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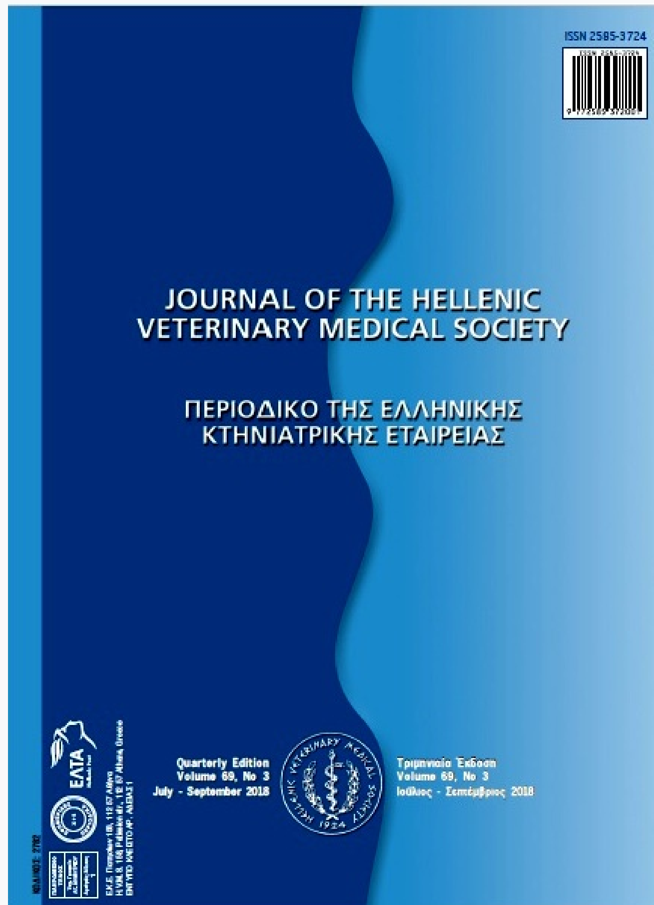

Comparative examination of the serological response to bluetongue virus in diseased ruminants by competitive and double recognition enzime-linked immunosorbent assays

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Research article
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Comparative examination of the serological response to bluetongue virus in diseased ruminants by competitive and double recognition enzime-linked immunosorbent assays

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ABSTRACT. Bluetongue (BT) is a viral non-contagious disease of ruminants which is transmitted by insects of the genus *Culicoides.* In recent years, BT has been a serious threat to livestock and to the economies of European countries. In Serbia the disease appeared for the first time in 2001, and after a 12 year period of freedom, it broke out again in 2014. Considering the actuality of this infectious disease, especially the need for prompt and rapid diagnostics, the aim of this paper was to determine the possibility of detecting the serological response in sheep and cattle with manifested clinical signs of the disease using two different methods: double recognition enzyme-linked immunosorbent assay (sELISA) and competitive enzyme-linked immunosorbent assay (cELISA). A total of 105 blood serum samples of cattle and sheep, which had exhibited clinical signs of BT during 2014, were taken for examination from a serum bank. Out of 74 blood serum samples of sheep and 31 blood serum samples of cattle, 52 samples of sheep and 18 samples of cattle tested positive using sELISA, while 50 samples of sheep and 18 samples of cattle gave positive reactions with cELISA. The results confirm the high sensitivity of sELISA which detected 4% more seropositive sheep in comparison with cELISA. Using Cohen's *kappa* statistical analysis, almost perfect agreement was determined between the results (k>0,81) obtained by cELISA and sELISA.

Keywords*:* bluetongue, cELISA, sELISA, ruminants, Serbia

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INTRODUCTION

Bluetongue (BT) is a viral non-contagious disease of ruminants which is transmitted by *Culicoides* biting midges. The agent belongs to the genus *Orbivirus*, family *Reoviridae*. All ruminants are susceptible to the infection, but clinical signs are most often manifested in sheep and white-tailed deer (*Odocoileus virginianus*) (Johnson et al., 2006; Sprelova and Zendulkova, 2011). The disease is manifested as an acute, chronic or subclinical condition. After an incubation period of four to eight days, clinical signs in the form of fever, apathy, tachypnea, and hyperaemia of the lips and nostrils, with excessive salivation and serous nasal discharge, appear in infected sheep. The clinical manifestation of the disease is influenced by the strain of the virus (Sprelova and Zendulkova, 2011). So far, 27 bluetongue virus (BTV) serotypes have been identified world-wide (van Rijn et al., 2016).

The economic losses may be direct such as death, abortions, weight loss or reduced milk yield and meat production inefficiency, and indirect as a result of export restrictions for live animals, semen and animal products. The annual world-wide losses due to BT have been estimated at 3 billion US$ (Tabachnick, 1996). In recent years BT has constituted a serious threat to the livestock and agricultural economies of European countries. In Serbia the disease appeared for the first time in 2001 (Đuričić et al., 2004), and after a 12 year period of freedom, it broke out again in 2014, when the virus was spreading rapidly throughout the countries of the Balkan Peninsula (Ostojić et al., 2014).

Serological tests for the detection of specific antibodies to the agent are very important diagnostic methods. The current OIE manual (OIE, 2014) describes the complement fixation test (CFT), agar gel imunodiffusion (AGID), competitive ELISA (cELISA) and indirect ELISA (iELISA). The bluetongue competitive or blocking ELISA was developed to detect specific antibodies against BTV without detecting cross-reacting antibodies to other orbiviruses. The specificity is the result of using serogroup-reactive monoclonal antibodies which bind to the amino-terminal region of the major core protein VP7 (Lunt et al., 1988; Afshar et al., 1989).

Considering the actuality of this infectious disease, especially the need for prompt and rapid diagnostics, as well as the lack of data in the current OIE Manual about the use of sELISA, the aim of the investigations was to compare the possibility of detecting the serological response in sheep and cattle with manifested clinical signs of BT, using two different enzyme-linked immunosorbent assays: the double recognition enzyme-linked immunosorbent assay (sELISA) and the competitive enzyme-linked immunosorbent assay (cELISA).

MATERIAL AND METHODS

Animals

The material for examination consisted of blood serum samples of sheep and cattle originating from South Banat, Serbia, the plains area that is bounded by the river Danube in the south. The numbers of sheep, goats and cattle is estimated at 45,000, 5,000 and 28,000, respectively. The investigation included 74 blood serum samples of sheep from 10 communities belonging to four municipalities and 31 blood serum samples of cattle from six communities within three municipalities. The samples originated from 19 sheep farms and 13 cattle farms.

Sampling

Samples of sheep and cattle, which had exhibited clinical signs of bluetongue during the 2014 epizootic were taken for the investigations from the serum bank of the Veterinary Specialised Institute “Pančevo”. During that epizootic, the blood of ruminants with manifested clinical signs of BT was sampled for examinations in accordance with the government protocol which implied that, when the signs of the disease appeared in a community for the first time, blood samples of the diseased animals were examined for the presence of viral RNA by RT-PCR (reverse transcription polymerase chain reactions) and also serologically for the presence of antibodies against the agent by ELISA. In the latter cases suspected ruminants were tested only serologically for the presence of specific antibodies. After the examinations, the samples were saved in the serum bank.

Serological examination

The presence of antibodies against BTV was exam-

ined using the sELISA produced by *Ingenasa*, Spain and the cELISA produced by *VMRD*, USA. Both tests had been previously verified in the laboratory, based on repeatability, by testing positive and negative internal control samples in six replicates, and reproducibility, by examining the same replicates of the positive and negative controls under the same conditions at a seven-day interval. The coefficients of variation (CVs) for both tests were <10%. Since the samples originated from animals with manifested clinical signs and from communities in which the first suspicious cases were diagnosed by the detection of viral RNA by RT-PCR, they were interpreted as positive if they gave a positive reaction in either of the ELISA methods.

Double recognition enzyme-linked immunosorbent assay

According to the instructions of the manufacturer, the sELISA kit has been designed to detect antibodies against BTV in sheep, goats and cattle. The sensitivity and specificity of the assay are 100% and 99.8%, respectively. Microtiter plates are coated with VP7 protein of BTV. After adding a sample to the well, if it contains BTV specific antibodies, they will bind to the antigen. When VP7 protein conjugated with peroxidase is added, they will catch the labeled VP7. In such a way antibodies are caught between two antigens (double recognition). Presence or absence of labeled VP7 will be detected by the addition of a substrate which, in the presence of the peroxidase, will develop a colorimetric reaction.

Competitive enzyme-linked immunosorbent assay

The cELISA has been designed to detect BTV antibodies in ruminant sera. The sensitivity and specificity of the assay are 100% and 99%, respectively. If present in samples, antibodies inhibit the binding of horseradish peroxidase-labeled bluetongue virus-specific monoclonal antibody to BT viral antigen coated on the plastic wells. Binding of the horseradish peroxidase-labeled monoclonal antibody conjugate is detected by the addition of a substrate and quantified by subsequent colour product development.

Statistical analysis

The agreement between the two ELISA tests was evaluated using Cohen's *kappa* statistical analysis. The calculation of the k (*kappa*) value is based on the difference between how much the agreement is actually present ("observed" agreement) compared to how much the agreement would be expected to be present by chance alone ("expected" agreement). The common interpretations of kappa are as follows: < 0 Less than a chance agreement; 0.01–0.20 Slight agreement; 0.21–0.40 Fair agreement; 0.41–0.60 Moderate agreement; 0.61–0.80 Substantial agreement; >0.80 Almost perfect agreement.

RESULTS

Out of 74 blood serum samples of sheep, 50 samples tested positive by cELISA, while 52 samples gave a positive reaction in sELISA. Out of 31 blood serum samples of cattle 18 samples tested positive in both ELISA tests (Table 1). It was determined, by applying the *kappa* statistical analysis, that there was almost perfect agreement (k>0,81) between the cELISA and sELISA for both classes of sample. The *kappa* value for blood serum samples of sheep is 0.93, whereas for blood serum samples of cattle the *kappa* value is 1.00

Table 1. Results of the examination for the presence of antibodies against bluetongue virus in diseased sheep and cattle using cELISA and sELISA

Number of samples	cELISA		sELISA	
	Number of seropositive samples	**Percent of seropositive samples**	**Number of seropositive samples**	**Percent of seropositive samples**
Sheep				
74	50	67.57%	52	70.27%
Cattle				
31	18	58.10%	18	58.10%

(Table 2).

Seropositive sheep were discovered in 15 out of 19 tested farms while seropositive cattle were discovered in 11 out of 13 tested farms. All of the clinically suspected animals were confirmed serologically in 10 sheep farms and in 7 cattle farms.

DISCUSSION

While the cELISA is described as a specific method for the serological diagnosis of bluetongue, the possibility of using sELISA is not cited in the current edition of the OIE Manual (OIE, 2014). This was one of the reasons for comparing cELISA and sELISA tests in our investigations. The *kappa* statistical analysis excludes the possibility of the high percentage agreement between the tests obtained in the present investigation being present by chance.

Several studies confirmed a high sensitivity of sELISA in relation to the cELISA tests of different manufacturers (Oura et al., 2009; Eschbaumer et al., 2011; Niedbalski, 2011). Comparing the values relevant for the assessment of the reaction, obtained for two samples that gave different reactions in applied ELISA methods (Table 3), it can be seen that they are close to the cut off values in both tests indicating their different sensitivity. In addition, it was taken into account for the interpretation of the results that the samples originated from animals that had manifested signs of BT and were from settlements in which the agent had been proven to be present by the RT-PCR method.

Afshar et al. (1987) determined, using different serological methods, that cELISA was superior to iELISA in the detection of anti-BTV antibodies in the sera and whole blood samples from both cattle and sheep early after infection with BTV. Similar to our results obtained in naturally infected ruminants, Oura et al. (2009) reported that sELISA tests of different manufacturers were more sensitive in detecting

Table 2. Examination of the agreement between the results obtained by cELISA and sELISA using *kappa* statistical analysis

	Sheep, total of 74 samples			**Cattle, total of 31 samples**		
	sELISA			**sELISA**		
cELISA	positive	negative	total	positive	negative	total
Positive	a = 50	b = 0	$m_1 = 50$	a = 18	b = 0	$m_1 = 18$
Negative	c = 2	d = 22	$m_0 = 24$	c = 0	d = 13	$m_0 = 13$
Total	$n_1 = 52$	$n_0 = 22$	n = 74	$n_1 = 18$	$n_0 = 13$	n = 31
***Kappa* statistical analysis**	$pe = [(n_1/n)x(m_1/n)] + [(n_0/n)x(m_0/n)]$ pe = 0.572 po = (a + d)/n = 0.97 k = (po-pe)/(1-pe) = 0.93			$pe = [(n_1/n)x(m_1/n)] + [(n_0/n)x(m_0/n)]$ pe = 0.33 po = (a + d)/n = 1 k = (po-pe)/(1-pe) = 1		

po = the observed agreement; pe = the expected agreement; k = *kappa* value

Table 3. Comparison of the results obtained by cELISA and sELISA for the samples that gave different reactions

No	**cELISA**				**sELISA**			
	OD value	**S/N %**	**Cut off** (S/N%)	**Result**	**OD value**	**PP%**	**Cut off** (PP%)	**Result**
1	0.39	58.22	positive	-	0.49	25.80	positive	+
2	0.41	57.69	< 50%	-	0.37	21.30	>15%	+

OD value = optical density; S/N% = the OD value of a sample in relation to the OD value of the negative control; PP% = the OD value of a sample in relation to the OD value of the positive control

antibodies in vaccinated sheep than cELISA methods. Niedbalski et al. (2011) investigated the performances of commercial ELISAs in cattle vaccinated or infected with BTV serotype 8. The authors found that the relative sensitivity for cELISA, *VMRD* and sELISA, *Ingenasa* in vaccinated cattle amounted 69.5 and 98.3%, respectively, while the relative sensitivity for infected cattle with BTV serotype 8 was 98.6% for *VMRD* and 100% for *Ingenasa*. Similarly, in naturally infected ruminants with serotype 4 in our investigations, the difference in relative sensitivity between tests was small and amounted to 4% for sheep and 0% for cattle. The present investigations coupled with the results of Niedbalski et al. (2011) lead to conclusion that neither the different BTV serotypes (the serotypes 4 and 8) nor the species of ruminants naturally infected influence on the relative sensitivity of sELISA (*Ingenasa*).

The relatively small percentage of serologically confirmed clinical cases in the present investigations can be explained by the possibility that some herds of sheep were infected with contagious ecthyma virus, which had previously been diagnosed in the area of investigation. That is the most likely reason for negative reactions for all of the tested samples in four sheep farms. For some negative results, the reason could be found in the fact that the veterinarians, beside the samples of the animals that exhibited clinical signs typical of BT, also took a number of samples from animals which exhibited nonspecific signs that were not caused by BTV.

Unlike the epizootic of 2001, during which clinical signs had not been recorded in cattle (Debeljak et al., 2003), the results of this study show that a large percentage of infected cattle exhibited clinical signs during the epizootic of 2014. Comparing the population numbers with the number of serologically confirmed clinical cases of the disease in cattle and sheep in the area of investigation, no differences in susceptibility between the ruminant species examined could be seen. The fact that these two epizootics were caused by different serotypes of the agent, the former by BTV serotype 9 and the latter by BTV serotype 4, could provide an explanation for differences in the susceptibility of cattle to bluetongue.

CONCLUDING REMARKS

Using Cohen's *kappa* statistical analysis, almost perfect agreement (k>0.81) was determined between the results obtained by cELISA and sELISA in detecting the serological response in ruminants with manifested clinical signs of BTV. The high percentage agreement between the results obtained by the two different ELISA methods shows that either of them can reliably be used for the detection of antibodies against BTV in blood sera of naturally infected sheep and cattle.

CONFLICT OF INTEREST

The authors declare no conflict of interest

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