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1 **Emergence of bluetongue virus serotype 6 in Europe – German field data and**
 2 **experimental infection of cattle**

3

4 Michael Eschbaumer¹, Bernd Hoffmann¹, Andreas Moss², Giovanni Savini³,
 5 Alessandra Leone³, Patricia König¹, Johanna Zemke¹, Franz Conraths⁴, and Martin
 6 Beer^{1,*}

7

8 ¹Institute of Diagnostic Virology, Friedrich-Loeffler-Institut, Südufer 10, 17493

9

Greifswald-Insel Riems, Germany

10

²LAVES - Veterinary Institute Oldenburg, Philosophenweg 38,

11

26121 Oldenburg, Germany

12

³Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise “G. Caporale”, Via

13

Campo Boario, 64100 Teramo, Italy

14

⁴Institute of Epidemiology, Friedrich-Loeffler-Institut, Seestraße 55,

15

16868 Wusterhausen, Germany

16

17

18

*Corresponding author:

19

Telephone: +49 38351 7200, Fax: +49 38351 7226, E-mail: martin.beer@fli.bund.de

20

21 **Abstract**

22

23 In late 2008, bluetongue virus (BTV) serotype 6 (BTV-6), which had never occurred in
24 Europe before, was first detected in the Netherlands and Germany. While the origin of
25 the virus remains unknown, the prevalence of infections in cattle was investigated in a
26 virological (N= 28,658) and serological (N= 2,075) field survey in Lower Saxony,
27 where 45 cases confined to the district Grafschaft Bentheim were found. Blood from
28 affected animals was used for the experimental infection of three cattle with different
29 BTV antibody status, leading to sustained viraemia in one animal naïve for BTV. Of
30 two animals that had detectable antibodies against BTV serotype 8, one became
31 transiently infected and seroconverted for BTV-6 while the other did not react. In
32 conclusion, while only a very limited spread of BTV-6 could be observed in the field,
33 experimental infection of cattle did not show substantial differences of the course of
34 infection in comparison to other BTV serotypes.

35

36

37 **Keywords:** bluetongue virus / btv-6 / germany / bovine / field data

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39

40

41 1. Introduction

42

43 1.1. Bluetongue in Europe

44

45 Bluetongue (BT) is an arthropod-borne disease of ruminants and camelids caused by
46 any of 24 established serotypes of *Bluetongue virus* (BTV), an *Orbivirus* of the family
47 *Reoviridae*. It is principally transmitted by haematophagous *Culicoides* midges
48 (Schwartz-Cornil *et al.*, 2008). Bluetongue virus did not regularly occur in mainland
49 Europe before 1998. Since then, serotypes 1, 2, 4, 9 and 16 have been circulating in
50 Southern Europe (Saegerman *et al.*, 2008). In recent years, a BTV-1 strain of Algerian
51 origin has expanded northwards across the Iberian Peninsula and France and its
52 intrusion into the Benelux and Germany appears imminent (Hateley, 2009). After its
53 first detection in the Netherlands in 2006, BTV-8 has spread across Central Europe and
54 beyond in three seasons, eventually reaching as far as Norway and Israel. Vaccination
55 campaigns against BTV-1 and -8 are ongoing. In the beginning of 2008, a putative new
56 BTV serotype, “Toggenburg orbivirus”, has been detected in goats in Switzerland
57 (Hofmann *et al.*, 2008) and later that year, BTV serotypes 6 and 11 (ISID ProMED-
58 mail, 2008b; ISID ProMED-mail, 2009) appeared in Europe for the first time.

59

60 1.2. BTV-6 in the Netherlands and Germany

61

62 In October 2008, BTV was detected in the Netherlands in animals previously vaccinated
63 against BTV-8. Routine sequencing of real-time RT-PCR amplicons yielded only 95%
64 nucleotide identity of genome segment 10 to the European strain of BTV-8, and the
65 introduction of a new strain or serotype was suspected by the Dutch national reference
66 laboratory for bluetongue (NRL BT) at the Central Veterinary Institute of Wageningen
67 UR (CVI), Lelystad (ISID ProMED-mail, 2008a).

68 The European Community reference laboratory (CRL) at the Institute for Animal
69 Health, Pirbright, UK, was able to isolate the virus from a Dutch sample and identified
70 it as BTV-6, with over 99.9% segment 2 (VP2) sequence identity to the South African
71 modified-live virus (MLV) vaccine strain (ISID ProMED-mail, 2008b-c). Vector-borne
72 spread of vaccine viruses has been reported before (Savini *et al.*, 2008; Listeš *et al.*,
73 2009). A BTV-6 restriction zone was created, entailing a mandatory screening of all
74 animals to be moved outside the zone.

75 The eastern part of the restriction zone covered German territory. Here, the first case of
76 BTV-6 was confirmed by the NRL BT at the Friedrich-Loeffler-Institut (FLI), Insel
77 Riems, on November 5, 2008.

78 Subsequently, a field survey was undertaken to determine the virological and
79 serological prevalence of BTV-6 in the affected area. At the NRL, initial attempts to
80 isolate BTV-6 from submitted samples in cell culture and embryonated chicken eggs
81 did not succeed. Since inoculation of susceptible animals is considered the most
82 sensitive method (Clavijo *et al.*, 1999), an animal experiment was conducted to obtain a
83 virus isolate and investigate the virulence of BTV-6 in cattle.

84

85 **2. Materials and Methods**

86

87 **2.1. Field survey**

88

89 Initially, infection with BTV-6 was detected in four cattle in three holdings in the
90 district Graftschaft Bentheim, Lower Saxony, Germany. Further samples were taken
91 from these animals in December 2008 and January 2009 and epidemiological inquiries
92 were performed in the holdings by epidemiologists of the FLI.

93 Regional laboratories in Lower Saxony conducted a survey to study the spatial
94 distribution of the infection. The majority of samples were taken along the Dutch border
95 and around the initially affected holdings, where the risk of exposure to BTV-6 infected
96 midges was highest. In total, 28,658 animals were investigated using the “pan-BTV”
97 group-specific real-time RT-PCR assay by Toussaint *et al.* (2007). Initially, samples
98 from BTV-positive animals were then evaluated at the NRL with real-time RT-PCR
99 assays specific for BTV-6 and -8 (Hoffmann *et al.*, 2009b). For ongoing surveillance,
100 these assays were outsourced to the regional laboratories in late November.

101 Serum samples (N= 2,075) from all susceptible animals in the first three affected
102 holdings and all holdings within a radius of 1 kilometre were sent to the bluetongue
103 reference laboratory of the World Organisation for Animal Health (OIE) at the Istituto
104 Zooprofilattico Sperimentale dell'Abruzzo e del Molise “G. Caporale” (IZSAM),
105 Teramo, Italy, for BTV neutralisation assays (Gard and Kirkland, 1999).

106

107 **2.2. Animal experiment**

108

109 **2.2.1. Animals and experimental design**

110

111 Three male Holstein Frisian calves were obtained locally and transferred to the BSL-3
112 facility of the FLI on Insel Riems, where they were confirmed to be free of circulating
113 BTV genome by real-time RT-PCR analysis of blood samples (Toussaint *et al.*, 2007).
114 They came from a region where BTV has never been reported. They were purchased as
115 immunised against BTV-8 with two doses of an inactivated vaccine (BTVPUR® AISap
116 8, Merial S.A.S., Lyon, France) but displayed different levels of BTV-specific
117 antibodies (Table I). The calves had been vaccinated with a gE-deleted BHV-1 marker
118 vaccine. They had detectable antibodies against BVDV, and were negative for BVDV
119 antigen.

120 On day 0 of the study, the animals were inoculated intravenously and subcutaneously
121 (multiple sites in the shoulder region) with BTV-6 positive blood pooled from samples
122 submitted to the NRL. None of the samples contained detectable BTV-8 RNA (data not
123 shown). Packed blood cells of the inoculum had been repeatedly washed with
124 phosphate-buffered saline solution (PBS) and then ultrasonically disrupted. The blood
125 preparation was tested free of bacterial contamination and it had a threshold cycle (C_t)
126 value of 26.87 in the BTV-6 specific real-time RT-PCR (Hoffmann *et al.*, 2009b).

127 After inoculation, blood samples were taken at regular intervals. Blood was drawn by
128 jugular puncture and collected in tubes containing potassium EDTA or clot activator,
129 respectively (Monovette, Sarstedt, Nümbrecht, Germany). Samples were stored at 4 °C

130 until analysis. During the entire study, rectal body temperatures were taken daily, and
131 the calves were monitored for clinical signs.

132

133

134 **2.2.2. Serology**

135

136 Serological data were collected from samples taken on days 0, 7, 14, 21 and 60 after
137 infection, using a commercially available double-recognition (DR) ELISA
138 (PrioCHECK® BTV DR, Prionics Deutschland GmbH, Planegg-Martinsried, Germany)
139 to detect BTV group-specific antibodies against the highly conserved core protein VP7.
140 Virus neutralisation assays against German isolates of BTV-8 and -6 were performed as
141 described previously (Eschbaumer *et al.*, 2009). The BTV-6 isolate was obtained by cell
142 culture inoculation with blood taken from calf 605 on day 7 post-infection (dpi) and
143 subsequently passaged on Vero cells (RIE15, Collection of Cell Lines in Veterinary
144 Medicine [CCLV], FLI, Insel Riems, Germany).

145

146

147 **2.2.3. Detection of viral RNA and virus isolation**

148

149 Total RNA from whole blood samples was extracted manually using a commercial kit
150 (QIAamp Viral RNA Mini Kit, QIAGEN, Hilden, Germany). The amount of BTV
151 genome in the samples was determined using the “pan-BTV” assay by Toussaint *et al.*
152 (2007) and the in-house BTV-6 and BTV-8 assays that had also been used for the field
153 samples (Hoffmann *et al.*, 2009b). The “pan-BTV” assay amplifies a highly conserved

154 sequence at the 5' end of BTV genome segment 5, while the serotype-specific assays are
155 directed to genome segment 2, encoding the outer shell protein VP2. One-step RT-PCR
156 was carried out using a commercial kit (iScript One-Step RT-PCR Kit for Probes, Bio-
157 Rad Laboratories, Hercules, CA, USA) in an Mx3005P QPCR system (Stratagene, La
158 Jolla, CA, USA).

159 For RT-PCR-positive samples, virus isolation was performed by a method adapted from
160 Clavijo *et al.* (1999), using embryonated chicken eggs as well as Vero (RIE15, CCLV)
161 and baby hamster kidney (BHK) 21 clone 13 cells (RIE179, CCLV). Briefly, blood
162 cells were packed, washed repeatedly with PBS and ultrasonically disrupted on ice.
163 Intravenous inoculation of 10-day-old chicken embryos and adsorption onto 75%
164 confluent cell layers was performed in parallel. After 5 days of incubation, embryo
165 organ lysate and cell culture supernatants were blindly passaged in cell culture. When a
166 cytopathic effect developed in the second passage, BTV-6 replication was confirmed by
167 serotype-specific real-time RT-PCR.

168

169

170 **3. Results**

171

172 **3.1. Epidemiological investigations and field survey**

173

174 All three initially affected holdings were dairy farms whose animals had not been in
175 contact with other cattle or sheep either on the farm or on pasture. Two farms had their
176 own breeding stock, and the third had only bought animals from Lower Saxony in 2008.
177 Animals on the farms were healthy and well kept. Record-keeping was complete and
178 consistent, and there was no indication of the illegal use of modified-live bluetongue
179 vaccines. The distance to the closest affected holding in the Netherlands was found to
180 be only 17 kilometres, while the farthest was 52 kilometres away. No direct
181 epidemiological link between the farms could be established.

182 Of the 28,658 animals in Lower Saxony that were tested by regional laboratories, 3,753
183 (12.71-13.49%, 95% confidence interval) were positive in the group-specific “pan-
184 BTV” real-time RT-PCR. Among them, 45 BTV-6 positive animals (overall prevalence
185 0.11-0.21%) in 23 holdings (Figure 1) were found. In at least two cases, BTV-6 and
186 BTV-8 genome was detected in the same animal by serotype-specific real-time RT-PCR
187 analysis (data not shown).

188 For 36 BTV-6 positive animals identified by the NRL in November 2008, C_t values
189 ranged from 25.56 to 34.72 (average 30.38). Table II shows the results of the
190 longitudinal virological monitoring of the first four cases together with serological data
191 from the first sampling in November. The amount of viral genome detected in these
192 animals slowly decreased within the survey period of 10 weeks. All four animals were
193 negative in the BTV-8 specific real-time RT-PCR.

194 Based on data from the German animal identification and registration system (HI-Tier;
195 Kroschewski *et al.*, 2006), BTV-8 vaccine coverage in the district Grafschaft Bentheim
196 was 96% for female cattle, 69% for sheep and 47% for goats. In the 2,075 sera tested at
197 IZSAM, only antibodies against BTV-8 and -6 were found (Table III), while all samples
198 were negative for serotypes 1, 2, 4, 9, 15 and 16. Neutralising antibodies against BTV-8
199 were detected in 1,218 (56.55-60.83%) cases and BTV-6-specific antibodies in 39
200 animals (1.34-2.56%), 27 of which also had neutralising antibodies against BTV-8
201 (0.86-1.89%). Neutralising titres against BTV-8 ranged from 1:10 to over 1:1280, BTV-
202 6 titres were between 1:10 and 1:640.

203

204 **3.2. Animal experiment**

205

206 While the calves had reportedly been vaccinated against BTV-8 in mid-2008 with two
207 doses of an inactivated vaccine, their serological status was diverse (Table I). After
208 inoculation with BTV-6, two animals (calves 605 and 588) displayed measurable virus
209 replication (as detected by real-time RT-PCR, Table IV) and subsequently
210 seroconverted for BTV-6 after two and three weeks, respectively, together with a
211 marked increase in group-specific antibodies (Table I). Calf 607 failed to react both in
212 the real-time RT-PCR (data not shown) and by serology.

213 Calf 588 was only transiently infected and had already fully cleared the virus four
214 weeks after infection, but the viraemia in calf 605 lasted over six weeks (Table IV). The
215 animal remained BTV-6-positive in the real-time RT-PCR for over 100 days until the
216 end of the study period. BTV-8 was not detected in any animal (data not shown).

217 Between days 5 and 9 after infection, calf 605 had a slightly elevated body temperature

218 and calf 588 showed a similar increase in temperature from day 10 to 12 (data not
219 shown), while no other clinical signs could be observed.

220

221

222 **4. Discussion**

223

224 Among tens of thousands of animals that were screened in the field survey, only a small
225 number was found to be positive for BTV-6 RNA or antibodies. These were mostly
226 isolated cases in holdings scattered throughout a single district in the German federal
227 state of Lower Saxony, close to the border with the Netherlands, where similar findings
228 were made (MinLNV, 2009a). Given the BTV screening measures that were in place in
229 2008, BTV-6 most likely was detected much earlier after its introduction than BTV-8
230 had been, suggesting an entry late in the season. Apparently it has only been able to
231 propagate to a very limited extent, while BTV-8 had spread rapidly after its introduction
232 in 2006 (Hateley, 2009). Together with the reported genetic similarities, this gave rise to
233 the theory that BTV-6 was an attenuated strain introduced either by the illegal local use
234 of a modified live vaccine (ISID ProMED-mail, 2008d) or the unrecognized import of
235 animals that had been vaccinated with a modified live vaccine in a third country and
236 were still viraemic for BTV-6 when they arrived in Europe (MinLNV, 2009a).

237 Serological evidence from areas affected by BTV-6 provided no indication of a recent
238 use of the commercially available multivalent MLV vaccine. No antibodies against
239 serotypes other than BTV-6 and -8 were detected in either the Netherlands (MinLNV,
240 2009a) or Germany (this study). Specifically, there were no traces of other serotypes
241 from bottle A of the multivalent vaccine package (1, 4, 12 and 14) (Dungu *et al.*, 2004),

242 which would have been the most likely source of the BTV-6 vaccine strain. This
243 however has no bearing on another possible explanation, the accidental introduction of
244 vectors already infected with BTV-6 as stowaways in global trade (Mintiens *et al.*,
245 2008).

246 According to the CRL, genome segment 2 of the Netherlands isolate is almost identical
247 to the South African BTV-6 vaccine strain (ISID ProMED-mail, 2008d). Whether this
248 isolated finding is particularly meaningful remains open to debate. The manufacturer of
249 the vaccine (Onderstepoort Biological Products, Onderstepoort, South Africa) maintains
250 that based on segment 2 alone, all African strains of BTV-6, including the vaccine, are
251 closely related (ISID ProMED-mail, 2008e). Such a high degree of homology was not
252 seen in European BTV-8, however, which shares 93% of nucleotides of segment 2 with
253 the South African BTV-8 vaccine strain, and 97% with a Nigerian isolate (Maan *et al.*,
254 2008). For other segments of European BTV-6, a high similarity to the South African
255 reference strain is claimed, but the origin of segment 10 is unknown (MinLNV, 2009b)
256 and reassortment has been suspected (Saegerman and Pastoret, 2009). Only four
257 isolated sequences of individual genome segments of BTV-6 are presently available
258 online, and no sequence of either the BTV-6 vaccine strain or the recent European
259 isolates has been made public (U.S. National Center for Biotechnology Information,
260 GenBank, <http://www.ncbi.nlm.nih.gov/Genbank/>).

261 But regardless of its origin, the question remains if the very limited spread of BTV-6
262 that has been observed in 2008 is due to attenuation of the virus itself. Judging from our
263 data, other factors could have contributed to that outcome. Our experiment suggests that
264 the susceptibility to BTV-6 infection of cattle is influenced by previous exposure to
265 BTV-8, but this needs to be investigated in a broader study using defined challenge

266 doses of BTV-1 and -6. In the present experiment, it is difficult to estimate the
267 infectious dose contained in the inoculum. The dates of infection of the donor animals
268 remain unknown, but the low amount of BTV-6 genome in the pooled samples (no C_t
269 value lower than 25, data not shown) and the failure to isolate virus by other means
270 suggest that the blood had been collected at a late stage of infection, resulting in limited
271 infectivity. While BTV-infected ruminants can remain positive in the RT-PCR and even
272 viraemic for weeks or months, the concentration of infectious particles in the blood
273 decreases over time (Singer *et al.*, 2001; MacLachlan *et al.*, 2009; Hoffmann *et al.*,
274 2009a). In a related animal experiment at the CVI, using a BTV-6 field sample from a
275 Dutch cow, at first only one of six animals could be infected, which also suggests a low
276 infectious dose. When fresh blood taken from the latter animal at 10 dpi was inoculated
277 in a second experiment, however, 11 out of 11 animals displayed virus replication (Van
278 Rijn, 2009).

279 Nevertheless, within the scope of our experiment, the course of BTV-6 infection once
280 established in the naïve calf was similar to BTV-8. No data are available for the BTV-6
281 MLV vaccine strain, but the longest duration of viraemia reported after vaccination of
282 cattle with other MLV vaccines was 28 days (Monaco *et al.*, 2004) – less than two
283 thirds of the viraemic period in calf 605. Long-lasting viraemia has been observed with
284 an insufficiently attenuated strain of BTV-16 (Savini *et al.*, 2008), and up to 63 days of
285 viraemia have been described after experimental infection of cattle with virulent BTV
286 strains (Singer *et al.*, 2001; Dal Pozzo *et al.*, 2009). On the other hand, the high number
287 of culture passages commonly used in the production of MLV vaccines (Savini *et al.*,
288 2008) might actually facilitate the subsequent reisolation of vaccine viruses *in vitro*.

289 At its peak, the amount of BTV-6 genome detected in calf 605 did not notably differ
290 from viral genome levels observed in BTV-8 animal experiments (Darpel *et al.*, 2007).
291 This similarity also extends to the persistence of detectable viral RNA in peripheral
292 blood of calf 605 and the only gradual decrease of circulating BTV-6 genome in the
293 presence of neutralising antibodies that was observed in field-infected animals (Table
294 II) (Dal Pozzo *et al.*, 2009). Viral RNA remains detectable in the blood for extended
295 periods of time because BTV persists in invaginations in the cell membrane of
296 erythrocytes (Schwartz-Cornil *et al.*, 2008), a trait probably shared by both field and
297 vaccine strains.

298 With regard to the absence of any clinical symptoms other than elevated body
299 temperature, it has to be noted that in our experience and based on experimental data
300 reported by other groups (Darpel *et al.*, 2007), clinical bluetongue disease in cattle does
301 occur, but it is not a regular finding in Holstein Frisians experimentally infected with
302 BTV-8 either. Conversely, it has been shown that even vaccine strains can cause disease
303 (Savini *et al.*, 2008), if only in sheep (Veronesi *et al.*, 2005). Exposure to sunlight might
304 play a role in bluetongue pathogenesis, which could contribute to the reduced severity
305 of disease observed after experimental infection in high-containment animal housing
306 (Verwoerd and Erasmus, 2004). The initial detection of BTV-6 in the Netherlands, on
307 the other hand, had been precipitated by clinical disease observed in the field (ISID
308 ProMED-mail, 2008d; Hateley, 2009).

309 There is no doubt that after two years of the BTV-8 epidemic and with mass
310 vaccinations beginning in mid-2008, BTV-6 faced by no means an animal population
311 naïve to BTV (Saegerman and Pastoret, 2009). To the contrary, the western North
312 European Plain shared by Belgium, the Netherlands and Germany arguably was the

313 region with the highest cumulative incidence of BTV-8 in Europe. In the field survey
314 presented here, over half of the animals had neutralising antibodies to BTV-8, and more
315 than one tenth were still positive in the real-time RT-PCR. Even if cross-neutralisation
316 between serotypes is low, there is evidence of group-specific cell-mediated immunity
317 after infection (Schwartz-Cornil *et al.*, 2008). Given these unfavourable conditions, the
318 fact that BTV-6 was able to spread at all does not suggest attenuation.

319

320

321 **5. Conclusion**

322

323 The final evaluation as well as the measures taken by animal health authorities
324 concerning the emergence of BTV-6 (SCoFCAH, 2009) are debatable from a scientific
325 point of view (Saegerman and Pastoret, 2009). If BTV-6 does not return in 2009, it will
326 be difficult to say with any certainty whether this was because of its vaccine origin or
327 simply because BTV-8 had come first.

328 In either case, one troubling fact remains: since 2006, three different serotypes of BTV
329 have first been detected in the same geographical region, without any previous notion of
330 their presence on European soil and without any definite explanation for their
331 introduction (Mintiens *et al.*, 2008; MacLachlan *et al.*, 2009). Finding and closing that
332 loophole is crucial for animal health in Europe, particularly with regard to the looming
333 threat of other, more lethal, orbivirus diseases of domestic animals like African horse
334 sickness.

335

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337

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483 **Table captions**

484

485 Table I: Serological data from the animal experiment

486 Values given for virus neutralisation assays (NA) are 50% neutralising doses against the
487 indicated serotype, double-recognition (DR) ELISA values denote the reciprocal of the
488 last serum dilution that gave a positive result.

489

490 Table II: Two-month follow-up on the first four BTV-6 positive animals

491 Threshold cycle (C_t) values given were obtained with a real-time RT-PCR assay
492 specific for BTV-6. Values for neutralisation assays (NA) are 50% neutralising doses
493 for the indicated serotype.

494

495 Table III: Results of BTV neutralisation assays for field samples

496 No neutralising antibodies against serotypes 1, 2, 4, 9, 15 or 16 were detected in any
497 sample. Percentages are given as a 95% confidence interval.

498

499

500 Table IV: Real-time RT-PCR and virus isolation from samples from the animal

501 experiment

502 Threshold cycle (C_t) values and results of the second passage of the virus isolation are
503 given where applicable, ND = not determined. No BTV-8 genome was detected in any
504 animal and no BTV-6 genome was detected in calf 607 (data not shown).

505

506 **Figure captions**

507

508 Figure 1: Cattle herds with BTV-6 infections in Germany

509

510 Dots (blue) mark the locations of affected holdings. The thick line (red) is the Dutch-

511 German border.

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Table I

Animal	Assay	Day 0	Day 7	Day 14	Day 21	Day 60
Calf 588	DR ELISA	8	8	16	2048	1024
	NA BTV-6	0	0	0	10	100
	NA BTV-8	4	3	3	2	0
Calf 605	DR ELISA	0	0	32	128	64
	NA BTV-6	0	0	3	40	320
	NA BTV-8	0	0	0	0	0
Calf 607	DR ELISA	64	64	64	64	32
	NA BTV-6	0	0	0	0	0
	NA BTV-8	8	6	4	4	3

Table II

Animal	Virus neutralisation		BTV-6 real-time RT-PCR		
	BTV-6	BTV-8	5 Nov 08	8 Dec 08	22 Jan 09
1328	40	0	C _t 26.86	C _t 26.83	C _t 29.00
1338	40	0	C _t 26.82	C _t 27.00	C _t 30.46
2635	40	160	C _t 30.66	C _t 29.36	C _t 30.14
9707	40	40	C _t 29.85	C _t 27.65	C _t 29.23

Table III

Sample origin	No. of samples	BTV-8 pos.	BTV-6 pos.	Both
First 3 holdings	858	574	23	15
1 km zone	1217	644	16	12
Total:	2075	1218	39	27
		(56.55-60.83%)	(1.34-2.56%)	(0.86-1.89%)

Table IV

Day	Calf 588			Calf 605		
	pan-BTV real-time	BTV-6 real-time	virus isolation	pan-BTV real-time	BTV-6 real-time	virus isolation
0	No C _t	No C _t	ND	No C _t	No C _t	ND
5	No C _t	No C _t	ND	No C _t	No C _t	ND
7	No C _t	No C _t	ND	29.41	28.26	positive
8	No C _t	No C _t	ND	27.44	26.86	ND
9	35.62	32.23	negative	27.03	26.43	ND
10	32.81	31.14	negative	26.03	25.29	ND
12	No C _t	34.35	negative	26.53	28.02	positive
13	33.28	32.22	negative	23.66	21.36	ND
14	32.75	31.94	negative	22.79	22.00	ND
17	32.95	32.56	positive	26.39	23.95	ND
19	33.76	34.04	negative	26.04	24.81	positive
21	35.78	No C _t	negative	27.85	26.93	positive
24	No C _t	No C _t	ND	29.06	27.94	positive
27	38.23	No C _t	ND	30.11	29.35	positive
31	No C _t	No C _t	ND	29.92	29.35	positive
34	No C _t	No C _t	ND	30.97	29.03	positive
38	No C _t	No C _t	ND	31.10	29.90	positive
45	No C _t	No C _t	ND	30.29	29.40	positive
52	No C _t	No C _t	ND	32.74	29.93	negative
60	No C _t	No C _t	ND	30.82	27.84	negative
69	No C _t	No C _t	ND	29.55	27.88	negative
77	No C _t	No C _t	ND	30.66	29.90	ND
84	No C _t	No C _t	ND	31.30	30.69	ND
91				32.06	32.15	ND
98				31.32	30.56	ND
105				31.83	31.18	ND

Figure 1

