

Detection of bluetongue virus antibodies in sheep from Paraná, Brazil

Detecção de anticorpos para o vírus da língua azul em ovinos do estado do Paraná, Brasil

Maria Carolina Ricciardi Sbizera¹; Luiz Fernando Coelho da Cunha Filho^{2*}; Michele Lunardi³; Simone Fernanda Nedel Pertile²; Thais Helena Constantino Patelli⁴; José Victor Pronievicz Barreto¹; Edviges Maristela Pituco⁵

Highlights:

Bluetongue, an infectious and non-contagious disease, is prevalent in sheep of Paraná. .
Based on our findings, cELISA is more sensitive and superior than AGID.
Climate plays an important role in occurrence and spread of the disease.

Abstract

Bluetongue (BT) is an infectious and non-contagious disease caused by bluetongue virus (BTV) belonging to the genus *Orbivirus*. It is transmitted by a hematophagous vector, *Culicoides sp.*, to ruminants, particularly to sheep, which are most susceptible to this disease. The main serological tests are agar gel immunodiffusion (AGID), which is recommended by the World Organization for Animal Health (OIE), and the competitive enzyme-linked immunosorbent assay (cELISA), which has the advantage of no cross-reaction with other orbiviruses. The aim was to compare the results of these two tests by conducting them on sera collected from sheep in the state of Paraná, Brazil. From March to October 2017, serum samples were collected from 270 sheep from 10 farms in six mesoregions of Paraná. The samples were subjected to AGID and cELISA to detect antibodies against BTV. Based on the test results, we classified the sheep as low, moderate, and high occurrence. The results demonstrated that 64.81% (175/270) of the sheep were seropositive through the cELISA test, showing a high occurrence, and 41.11% (111/270) were seropositive through the AGID test, indicating a moderate occurrence. The concordance between the tests was moderate (0.51) as determined by the Kappa coefficient. Among the studied farms, 90% (9/10) presented at least one seropositive sheep, and the number of animals tested positive by the cELISA test was higher than those by the AGID test. Favorable climate, which favors the presence and multiplication of the culicoid vector and the occurrence of infection, was the biggest predominant factor responsible for the obtained results. The low occurrence in farms with milder climate suggest that the presence of antibodies also occurs due to the low pathogenicity of circulating serotypes in the different mesoregions studied. It is concluded that BTV infection is present in the sheep herds in Paraná, and the occurrence was moderate detected by AGID test and high detected by cELISA test.

Key words: *Culicoides*. Diagnosis. Occurrence. *Orbivirus*. Small ruminants.

¹ Discentes do Curso de Mestrado do Programa de Pós-Graduação em Saúde e Produção Animal, Universidade Pitágoras Unopar, UNOPAR, Araçongas, PR, Brasil. E-mail: mcrcsbizera@gmail.com; jose.proni@hotmail.com

² Profs. Drs., Programa de Pós-Graduação em Saúde e Produção Animal, UNOPAR, Araçongas, PR, Brasil. E-mail: vtluiz.cunha@gmail.com; s.pertile@zootecnista.com.br

³ Prof^a Dr^a, Universidade de Cuiabá, UNIC, Cuiabá, MT, Brasil. E-mail: michelelunardi@gmail.com

⁴ Prof^a Dr^a, Universidade Estadual do Norte do Paraná, UENP, Bandeirantes, PR, Brasil. E-mail: thaispatelli@uenp.edu.br

⁵ Pesquisadora, Dr^a, Laboratório de Vírus de Bovídeos, Instituto Biológico de São Paulo, IB, São Paulo, SP, Brasil. E-mail: empituco@gmail.com

* Author for correspondence

Resumo

A língua azul (LA) é uma enfermidade infecciosa e não contagiosa causada por um vírus (VLA) do gênero *Orbivirus*, transmitida por vetores hematófagos *Culicoides* sp., aos ruminantes sobretudo aos ovinos, espécie mais susceptível. Os principais testes sorológicos utilizados são a Imunodifusão em Gél de Ágar (IDGA), preconizada pela OIE, e o teste Imunoensaio Enzimático Competitivo (ELISAc), sendo que este tem como vantagem não ocorrer reação cruzada contra outros orbivirus. O objetivo do trabalho foi detectar a presença de anticorpos contra o VLA em ovinos no estado do Paraná através dos testes diagnósticos IDGA e ELISAc. Durante os meses de março a outubro de 2017, colheu-se sangue de 270 ovinos, em 10 propriedades localizadas em seis mesorregiões paranaenses. As amostras foram submetidas aos testes de IDGA e ELISAc para detecção de anticorpos contra o VLA. Baseado nos resultados classificaram-se os rebanhos como baixa, moderada ou elevada ocorrência. Os resultados demonstraram elevada ocorrência através do teste de ELISAc, que apresentou 64,81% (175/270) de ovinos positivos, e moderada ocorrência através do IDGA, com 41,11% (111/270) de ovinos soropositivos. A concordância obtida entre os testes foi moderada (0,51) através do coeficiente Kappa. O número de ovinos reagentes no exame de ELISAc foi maior que o teste de IDGA em todas as propriedades positivas, demonstrando ser superior. O clima propício foi um dos fatores favoráveis para as ocorrências observadas, pois favorece a presença e multiplicação do vetor *Culicoide* e a ocorrência da infecção. A baixa ocorrência nas propriedades com clima mais ameno sugere que a presença de anticorpos provavelmente ocorra também pela baixa patogenicidade dos sorotipos circulantes nas diferentes mesorregiões estudadas. Conclui-se que há infecção de VLA no rebanho ovino paranaense, e a detecção de anticorpos para o VLA foi moderada, através do teste de IDGA, e elevada, através do teste ELISAc.

Palavras-chave: *Culicoides*. Diagnóstico. Ocorrência. *Orbivirus*. Pequenos ruminantes.

Introduction

The bluetongue (BT) disease is an infectious, non-contagious disease, which should be mandatorily reported to the authorities (World Organisation for Animal Health [OIE], 2018). It is caused by *Orbivirus* bluetongue virus (BTV), which belongs to the Reoviridae family. Its 27 serotypes have been characterized worldwide (Roy, 2008; Jenckel et al., 2015).

This virus is transmitted by hematophagous vectors, particularly by midges of *Culicoides* sp. Domestic and wild ruminants are generally infected. Sheep are the most susceptible species (Dorneles et al., 2012). Factors, such as nutritional and immune status, stress, temperature (Maclachlan, 2004), age and race (Lobato, Guedes, & Matos, 2015), and the pathogenicity of the serotype (Purse, Carpenter, Venter, Bellis, & Mullens, 2015), influence the severity of infection.

The disease is distributed worldwide, coinciding with the presence of vectors. It is prevalent approximately between 50 °N and 35 °S latitudes, which includes climatic, tropical and subtropical, and temperate regions of the globe (Coetsee, Stokstad, Venter, Myrmel, & Van Vururen, 2012). High or moderately high temperatures predominate in most of these regions. In addition, vector development and proliferation is favorable in these regions (Dorneles et al., 2012). In Brazil, several studies have shown nationwide prevalence of bluetongue in different domestic and wild ruminant species (Costa, Lobato, Herrmann, Leite, & Haddad, 2006; Alves et al., 2009; Nogueira, Pituco, Stefano, Curci, & Cardoso, 2009; Tomich et al., 2009; Souza, Costa, Martinez, Costa, & Pinheiro, 2010; Mota et al., 2011; Dorneles et al., 2012; Pinheiro et al., 2013; Sbizera et al., 2017; Batista et al., 2018; Silva et al., 2018; Morikawa et al., 2018).

In Brazil, two outbreaks of this disease occurred in the state of Paraná in 2001 and 2002 (Clavijo et al., 2002; Lager, 2004); however, there is no data on the prevalence available in this state.

BTV infection in sheep is usually associated with clinical signs such as fever, nasal discharge, sialorrhea, oral injury, face and tongue edema, depression, anorexia, and muscle weakness (Gang-Ma et al., 2017). In addition, reproductive disorders such as abortion, stillbirth, and progeny malformations have been observed (Pinheiro et al., 2013). The economic impact associated with this disease stems not only from direct losses in the affected herds but also from restrictions on trade of animals and their products (Dorneles et al., 2012).

The main laboratorial methods for the diagnosis of Blue Tongue recommended by the World Organization for Animal Health (OIE) are based on isolation and identification of the agent, and on serological tests for certification of animals for international transit purposes, where the most used ones are the Agar Gel Immunodiffusion (AGID) and the Competitive Enzyme Immunoassay (cELISA) (Souza et al., 2010).

The AGID test has been one of the standard tests recommended by OIE for the certification of ruminant animals for international transit purposes since 1982; it is economical and simple to perform. However, its disadvantage is the possibility of cross-reaction with other viruses belonging to the genus Orbivirus (OIE, 2018); for example, the virus causing epizootic hemorrhagic cervical disease may show cross reaction in this test (Souza et al., 2010). Unfortunately, the AGID test is capable of determining only seropositive or seronegative animals, and it does not identify the serotypes present in a given seroreactive sample (Costa et al., 2006).

The cELISA test was developed to detect antibodies against the VP7 protein, which is present in the inner layer of the viral particle and contains the major group-specific antigenic determinants (OIE, 2018). Thus, the cELISA test does not show

cross-reaction against other orbiviruses (OIE, 2018), and it is recommended mainly to confirm positive results of the AGID test. Because of its sensitivity (Shringi & Shringi, 2005; Velic, Velic, Bajrovic, Dukic, & Cano, 2004), cELISA has been recommended as an official OIE test for serological monitoring of antibodies against BTV in small ruminants (Shringi & Shringi, 2005). Nonetheless, AGID is still the main serological test used for screening animals during transit because it is easy to perform. As no seroepidemiological study of BT has been conducted in the state of Paraná, we aimed to detect the presence of antibodies against BTV in sheep in Paraná using AGID and cELISA.

Materials and Methods

The study was conducted between March and October 2017. Initially, sheep blood was collected from 10 randomly selected farms, which had already been monitored in 2014 using AGID. Based on the data from 2014, the farms were classified as follows: low occurrence (0%–9.99% positive sheep; n = 2), moderate occurrence (10%–49.99% positive sheep; n = 4), and high occurrence (50%–100% positive sheep; n = 4). We classified the farms in this manner because there is no specification in the literature regarding the classification of sheep farms based on the occurrence of BTV infection in Brazil.

Overall, this study covered farms located in the municipalities of Araruna, Bom Sucesso, Cândói, Castro, Colombo, Congoinhas, Mandaguari, Maringa, Rancho Alegre, and Rosario do Ivaí, which are situated in six mesoregions of Paraná (Western Center, East Center, South Center, Curitiba, North Central, and North Pioneer). These regions have 60% of the state herd (Secretaria de Estado da Agricultura e do Abastecimento [SEAB], 2017) and are located between latitudes 22 °S and 26 °S.

The climate in these farms is subtropical and is classified according to Koppen (Instituto Agronômico do Paraná [IAPAR], 2017) as: Cfa,

which cover most of the state, with hot summer and well-distributed rainfall throughout the year, and temperature over 22°C; and Cfb, with temperate summer and temperature does not exceed 22°C.

The minimum number of serum samples ($n = 270$) to be tested was calculated using the EpiInfo 7® program, considering that the average prevalence observed in 2014 was 30% (95% confidence interval and 6% standard error). The animals were randomly selected so that 10% of the total number of sheep were sampled from each farm.

The sampled animals belonged to Texel, Dorper, White Dorper, and Santa Inês races, were of both sexes and different ages, had no characteristic clinical signs of BT and could be naturally infected with BTV. Blood was collected in a vacuum test tube after local antiseptics and venipuncture of the jugular vein. It was stored without anticoagulant to obtain serum. The tubes were centrifuged and the sera were separated. The sera were then split into two 1.5 mL microtubes (duplicates), labelled, and frozen until further use.

For the detection of BTV antibodies, the sera were subjected to AGID (Bluetongue Virus Antibody Test Kit AGID-VMRD®) and cELISA test kits. The AGID test was performed in the microbiology laboratory of the Veterinary Hospital of the Northern University of Paraná, campus of Arapongas - PR, using the manufacturer's protocol. The samples that formed the precipitation line were classified as positive. To validate the kit, a known positive blood serum sample was used; this sample was collected during the 2001 outbreak in Paraná (Clavijo et al., 2002) and was provided by the Paraná Agricultural Defense Agency, Curitiba. The other duplicate sample was sent to the Bovine Virus Laboratory of the São Paulo Biological Institute, where the cELISA test was performed using a commercial Bluetongue Virus Antibody Test Kit and following manufacturer's instructions. The samples that showed optical density values of <50% of the mean

optical density values of the negative control were considered positive. Two standard samples were used as controls (one positive and one negative).

To compare the results of the AGID and cELISA tests, a Kappa coefficient of agreement was calculated using the epiDisplay package of the R software (Chongsuvivatwong, 2018).

All procedures used in this study were approved by the Animal Use Ethics Committee of the Northern University of Paraná, Arapongas campus, under protocol 006/16.

Results and Discussion

According to the results it was decided to classify the farm as low occurrence (0-9.99% positive sheep), moderate occurrence (10-49.99% positive sheep) and high occurrence (50-100% positive sheep). This was classified in this manner because there is no specification in the literature regarding the classification of sheep farms based on BTV infection in Brazil.

Of all the sheep tested, 64.81% (175/270) had antibodies against BTV as shown by the cELISA test, indicating high occurrence. On the contrary, 41.11% (111/270) were shown seroreactive by the AGID test, indicating moderate occurrence (Table 1). Of all the samples analyzed, 40% (108/270) of the sheep were seroreactive and 34.07% (92/270) were unreactive in both tests. Discordance was observed in only 1.11% (3/270) animals, which were positive only in the AGID test, and in 24.81% (67/270) animals that were positive only in the cELISA test.

The number of animals found to be positive in the cELISA test was higher than those in the AGID test across all the farms that had seroreactive sheep. Further, the Kappa coefficient between the results of both tests showed a moderate agreement (0.50) (Thrusfield, 2004).

Table 1
Distribution of number and percentage of sheep detected seroreactive using the cELISA and AGID tests

County/ Farm	Climate classification	No. of serum samples analyzed	Positive serum samples	
			cELISA	AGID
Araruna	Cfa	30	20 (66.67%)	12 (40.00%)
Bom Sucesso	Cfa	26	24 (92.30%)	20 (76.92%)
Candói	Cfb	26	22 (84.62%)	16 (61.53%)
Castro	Cfb	40	4 (10.00%)	1 (2.50%)
Colombo	Cfb	27	0 (0%)	0 (0%)
Cornélio Procópio	Cfa	29	25 (86.21%)	12 (41.38%)
Mandaguari	Cfa	30	25 (83.33%)	19 (63.33%)
Maringá	Cfa	30	25 (83.33%)	11 (36.67%)
Rancho Alegre	Cfa	9	7 (77.78%)	2 (22.22%)
Rosário do Ivaí	Cfa	23	23 (100%)	18 (78.26%)
Total	-	270	175 (64.81%)	111 (41.11%)

Probably, unlike the AGID test, the cELISA test was able to detect seroreactive sheep even when the antibody concentration was low. Several researchers have reported that the AGID test can be difficult to interpret because it is qualitative and not quantitative, has low sensitivity mainly in samples with low antibody titration, and may cross-react with other orbiviruses (Ward & Thurmond, 1995; Chandel, Chauhan, & Kher, 2003). In this study, only 1.11% (3/270) of the sheep were positive in AGID alone; this discrepancy between the two tests could be because of a cross reaction with other orbiviruses.

In this study, the cELISA test could detect 97.29% of sheep that were found seroreactive by AGID. Further, it detected 64 animals that were not positive in AGID, indicating to be more reliable. Similar results were observed by Shringi and Shringi (2005) when they observed that cELISA detected 100% of the sheep found to be seropositive by AGID and 37 more sheep that were not found to be positive by AGID. Although AGID has been recommended by OIE since 1982 because of its practicality and lower cost per sample, it is clear that it is not as sensitive as cELISA and is therefore being replaced by cELISA (Shringi & Shringi, 2005).

In regions close to where the present study was conducted, in the Northern Central Mesoregion of Paraná, Negri et al. (2016) detected antibodies against BTV in dairy cattle using cELISA and the virus neutralization technique. They found 100% (633/633) and 64.61% (409/633) positive samples, respectively, demonstrating the superiority of the cELISA test.

Corroborating the findings of the present study, Nogueira et al. (2009) compared the cELISA and AGID techniques in the state of São Paulo and found 74.3% (744/1002) of the sheep to be positive by cELISA and 65% (651/1002) by AGID, demonstrating a high occurrence of BTV antibodies in sheep (50%–100% positive animals). Their observations also showed more sensitive antibody detection by the cELISA test and demonstrated the superiority of this test. Many dissertations have also reinforced the reliability of cELISA compared to AGID (Biihrer, 2017; Kawanami, 2017). In the state of São Paulo, Kawanami (2017) observed the occurrence of BTV antibodies in several ruminant species. They found that in sheep, 60.69% (105/173) and 55, 49% (96/173) animals were positive, by cELISA and AGID, respectively. In Minas Gerais, Biihrer (2017) observed 56.44% (171/303) positive sheep by cELISA and 54.13% (164/303) by AGID.

Similar results of high BTV occurrence were obtained in the states of Paraná, São Paulo, and Minas Gerais (Coetzee et al., 2012). This is because of the similarity between climates; all farms in these studies were distributed within the BTV occurrence zone of 50 °N to 35 °S. The farms studied in this report were located between 22 °S and 26 °S, which also falls within the BTV occurrence zone. In addition, our results are consistent with those reported in the literature, which indicate high serum prevalence of BTV in subtropical regions, such as Paraná. Most of the territory in this region has hot summers and well distributed rainfall throughout the year; this condition combined with the absence of dry season favors the presence and multiplication of the Culicoid vector and the infection occurs throughout the year (Lobato et al., 2015).

The results obtained by serological tests showed that 90% of the farms (9/10) presented at least one seropositive sheep, and only the farm located in the municipality of Colombo, metropolitan region of Curitiba, did not show BTV antibodies in any of the tests. These results demonstrate that, although clinical signs are not present, the virus is present in the sheep across most of the farms in Paraná, showing that the virus is spreading silently. Similar results were observed by Venditti (2009) in the state of São Paulo, where 85% (11/13) of the farms presented at least one seroreactive sheep using both tests. Further, Nogueira et al. (2009) in São Paulo and Biihrer (2017) in Minas Gerais reported that 100% of the studied farms had BTV-seroreactive animals detected by both cELISA and AGID.

Overall, our results show that the regions classified as Cfa (hot summers, well distributed rainfall throughout the year, and average temperature above 22 °C) present higher occurrence of BTV than the regions classified as Cfb (mild summers with average temperature not exceeding 22 °C) (Table 1). The farm located in Colombo, for example, has Cfb climate and has no BTV seroreactive sheep. A previous study has shown that as temperature decreases, BTV infection and transmission rates

decrease (Mellor, 2000), which possibly explains the lower detection of antibodies in Colombo. Medlock and Leack (2015) have reported that BT and other Culicoid-transmitted diseases occur more or less frequently all over the globe, mainly because of climatic variations.

Despite the low occurrence of BTV observed in a farm located in a Cfb region, the first outbreak of the disease reported in Brazil in 2001 occurred in the same mesoregion (Metropolitana de Curitiba). At the time, Clavijo et al. (2002) isolated BTV serotype 12 from sheep and goats. Later, Negri et al. (2015) identified serotype 4 from clinically healthy cattle. Recently, serotypes 3, 14, 18, 19, and 22 (OIE, 2016) have been isolated after the death of bororo deer in an ecological reserve in Foz do Iguaçu, Paraná. This finding suggests that the presence of antibodies associated with the absence of clinical signs cannot exclusively be justified by the climatic variations; low pathogenicity of circulating serotypes in different farms could also be a reason behind it.

Conclusions

Finally, it can be concluded that BTV infection is present in the sheep herds of Paraná. The occurrence was moderate as detected by the AGID test and high as detected by the cELISA test. Further study on the climatic variations and differences in serotypes present in different mesoregions is warranted to maintain disease surveillance and prevent further outbreaks.

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