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## **Evaluation of antibody-ELISA and real time RT-PCR for the diagnosis and profiling of bluetongue virus serotype 8 during the epidemic in Belgium in 2006**

Frank Vandebussche, Tine Vanbinst, Bart Verheyden, Wesley van Dessel, Lien Demeestere, Philippe Houdart, Guido Bertels, Nicolas Praet, Dirk Berkvens, Koen Mintiens, et al.

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2 **bluetongue virus serotype 8 during the epidemic in Belgium in 2006.**

3

4

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27 **Abstract**

28 In 2006 bluetongue (BT) emerged for the first time in North-Western Europe. Reliable  
29 diagnostic tools are essential in controlling BT but data on the diagnostic sensitivity (Se) and  
30 specificity (Sp) are often missing. This paper aims to describe and analyse the results obtained  
31 with the diagnostics used in Belgium during the 2006 BT crisis. The diagnosis was based on a  
32 combination of antibody detection (competitive ELISA, cELISA) and viral RNA detection by  
33 real-time RT-PCR (RT-qPCR). The performance of the cELISA as a diagnostic tool was  
34 assessed on field results obtained during the epidemic and previous surveillance campaigns.  
35 As the infectious status of the animals is unknown during an epidemic, a Bayesian analysis  
36 was performed. Both assays were found to be equally specific (RT-qPCR: 98.5%; cELISA:  
37 98.2%) while the diagnostic sensitivity of the RT-qPCR (99.5%) was superior to that of the  
38 cELISA (87.8%). The assumption of RT-qPCR as standard of comparison during the BTV  
39 epidemic proved valid based on the results of the Bayesian analysis. A ROC analysis of the  
40 cELISA, using RT-qPCR as standard of comparison, showed that the cut-off point with the  
41 highest accuracy occurred at a percentage negativity of 66, which is markedly higher than the  
42 cut-off proposed by the manufacturer. The analysis of the results was further extended to  
43 serological and molecular profiling and the possible use of profiling as a rapid  
44 epidemiological marker of the BTV in-field situation was assessed. A comparison of the  
45 serological profiles obtained before, during and at the end of the Belgian epidemic clearly  
46 showed the existence of an intermediate zone which appears soon after BTV (re)enters the  
47 population. The appearance or disappearance of this intermediate zone is correlated with virus  
48 circulation and provides valuable information, which would be entirely overlooked if only  
49 positive and negative results were considered.

50

51 **Keywords**

52 Bluetongue, profiling, cELISA, real-time RT-PCR, ROC analysis, Bayesian analysis

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## 53 1. Introduction

54 Bluetongue (BT) is a non-contagious disease of domesticated as well as wild ruminants that  
55 induces variable clinical signs depending on the species and the breed (MacLachlan, 1994;  
56 Verwoerd and Erasmus, 2004). The disease is caused by the bluetongue virus (BTV) which is  
57 the type species of the genus *Orbivirus* within the family *Reoviridae* (Mertens et al., 2004).  
58 BTV is an arbovirus and is only transmitted by certain species of the genus *Culicoides*  
59 (Mellor et al., 2000; Tabachnick, 2004). Its reliance upon an arthropod vector that is normally  
60 exclusively found in tropical and temperate areas restricted BT for a long time to America,  
61 Australia, Africa and some regions of Asia (Walton, 2004). However, probably due to  
62 climatical changes BTV recently spread northwards into the Mediterranean Basin (Toussaint  
63 et al., 2006a), where 5 serotypes of bluetongue (1, 2, 4, 9 and 16) have been identified in more  
64 than 12 countries (Purse et al., 2005). In the summer of 2006, BTV emerged for the first time  
65 in North-Western Europe and quickly disseminated over large parts of the Netherlands,  
66 Belgium, Germany and France (OIE Animal Health Department, 2006; Communication  
67 Directorate General, 2006; Toussaint et al., 2006b). Virus isolation and subsequent  
68 characterization demonstrated that the epidemic was caused by a BTV serotype 8 strain  
69 (European CRL, 2006; Toussaint et al., 2007b) that had previously only been found in the  
70 Republic of South Africa, Nigeria, Kenya, Central America, the Caribbean and indications  
71 through the presence of antibodies against BTV 8 in India and Malaysia (Hassan, 1992,  
72 Uppal, 1992, Gibbs and Greiner, 1994; Mo et al., 1994; Gerdes, 2004; Lager, 2004; Bréard et  
73 al., 2005). Apart from the classical clinical signs in sheep, the BTV8 epidemic was especially  
74 characterized by clear clinical signs in cattle (Thiry et al., 2006).

75

76 An introduction of BTV in livestock causes substantial economic losses due to the disease  
77 itself and, more substantially, to the complete trade block between infected and non-infected

78 areas (Calistri et al., 2004; MacLachlan and Osburn, 2006). As with all other former list A  
79 diseases of the World Organisation for Animal Health (OIE - Office International des  
80 Epizooties), fast and reliable diagnostic tools are essential in controlling BT. Over the past  
81 decades, various serological (agar gel immunodiffusion, competitive ELISA (cELISA)) and  
82 virological (virus isolation, RT-PCR) methods have been described and recognised by the  
83 OIE (OIE, 2004) as prescribed or alternative tests. Although the validation process  
84 recommended by the OIE clearly identifies 5 stages (OIE, 2004 – Chapter 1.1.3. adopted May  
85 2006), validation of most diagnostic tests is limited to the first two stages: i) essential  
86 prerequisites such as checking the ‘fit for purpose’, the feasibility and the normalisation of  
87 results and ii) part 1 of assay validation with determination of repeatability and analytical  
88 sensitivity and specificity. Especially, data on the diagnostic sensitivity and diagnostic  
89 specificity are often missing which hampers the evaluation of the test accuracy. For epizootic  
90 diseases, like BT, both false positive and false negative results are of great concern to  
91 international trade (De Clercq, 1995). Other major constraints to the validation process of BT  
92 diagnostics are the lack of an easy-to-use gold standard (now defined as ‘standard of  
93 comparison’ by OIE) for reliable detection of infectious virus in infected animals and the fact  
94 that the detection of specific anti-BTV antibodies or BTV-RNA does not necessarily imply  
95 the presence of infectious BTV (Katz et al., 1994; MacLachlan, 2004).

96  
97 This paper aims to describe and analyse the results obtained from the diagnostics used in  
98 Belgium during the 2006 BT crisis. The performance characteristics of a cELISA and a real-  
99 time RT-PCR (RT-qPCR) as diagnostic tools are assessed and discussed. The analysis of the  
100 results is not limited to the typical dichotomised classification of positives and negatives  
101 based on a fixed cut-off value (CO), but is extended to serological and molecular profiling  
102 through the analysis of frequency distributions. Bergmann et al. (2003) proved that profiling



103 is particularly important for recognising hidden information in the intermediate zone when  
104 assessing foot-and-mouth disease virus persistence at the herd level. Earlier, De Clercq (1998)  
105 already showed that profiling could be used at the individual animal level to distinguish  
106 genuine positives from singleton reactors for swine vesicular disease. In the current study  
107 frequency distributions of reactivity levels of specific anti-BTV antibodies and BTV-RNA in  
108 cattle and sheep are analysed. The resulting profiles are correlated to the epidemiological  
109 status and distinct distributions of reactivity patterns reflecting the various epidemiological  
110 situations are attained. This study demonstrates the possible use of profiling as a rapid  
111 epidemiological marker of the BTV in-field situation.

112

## 113 **2. Methods**

### 114 2.1. Biological samples

#### 115 2.1.1. Serum, blood and tissue samples from BT suspected animals

116 From August 18<sup>th</sup> until December 31<sup>st</sup> 2006 a total of 1573 serum samples (906 cattle; 667  
117 sheep) and 765 (539 cattle; 226 sheep) EDTA blood samples from animals showing typical  
118 BT clinical signs (suspicion) were analysed with a commercial cELISA and/or a RT-qPCR,  
119 respectively (see 2.2.). In total 674 samples were tested in both assays and used to determine  
120 the relative sensitivity and specificity (test validation). All samples were used for analysing  
121 the frequency distribution of the antibody response values and the RNA levels (profiling). In  
122 addition to these serum and blood samples, a wide variety of tissue samples such as spleen,  
123 lymph nodes, lungs, and oral lesions (N = 80) from severely or fatally infected animals were  
124 presented for analysis by RT-qPCR with spleen and lymph nodes being most abundant.

125

#### 126 2.1.2. Serum and blood samples from different screening programmes

127 (i) Due to the emergence of BTV in Europe (Purse et al., 2005) and in the framework of an  
128 early warning system for epizootic diseases, pro-active measures were taken in Belgium such  
129 as the implementation of a BT serological cELISA in the National Reference Laboratory in  
130 2003. Comparable to a survey performed in Switzerland, but without the attempt to  
131 substantiate freedom of BTV infection, sera were taken at randomly from a bovine  
132 herpesvirus-1 screening programme in Belgium in 2004 (N= 338) and 2005 (N= 365) and  
133 checked for the presence of anti-BTV antibodies (Boelaert et al., 2000; Cagienard et al.,  
134 2006).

135 The samples described in (i) and (ii) were used for antibody response profiling.

136 (ii) Early September 2006, all provinces of Belgium minus the area that was infected at the  
137 end of August (20 km zone; Fig. 1) were checked for freedom of BTV infection. To this end,  
138 991 serum samples were collected from randomly selected farms stratified per province (150  
139 farms per province, 841 cattle samples in total) and from artificial insemination centres (136  
140 cattle and 14 sheep samples) and tested for the presence of antibodies against BTV.

141 (iii) At the end of January 2007, a national cross-sectional survey was conducted to determine  
142 the serological prevalence of BTV in Belgium. Three hundred and eighty five farms were  
143 selected, again stratified per province, at which serum samples and EDTA blood samples  
144 were collected from all animals older than 2 years. To assess the frequency distribution of  
145 antibody response values and of RNA levels, 5752 serum samples as well as 637 EDTA blood  
146 samples from 75 randomly selected cattle farms were further analysed.

147

#### 148 2.1.3. Paired blood samples

149 According to 'Procedure J124' of the Belgian Federal Agency for the Safety of the Food  
150 Chain (Food Agency), a BTV-infected farm could only be declared free of BT if one month  
151 post (T2) the initial sampling (T1) (i) none of the animals showed clinical signs and (ii) all

152 animals positive by RT-qPCR at T1 were negative at T2. Animals still positive at T2 were  
153 only declared free of the disease after a second one-month waiting period. During the  
154 epidemic, 75 of these T1/T2 paired blood samples were collected and analysed by RT-qPCR.

155

## 156 2.2. Diagnostic assays

### 157 2.2.1. Bluetongue virus antibody competitive ELISA

158 Anti-BTV antibodies were detected using the 'ID Screen<sup>®</sup> Bluetongue Competition' assay  
159 according to the manufacturer's instructions (ID VET, 570 rue des Bouissettes - 34070  
160 Montpellier – FRANCE). Besides the kit controls, a two-fold dilution series of an anti-BTV  
161 antibody positive reference serum developed and tested by the Centre de Coopération  
162 Internationale en Recherche Agronomique pour le Développement (CIRAD, Montpellier,  
163 France) was included as working standard in each assay in order to monitor the performance  
164 of the cELISA in time as described for foot-and-mouth disease by Goris and De Clercq  
165 (2005). Results were expressed as % negativity (PN) compared to the negative kit control and  
166 transferred to a positive, doubtful or negative result according to the cut-off settings provided  
167 by the manufacturer ( $PN \leq 35$  is positive;  $35 < PN \leq 45$  is doubtful;  $PN > 45$  is negative).

168

### 169 2.2.2. Fluorogenic RT-qPCR specific for BTV segment 5 and beta-actin

170 During the BT epidemic 2006 in Belgium a quantitative reverse-transcription PCR targeting  
171 BTV segment 5 (RT-qPCR\_S5) was used to detect BT viral RNA in blood samples and tissue  
172 samples. Each test was performed in parallel with the RT-qPCR\_ACT to amplify beta-actin  
173 mRNA as internal control. Both assays were carried out according to Toussaint et al. (2007a)  
174 with slight modifications. Briefly, total RNA was purified from 400  $\mu$ l of red blood cells or 25  
175 mg of tissue by Trizol extraction (Invitrogen) and denatured by heating for 3 min at 95 °C  
176 with 10% dimethyl sulfoxide (Sigma-Aldrich). Reverse transcription (RT) reactions were

177 carried out using the Taqman reverse transcription reagents according to the manufacturer's  
178 instructions (Applied Biosystems). Real-time qPCR reactions consisted of 1x concentrated  
179 Taqman fast universal PCR master mix (Applied Biosystems), 375 nM (beta-actin) or 500 nM  
180 (bluetongue) of each primer, 250 nM of the Taqman probe conjugated to FAM at the 5' end  
181 and to TAMRA at the 3' end and 5 µl cDNA. Cycling conditions were as follows: 1 cycle at  
182 95 °C for 20 s, followed by 45 cycles of 1 s at 95 °C and 20 s at 60 °C. For both assays the  
183 cut-off was set at a Ct-value of 40 as previously determined by Toussaint et al. (2007a).

184

### 185 2.3. Data analysis

186 For BT the VI is a non-easy-to-perform assay but the RT-qPCR was previously shown to be  
187 also highly accurate (Toussaint et al., 2007a; Yang and Rothman, 2004). Therefore the  
188 relative performance of the cELISA as a diagnostic assay was compared to the RT-qPCR\_S5  
189 using a 2x2 contingency table. The 95% confidence intervals of diagnostic sensitivity and  
190 specificity for the cELISA were calculated using Bayesian inference as described by Goris et  
191 al. (2007a). Moreover, since the true BTV infection status of the suspected BTV-infected  
192 animals is in fact unknown and RT-qPCR is not an officially accepted standard of  
193 comparison, a Bayesian model allowing the integration of field data and of expert opinion  
194 (prior information), was developed using the WinBUGS Version 1.4 as described by Goris et  
195 al. (2007b). Subsequently, a receiver operating characteristic (ROC) analysis was carried out  
196 using MedCalc 9.0 software to assess the performance of the cELISA in more detail. The  
197 sensitivity (i.e. true positive rate) is plotted against 100-specificity (i.e. false positive rate).  
198 Each point on the ROC curve thus represents a sensitivity/specificity pair corresponding to a  
199 particular CO i.e. decision threshold. The optimum CO for diagnostic purposes is the point  
200 closest to the upper left corner (100% sensitivity, 100% specificity) corresponding to the  
201 value with the highest accuracy (minimal false negative and false positive results) (Zweig and

202 Campbell, 1993). This analysis was performed on results obtained from samples tested in both  
203 RT-qPCR and cELISA (N=674, see 2.1.1.).

204 The viral load in the paired blood samples was compared with a paired t-test. Prior to  
205 analysis, the assumptions of normality and equal variance were assessed using the  
206 Kolmogorov-Smirnov and Levene's test, respectively. For all tests, statistical significance was  
207 set at  $P \leq 0.05$ .

208

### 209 **3. Results**

#### 210 3.1. Samples tested in cELISA and RT-qPCR

211 A total of 674 animals showing clinical signs were tested for the presence of anti-BTV  
212 antibodies as well as BTV RNA. A pair-wise comparison of the results indicated a high  
213 degree of concordance between cELISA and RT-qPCR with 616 (91%) animals being  
214 negative or positive in both tests (Table 1). Interestingly, all animals with a doubtful cELISA  
215 result (PN: 36-45) were clearly positive by RT-qPCR with Ct-values ranging between 21.8  
216 and 30.3 (Table 2). In 46 animals BTV infection was only detected by RT-qPCR. Out of  
217 these, 22 animals exhibited Ct-values  $<33.0$  and a PN value ranging from 46 to 85 indicating  
218 a recent infection in which the immune response is just mounting (Table 2). The Ct-values of  
219 the remaining 24 animals with a PN value  $>85$  ranged from high to low (Ct: 22.5-38.9).

220 Surprisingly, 3 animals were positive by cELISA but clearly negative by RT-qPCR. Given the  
221 high antibody titres that were detected (PN  $< 20$ ), these cases most likely represent false-  
222 negative RT-qPCR results instead of false-positive ELISA results. Resampling of one of these  
223 animals only one week later, indeed, yielded a clear positive RT-qPCR result (Ct: 27.9).

224 Furthermore, in all 3 cases the internal beta-actin control was positive. Retesting of 2 of the  
225 samples yielded the same result ruling out human error. After diluting the samples 1:10 a

226 clear positive signal (Ct: 31.0) was obtained for 1 of these samples. The third sample could  
227 not be retested and therefore no clear follow up could be done.

228 The relative sensitivity and specificity of the cELISA were estimated from the 2x2  
229 contingency table (Table 1) using the RT-qPCR as non-official standard of comparison (gold  
230 standard). Considering the doubtful cELISA results as positives, the cELISA has a relative  
231 sensitivity (Se) of 87.4 % (95% CI: 83.5-90.4) and a relative specificity (Sp) of 99.0 %, (95%  
232 CI: 97.2-99.6). Detailed analysis using only cattle sera revealed that the cELISA displayed a  
233 relative Se of 88.0 % (95% CI: 83.7-91.7) and a relative Sp of 98.0 %, (95% CI: 95.7-99.5)  
234 while for sheep respectively values of 84.5 % (95% CI: 77.4-90.4) and 99.1 %, (95% CI:  
235 96.6-100.0) were found. The overlapping confidence intervals clearly indicate that no  
236 significant differences in test performance characteristics could be demonstrated for both  
237 species. However, the true BTV infection status of the 674 sampled animals was unknown  
238 and RT-qPCR is not an accepted gold standard test indicating that a latent class analysis  
239 should be performed to accurately predict the test performance parameters. The developed  
240 Bayesian model was validated both within and outside WinBUGS and yielded estimates for  
241 the test characteristics (posterior information) in the absence of a gold standard and assuming  
242 conditional dependence of the cELISA and the RT-qPCR test. Both assays were found to be  
243 equally specific with a diagnostic specificity (DSp) estimate for RT-qPCR of 98.5% (95% CI:  
244 97.1-100.0) and for cELISA of 98.2% (95% CI: 96.3-99.6). The diagnostic sensitivity (DSe)  
245 of the RT-qPCR (99.5%, 95%CI: 99.0-100.0) was superior to that of the cELISA (87.8%,  
246 95%CI: 85.1-91.1). Separate Bayesian analysis for cattle and sheep showed no significant  
247 difference in DSe or DSp (Table 3). The combination of a rather low Se with a high Sp results  
248 in a fairly high likelihood ratio for a positive test result (LR+) of 90, meaning that, during an  
249 epidemic, a positive cELISA result is ninety times as likely to be seen in a suspected animal  
250 with positive RT-qPCR result as opposed to one without a positive RT-qPCR result (Collins,

251 2002). The performance of the cELISA as a diagnostic test was further assessed via a ROC  
252 analysis using the RT-qPCR as a reference test which was a justified assumption based on the  
253 high RT-qPCR sensitivity and specificity estimates observed using Bayesian analysis. The  
254 area under the curve (Fig. 2) was 0,974 with a 95% confidence interval between 0,959 and  
255 0,985 indicating a high accuracy. The cut-off point at which both sensitivity and specificity  
256 were optimal for diagnostic purposes (see 2.3.) occurred at a PN value of 66, which is  
257 markedly higher than the cut-off proposed by the manufacturer. Using this cut-off point, the  
258 sensitivity and specificity of the test were estimated to be 91,2% (95% CI: 87,8-93,9) and  
259 97,3% (95% CI: 94,8-98,8), respectively.

260

### 261 3.2. Antibody competition ELISA

262 To illustrate the antibody reactivity patterns, the frequency distribution analysis and  
263 interpretation of the cELISA results were based on profile distribution of PN values divided  
264 into 10 PN classes. The PN distribution profile of the results from the screening programmes  
265 of 2004 (N = 338) and 2005 (N = 365) were highly similar and showed a unimodal,  
266 negatively skewed shape (Fig 3A). As it is highly unlikely that BTV was already present in  
267 Belgium at that time, this distribution profile can be considered as representative for a BTV-  
268 naïve population. A similar distribution profile was obtained for the cattle population in the  
269 area checked for freedom of BTV infection in September 2006 and for the cattle and sheep of  
270 the artificial insemination centres in the same area (in total N = 991; Fig. 3A).

271 A completely different distribution profile was observed for the suspected cattle/sheep  
272 population (N = 1573), which showed a clear bimodal shape (Fig 3B). This bimodal profile  
273 indicates that distinct negative categories coexisted with positive ones. Apart from the  
274 appearance of this additional mode corresponding to BTV infected animals, a markedly  
275 higher number (N = 46) of sera was noticed in the intermediate zone spanning the cut-off

276 class (PN: 36-45) and the near cut-off PN classes (PN: 46-55 and 56-65). With the exception  
277 of the number of negatives, hardly any difference could be observed in the distribution profile  
278 of cattle and sheep.

279 Comparing the frequency distribution profile of the 5752 animals from 75 randomly selected  
280 farms from the January 2007 survey (Fig. 3C) with the profile from the suspected animals  
281 (Fig. 3B) showed that almost all positive animals had shifted towards the lowest PN class  
282 (PN: 0-5; strong positives).

283

### 284 3.3. Real time RT-PCR (RT-qPCR)

285 To allow for statistical analysis, all samples for which no Ct-value could be determined for  
286 BTV were given a value of 45.1. The RT-qPCR results were divided in 8 classes according to  
287 their Ct-value. The Ct frequency distribution profile of the 765 EDTA blood samples from  
288 animals showing BT clinical signs exhibited a bimodal shape for both sheep and cattle (Fig.  
289 4). The position of the positive mode, however, differed depending on the host species (peak  
290 for sheep at 26-28, for cattle at 32-34) suggesting that the virus accumulates to higher levels  
291 in sheep than in cattle. It is interesting to note that only a limited number of samples were  
292 found in Ct-classes near the cut-off value (i.e. Ct: 35-37 and 38-40), in contrast to the  
293 frequency distribution of the samples taken from non-suspected animals at the end of January  
294 2007 (N = 673) where the positive mode of the Ct-values shifted towards the Ct classes near  
295 the cut-off (Fig. 4). The frequency distribution profile of Ct-values from the tissue samples  
296 was very similar to the profile of the blood samples of sheep with the exception that the viral  
297 RNA load in the tissue samples was generally lower (Fig. 4). Compared to other tissue  
298 samples, best results were obtained with spleen and lymph nodes.

299

### 300 3.4. Paired samples



301 Seventy-five EDTA blood samples were analysed by RT-qPCR at the time of clinical  
302 suspicion (T1) and one month later (T2). The difference in Ct value at T1 and T2 (T1-T2)  
303 ranged from  $\Delta Ct = +7.50$  (recent infection) to  $-9.90$  (non-recent infection). The mean Ct-value  
304 at T1 was 30.64 (95% CI: 29.79-31.49) and 33.35 (95% CI: 32.66-34.04) at T2. The mean  
305 increase of 2.71 Ct (95% CI: 1.87-3.56) one month post initial sampling indicates a decrease  
306 in the BTV RNA level present in the blood (less positive). The difference was highly  
307 significant ( $P < 0.001$ ).

308

#### 309 **4. Discussion**

310 With globalisation of trade in animals, it is of the utmost importance to convince trading  
311 partners that the exporting country is free of animals carrying infectious BTV particles, which  
312 could be transmitted through competent vectors (MacLachlan and Osburn, 2006). This  
313 requirement challenges the surveillance systems and, consequently, several indicators are of  
314 great relevance to assist in the decision-making policy, among which are diagnostic  
315 parameters related to the recognition of the presence of infectious BTV. As molecular  
316 diagnostic tools such as PCR can demonstrate the presence of BTV RNA in an infected  
317 animal long time after the virus has become non-infectious (MacLachlan et al., 1994;  
318 MacLachlan, 2004), the diagnosis of infectious BTV particles has traditionally been  
319 accomplished through the direct demonstration and identification of the causative agent by  
320 isolation procedures on embryonated chicken eggs and subsequent cell culture passages  
321 (Zientara et al., 2006). These procedures are, however, time-consuming and may fail to detect  
322 low levels of infectious virus, which impairs their use in any surveillance programme.  
323 Serological and molecular profiling could therefore play a major role to substantiate absence  
324 or presence of viral activity on the condition that the diagnostic performance characteristics of  
325 the tests used are known.

326

327 To assess the performance of the cELISA as a diagnostic tool, a comparative assessment was  
328 made with the results obtained by RT-qPCR. As expected, most results were concordant. The  
329 rather low relative Se (87%) of the cELISA in clinically suspected animals, is most likely due  
330 to the fact that samples were taken at an early stage of infection when the humoral antibody  
331 response against BTV is still limited (antibodies can only be detected 6-10 days post  
332 infection; Koumbati et al., 1999). Both the relative Se and Sp of the 'ID Screen<sup>®</sup> Bluetongue  
333 Competition' assay used in this study were higher than the values found for the VMRD  
334 ELISA kit (Biteau-Coroller et al., 2006) and the BDSL assay (Cagienard et al., 2006).  
335 Accurate estimates for Se and Sp are essential for the interpretation of laboratory results and  
336 to calculate the number of samples to be taken in a survey. The assumption of RT-qPCR as  
337 reference test or standard of comparison during the first couple of months of the BTV  
338 epidemic proved valid based on the results of the Bayesian analysis, as RT-qPCR combines a  
339 near to perfect diagnostic sensitivity with a high diagnostic specificity.

340

341 Based on a visual inspection of the frequency distributions of all naïve populations (Fig. 2A),  
342 a cut-off of 65 PN could be suggested for the cELISA, resulting in only 3 false-positives. The  
343 relative Se and Sp of the cELISA would accordingly change to 91,0% (95%CI: 87,5-93,5) and  
344 97,4% (95%CI: 94,9-98,7) respectively with a decreased LR+ of 35. Consequently the  
345 number of false positives will slightly increase but more importantly during an epidemic the  
346 number of false negatives will decrease by 13. A ROC analysis indeed confirmed that the  
347 optimal cut-off for diagnostic purposes is situated around a PN-value of 66. Although Biteau-  
348 Coroller et al. (2006) suggested that cELISA is only suitable as a screening test, our results  
349 clearly demonstrate that it can also be used as a diagnostic tool to determine the disease at the  
350 individual level once a BT epizootic is confirmed. Considering the fit-for-purpose principal,

351 we suggest using the cELISA at a cut-off of 66 PN as a diagnostic tool and with a cut-off of  
352 45 PN for screening.

353

354 Most probably 3 false negative RT-qPCR results were obtained. Since BTV RNA could also  
355 be detected in one of the samples when the RNA was diluted 10 times, the initial false-  
356 negative result was presumably due to the presence of contaminating inhibitors. Interestingly,  
357 the internal control (beta-actin RNA) was easily detected in both the undiluted and diluted  
358 sample, which indicates that the sample contained a small but sufficient amount of specific  
359 contaminants to completely inhibit the amplification of BTV RNA. In at least one of the other  
360 cases, the animal was probably infected long time before sampling and had already cleared  
361 the virus from its blood stream. The corresponding sample was received in October 2006 and  
362 the viral RNA load in several animals on the same farm was rather low (Ct: 35-37) while the  
363 antibody titres were very high (PN: 0-5), which is indicative of a late infection based on our  
364 profiling results (see below). All together, the present study clearly suggests that the number  
365 of false-negative RT-qPCR results is rather low. Although it is impossible to estimate the  
366 number of combined cELISA/RT-qPCR false-negatives in case of early infections, the  
367 number is most probably also very low as in the majority of cases BTV RNA can already be  
368 detected before the onset of clinical signs (MacLachlan, 2004).

369

370 In sheep 66% of the notified cases were confirmed by cELISA, whereas in cattle 50% of the  
371 cases were apparently misdiagnosed on the basis of clinical signs (Fig. 2B). This percentage  
372 confirmed cases in sheep at the end of the 2006 epidemic was considerably higher compared  
373 to the early phase of the epidemic when it was only 29% (Toussaint et al., 2007b), which was  
374 probably due to the organisation from September 2006 onwards of several informational  
375 meetings on the topic of BT for field veterinarians. The noted improvement in BT clinical

376 diagnosis, thus, demonstrates the importance of such initiatives at national/regional level.  
377 However, during these training sessions, strong emphasis was put on the special situation in  
378 cattle (clear clinical signs), which might have caused an over-notification of cases in cattle as  
379 the percentage confirmed cases decreased from 68% to 50% (Toussaint et al., 2007b).  
380  
381 During the BT 2006 crisis in Belgium, a commercial cELISA test was used both as a  
382 screening and diagnostic tool. A comparison of the PN frequency distributions clearly  
383 indicated that the profile depends on the immune/health status of the population. In BTV-  
384 naïve populations none of the animals showed elevated levels of anti-BTV antibodies and,  
385 therefore, a typical unimodal profile is seen (Fig. 3A). Compared to the frequency distribution  
386 of BT cELISA results of cattle sera from French BTV-free areas using the VMRD ELISA kit  
387 between 2001 and 2004 (Biteau-Coroller et al., 2006), the distributions of the Belgian cattle  
388 sera from the surveys in 2004, 2005 and September 2006 are skewed more to the negative  
389 classes. Since the profile of the cattle population from the September 2006 survey is  
390 comparable to those from the surveys in 2004 and 2005, it is reasonable to conclude that this  
391 population was indeed still free of BTV infection at the time of sampling. As such, serological  
392 profiling could be a powerful tool to help decision makers substantiate the disease-free status  
393 of a region or zone.  
394 During the course of the epidemic the profile changed and became bimodal in shape with the  
395 additional mode corresponding to the infected animals (Fig. 3B). The profile of the suspected  
396 animals is comparable to the one previously found in a BTV infected area in Corsica (France)  
397 in 2002 (Biteau-Coroller et al., 2006). A comparison of the profiles obtained before (Fig. 3A)  
398 and during (suspected cases) (Fig. 3B) the Belgian epidemic shows the appearance of an  
399 intermediate zone in the profile of an infected population with numerous animals exhibiting  
400 PN values in the cut-off or near cut-off classes. This intermediate zone appears soon after

401 BTV enters into the population and is a clear indicator of virus transmission. Further  
402 comparison to the profile obtained at the end of the epidemic (January 2007 survey, Fig. 3C)  
403 shows a clear shift towards the lowest PN class (PN: 0-5) representing strong positives. The  
404 appearance or disappearance of the intermediate zone is thus correlated with virus circulation  
405 and provides valuable information, which would be entirely overlooked if only positive and  
406 negative results were being considered. Checking for the appearance of reactors in this  
407 intermediate range could be used as an early warning criterion in the follow-up of sentinel  
408 herds. In contrast, shifting to the most positive antibody class at the end of an epidemic  
409 indicates the decrease or disappearance of virus transmission by the midges.

410

411 Due to the high cost and length of the procedure, RT-qPCR was only used as a diagnostic tool  
412 throughout the crisis and not as a mass-screening tool. The frequency distribution profile of  
413 the Ct-values displayed a bimodal shape characteristic of an infected population (Fig 4). In  
414 contrast to the cELISA, the position of the positive mode differed depending on the host  
415 species. This finding supports our statement that profiling gives more information than a  
416 dichotomised (pos/neg) analysis used to calculate sensitivity and specificity and showing no  
417 difference between both species. In general, the virus accumulated to higher levels in the  
418 blood of sheep than that of cattle. This might explain the higher case fatality in sheep (42%)  
419 compared to cattle (18%) during the epidemic.

420 The Ct distribution profile of the tissue samples was similar to that observed for blood  
421 samples but with the peak corresponding to positive animals slightly shifted towards the right  
422 (Fig. 4). This suggests a lower viral load in or viral RNA recovery from tissue samples. Since  
423 the presented tissue samples were frequently of poor quality, as judged by the internal beta-  
424 actin control, this difference could at least in part be due to the degradation of the viral RNA.

425 This observation highlights the necessity of rapid transport of tissue samples under  
426 refrigerated conditions to avoid false negative results.

427

428 At the end of the Belgian BT epidemic in 2006, combined serological and molecular profiling  
429 was used to evaluate the laboratory results of an individual animal with clinical signs and  
430 suspected of BTV infection. When high antibody levels against BTV (PN 0-5) together with  
431 low BTV-RNA levels (high Ct values) were observed, it was concluded that the animal had  
432 become infected with BTV several weeks ago and that, therefore, the actual clinical signs  
433 most probably were not caused by a BTV infection. In contrast, an animal with a cELISA  
434 result of 10-35 PN and a Ct value <33, is highly indicative of a recent infection.

435

436 In order to declare a farm free of BTV all positive animals were resampled and retested one  
437 month post initial diagnosis. Analysis of 75 of these paired samples demonstrated that the  
438 viral load might change substantially over a one-month period. In general the viral load  
439 decreased with 2.71 Ct-values. Consequently, animals with a Ct-value of less than 35 at T1  
440 will certainly remain BTV-positive by RT-qPCR for more than one month. To avoid any  
441 unnecessary analysis it would, therefore, be more appropriate to only retest these animals after  
442 2 to 3 months. It is, however, also important to point out that the detection of viral RNA is not  
443 always sufficient to determine the infectious status of the animal (MacLachlan, 2004). Since  
444 the RT-qPCR used in this study is at least 100 times more sensitive than virus isolation  
445 (Toussaint et al., 2007a), a weak positive RT-qPCR result might indeed be clinically or  
446 epidemiologically irrelevant. This is particularly pertinent for non-recently infected animals,  
447 which often exhibit a low viral load in the presence of a high antibody titre. Further research  
448 is, therefore, urgently needed to correlate the viral load or Ct-value of an animal with its  
449 infectious status.

450

451 **5. Conclusion**

452 Based on the antibody frequency distribution and on the ROC analysis, it was concluded that  
453 ‘ID Screen<sup>®</sup> Bluetongue Competition’ ELISA should be used at a different cut-off both for  
454 diagnosis and for screening. The use of RT-qPCR as reference test during the epidemic,  
455 instead of the not-easy-to-use VI, is supported by the results of a Bayesian analysis. BT  
456 serological and molecular profiling through the analysis of frequency distributions of the  
457 results of a cELISA and a RT-qPCR with well-known performance characteristics provides  
458 valuable information for the control of BT that could be of use to decision makers. In order to  
459 declare a farm free of BTV, resampling of animals with a Ct-value of less than 35 one month  
460 after initial sampling seems pointless, unless Ct-values are correlated to the infectious status  
461 of the BTV.

462

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469

470 **References**

471 Bergmann, I.E., Neitzert, E., Malirat, V., Ortiz, S., Colling, A., Sanchez, C., Correa Melo, E.,  
472 2003. Rapid serological profiling by enzyme-linked immunosorbent assay and its use as  
473 an epidemiological indicator of foot-and-mouth disease viral activity. Arch. Virol. 148,  
474 891-901.

- 475 Biteau-Coroller, F., Gerbier, G., Stark, K.D., Grillet, C., Albina, E., Zientara, S., Roger, F.,  
476 2006. Performance evaluation of a competitive ELISA test used for bluetongue antibody  
477 detection in France, a recently infected area. *Vet. Microbiol.* 118, 57-66.
- 478 Boelaert, F., Biront, P., Soumare, B., Dispas, M., Vanopdenbosch, E., Vermeersch, J.P.,  
479 Raskin, A., Dufey, J., Berkvens, D., Kerkhofs, P., 2000. Prevalence of bovine herpesvirus-  
480 1 in the Belgian cattle population. *Prev. Vet. Med.* 45, 285-95.
- 481 Bréard, E., Sailleau, C., Hamblin, C., Zientara, S., 2005. Bluetongue virus in the french island  
482 of reunion. *Vet. Microbiol.* 106, 157-165.
- 483 Cagienard, A., Thur, B., Griot, C., Hamblin, C., Stark, K.D., 2006. No evidence of bluetongue  
484 virus in Switzerland. *Vet. Microbiol.* 116, 13-20.
- 485 Calistri, P., Giovannini, A., Conte, A., Nanninni, D., Santucci, U., Patta, C., Rolesu, S.,  
486 Caporale, V., 2004. Bluetongue in Italy: Part I. *Vet. Ital.* 40, 243-251.
- 487 Collins, M.T., 2002. Interpretation of a commercial bovine paratuberculosis enzyme-linked  
488 immunosorbent assay by using likelihood ratios. *Clin. Diagn. Lab. Immunol.* 9, 1367-  
489 1371.
- 490 Communication Directorate General, 2006. Bluetongue confirmed in France. EU Middy-  
491 express. [cited 2006 Aug 31]. Available from <http://europa.eu.int/rapid/>, reference:  
492 MEX/06/0831.
- 493 De Clercq, K., 1995. Diagnostic aspects of trade. Report of the Session of the FAO Research  
494 Group of the Standing Technical Committee of the European Commission for the Control  
495 of Foot-and-Mouth Disease, September 20-22, Vladimir, Russian Federation, p. 42-44.
- 496 De Clercq, K., 1998. Reduction of singleton reactors against swine vesicular disease virus by  
497 a combination of virus neutralisation test, monoclonal antibody-based competitive ELISA  
498 and isotype specific ELISA. *J. Virol. Methods* 70, 7-18.



- 499 European Commission Reference Laboratory (CRL) for Bluetongue, 2006. Bluetongue virus  
500 in the Netherlands identified as serotype 8 by Institute for Animal Health. ProMed.  
501 August 28, 2006. Accessed at <http://www.promedmail.org>, archive no.: 20060828.2448.
- 502 Gerdes, G.H., 2004. A south african overview of the virus, vectors, surveillance and unique  
503 features of bluetongue. *Vet. Ital.* 40, 39-42.
- 504 Gibbs, E.P., Greiner, E.C., 1994. The epidemiology of bluetongue. *Comp. Immunol.*  
505 *Microbiol. Infect. Dis.* 17, 207-220.
- 506 Goris, N., De Clercq, K., 2005. Quality assurance/quality control of foot and mouth disease  
507 solid phase competition enzyme-linked immunosorbent assay-Part II. Quality control:  
508 comparison of two charting methods to monitor assay performance. *Rev. Sci. Tech.* 24,  
509 1005-1016.
- 510 Goris, N., Merkelbach-Peters, P., Diev, V.I., Verloo, D., Zakharov, V.M., Kraft, H.P., De  
511 Clercq, K., 2007a. European Pharmacopoeia foot-and-mouth disease vaccine potency  
512 testing in cattle: between test variability and its consequences. *Vaccine* 25, 3373-3379.
- 513 Goris, N., Praet, N., Sammin, D., Yadin, H., Paton, D., Brocchi, E., Berkvens, D., De Clercq,  
514 K., 2007b. Foot-and-mouth disease non-structural protein serology in cattle: use of a  
515 Bayesian framework to estimate diagnostic sensitivity and specificity of six ELISA tests  
516 and true prevalence in the field. *Vaccine* 25, 7177-7196.
- 517 Hassan, A., 1992. Epidemiology of bluetongue virus infection in Malaysia. In: Walton, T.E.,  
518 Osburn, B.I. (Eds.), *Bluetongue, African horse sickness & Related Orbiviruses*. CRC  
519 Press, Boca Raton, pp. 155-161.
- 520 Katz, J., Alstad, D., Gustafson, G., Evermann, J., 1994. Diagnostic analysis of the prolonged  
521 bluetongue virus RNA presence found in the blood of naturally infected cattle and  
522 experimentally infected sheep. *J. Vet. Diagn. Invest.* 6, 139-42.

- 523 Koumbati, M., Mangana, O., Nomikou, K., Mellor, P.S., Papadopoulos, O., 1999. Duration of  
524 bluetongue viraemia and serological responses in experimentally infected European  
525 breeds of sheep and goats. *Vet. Microbiol.* 64, 277-285.
- 526 Lager, I.A., 2004. Bluetongue virus in south america: Overview of viruses, vectors,  
527 surveillance and unique features. *Vet. Ital.* 40, 89-93.
- 528 MacLachlan, N.J., 1994. The pathogenesis and immunology of bluetongue virus infection of  
529 ruminants. *Comp. Immunol. Microbiol. Infect. Dis.* 17, 197-206.
- 530 MacLachlan, N.J., Nunamaker, R.A., Katz, J.B., Sawyer, M.M., Akita, G.Y., Osburn, B.I.,  
531 Tabachnick, W.J., 1994. Detection of bluetongue virus in the blood of inoculated calves:  
532 Comparison of virus isolation, PCR assay, and in vitro feeding of *Culicoides variipennis*.  
533 *Arch. Virol.* 136, 1-8.
- 534 MacLachlan, N.J., 2004. Bluetongue: pathogenesis and duration of viraemia. *Vet. Ital.* 40,  
535 462-467.
- 536 MacLachlan, N.J., Osburn, B.I., 2006. Impact of bluetongue virus infection on the  
537 international movement and trade of ruminants. *J. Am. Vet. Med. Assoc.* 228, 1346-1349.
- 538 Mellor, P.S., Boorman, J., Baylis, M., 2000. *Culicoides* biting midges: Their role as arbovirus  
539 vectors. *Ann. Rev. Entomol.* 45, 307-340.
- 540 Mertens, P.P., Diprose, J., Maan, S., Singh, K.P., Attoui, H., Samuel, A.R., 2004. Bluetongue  
541 virus replication, molecular and structural biology. *Vet. Ital.* 40, 426-437.
- 542 Mo, C.L., Thompson, L.H., Homan, E.J., Oviedo, M.T., Greiner, E.C., Gonzalez, J., Saenz,  
543 M.R., 1994. Bluetongue virus isolations from vectors and ruminants in central america  
544 and the caribbean. Interamerican bluetongue team. *Am. J. Vet. Res.* 55, 211-215.
- 545 OIE, 2004. World Organisation for Animal Health - Manual of diagnostic tests and vaccines  
546 for terrestrial animals, 5th edition, OIE, Paris, 1178 pp.

- 547 OIE, 2006. World Organisation for Animal Health - Terrestrial animal health code, 15th  
548 edition, OIE, Paris.
- 549 OIE Animal Health Department, 2006. Bluetongue - Netherlands, Belgium, Germany-OIE.  
550 ProMed. August 21, 2006. Accessed at <http://www.promedmail.org>, archive no.:  
551 20060821.2353.
- 552 Purse, B.V., Mellor, P.S., Rogers, D.J., Samuel, A.R., Mertens, P.P., Baylis, M., 2005.  
553 Climate change and the recent emergence of bluetongue in Europe. *Nat. Rev. Microbiol.* 3,  
554 171-181.
- 555 Tabachnick, W. J., 2004. Culicoides and the global epidemiology of bluetongue virus  
556 infection. *Vet. Ital.* 40, 145-150.
- 557 Thiry, E., Saegerman, C., Guyot, H., Kirten, P., Losson, B., Rollin, F., Bodmer, M.,  
558 Czaplicki, G., Toussaint, J.F., De Clercq, K., Dochy, J.M., Dufey, J., Gillemans, J.L.,  
559 Messeman, K., 2006. Bluetongue in Northern Europe. *Vet. Rec.* 159, 327.
- 560 Toussaint, J.F., Kerkhofs, P., De Clercq, K., 2006a. Influence des changements climatiques  
561 globaux sur la progression des arboviroses. *Ann. Med. Vet.*, 150: 56-63.
- 562 Toussaint, J.F., Vandebussche, F., Mast, J., Demeestere, L., Goris, N., Van Dessel, W.,  
563 Vanopdenbosch, E., Kerkhofs, P., Zientara, S., Sailleau, C., Czaplicki, G., Depoorter, G.,  
564 Dochy, J.M., De Clercq, K., 2006b. Bluetongue in Northern Europe. *Vet. Rec.* 159, 327.
- 565 Toussaint, J.F., Sailleau, C., Bréard, E., Zientara, S., De Clercq, K., 2007a. Bluetongue virus  
566 detection by two real-time RT-qPCRs targeting two different genomic segments. *J. Virol.*  
567 *Methods* 140, 115-123.
- 568 Toussaint, J.F., Sailleau, C., Mast, J., Houdart, P., Czaplicki, G., Demeestere, L.,  
569 Vandebussche, F., Van Dessel, W., Goris, N., Bréard, E., Bounaadja, L., Thiry, E.,  
570 Zientara, S., De Clercq, K., 2007b. Bluetongue in Belgium, 2006. *Emerg. Infect. Dis.* 13,  
571 614-616. Available from <http://www.cdc.gov/EID/content/13/4/614.htm>

- 572 Uppal, P.K., 1992. Present position of AHS and BT in India. In: Walton, T.E., Osburn, B.I.  
573 (Eds.), *Bluetongue, African horse sickness & Related Orbiviruses*. CRC Press, Boca  
574 Raton, pp. 175-180.
- 575 Verwoerd, D.W., Erasmus, B.J., (2004). Bluetongue. In: Coetzer, J.A.W., Tustin, R.C. (Eds.),  
576 *Infectious diseases of livestock*. Oxford University Press, New York, pp. 1201-1220.
- 577 Walton, T.E., 2004. The history of bluetongue and a current global overview. *Vet. Ital.* 40,  
578 31-38.
- 579 Yang, S., Rothman, R.E., 2004. PCR-based diagnostics for infectious diseases: Uses,  
580 limitations, and future applications in acute-care settings. *Lancet Infect. Dis.* 4, 337-348.
- 581 Zientara, S., Bréard, E., Sailleau, C., 2006. Bluetongue: Characterization of virus types by  
582 reverse transcription-polymerase chain reaction. *Dev. Biol. (Basel)* 126, 187-196, 326-  
583 187.
- 584 Zweig, M.H., Campbell, G., 1993. Receiver-operating characteristic (roc) plots: A  
585 fundamental evaluation tool in clinical medicine. *Clin. Chem.* 39, 561-577.

586 **Tables**

587 Table 1: Contingency table of the results obtained by cELISA and RT-qPCR. Doubtful

588 cELISA results (N= 9) are added to the positives.

589

		RT-qPCR		
		Positive	Negative	Total
cELISA	Positive	320	3	323
	Negative	46	305	351
Total		366	308	674

590

591

592 Table 2: Combined frequency distributions of cELISA results and RT-qPCR results. Most  
 593 results were concordant but in 46 cases infection was only detected by RT-qPCR.  
 594 Nine doubtful cELISA results were all positive by RT-qPCR and in 3 cases no viral  
 595 RNA could be detected by RT-qPCR in spite of a positive cELISA result.  
 596

		RT-qPCR							
		20-22	23-25	26-28	29-31	32-34	35-37	38-40	>40
cELISA	0-5	4	14	28	48	58	18	5	2
	6-15	4	23	37	18	19	3		
	16-25	2	5	7	1	3			1
	26-35	1	4	3	1				
	36-45	1	2	4	2				
	46-55	2	2	1	1				3
	56-65	1	3	2	1				2
	66-75		1	2	1				5
	76-85			2	1	2			5
	>85	2	4	3	5	6	2	2	283

597  
 598

599 Table 3: Bayesian analysis using only cattle or sheep sera gave estimates for the test  
 600 characteristic of the cELISA and the RT-qPCR in the absence of a gold standard and  
 601 assuming conditional dependence between both tests. The overlapping confidence  
 602 intervals clearly indicate that no significant differences in test performance  
 603 characteristics could be demonstrated for both species.  
 604

		Sheep	Cattle
RT-qPCR	Se (95% CI)	99.55 (99.03 - 99.98)	99.50 (99.02 - 99.97)
	Sp (95% CI)	98.48 (97.07 - 99.93)	98.47 (97.07 - 99.92)
cELISA	Se (95% CI)	85.05 (77.96 - 91.14)	88.65 (85.28 - 92.29)
	Sp (95% CI)	98.46 (96.32 - 99.82)	97.80 (95.75 - 99.47)

605

606 **Figures**

607 Fig.1. Situation of bluetongue in Belgium at the end of August 2006 (Source FAVV). Sixty-  
608 five positive herds are shown in the first 20 km zone (black dots). The second 20 km zone was  
609 due to the herd in the North of France. The rest of Belgian was considered as one 100 km  
610 zone.

611

612 Fig. 2. ROC analysis: using the RT-qPCR as standard of comparison, the cut-off point at  
613 which both sensitivity and specificity were optimal occurred at a percentage negativity of 66.

614

615 Fig. 3A. Frequency distribution of cELISA results of serum samples from cattle taken in 2004  
616 (S 2004), in 2005 (S 2005) and early September 2006 (S 2006) in an area checked for  
617 freedom of infection and from cattle and sheep in artificial insemination centres (AIC 2006).

618

619 Fig. 3B. Frequency distribution of cELISA results of serum samples from cattle (Bo) and  
620 sheep (Ov) suspected of BT taken during the epidemic in 2006.

621

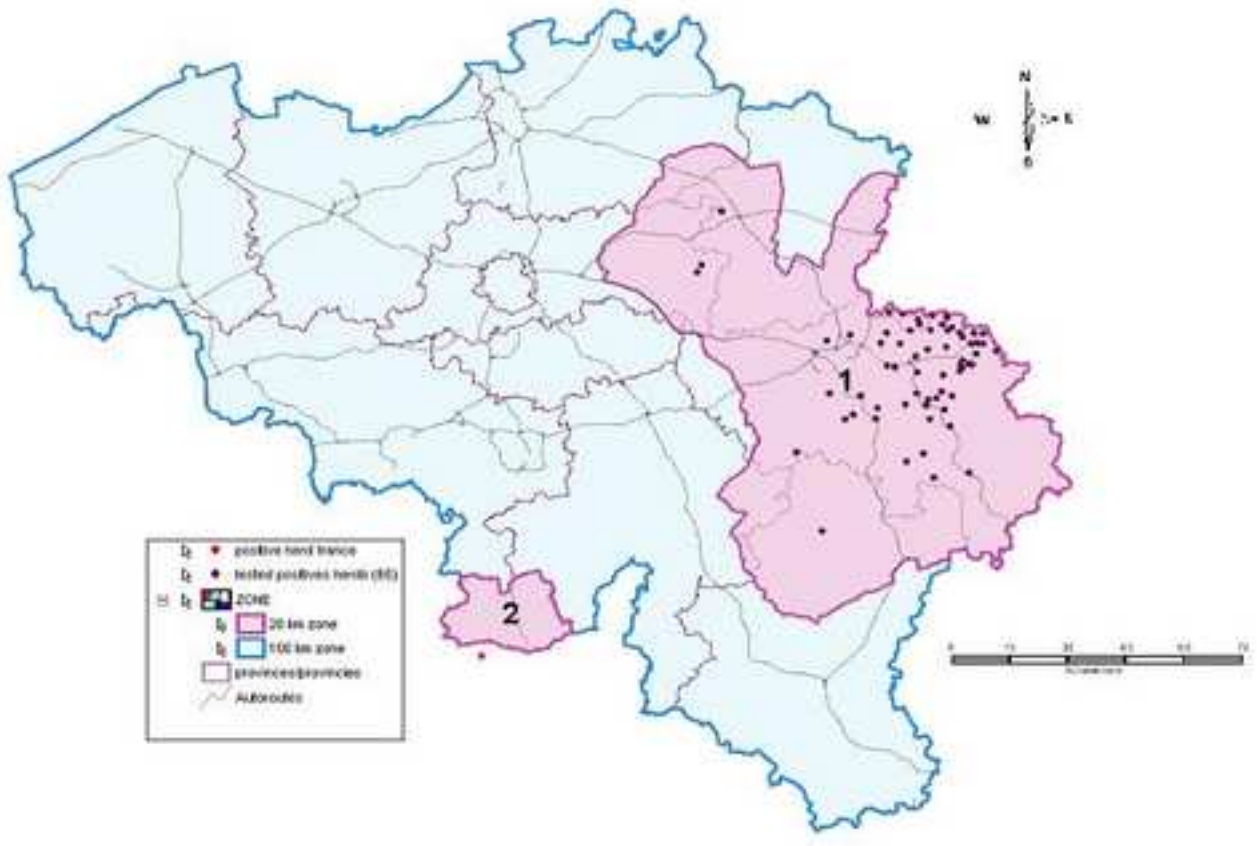
622 Fig. 3C. Frequency distribution of cELISA results of serum samples from cattle at the end of  
623 January 2007.

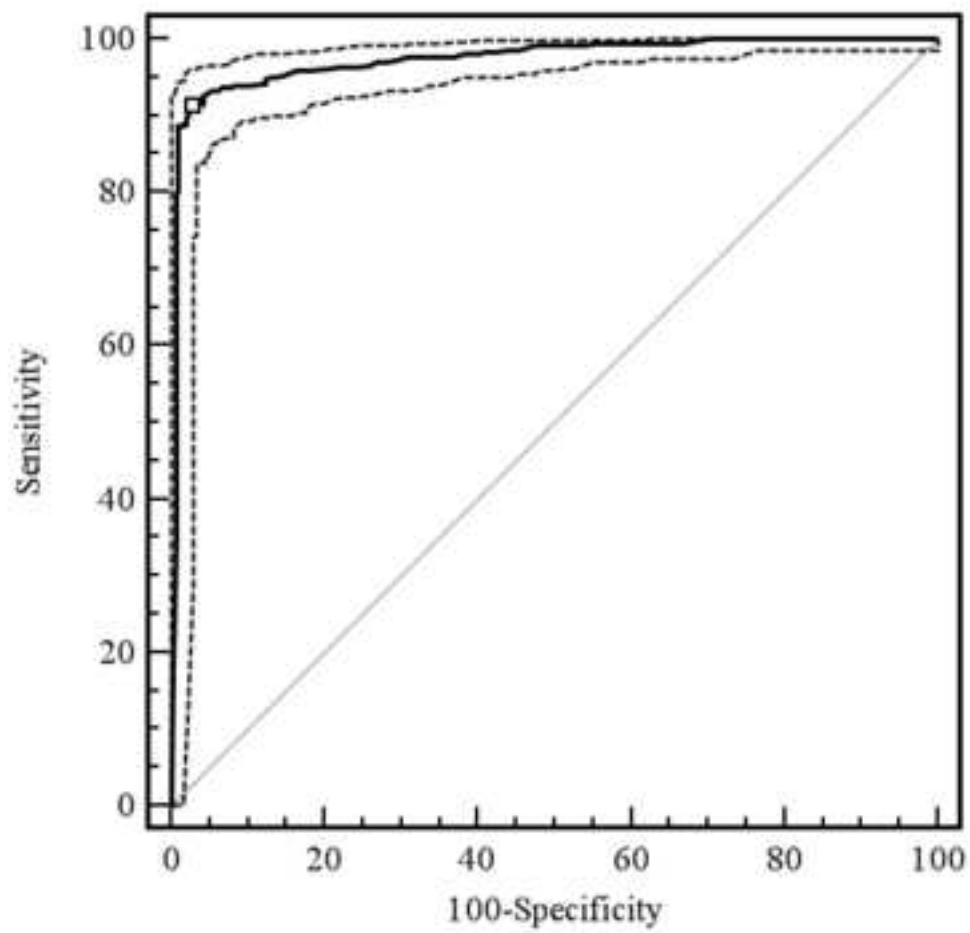
624

625 Fig. 4: Frequency distribution of RT-qPCR Ct-values on EDTA blood and tissue samples  
626 from cattle (Bo) and sheep (Ov) with clinical signs suspected of bluetongue during the  
627 bluetongue epidemic in 2006 and from cattle at the end of January 2007 (Bo Jan 2007).

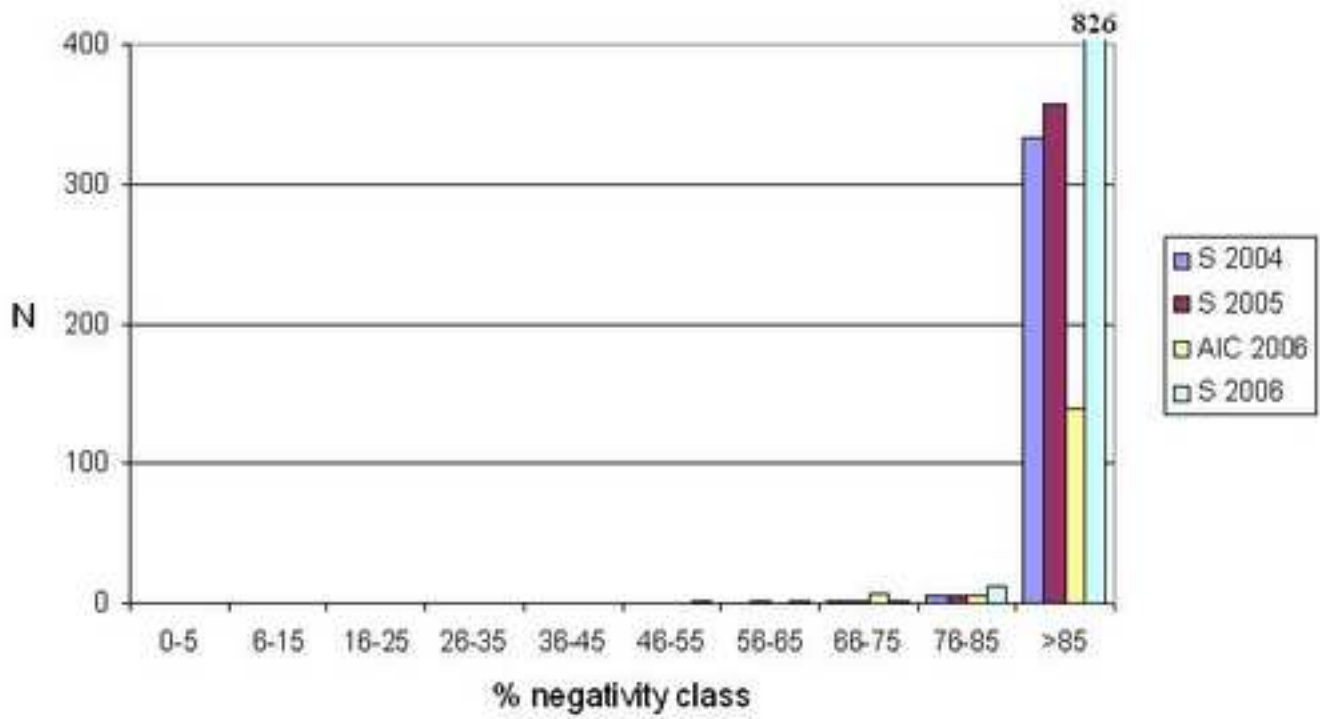


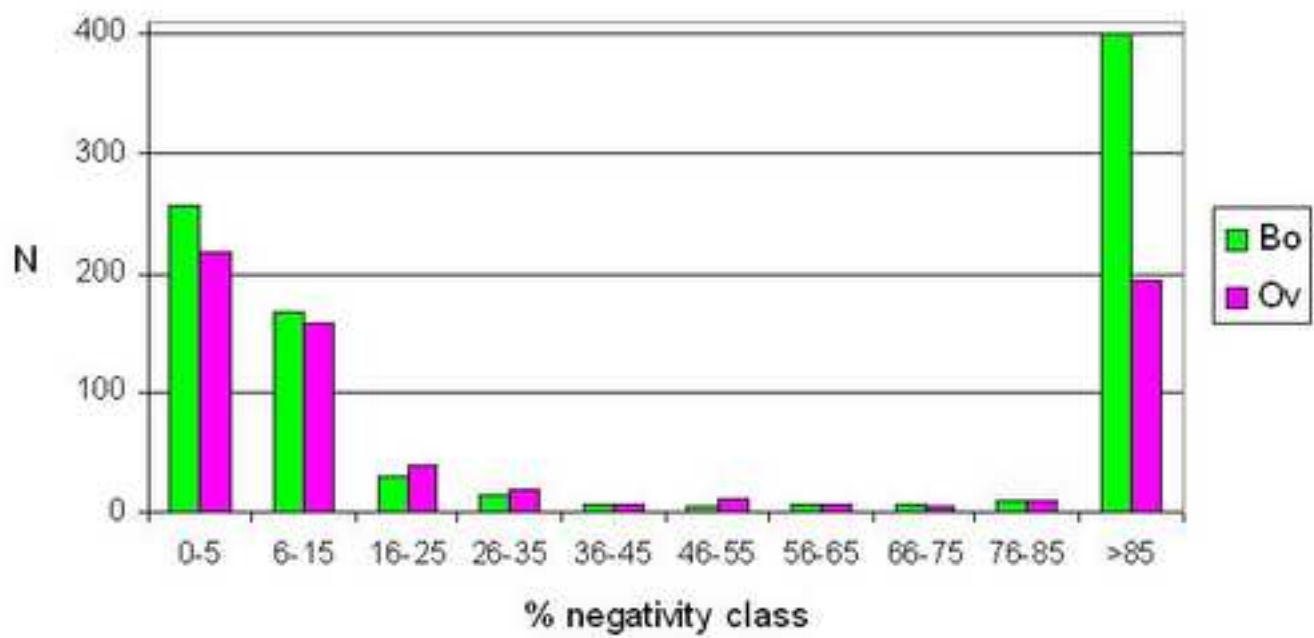
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