

# **Clinical pattern characterisation of cattle naturally infected by BTV-8**

## **Clinical characterisation of BTV-8 infected cattle**

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## **Abstract**

Forty-one cattle from seven Belgian farms and two French farms confirmed as infected with bluetongue virus serotype 8 (BTV-8) were monitored from the onset of clinical signs in order to describe the disease pattern and estimate the duration of blood RT-qPCR and cELISA positivity under field conditions. On each visit, blood samples were taken and a standardised

clinical form filled in for each animal. A clinical score was calculated for every week until the end of clinical signs. A classification and regression tree (CART) analysis was conducted to determine the most important clinical signs every week for the first seven weeks. The highest scores were recorded within two weeks of clinical onset. The first recorded clinical signs were quite obviously visible (tiredness and limited walking, conjunctivitis, lesions of nasal mucosa, nasal discharge). Skin lesions, a drop in milk production and weight loss appeared later in the course of the disease. A biphasic pattern regarding nasal lesions was noticed: the first peak concerned mainly congestive and ulcerative lesions, whereas the second peak mainly concerned crusty lesions. The median time estimated by survival analysis to obtain negative RT-qPCR results from the onset of clinical signs was 195 days (range 166 to 213 days) in the 23 cattle included in the analysis. Serological results remained strongly positive until the end of the study. These results should ensure more accurate detection of an emerging infectious disease and are of prime importance in improving the modelling of BTV-8 persistence in Europe.

Keywords: Bluetongue; BTV-8; Clinical signs; RT-qPCR; Cattle; cELISA

## **1. Introduction**

Bluetongue (BT) is a vector-borne viral disease of wild and domestic ruminants caused by a double-stranded RNA virus of the family *Reoviridae*, genus *Orbivirus* (Roy, 1992). The BT virus (BTV) consists of 26 serotypes (Maan et al., 2011), principally transmitted by several species of biting midges of the family *Ceratopogonidae*, genus *Culicoides* (Mellor and Wittmann, 2002). Since 1998, five distinct serotypes of BTV (1, 2, 4, 9 and 16) have spread across Southern and Central Europe (Mellor and Wittmann, 2002; Schwartz-Cornil et al., 2008). In August 2006, a sixth serotype—BTV-8—was first identified in Northern Europe,

from where it quickly spread throughout the Netherlands, Belgium, Luxembourg, Germany and Northern France (Darpel et al., 2007; Durand et al., 2010; Elbers et al., 2008b; Saegerman et al., 2008a; Saegerman et al., 2010; Toussaint et al., 2006). By the end of 2009, BTV-8 had spread to most countries in Western and Central Europe, including Austria, the Czech Republic, Denmark, Hungary, Italy, Luxembourg, Norway, Spain, Sweden and the United Kingdom.

Clinical signs of BTV are typically associated with sheep, as cattle generally remain asymptomatic (Guyot et al., 2007). The BTV-8 incursion in Europe caused cattle to show clinical signs that had never been reported previously (Landeg, 2007; Thiry et al., 2006). The clinical signs observed in this species in Europe have been described, but the corresponding stage of the disease was not specified as the information was very likely collected during occasional veterinarian inspections or reported by farmers (Elbers et al., 2008a; Elbers et al., 2009; Landeg, 2007; Le Gal et al., 2008; Thiry et al., 2006). Only experimental studies with a small sample of calves (Dal Pozzo et al., 2009; Darpel et al., 2007; Martinelle et al., 2011) have attempted to describe the course of the disease caused by BTV-8. On the other hand, cattle have been reported to remain positive to BTV by RT-qPCR for a long period of time (MacLachlan, 2004). This period has occasionally been estimated in field conditions (Katz et al., 1994) but more usually following experimental inoculations (Barratt-Boyes and MacLachlan, 1995). For BTV-8, no reliable field data are available (EFSA Panel on Animal Health and Welfare (AHAW), 2011).

We herein describe the results of a joint French-Belgian study to characterise the clinical course taken by BTV-8 infected cattle under field conditions. Blood samples were taken regularly to estimate the duration of blood RT-qPCR and cELISA positivity in cattle naturally infected by BTV-8.

## 2. Materials and methods

### 2.1. Animals

Animals included in the study were selected from zones where several BTV-8 outbreaks were confirmed in the 2006 summer for Belgium and in the 2008 summer for France. All the animals displaying BTV clinical signs for the first time (Guyot et al., 2008) on one farm and confirmed as BTV cases by RT-qPCR and cELISA were included in the study. In Belgium, the study was conducted on seven farms in the province of Liège, the most likely place of introduction of BTV-8 into Belgian ruminants (Saegerman et al., 2010). Twenty-one adult cattle (2.5 to 15 years old) of three different breeds (17 Holstein, 3 Red Pied Westphalian and 1 Limousine) were included. Eighteen were followed from August 2006 to October 2006 and three from August 2006 to April 2007.

Twenty adult cattle of Holstein, Normand and Simmental breeds were selected from two distinct farms located in the North of France. The clinical follow-up of the animals was conducted between August 2008 and July 2009.

There had been no records of bovine viral diarrhoea or infectious bovine rhinotracheitis occurrence in the selected farms. Farmers also declared that it was the first time they had observed that set of clinical signs in their animals. Therefore, considering the epidemiological context, it was unlikely that the observed clinical signs have been caused by other etiologies.

### 2.2. Follow-up of clinical signs and sampling

In Belgium, a standardised clinical form (Saegerman et al., 2008b) was filled in by three veterinarians on days 0 (clinical onset as noticed by the farmers), 7, 15, 21, 29 and 52. Blood samples were taken from three of these cattle on day 0 and then every 15 days in the first month and once every month until the first negative RT-qPCR result.

In France, two veterinarians were in charge of the clinical inspections and samplings on the two selected farms throughout the study. The same clinical standardised form used in

Belgium was filled in on each veterinarian visit. For some animals, the first visit occurred a few days after clinical onset and the date of appearance of clinical signs was reported by the farmer. For other animals, the first visit corresponded to the appearance of clinical signs. Afterwards, the selected animals were clinically inspected and blood samples were taken every week in the first month, every two weeks in the second and third months and then once every month. A year elapsed between the first visit and the last visit.

BTV clinical signs were classified into 19 categories (Table 1). The clinical score represents the sum of an animal's clinical signs. It was recorded every week from day 0 until the end of clinical signs for the 41 animals included in the study.

All blood samples were tested by RT-qPCR and cELISA.

## 2.3. Laboratory assays

The laboratory assays were conducted at the Laboratoire Nationale de Contrôle des Reproducteurs, Maisons-Alfort, France; the Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège and the Veterinary and Agrochemical Research Centre, Liège, Belgium.

### 2.3.1. BTV RT-qPCR

All the samples were evaluated with a pan-BTV RT-qPCR (Toussaint et al., 2007) targeting a segment 1 sequence, well conserved among BTV serotypes (BTV M ®, LSI, Lissieu, France). This RT-qPCR includes an internal control (a housekeeping RNA specific sequence). Briefly, 5 µL of RNA were denatured (3 minutes at 95°C) and incubated with 20 µL of the RT-qPCR mix in a thermocycler (ABIPRISM ® - APPLIED Biosystems). After reverse transcription (10 minutes at 45°C), a 40-cycle amplification was performed as follows: 1 cycle of 10

minutes at 95°C then 40 Cycles: 15 sec. at 95°C + 60 sec. at 60 °C. A result was considered positive when the cycle threshold [Ct] value < 40.

### 2.3.2. cELISA

BTV antibody levels were measured using the cELISA ‘ID Screen® Bluetongue Competition’ assay (ID VET, Montpellier, France) according to the manufacturer’s instructions. Results were expressed as % of negativity (PN) compared to the kit control and considered a positive, doubtful or negative result according to the cut-off settings provided by the manufacturer (PN ≤ 35 is positive; 35 < PN ≤ 45 is doubtful; PN > 45 is negative).

## 2.4. Statistical analyses

### 2.4.1. Classification and regression tree (CART) analysis

A CART analysis was conducted on the dataset for the first seven weeks (until the upper limit of the mean clinical score +/- standard deviation was below 1; see Figure 1) of the period observed, where the presence or absence of clinical signs during each week was used as the dependent variable and clinical signs were used as independent or predictor variables. Briefly, a CART analysis is a non-linear, non-parametric model that is fitted by binary recursive partitioning of multidimensional covariate space. Using CART 6.0 software (Salford Systems, San Diego, CA, USA), the analysis successively splits the dataset into increasingly homogeneous subsets until it is stratified to meet specified criteria. The Gini index was used as the splitting method, and a 10-fold cross-validation was used to test the predictive capacity of the obtained trees. For each node in a CART-generated tree, the “primary splitter” is the variable that best splits the node, maximizing the purity of the resulting nodes. Further details on CART are given in previously published articles (Breiman et al., 1984; Saegerman et al., 2004; Speybroeck et al., 2004; Porter et al., 2011; Saegerman et al., 2011).

#### 2.4.2. Survival analysis

All the 20 French cattle and three Belgian cattle — for which follow-up RT-qPCR results were available — were included in a survival analysis, used to estimate how long it takes for an event to occur. In this study, the event was the animal becoming negative to RT-qPCR. The duration of RT-qPCR positive blood results after the occurrence of the first clinical signs was determined using the non-parametric survival Kaplan-Meier method. Statistical analysis and graphs were performed with R (R development Core Team, 2009).

### 3. Results

#### 3.1. Follow-up of clinical signs

The highest scores in the 41 cattle (total of 415 observations) were recorded in the first two weeks after clinical onset (Figure 1). They gradually decreased until the 10<sup>th</sup> week, after which most of the animals showed no more clinical signs.

#### 3.2. CART analysis

To investigate the relative importance of clinical signs depending on the duration of the disease, a CART analysis was performed for the first seven weeks and the variable importance of clinical signs recorded for each week (Table 1). The most important clinical signs recorded first were conjunctivitis, lacrimation and peri-ocular dermatitis (variable importance of 100), tiredness, limited walking (variable importance of 92) and ulcerative lesions of nasal mucosa (variable importance of 32) and mucous, serous, aqueous nasal discharge (variable importance of 26). Ulcerative lesions of nasal mucosa were the most important clinical signs in the second and in the third week with addition of anorexia or loss of appetite. They remained important in the fourth week (variable importance of 91) and were

not prominent during the fifth and sixth week. In the seventh week lesions of nasal mucosa were recorded again as an important clinical sign (variable importance of 76) but lesions were mainly crusts instead of ulcerations. In the seventh week, the most important clinical sign was milk loss (variable importance of 100) followed by ulcerative lesions of nasal mucosa (variable importance of 76).

### 3.3. RT-qPCR and cELISA results

The Ct values for 23 animals for which RT-qPCR results were available are summarised in Figure 2. The lowest Ct value obtained was 20 within the first week of the onset of clinical signs. Between 24 and 31 weeks, all the animals had a  $Ct \geq 41$  (see following paragraph).

All the samples collected gave positive results in cELISA from the day of first sampling until the last date of sampling. For the 20 French cattle, PN values never exceeded 14% and the last blood samples were taken between 300 and 349 days after the appearance of clinical signs. For the three Belgian cattle, the last blood samples were taken on days 177, 177 and 213 respectively, and their PN values were below 10%.

### 3.4. Estimated time to RT-qPCR negative status using survival analysis

The Kaplan Meier survival curve shows that the time taken to obtain negative BTV-8 RT-qPCR results following the onset of clinical signs ranges from 166 to 213 days in the 23 cattle included in the survival analysis (Figure 3). The median time was of 195 days.

## 4. Discussion

A distinction must be made between clinical signs as they objectively occur during the course of a disease, and clinical signs that will lead to a veterinary call-out. Some moderate clinical



signs can easily remain unnoticed or are not severe enough to justify medication and are thus not recorded. This is the case for BTV-8 disease in cattle, which is considered a mild affection. A critical point is the observation pressure, which is directly related to the epidemiological context (a breeder will be more aware of an expected disease) and the potential economic losses, *i.e.* many diseases become apparent when animals are affected enough to significantly lower the breeder's income. Keeping these aspects in mind, it is likely that reported clinical signs of BTV-8 in cattle in the field up to now correspond to signs observed at stages of the disease when they were already prominent. Only a follow-up of cattle from the first detectable signs in the field could allow the course of the disease to be accurately described.

In our study, the first recorded clinical signs were quite obviously visible (conjunctivitis, lesions of nasal mucosa, nasal discharge). In comparison, in experimental infections the first clinical signs are usually oral inflammation of different grades, often unrelated to appetite loss (Dal Pozzo et al., 2009; Martinelle et al., 2011). A slight oral congestion would obviously remain unnoticed in the field whereas a thoroughly standardised clinical examination would clearly detect it, particularly since examinations in an experimental context would start before the very first signs had even occurred. It is interesting to note that in some experimental infections, when the clinical picture is mild, conjunctivitis may be the earliest recorded clinical sign (Martinelle et al., 2011). This could support the general mildness of the clinical cases encountered in the field. During the second week of clinical manifestations, ulcerative lesions of the nasal mucosa took over from ocular lesions. This confirms conjunctivitis as an acute lesion, whereas muzzle ulcerations tend to be chronic. No other clear clinical sign seemed to be reported during the second week. In the third week, nasal lesions remained in first place, but were then associated with anorexia or loss of appetite and ulcerative lesions of oral mucosa and excoriation. The latter is more likely a cause of anorexia or loss of appetite,

as oral inflammation is considered a early, long-lasting lesion in an experimental context, possibly leading to a lack of appetite and subsequently an examination of the oral cavity. In the fourth week, nasal lesions were preeminent, and weight loss noted as a consequence of loss of appetite. Weight loss took the lead during the fifth week. Milk loss probably started sooner, during the acute phase of the disease, but this loss can remain unnoticed several weeks before becoming clearly significant. Skin lesions of the udder, teat or vulva are also described as late lesions. Six weeks after the start of clinical manifestations, ocular lesions were again the most reported clinical entity. This could be related to the inclusion of “peri-ocular dermatitis”, which could also be regarded as a photosensitivity-like related lesion, already described as a late lesion, as opposed to conjunctivitis, which is clearly more acute. Milk loss was increasing, and swelling of the coronary band was also an important clinical sign. In the seventh week, milk loss asserted itself as the most frequently-reported clinical sign, followed by nasal lesions. A biphasic pattern regarding nasal lesions has already been reported in an experimental context (Martinelle et al., 2011), but with a shorter gap between the two peaks of nasal lesion manifestations. In our study, the first peak was more about congestive and ulcerative lesions, whereas the second week mainly concerned crusty lesions. Weight loss was still important during late weeks.

BTV nucleic acid may be detected by RT-PCR in the blood of infected cattle and sheep when the infectious virus can no longer be detected by virus isolation in cell culture or inoculation of susceptible sheep (MacLachlan et al., 2009; MacLachlan et al., 1994). BTV-8 RNA was detected by real time RT-PCR in the blood of calves up to 151-157 days post infection after experimental inoculation (Di Gialleonardo et al., 2011). In the current study, BTV-8 RNA was detected by real time RT-PCR up to 166 - 213 days after the onset of clinical signs, which is longer than described in the above experimental study. The persistence of BTV nucleic acid in the blood of infected ruminants has been linked to the normal lifespan of erythrocytes

(Barratt-Boyes and MacLachlan, 1995): *in vitro* virus particles persisted in invaginations of erythrocyte membranes (Brewer and MacLachlan, 1994). Erythrocytes are long-lasting cells and estimates of their normal circulatory lifespan range from 130 to 160 days in sheep and slightly longer in cattle (Katz et al., 1994). Long-term experimental studies conducted in sheep showed that BTV RNA was detected by real-time RT-PCR up to 133 days post-infection (three sheep inoculated with BTV-8) (Worwa et al., 2010). However, in another study, genome was continuously detected by RT-PCR in three sheep up to 111 - 222 days after infection with BTV-17 (Bonneau et al., 2002). It seems, therefore, that the range of BTV detection time by RT-PCR is similar in sheep and cattle. This observation should, however, be confirmed by future longitudinal studies including a larger population of naturally-infected sheep.

In conclusion, this longitudinal study gives important information concerning the clinical characterisation of cattle naturally infected with BTV-8 and their evolution over time. The collaboration between countries, the standardisation of clinical signs and the use of more advanced tools (e.g. classification and regression tree) could be the way to further collate information Europe-wide and ensure more accurate detection of emerging infectious diseases. Repeated sampling allowed us to estimate the duration of blood BTV-8 RT-qPCR positivity. The estimation of this duration is of prime importance for improving the modelling of BTV-8 persistence in Europe. Furthermore, the low PN cELISA values found in the naturally-infected animals included in the current study over a whole year suggest that these animals could be protected against future re-infections by BTV-8.

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398 Table 1. Variable importance in CART analysis during the first seven weeks

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400 Figure 1. Mean clinical score observed by week after the first clinical appearance (+/- SD)

401 X-axis: week(s) after the first clinical appearance; number of animals involved each period of  
402 time in brackets (during weeks 10 to 50, animals were observed several times). Y-axis: mean  
403 clinical score +/- standard deviation

404

405 Figure 2. RT-qPCR Ct value box plots for 23 cattle by week after the first clinical appearance

406 (a line indicates the median value, a box the inter-quartile range and bars the range)

407

408 Figure 3. Kaplan-Meier survival curve for time to obtain negative BTV-8 RT-qPCR results in

409 23 cattle

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		Variable importance						
		Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7
Clinical sign		1	2	3	4	5	6	7
Head	Conjunctivitis, lacrimation, peri-ocular dermatitis	100	38		33		100	
	Ulcerative lesions of nasal mucosa, crusts	32	100	98	91			76
	Mucous, serous, aqueous nasal discharge	26	1		100		28	
	Purulent nasal discharge		14		6		13	10
	Congestion, erythema, redness of buccal mucosa and/or muzzle		21	7	19		61	
	Ulcerative lesions of buccal mucosa, excoriation	11		24	44			
	Salivation, ptyalism, mouth foam			11	6		7	
	Swelling of the head, tongue, sub-maxillary area, jaws			18	22		16	
	Skin lesions of udder, teat or vulva	1			9	32	18	
	Swelling of coronary bands		7				62	3
	Lameness or generalised stiffness				2		5	3
	Anorexia or Loss of appetite			100	18	6	39	32
General	Tiredness, limited walking	92			2		47	
	Incapacity to stand up, prostration			2	1			3

Dyspnoea, buccal breathing, loud

breathing 22 5 19

Weight loss 62 100 5 41

Muscular atrophia 36

Anoestrus 53 9 5

Milk loss 34 69 78 100

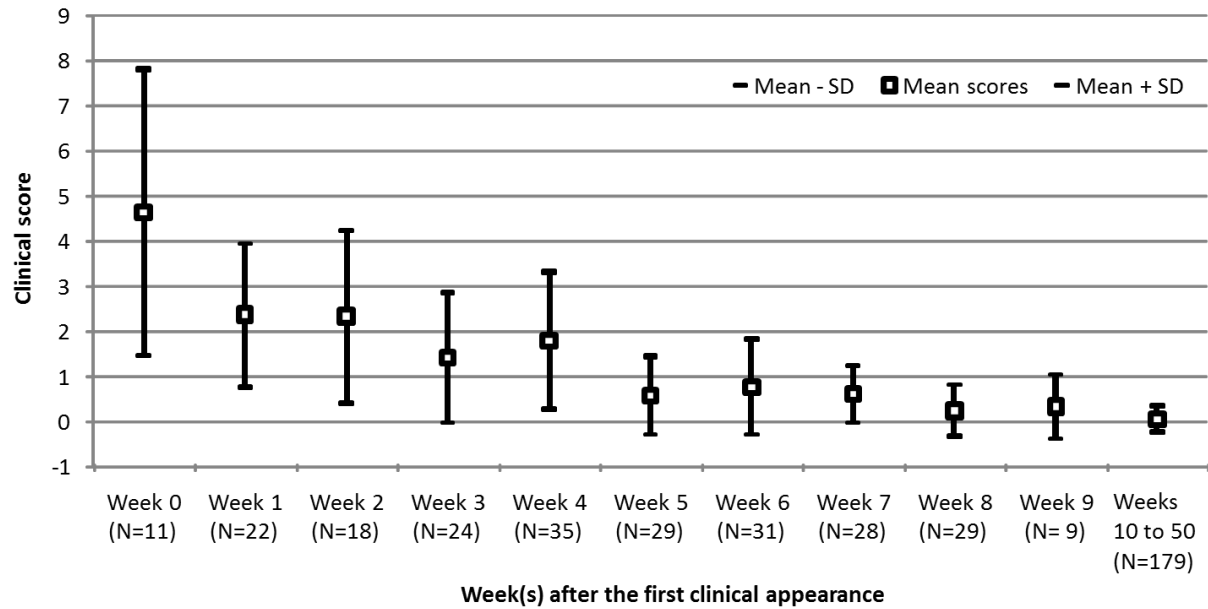
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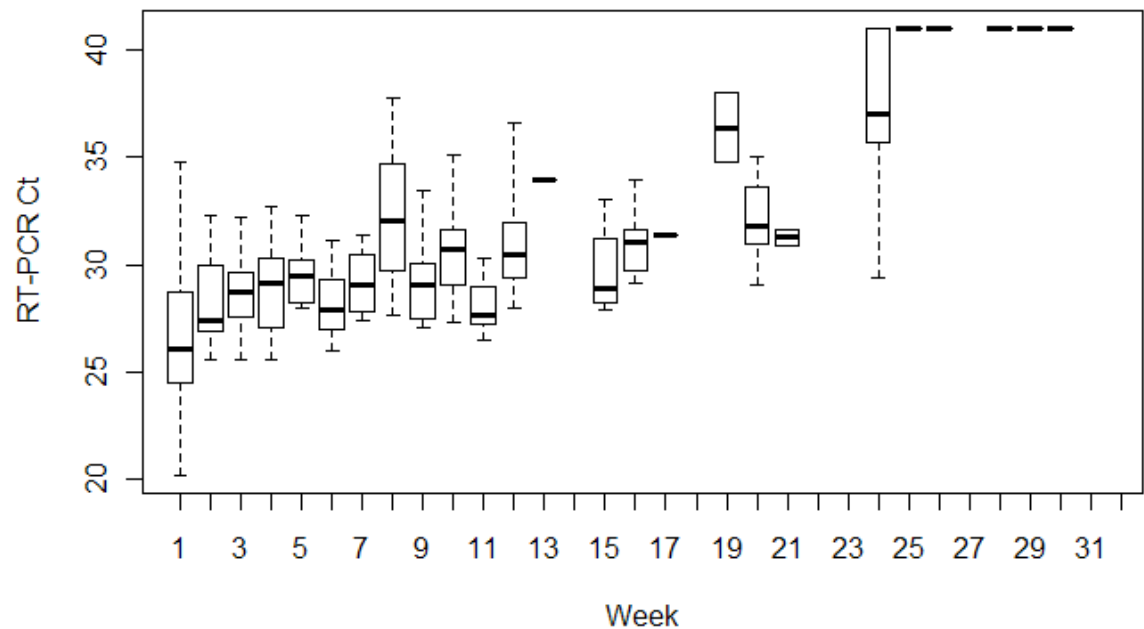
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**Figure 1.** Mean clinical score observed by week after the first clinical appearance (+/- SD)

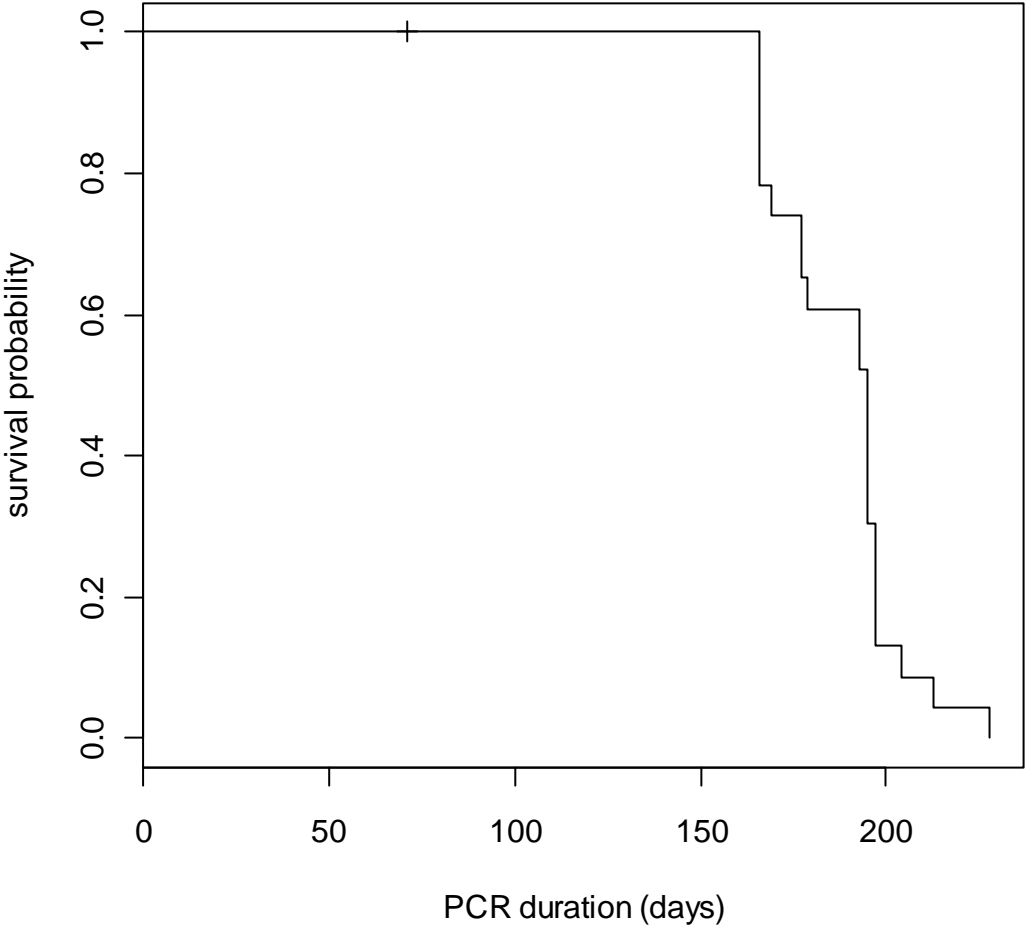
X-axis: week(s) after the first clinical appearance; lower limit of the interval; number of animals involved each period of time in brackets (during weeks 10 to 50, animals were observed several times). Y-axis: mean clinical score +/- standard deviation



**Figure 2.** RT-PCR Ct value box plots for 23 cattle by week after the first clinical appearance  
(a line indicates the median value, a box the inter-quartile range and bars the range)



424 **Figure 3.** Kaplan-Meier survival curve for time to obtain negative BTV-8 RT-PCR results in  
425 23 cattle



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