Full length article

Evaluation of two enzyme-linked immunosorbent assays for diagnosis of bluetongue virus in wild ruminants

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Abstract

Bluetongue (BT) is a reportable re-emerging vector-borne disease of animal health concern. Enzyme-linked immunosorbent assays (ELISA) are frequently used in BT surveillance programs in domestic ruminants, but their diagnostic accuracy has not been evaluated for wild ruminants, which can play an important role as natural reservoirs of bluetongue virus (BTV). The aim of this study was to assess two commercial ELISAs for BT diagnosis in wild ruminants using control sera of known BTV infection status and field samples. When control sera were tested, the double recognition ELISA (DR-ELISA) showed 100% sensitivity (Se) and specificity (Sp), while the competitive ELISA (C-ELISA) had 86.4% Se and 97.1% Sp. Using field samples, the selected latent-class analysis model showed 95.7% Se and 85.9% Sp for DR-ELISA, 58.2% Se and 95.8% Sp for C-ELISA and 84.2% Se for the serum neutralization test (SNT). Our results indicate that the DR-ELISA may be a useful diagnostic method to assess BTV circulation in endemic areas, while the C-ELISA should be selected when free-areas are surveyed. The discrepancy between control and field samples point out that the inclusion of field samples is required to assess the accuracy of commercial ELISAs for the serological diagnosis of BTV in wild ruminants.

Keywords: Bluetongue; ELISA; Diagnostic test evaluation; Wild ruminants; latent-class analysis; Serosurvey

1. Introduction

Bluetongue (BT) is a re-emergent vector-borne disease affecting domestic and wild ruminant species. This reportable disease has considerable socioeconomic impact associated to production losses, costs derived from implementation of control and vaccination programs and international trade restrictions [1–3]. Bluetongue virus (BTV; genus *Orbivirus*) is mainly transmitted between vertebrate hosts by biting midges of the genus *Culicoides* [4]. Up to date, 27 BTV serotypes have been identified, and since the beginning of the 2000s, a considerable number of them have been involved in outbreaks in livestock across Europe [5]. Despite control measures implemented in affected countries, BTV is still circulating in both endemic and non-endemic regions. Currently, this continent has restricted zones for BTV serotypes 1, 2, 3, 4, 8 and 16 [6].

BT surveillance in livestock is usually conducted by using serological methods. Among them, serum neutralization test (SNT) is considered a highly specific technique [7,8] which has been used as reference technique for testing other serological methods [9] and allows the serotype identification [10]. However, SNT sensitivity (Se) is estimated to be less than perfect [8]. Moreover, it is complex, expensive, time-consuming and sensitive to the quality of the sample. In contrast, enzyme-linked immunosorbent assays (ELISAs) are quick, easy to use, have a relatively low cost and allow the detection of all serotypes since they use the conserved viral protein 7 (VP7) as antigen [11]. For these reasons, ELISA methods are endorsed by the OIE [10] and are frequently used in BT surveillance programs in livestock.

Wild ruminants are susceptible to BTV infection and their potential role as natural reservoir has been evidenced [12–15]. The epizootic cycle of BTV among wild ruminants and

competent vectors is considered an important factor in the maintenance of the virus in certain regions [16], being of particular interest in areas where these species coexist with livestock [15]. BTV circulation in wild ruminants, especially when high densities occur, make them useful as sentinels [17], particularly in areas where vaccination programs have been implemented in livestock.

Different commercial ELISAs have been used to detect anti-BTV antibodies in wild ruminants in previous studies [15,18–20]. However, the success of surveillance in these species can be compromised by the accuracy of the diagnostic techniques employed. Diagnostic validity of ELISA methods for the detection of antibodies against BTV have been evaluated in livestock in numerous studies [21], but rarely in wild ruminants [8,22]. Hence, we investigate the accuracy of two commercial ELISAs, namely double recognition ELISA (DR-ELISA) and competitive ELISA (C-ELISA), for the diagnosis of BTV in wild ruminant species. The aims of the study were (1) to compare the diagnostic accuracy of DR-ELISA and C-ELISA using control sera from wild ruminants of known BTV infection status, and (2) to test the performance of both ELISAs and SNT, using serum samples from wild ruminants of unknown infection status collected under field conditions.

2. Materials and methods

2.1. Control samples

A total of 57 sera of known BTV infection status were used as control samples. Positive control sera were obtained from 14 Spanish ibex (*Capra pyrenaica hispanica*) and eight red deer (*Cervus elaphus*) from two experimental studies (for further details, see [23,24]). Negative controls (19 red deer and 16 Spanish ibex) used in the same previous

studies were also included and they came from BTV-free areas in which outbreaks have not been reported. Both positive and negative controls were verified by SNT and real-time reverse transcription polymerase chain reaction [23,24].

2.2. Field samples

A total of 264 free-ranging wild ruminants including 171 red deer, 58 fallow deer (*Dama dama*) and 35 European mouflon (*Ovis aries musimon*), were sampled in Southern Spain (36ºN to 38º60´N, 1º75´W to 7º25´W) between the hunting seasons 2007/2008 and 2014/2015. Samples were obtained in a region and time period with a wide circulation of BTV in livestock (RASVE 2019). Blood samples were taken by puncture of the endocranial venous sinuses, as previously described [26]. Samples were placed into sterile tubes without anticoagulant and centrifuged at 400 *g* for 15 minutes. Sera were stored at -20 ºC until analyses.

2.3. Serological tests

All sera were tested using two commercial ELISA kits: DR-ELISA (INGEZIM BTV DR 12.BTV.K0, INGENASA, Spain) and C-ELISA (ID Screen Bluetongue Competition ELISA kit, IDVET, France). Both ELISAs were performed following the manufacturers' instructions. Sensitivity (Se) and specificity (Sp) values provided by the manufacturers were 100% and 97.3% for DR-ELISA and 100% and 100% for C-ELISA, respectively. The cut-off of positive readings was calculated according the instructions of each test:

DR-ELISA: Positive sample = optical density (OD) sample > 0.15 x OD positive control

C-ELISA: Positive sample = OD sample / OD negative control x 100 > 40%

With the aim of evaluating the repeatability of the assay, the same positive and negative controls were included in duplicate in every plate of the study (which were tested on the same day and on different days, as well as by different operators). Additionally, field samples were tested by SNT for the detection of antibodies against BTV-1, BTV-4 and BTV-8 serotypes, which are the serotypes that have been circulating in the sampling area in the last two decades [25]. The SNT protocol was performed as previously described [10]. Briefly, serum samples were inactivated at 56°C for 30 minutes prior to analysis. Sera were diluted (1:2-1:256) in MEM (Eagle's minimum essential medium) and mixed with 100 $TCID_{50}$ (50% tissue culture infective doses) of each reference strain, BTV-1, BTV-4 and BTV-8. Plates were incubated for 1 hour 30 minutes at 37°C. Finally, 100 µL of a Vero E6 cells suspension (1.5×10^4 cells/well) were added in cell growth media (MEM supplemented with 15% foetal calf serum, 300 µg L-glutamine/mL, 300 U penicillin/mL and 300 µg streptomycin/mL). The mixture was further incubated for 6-7 days at 37ºC until a cytopathic effect (CPE) was developed in control wells containing 100 $TCID_{50}$ of virus and no serum. Only samples that showed neutralization (absence of CPE) at dilutions ≥ 1:4 were considered positive [24]. Controls for cytotoxicity in the absence of virus were included for each analysis at a dilution of 1:2.

2.4. Statistical analysis

For both ELISAs, Se and Sp values were calculated from control sera. The package "OptimalCutpoints" of the statistical software R [27] was used to obtain Se and Sp with 95% confidence intervals (CI 95%), differential positive and negative rates, and the area under the curve (AUC) determined by receiver operating characteristic analysis [28]. AUC was calculated just for obtaining a single numerical estimate of the overall accuracy of the ELISAs using the control sera. The agreement between ELISA tests was measured by the kappa index

149 (κ) using the R package "epiR" and differences between methods were analysed by the
150 McNemar's test for correlated proportions in subgroups of positive and negative animals,
151 respectively.

152 The overall agreement and the kappa index of both ELISAs and SNT were also
153 calculated from the field samples. These sera were also used to estimate the Se and Sp of
154 ELISAs and SNT by latent class analysis (LCA) considering three tests and one population.
155 The scripts used for the analysis were described previously [29]. A conditional independence
156 (CID) assumption was initially considered for LCA models. However, since the compared
157 tests are based in the detection of the same biological property, implications of conditional
158 dependence (CD) were also explored by running separate models that accounted for model
159 co-variance, which was calculated and implemented as described previously [30]. The prior
160 information for the ELISA parameters was obtained from the results of control sera. For SNT
161 Se, prior information was estimated from previous results in the literature [8] (mean = 90%;
162 85% sure > 85%) assuming a beta distribution (62,7). SNT Sp was assumed to be 100% based
163 on the assumption that the test is highly specific and showed perfect specificity in previous
164 studies with wild ruminants [8]. In addition, CID and CD models with vague priors were also
165 run to explore the impact of changing priors. The models were compared and selected
166 considering the Deviance Information Criterion (DIC) [31]. For each model, three chains
167 were run simultaneously from different initial starting points. A total of 120,000 iterations
168 were used with a burn-in of 10,000 iterations and a thinning of five. Mean values and 95%
169 posterior credibility intervals (PCI) were extracted from the posterior distribution of the
170 different parameters of the model. Models were fitted with the software JAGS version 2.2.0
171 (http://mcmc-jags.sourceforge.net/). Convergence was assessed by visual inspection of the

trace plots of the sampled parameters and autocorrelation plots, and with the Geweke diagnostic [32] and the Heidelberger and Welch diagnostic [33] using the R package "coda".

3. Results

3.1. Control samples

Results of Se and Sp for ELISAs using the control sera are shown in Table 1. DR-ELISA showed a 100% of Se and Sp, while C-ELISA showed Se = 86.4% and Sp = 97.1%. No statistical differences were found between both ELISAs, neither in the subpopulation of positives (p = 0.248) nor in the subpopulation of negative sera (p = 0.999) and the tests presented a very good concordance with a high kappa index (κ = 0.85; CI 95%: 0.71-0.99).

3.2. Field samples

Of the 264 field sera, 198 showed positive results to the DR-ELISA (75.0%), 108 to the C-ELISA (40.9%) and 157 to SNT (59.5%). Results between ELISAs showed a fair agreement, with κ = 0.31 (CI 95%: 0.22-0.39). The DR-ELISA detected the highest number of positives samples, including a high percentage of sera that tested negative to other techniques (44.9% to SNT, 60.9% to C-ELISA). On the contrary, the C-ELISA detected the highest number of negatives samples including a high percentage of sera that tested positive to other methods (60.9% to DR-ELISA and 42.7% to SNT) (Table 2).

The LCA model that showed the best performance and DIC values was the informative CID model. When CD was considered, the models showed less convergence and higher levels of correlation than when CID was assumed, with and without informative priors. Estimates from CD models presented larger PCI that overlapped with CID models. Estimates of Se and Sp derived from the selected CID model are shown in Table 3. The informative CID

model showed a high Se (95.7%; PCI 95%: 92.4-98.1%) but moderate Sp (85.9%; PCI 95%: 73.6-98.4%) for the DR-ELISA. In contrast, the C-ELISA had very low Se (58.2%; PCI 95%: 50.7-65.7%), but Sp was high (95.8%; PCI 95%: 90.8-99.1%). SNT Se was 84.2% (PCI 95%: 77.5-90.2%).

4. Discussion

Wild ruminants have been proposed as and suitable sentinel species for monitoring BTV, particularly in regions where livestock are vaccinated [17]. The importance to include these potential wild reservoirs in BT surveillance programs highlights the need of validated serological methods for every epidemiological context. Despite the performance of commercial ELISAs for detection of anti-BTV antibodies has been evaluated in livestock in different studies [21], to the author's knowledge, the accuracy of these techniques has not been assessed in wild ruminant species. In the present study, we have assessed two commercial ELISAs using control and field sera from wild ruminants. Both DR-ELISA and C-ELISA showed high Se and Sp values and a strong concordance between them when control sera were tested. These findings are in accordance with those previously obtained in domestic ruminants with a known BTV infection status using the same commercial ELISA tests [34,35]. Similarly, experimental studies on challenged ruminant and camelid species showed an overall good performance of both ELISAs [36–38].

When field samples were analysed, results between ELISAs presented poor agreement. Interestingly, the discrepancies of both ELISAs have an opposite nature when they were compared to SNT. DR-ELISA presented the highest number of positive sera, but with a high proportion of samples testing negative to SNT (44.9%). Similar findings were obtained in previous serosurvey studies in wild ruminant species using the same tests [19,24]. The differentiated ability to detect recent infections [36–38], the targeting of the different antibody

populations [39], cytotoxicity reactions of sera with low titres of specific neutralizing antibodies or the circulation of serotypes not included in the SNT are possible factors implicated in the discrepancies observed. On the other hand, a high number of negative sera by C-ELISA showed positive results by SNT (42.7%). Since SNT is considered a very high specific technique [7,8], this finding suggests false negative results by C-ELISA. Further investigations are required to determine the precise nature of the discrepancies found among the three analysed serological methods.

The differences observed between control and field samples point out how field conditions can affect the accuracy of the diagnostic methods. In the control group, samples are usually collected from live captive individuals, while samplings in free-ranging animals present difficulties that may lead to poor sera quality, which subsequently may affect the performance of the diagnostic tests [19,40]. Blood collection in wild ruminants is frequently performed in hunted-harvested animals and sera are frequently taken hours after death. Moreover, the sampling method used in these species has been showed to have influence in the quality of samples [26]. In this regard, haemolytic sera affect SNT performance due to the presence of cytotoxicity and cloudy suspensions that may influence the final outcome [15,19]. In the same way, when repeated freeze-thawing cycles are applied to haemolytic sera, the ELISA performance may also be affected [40]. Our results indicate that, in order to have a better estimation of the diagnostic tests performance, these methods should be evaluated including not only control sera but also field samples.

The LCA model showed a lower accuracy of both ELISAs compared to the results when control sera were evaluated. The results also differed with those indicated by the manufacturers and with those reported in livestock under field conditions. In domestic ruminant species, Se values ranged from 98.2 to 100% and reached 99.5% Sp for DR-ELISA,

while Se and Sp values for C-ELISA ranged from 87.8 to 100% and from 98.2% to 99.3%, respectively [34,35,41]. In addition, the LCA also showed marked differences between ELISAs. DR-ELISA showed a high Se, even higher to SNT, but Sp was moderated, while the C-ELISA showed low Se and high Sp. These results indicate that commercial ELISAs can be used for BTV surveillance in wild ruminants with appropriate considerations. In absence of a gold-standard method for detection of antibodies against BTV, the serological test with highest Se is preferred in endemic areas to reveal the exposure to the virus. In this context, the DR-ELISA should be selected, though a subsequent verification of the DR-ELISA positive samples using other methods such as SNT may be contemplated to ensure the results. In contrast, the C-ELISA is less suitable for BTV monitoring in endemic areas, since it could largely underestimate the number of infected animals. Nevertheless, when free-areas are assessed, the test with highest Sp must be used to avoid false positive results that lead to an incorrect sanitary classification of the area. In this epidemiological scenario, the C-ELISA should be preferably selected as diagnostic method.

In conclusion, the results obtained in the present study indicate that the accuracy of commercial ELISA methods for diagnosis of BTV in wild ruminants requires of a particular approach and it cannot be inferred straightforwardly from their performance in livestock. When control sera were analysed, both ELISAs showed a good diagnostic validity, comparable to their performance in domestic ruminants. However, in field conditions, the Se and Sp differed, which indicates that their use should be evaluated according to the epidemiological scenario. In this regard, the DR-ELISA may be a useful method to assess BTV circulation in endemic areas, while the C-ELISA Sp should be selected when free-areas are surveyed. The discrepancy between control and field samples reveals that control sera are not enough to know the performance of an ELISA and the inclusion of field samples should

be taken into account when the diagnostic validity is assessed. Further investigations including the analysis of different wild ruminant or camelid species, age classes or different immunological status are warranted to optimize the accuracy of the parameters obtained in this study.

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Table 1. Accuracy of two commercial ELISAs (DR-ELISA and C-ELISA) by testing control sera samples.

Parameter	DR-ELISA	C-ELISA
Se (CI 95%) (n = 22)	100 (97.3-100)	86.4 (69.8-100)
Sp (CI 95%) (n = 35)	100 (98.6-100)	97.1 (90.2-100)
DPR (CI 95%)	-	30.2 (4.3-210.1)
DNR (CI 95%)	-	0.1 (0.05-0.4)
AUC (CI 95%)	1	0.918 (0.839-0.996)

DR-ELISA, double recognition ELISA; C-ELISA, competitive ELISA; Se, sensitivity; Sp, specificity; DPR, differential positive rate, DNR: differential negative rate, AUC: area under the curve

Table 2. Comparative results of DR-ELISA, C-ELISA and SNT when testing field sera (n= 264).

		SNT				C-ELISA			
		Positive (%)	Negative (%)	Overall agreement	Kappa (CI 95%)	Positive (%)	Negative (%)	Overall agreement	Kappa (CI 95%)
DR-ELISA	Positive (%)	150 (95.5)	48 (44.9)	0.79	0.58 (0.44-0.64)	103 (95.4)	95 (60.9)	0.62	0.31 (0.22-0.39)
	Negative (%)	7 (4.5)	59 (55.1)			5 (4.6)	61 (39.1)		
C-ELISA	Positive (%)	90 (57.3)	18 (16.8)	0.68	0.38 (0.28-0.48)	-	-	-	-
	Negative (%)	67 (42.7)	89 (83.2)			-	-	-	-

DR-ELISA, double recognition ELISA; C-ELISA, competitive ELISA; SNT, serum neutralization test; CI, confidence interval

410 Table 3. Results of the model (informative, conditionally independent) for DR-ELISA,
411 C- ELISA and SNT when assessing field sera (n = 264).

Variable	Mean	PCI 95%	Priors
DR-ELISA Se	95.7%	92.4-98.1	beta[23,1]
C-ELISA Se	58.2%	50.7-65.7	beta[20,4]
SNT Se	84.2%	77.5-90.2	beta[62,7]
DR-ELISA Sp	85.9%	73.6-98.4	beta[36,1]
C-ELISA Sp	95.8%	90.8-99.1	beta[35,2]
SNT Sp	100%	100%	[a]
DIC	872.984		

412 [a] Assumed to be 100%

413 DR-ELISA, double recognition ELISA; C-ELISA, competitive ELISA; SNT, serum
414 neutralization test; PCI, posterior credibility intervals; Se, sensitivity; Sp, specificity;
415 DIC, deviance information criterion

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