0 dpp <sup>d</sup> )		(g/L)	(+ in dpp)	(dpp)			
Infected	967 (1) <sup>k</sup>	3-†	12-†	22	<b>983</b>	11	-	-	col-I <sup>f</sup>	8	-	-	67
	968 (2)	3-†	12-†	22	<b>984</b>	13	-	-		7	-	1-16	65
	970 (4)	3-12	12-†	29	<b>986</b>	17	-	-		ND <sup>g</sup>	-	-	1
	969 (3)	3-†	12-†	33	<b>988</b>	25	-	-	col-NI+bld-I	4	-	1-53	53
	975 (5)	3-†	12-†	42	<b>991</b>	40	-	-		4	-	-	38
	978 (7)	3-†	9-†	33	<b>987</b>	23	+	-	col-NI	1	0-2	-	2
	977 (6)	2-†	12-†	23	<b>X<sup>h</sup></b>								
Non-Infected	972	-	-	22	<b>985</b>	16	-	-	col-I	10	-	1-20	62
	974	-	-	36	<b>989</b>	30	-	-		1	-	12	48
	973	-	-	36	<b>990</b>	33	-	-		9	-	1-32	46
	971 <sup>l</sup>	-	-	9	<b>979</b>	8	-	-	col-NI+bld-I	5	8-70	35-70	70
					<b>980</b>	8	-	-		3	-	-	70
	966	-	-	15	<b>981</b>	11	-	-		3	-	-	67
	964	-	-	22	<b>982</b>	11	-	-		13	-	-	67
	965	-	-	71	<b>992</b>	58	-	-	12	-	-	10	
963	-	-	15	<b>X<sup>i</sup></b>									
Excluded	976	-	-	-3	<b>X<sup>j</sup></b>	-3							

<sup>a</sup> ID = identification number

<sup>b</sup> † = day of euthanization/death

<sup>c</sup> dpi = days post infection

<sup>d</sup> dpp = days post partum

<sup>e</sup> The concentration (in gram per liter) of total Immunoglobulin G was tested in serum from the calves three days post partum, as an indicator for the intake of antibodies from colostrum.

<sup>f</sup> feedgroups: col-I: colostrum from infected dam; col-NI+bld-I: colostrum from non-infected dam spiked with blood from infected dam; col-NI: colostrum from non-infected dam

<sup>g</sup> ND = not done, this calf lived for only 1 day.

<sup>h</sup> caesarian section, calf found dead antedated at least 2 days

<sup>i</sup> torsio uteri, calf found dead with caesarian

<sup>j</sup> This calf was born before the onset of the experiment and therefore excluded

<sup>k</sup> the numbers between brackets refer to clinical signs of the infected dams in table I and Fig. 1

<sup>l</sup> Dam 971 gave birth to a twin, calves 979 and 980.