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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

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Abstract

In 2006 bluetongue (BT) emerged for the first time in North-Western Europe. Reliable diagnostic tools are essential in controlling BT but data on the diagnostic sensitivity (Se) and specificity (Sp) are often missing. This paper aims to describe and analyse the results obtained with the diagnostics used in Belgium during the 2006 BT crisis. The diagnosis was based on a combination of antibody detection (competitive ELISA, cELISA) and viral RNA detection by real-time RT-PCR (RT-qPCR). The performance of the cELISA as a diagnostic tool was assessed on field results obtained during the epidemic and previous surveillance campaigns. As the infectious status of the animals is unknown during an epidemic, a Bayesian analysis was performed. Both assays were found to be equally specific (RT-qPCR: 98.5%; cELISA: 98.2%) while the diagnostic sensitivity of the RT-qPCR (99.5%) was superior to that of the cELISA (87.8%). The assumption of RT-qPCR as standard of comparison during the bluetongue virus (BTV) epidemic proved valid based on the results of the Bayesian analysis. A ROC analysis of the cELISA, using RT-qPCR as standard of comparison, showed that the cut-off point with the highest accuracy occurred at a percentage negativity of 66, which is markedly higher than the cut-off proposed by the manufacturer. The analysis of the results was further extended to serological and molecular profiling and the possible use of profiling as a rapid epidemiological marker of the BTV in-field situation was assessed. A comparison of the serological profiles obtained before, during and at the end of the Belgian epidemic clearly showed the existence of an intermediate zone which appears soon after BTV (re)enters the population.

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The appearance or disappearance of this intermediate zone is correlated with virus circulation and provides valuable information, which would be entirely overlooked if only positive and negative results were considered.
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1. Introduction

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

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

This paper aims to describe and analyse the results obtained from the diagnostics used in Belgium during the 2006 BT crisis. The performance characteristics of a cELISA and a real-time RT-PCR (RT-qPCR) as diagnostic tools are assessed and discussed. The analysis of the results is not limited to the typical dichotomised classification of positives and negatives

based on a fixed cut-off value, but is extended to serological and molecular profiling through the analysis of frequency distributions. Bergmann et al. (2003) proved that profiling is particularly important for recognising hidden information in the intermediate zone when assessing foot-and-mouth disease virus persistence at the herd level. Earlier, De Clercq (1998) already showed that profiling could be used at the individual animal level to distinguish genuine positives from singleton reactors for swine vesicular disease. In the current study frequency distributions of reactivity levels of specific anti-BTV antibodies and BTV RNA in cattle and sheep are analysed. The resulting profiles are correlated to the epidemiological status and distinct distributions of reactivity patterns reflecting the various epidemiological situations are attained. This study demonstrates the possible use of profiling as a rapid epidemiological marker of the BTV in-field situation.

2. Methods

2.1. Biological samples

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

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

2.1.2. Serum and blood samples from different screening programmes

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

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

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

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

2.1.3. Paired blood samples

According to 'Procedure J124' of the Belgian Federal Agency for the Safety of the Food Chain (Food Agency), a BTV-infected farm could only be declared free of BT if 1 month post (T2) the initial sampling (T1) (i) none of the animals showed clinical signs and (ii) all animals positive by RT-qPCR at T1 were negative at T2. Animals still positive at T2 were only declared free of the disease after a second 1-month waiting period. During the epidemic, 75 of these T1/T2 paired blood samples were collected and analysed by RT-qPCR.

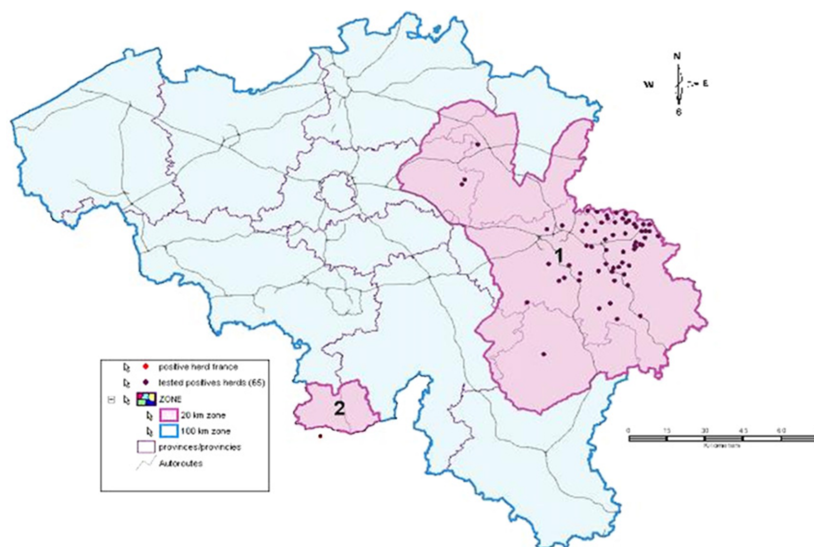

Fig. 1. Situation of bluetongue in Belgium at the end of August 2006 (Source FAVV). Sixty-five positive herds are shown in the first 20 km zone (black dots). The second 20 km zone was due to the herd in the North of France. The rest of Belgium was considered as one 100 km zone.

2.2. Diagnostic assays

2.2.1. Bluetongue virus antibody competitive ELISA

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

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

During the BT epidemic 2006 in Belgium a quantitative reverse-transcription PCR targeting BTV segment 5 (RT-qPCR_S5) was used to detect BT viral RNA in blood samples and tissue samples. Each test was performed in parallel with the RT-qPCR_ACT to amplify beta-actin mRNA as internal control. Both assays were carried out according to Toussaint et al. (2007a) with slight modifications. Briefly, total RNA was purified from 400 μl of red blood cells or 25 mg of tissue by Trizol extraction (Invitrogen) and denaturated by heating for 3 min at 95 °C with 10% dimethyl sulfoxyde (Sigma–Aldrich). Reverse transcription (RT) reactions were carried out using the Taqman reverse transcription reagents according to the manufacturer's instructions (Applied Biosystems). Real-time qPCR reactions consisted of 1× concentrated Taqman fast universal PCR master mix (Applied Biosystems), 375 nM (beta-actin) or 500 nM (bluetongue) of each primer, 250 nM of the Taqman probe conjugated to FAM at the 5′ end and to TAMRA at the 3′ end and 5 μl cDNA. Cycling conditions were as follows: 1 cycle at 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 cut-off was set at a Ct-value of 40 as previously determined by Toussaint et al. (2007a).

2.3. Data analysis

Bluetongue virus isolation is a non-easy-to-perform assay but the RT-qPCR was previously shown to

Table 1
Contingency table of the results obtained by cELISA and RT-qPCR. Doubtful cELISA results ($N = 9$) are added to the positives

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

be also highly accurate (Yang and Rothman, 2004; Toussaint et al., 2007a). Therefore the relative performance of the cELISA as a diagnostic assay was compared to the RT-qPCR_S5 using a 2×2 contingency table. The 95% confidence intervals of diagnostic sensitivity and specificity for the cELISA were calculated using Bayesian inference as described by Goris et al. (2007a). Moreover, since the true BTV infection status of the suspected BTV-infected animals is in fact unknown and RT-qPCR is not an officially accepted standard of comparison, a Bayesian model allowing the integration of field data and of expert opinion (prior information), was developed using the WinBUGS Version 1.4 as described by Goris et al. (2007b). Subsequently, a receiver operating characteristic (ROC) analysis was carried out using MedCalc 9.0 software to assess the performance of the cELISA in more detail. The sensitivity (i.e. true positive rate) is plotted against 100-specificity (i.e. false positive rate). Each point on the ROC curve thus represents a sensitivity/specificity pair corresponding to a particular cut-off value i.e. decision threshold. The optimum cut-off for diagnostic purposes is the point closest to the upper left corner (100% sensitivity, 100% specificity) corresponding to the value with the highest accuracy (minimal false negative and false positive results) (Zweig and Campbell, 1993). This analysis was performed on results obtained from samples tested in both RT-qPCR and cELISA ($N = 674$, see Section 2.1.1).

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

3. Results

3.1. Samples tested in cELISA and RT-qPCR

A total of 674 animals showing clinical signs were tested for the presence of anti-BTV antibodies as well as BTV RNA. A pair-wise comparison of the results indicated a high degree of concordance between cELISA and RT-qPCR with 616 (91%) animals being negative or positive in both tests (Table 1). Interestingly, all animals with a doubtful cELISA result (PN: 36–45) were clearly positive by RT-qPCR with Ct-values ranging between 21.8 and 30.3 (Table 2). In 46 animals BTV infection was only detected by RT-qPCR. Out of these, 22 animals exhibited Ct-values <33.0 and a PN value ranging from 46 to 85 indicating

Table 2
Combined frequency distributions of cELISA results and RT-qPCR results

	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

Most results were concordant but in 46 cases infection was only detected by RT-qPCR. Nine doubtful cELISA results were all positive by RT-qPCR and in three cases no viral RNA could be detected by RT-qPCR in spite of a positive cELISA result.

a recent infection in which the immune response is just mounting (Table 2). The Ct-values of the remaining 24 animals with a PN value >85 ranged from high to low (Ct: 22.5–38.9). Surprisingly, three animals were positive by cELISA but clearly negative by RT-qPCR. Given the high antibody titres that were detected (PN < 20), these cases most likely represent false-negative RT-qPCR results instead of false-positive ELISA results. Resampling of one of these animals only 1 week later, indeed, yielded a clear positive RT-qPCR result (Ct: 27.9). Furthermore, in all three cases the internal beta-actin control was positive. Retesting of two of the samples yielded the same result ruling out human error. After diluting the samples 1:10 a clear positive signal (Ct: 31.0) was obtained for one of these samples. The third sample could not be retested and therefore no clear follow-up could be done.

The relative sensitivity and specificity of the cELISA were estimated from the 2×2 contingency table (Table 1) using the RT-qPCR as non-official standard of comparison (gold standard). Considering the doubtful cELISA results as positives, the cELISA has a relative sensitivity (Se) of 87.4% (95% CI: 83.5–90.4) and a relative specificity (Sp) of 99.0% (95% CI: 97.2–99.6). Detailed analysis using only cattle sera revealed that the cELISA displayed a relative Se of 88.0% (95% CI: 83.7–91.7) and a relative Sp of 98.0% (95% CI: 95.7–99.5) while for sheep respective values of 84.5% (95% CI: 77.4–90.4) and 99.1%, (95% CI: 96.6–100.0) were found. The overlapping confidence intervals clearly indicate that no significant differences in test performance characteristics could be demonstrated for both species. However, the true BTV infection status of the 674 sampled animals was unknown and RT-qPCR is not an accepted gold standard test indicating that a latent class analysis should be performed to accurately predict the test performance parameters. The developed Bayesian model was validated both within and outside WinBUGS and yielded estimates for the test characteristics (posterior information) in the absence of a gold standard and assuming conditional dependence of the cELISA and the RT-qPCR test. Both assays were found to be equally specific with a diagnostic specificity (DSp) estimate for RT-qPCR of 98.5% (95% CI: 97.1–100.0) and for cELISA of 98.2% (95% CI: 96.3–99.6). The diagnostic sensitivity (DSe) of the RT-qPCR (99.5%, 95%CI: 99.0–100.0) was superior to that of the cELISA (87.8%, 95%CI: 85.1–91.1). Separate Bayesian analysis for cattle and sheep showed no significant difference in DSe or DSp (Table 3). The combination of a rather low Se with a high Sp results in a fairly high likelihood ratio for a positive test result (LR+) of 90, meaning that, during an epidemic, a positive cELISA result is ninety times as likely to be seen in a suspected animal with positive

Table 3
Bayesian analysis using only cattle or sheep sera gave estimates for the test characteristic of the cELISA and the RT-qPCR in the absence of a gold standard and assuming conditional dependence between both tests

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

The overlapping confidence intervals clearly indicate that no significant differences in test performance characteristics could be demonstrated for both species.

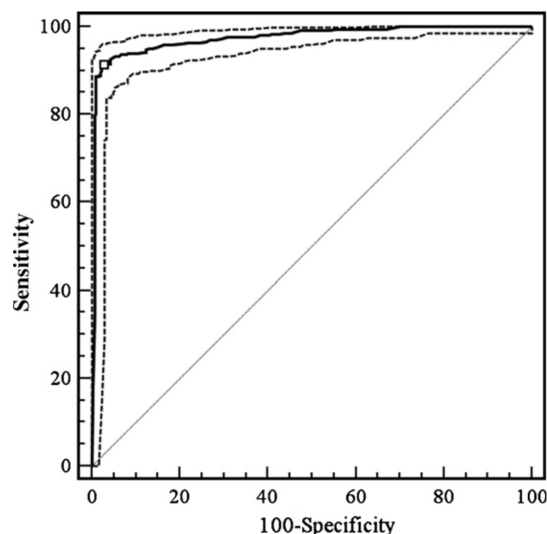

Fig. 2. ROC analysis: using the RT-qPCR as standard of comparison, the cut-off point at which both sensitivity and specificity were optimal occurred at a percentage negativity of 66.

RT-qPCR result as opposed to one without a positive RT-qPCR result (Collins, 2002). The performance of the cELISA as a diagnostic test was further assessed via a ROC analysis using the RT-qPCR as a reference test which was a justified assumption based on the high RT-qPCR sensitivity and specificity estimates observed using Bayesian analysis. The area under the curve (Fig. 2) was 0.974 with a 95% confidence interval between 0.959 and 0.985 indicating a high accuracy. The cut-off point at which both sensitivity and specificity were optimal for diagnostic purposes (see Section 2.3) occurred at a PN value of 66, which is markedly higher than the cut-off proposed by the manufacturer. Using this cut-off point, the sensitivity and specificity of the test were estimated to be 91.2% (95% CI: 87.8–93.9) and 97.3% (95% CI: 94.8–98.8), respectively.

3.2. Antibody competition ELISA

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

A completely different distribution profile was observed for the suspected cattle/sheep population ($N = 1573$), which showed a clear bimodal shape (Fig. 3B). This bimodal profile indicates that distinct negative categories coexisted with positive ones. Apart from the appearance of this additional mode corresponding to BTV infected animals, a markedly higher number ($N = 46$) of sera was noticed in the intermediate zone spanning the cut-off class (PN: 36–45) and the near cut-off PN classes (PN: 46–55 and 56–65). With the exception of the number of negatives, hardly any difference could be observed in the distribution profile of cattle and sheep.

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

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

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

3.4. Paired samples

Seventy-five EDTA blood samples were analysed by RT-qPCR at the time of clinical suspicion (T1) and 1 month later (T2). The difference in Ct value at T1 and T2 (T1 − T2) ranged from $\Delta Ct = +7.50$ (recent infection) to −9.90 (non-recent infection). The mean Ct-value at T1 was 30.64 (95% CI: 29.79–31.49) and 33.35 (95% CI: 32.66–34.04) at T2. The mean increase of 2.71 Ct (95% CI: 1.87–3.56) 1 month post-initial sampling indicates a decrease in the BTV RNA level present in the blood (less positive). The difference was highly significant ($P < 0.001$).

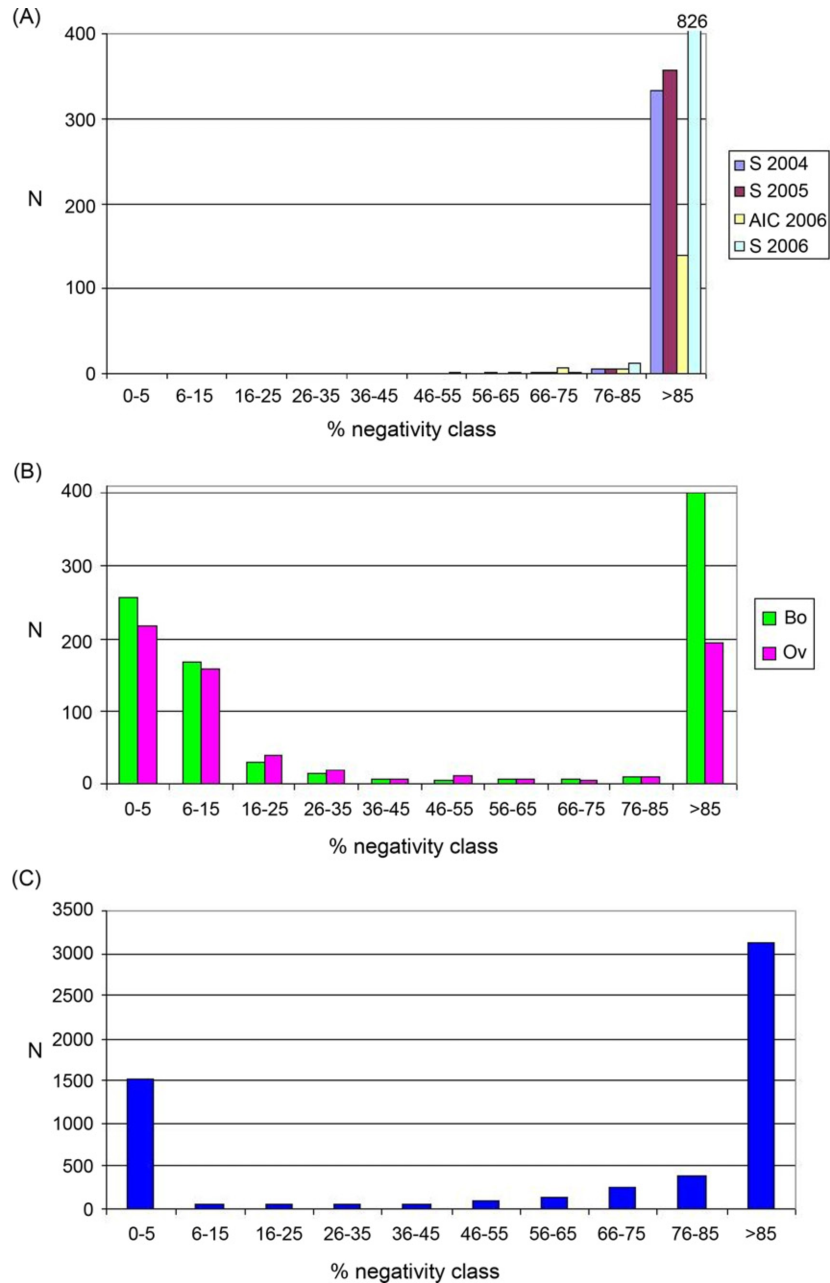

Fig. 3. (A) Frequency distribution of cELISA results of serum samples from cattle taken in 2004 (S 2004), in 2005 (S 2005) and early September 2006 (S 2006) in an area checked for freedom of infection and from cattle and sheep in artificial insemination centres (AIC 2006). (B) Frequency distribution of cELISA results of serum samples from cattle (Bo) and sheep (Ov) suspected of BT taken during the epidemic in 2006. (C) Frequency distribution of cELISA results of serum samples from cattle at the end of January 2007.

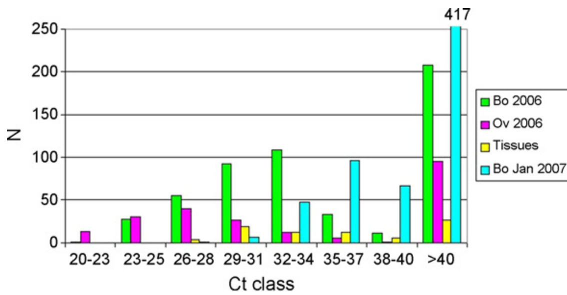

Fig. 4. Frequency distribution of RT-qPCR Ct-values on EDTA blood and tissue samples from cattle (Bo) and sheep (Ov) with clinical signs suspected of bluetongue during the bluetongue epidemic in 2006 and from cattle at the end of January 2007 (Bo Jan 2007).

4. Discussion

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

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

Based on a visual inspection of the frequency distributions of all naïve populations (Fig. 2A), a cut-off of 65 PN could be suggested for the cELISA, resulting in only three false-positives. The relative Se and Sp of the cELISA would accordingly change to 91.0% (95%CI: 87.5–93.5) and 97.4% (95%CI: 94.9–98.7) respectively with a decreased LR+ of 35. Consequently the number of false positives would slightly increase but more importantly during an epidemic the number of false negatives would decrease by 13. A ROC analysis indeed confirmed that the optimal cut-off for diagnostic purposes is situated around a PN-value of 66. Although Biteau-Coroller et al. (2006) suggested that cELISA is only suitable as a screening test, our results clearly demonstrate that it can also be used as a diagnostic tool to determine the disease at the individual level once a BT epizootic is confirmed. Considering the fit-for-purpose principal, we suggest using the cELISA at a cut-off of 66 PN as a diagnostic tool and with a cut-off of 45 PN for screening.

Most probably three false negative RT-qPCR results were obtained. Since BTV RNA could also be detected in one of the samples when the RNA was diluted 10 times, the initial false-negative result was presumably due to the presence of contaminating inhibitors. Interestingly, the internal control (beta-actin RNA) was easily detected in both the undiluted and diluted sample, which indicates that the sample

contained a small but sufficient amount of specific contaminants to completely inhibit the amplification of BTV RNA. In at least one of the other cases, the animal was probably infected long time before sampling and had already cleared the virus from its blood stream. The corresponding sample was received in October 2006 and the viral RNA load in several animals on the same farm was rather low (Ct: 35–37) while the antibody titres were very high (PN: 0–5), which is indicative of a late infection based on our profiling results (see below). All together, the present study clearly suggests that the number of false-negative RT-qPCR results is rather low. Although it is impossible to estimate the number of combined cELISA/RT-qPCR false-negatives in case of early infections, the number is most probably also very low as in the majority of cases BTV RNA can already be detected before the onset of clinical signs (MacLachlan, 2004).

In sheep 66% of the notified cases were confirmed by cELISA, whereas in cattle 50% of the cases were apparently misdiagnosed on the basis of clinical signs (Fig. 2B). This percentage confirmed cases in sheep at the end of the 2006 epidemic was considerably higher compared to the early phase of the epidemic when it was only 29% (Toussaint et al., 2007b), which was probably due to the organisation from September 2006 onwards of several informational meetings on the topic of BT for field veterinarians. The noted improvement in BT clinical diagnosis, thus, demonstrates the importance of such initiatives at national/regional level. However, during these training sessions, strong emphasis was put on the special situation in cattle (clear clinical signs), which might have caused an over-notification of cases in cattle as the percentage confirmed cases decreased from 68% to 50% (Toussaint et al., 2007b).

During the BT 2006 crisis in Belgium, a commercial cELISA test was used both as a screening and diagnostic tool. A comparison of the PN frequency distributions clearly indicated that the profile depends on the immune/health status of the population. In BTV-naïve populations none of the animals showed elevated levels of anti-BTV antibodies and, therefore, a typical unimodal profile is seen (Fig. 3A). Compared to the frequency distribution of BT cELISA results of cattle sera from French BTV-free areas using the VMRD ELISA kit between 2001 and 2004 (Biteau-Coroller et al., 2006), the distributions of the Belgian cattle sera from the surveys in 2004, 2005 and September 2006 are skewed more to the negative classes. Since the profile of the cattle population from the September 2006 survey is comparable to those from the surveys in 2004 and 2005, it is reasonable to conclude that this population was indeed still free of BTV infection at the time of sampling. As such, serological profiling could be a powerful tool to help decision makers substantiate the disease-free status of a region or zone.

During the course of the epidemic the profile changed and became bimodal in shape with the additional mode corresponding to the infected animals (Fig. 3B). The profile of the suspected animals is comparable to the one previously found in a BTV infected area in Corsica (France) in 2002 (Biteau-Coroller et al., 2006). A comparison of the profiles obtained before (Fig. 3A) and during (suspected cases) (Fig. 3B) the Belgian epidemic shows the appearance of an intermediate zone in the profile of an infected population with numerous animals exhibiting PN values in the cut-off or near cut-off classes. This intermediate zone appears soon after BTV enters into the population and is a clear indicator of virus transmission. Further comparison to the profile obtained at the end of the epidemic (January 2007 survey, Fig. 3C) shows a clear shift towards the lowest PN class (PN: 0–5) representing strong positives. The appearance or disappearance of the intermediate zone is thus correlated with virus circulation and provides valuable information, which would be entirely overlooked if only positive and negative results were being considered. Checking for the appearance of reactors in this intermediate range could be used as an early warning criterion in the follow-up of sentinel herds. In contrast, shifting to the most positive antibody class at the end of an epidemic indicates the decrease or disappearance of virus transmission by the midges.

Due to the high cost and length of the procedure, RT-qPCR was only used as a diagnostic tool throughout the crisis and not as a mass-screening tool. The frequency distribution profile of the Ct-values displayed a bimodal shape characteristic of an infected population (Fig. 4). In contrast to the cELISA, the position of the positive mode differed depending on the host species. This finding supports our statement that profiling gives more information than a dichotomised (pos/neg) analysis used to calculate

sensitivity and specificity and showing no difference between both species. In general, the virus accumulated to higher levels in the blood of sheep than that of cattle. This might explain the higher case fatality in sheep (42%) compared to cattle (18%) during the epidemic.

The Ct distribution profile of the tissue samples was similar to that observed for blood samples but with the peak corresponding to positive animals slightly shifted towards the right (Fig. 4). This suggests a lower viral load in or viral RNA recovery from tissue samples. Since the presented tissue samples were frequently of poor quality, as judged by the internal beta-actin control, this difference could at least in part be due to the degradation of the viral RNA. This observation highlights the necessity of rapid transport of tissue samples under refrigerated conditions to avoid false negative results.

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

In order to declare a farm free of BTV all positive animals were resampled and retested 1-month post-initial diagnosis. Analysis of 75 of these paired samples demonstrated that the viral load might change substantially over a 1-month period. In general the viral load decreased with 2.71 Ct-values. Consequently, animals with a Ct-value of less than 35 at T1 will certainly remain BTV-positive by RT-qPCR for more than 1 month. To avoid any unnecessary analysis it would, therefore, be more appropriate to only retest these animals after 2–3 months. It is, however, also important to point out that the detection of viral RNA is not always sufficient to determine the infectious status of the animal (MacLachlan, 2004). Since the RT-qPCR used in this study is at least 100 times more sensitive than virus isolation (Toussaint et al., 2007a), a weak positive RT-qPCR result might indeed be clinically or epidemiologically irrelevant. This is particularly pertinent for non-recently infected animals, which often exhibit a low viral load in the presence of a high antibody titre. Further research is, therefore, urgently needed to correlate the viral load or Ct-value of an animal with its infectious status.

5. Conclusion

Based on the antibody frequency distribution and on the ROC analysis, it was concluded that 'ID Screen® Bluetongue Competition' ELISA should be used at a different cut-off both for diagnosis and for screening. The use of RT-qPCR as reference test during the epidemic, instead of the not-easy-to-use VI, is supported by the results of a Bayesian analysis. BT serological and molecular profiling through the analysis of frequency distributions of the results of a cELISA and a RT-qPCR with well-known performance characteristics provides valuable information for the control of BT that could be of use to decision makers. In order to declare a farm free of BTV, resampling of animals with a Ct-value of less than 35 1 month after initial sampling seems pointless, unless Ct-values are correlated to the infectious status of the BTV.

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