


Prevalence and risk factors for bluetongue in the State of São Paulo, Brazil

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Abstract

Bluetongue (BT), caused by *Bluetongue virus* (BTV), is a disease that affects ruminants such as cattle, sheep, goats and deer. BTV is transmitted by female midges of the genus *Culicoides*. In Brazil, information on the prevalence of BTV in cattle is limited, so the objective of this work was to identify BTV serotypes in cattle. The State of São Paulo was divided into seven cattle-producing regions, and in each of them, 300 cattle farms were randomly selected. One animal from each farm (out of a total of 1,598 farms) was selected and its sera tested by virus neutralization technique against BTV serotypes (1–24 and 26) for determining antibody titre. Moreover, for each sampled farm, an epidemiological questionnaire was submitted to verify the type of cattle production and the zootechnical and sanitary practices carried out, which could be associated with a higher risk of BTV infection. In this study, antibodies (percentage, [95% confidence interval]) were identified against 11 serotypes: BTV-1 (22.15%, [15.72–27.92]), BTV-2 (31.03%, [26.65–37.98]), BTV-3 (18.96%, [12.42–24.90]), BTV-4 (24.90% [19.41–29.12]), BTV-9 (6.82%, [1.45–11.72]), BTV-12 (7.50%, [2.82–12.51]), BTV-17 (23.90%, [17.35–29.35]), BTV-19 (10.20%, [4.62–5.56]), BTV-21 (30.66%, [25.00–36.00]), BTV-22 (12.14%, [5.91–18.55]), BTV-26 (57.00%, [51.41–63.59]). In this study, for the first time in Brazil serological evidence of the presence of serotypes BTV-2, BTV-9, BTV-21 and BTV-26 is reported. The variable 'new cattle entering herd' was considered a risk factor for the occurrence of infection (OR = 2.183, 95% CI = 1.6–2.9).

Keywords: *Bluetongue virus*, Brazil, seroprevalence.

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Introduction

Bluetongue (BT) is an infectious viral disease affecting wild and domestic ruminants. It is caused by *Bluetongue virus* (BTV), a species of the genus *Orbivirus* transmitted mainly by adult female *Culicoides* spp. (Diptera: Ceratopogonidae) (Takamatsu *et al.* 2003).

The BTV genome consists of 10 linear segments of dsRNA (Seg-1 to Seg-10); it encodes seven structural proteins (VP-1 to VP-7) and five non-structural proteins (NS1, NS2, NS3/NS3a, NS4 and S10-ORF2) and the virus particle is surrounded by three

concentric protein layers (Maan *et al.* 2007; Attoui *et al.* 2012). VP-2 and VP-5 are the most variable proteins in BTV, and VP-2 contains most of the epitopes responsible for the specific antibody neutralization reaction generated by the vertebrate host, and is therefore used to identify different serotypes of BTV (Maan *et al.* 2011). To date, 27 serotypes have been widely recognized, and two new putative serotypes, BTV-28 and BTV-29, have been proposed (Maan *et al.* 2015; Bumbarov *et al.* 2016; Sun *et al.* 2016). Infection in cattle and goats is usually asymptomatic, but the disease can cause severe clinical signs and death in sheep and deer (Darpel *et al.* 2007). BT is

included in the list of diseases of immediate notification to the World Organization for Animal Health, due to the considerable socioeconomic concern and great importance for the international trade of animals and their byproducts (OIE 2017a,b). Economic losses associated with BTV infection are directly caused by reductions in animal productivity, death and commercial losses due to restrictions of animal movements (Walton 2004). Mortality, abortion, medication, veterinary and additional labour services, as well as the drop of production registered in 6 months, led to a loss of US\$ 6,700 (Balara *et al.* 2014a,b).

In South America, BTV was isolated in Argentina (BTV-4), French Guiana (BTV-1, -2, -6, -10, -12, -13, -17 and -24) and Ecuador (BTV-9, -13, -18) (Legisa *et al.* 2013; Viarouge *et al.* 2014; Verdezoto *et al.* 2017). Serotypes BTV-1, -3, -4, -12, -14, -17, -18, -19 and -22, associated with the deaths of pygmy brocket deers (*Mazama nana*), were also identified in Brazil, in 2015 and 2016 (Grocock & Campbell 1982; Guimarães *et al.* 2017).

The State of São Paulo is located in the southeastern region of Brazil (21°49'47"S, 49°12'27"W). It is characterized by a hot and humid climate throughout the year. It has a cattle population which represents approximately 5% of the national cattle population, corresponding to the largest meat consumer market in the country (ABIEC, 2015). Although there are several serological studies confirming that BT is endemic in the State (Nogueira *et al.*, 2016; Lobato *et al.* 2015), there is still a knowledge gap regarding the serotypes circulating in Brazil.

In this regard, this study aims to estimate the prevalence of different BTV serotypes and to identify the risk factors for BT in the State of São Paulo, Brazil.

Material and methods

A cross-sectional study was conducted between May and November 2011, in which the State of São Paulo was divided into seven cattle-producing regions (Fig. 1), according to the different breeding, operational and logistic systems as determined by the Animal Health Service of the state of São Paulo

(Coordenadoria de Defesa Agropecuária – CDA). In each region, serum samples were collected from female bovines aged 24 months or older in the context of the bovine brucellosis control study in the State of São Paulo (Dias *et al.* 2016).

This study was carried out following the ethical guidelines adopted by the Brazilian Society of Laboratory Animal Sciences and the Brazilian College of Animal Experimentation (SBCAL/COBEA), and was approved by the Ethics Committee of the Biological Institute, São Paulo, Protocol Number 66/08.

In the Bovine Viruses Laboratory (LVB), of the Center for Research and Development of Animal Health of the Biological Institute in São Paulo, Brazil, a solid phase ELISA (CFS ELISA) screening test (Panaftosa, Rio de Janeiro) was performed as a selection criterion. One positive animal was selected from 1598 farms (total 1598 sera) and tested by virus neutralization test (VNT) at the OIE Reference Laboratory for Bluetongue, Teramo (Italy) to evaluate the presence of neutralizing antibodies against BTV serotypes (1–24 and 26).

Briefly, sera are heat-inactivated at 56°C for 30 min in a bain-marie before testing. Subsequently, they were serially diluted twofold in an appropriate medium (MEM added with antibiotics and foetal calf serum) from an initial dilution of 1:10 up to 1:1280. Subsequently, an equal volume of 50 µL OIE standard reference BTV serotypes (100–300 TCID₅₀) (reference strains obtained from Onderstepoort Veterinary Institute, South Africa) was added to each well. After 1 h incubation at 37°C in 5% CO₂, approximately 10⁴ VERO cells were added per well in a volume of 100 µL of MEM containing antibiotics and plates incubated as previously described. Positive and negative control sera, virus control wells and cell control wells were included in each neutralisation session. Beginning from the third day, plates were scored for the degree of cytopathic effect (CPE) observed in the virus control wells (back titration from 100 to 300 TCID₅₀) and for the titre of the reference positive (expected titre ± 1 dilution factor) and negative sera (no neutralisation). A sample was considered positive when it showed more than 75% of CPE neutralisation at the lowest dilution (1:10). The serum titre was defined as the highest

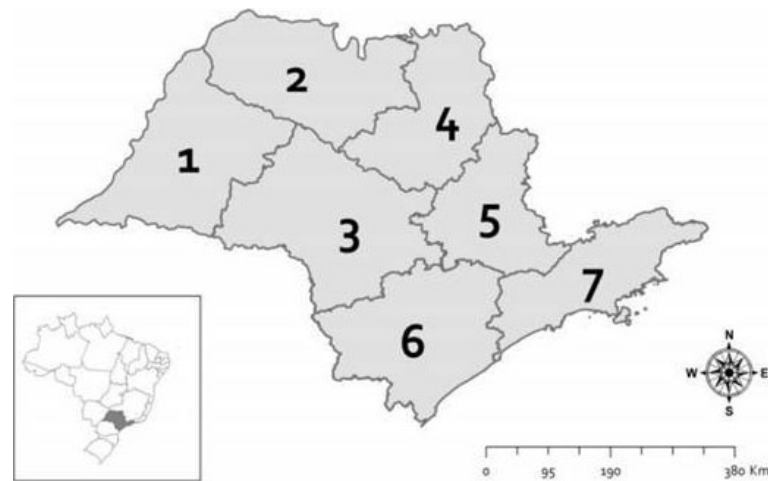


Fig. 1 Map of the State of São Paulo showing the division into regions (Dias *et al.* 2009).

serum dilution capable of neutralising more than 75% CPE in the tissue culture. Antibody titre was expressed as the log₁₀ of the reciprocal of the highest serum dilution able to inhibit at least 75% of the virus CPE (Savini *et al.* 2004).

Risk factors

For each sampled farm, a questionnaire was completed to generate data on its sanitary and zootechnical practices. All information generated in the field and in the laboratory was entered into a database. In this cross-sectional study, the risk factors evaluated were: (i) type of cattle production (beef, dairy or mixed); (ii) type of operation (confined, semi-confined and extensive); (iii) type of milking (manual, mechanical at the foot of the cow, mechanical in a milking parlour); (iv) use of artificial insemination and/or natural breeding; (v) presence of wild animals, including deer; (vi) presence of sheep/goats; (vii) abortions in the last 12 months; (viii) sale of animals for breeding; (ix) slaughter of adult animals; (x) new cattle entering herd; (xi) purchase of breeding stock; (xii) pasture rental; (xiii) sharing rights of way, such as access to water and salt; (xiv) pasture in common with other properties; (xv) presence of flooded areas that cattle have access to; (xvi) concentration of cattle in small areas; (xvii) calving pens; (xviii) classification of property; (xix) veterinary care. For

the study of the risk factors associated with BTV, the epidemiological questionnaire variables were first submitted to an exploratory data analysis using the chi-square test χ^2 (univariate). Variables with a significance level of 80% or greater ($P \leq 0.20$) were added to the final multivariate logistic regression model (regression). Risk factors for BTV were considered as variables with a significance level greater than or equal to 95% ($P \leq 0.05$) in the final model. All calculations were made using SPSS 2.2 and EpiInfo 7.0 software.

Results

All animals screened were ELISA positive. Neutralizing antibodies (percentage, [95% confidence interval]) were identified for 11 serotypes: BTV-1 (22.15%, [15.72–27.92]), BTV-2 (31.03%, [26.65–37.98]), BTV-3 (18.96%, [12.42–24.90]), BTV-4 (24.90% [19.41–29.12]), BTV-9 (6.82%, [1.45–11.72]), BTV-12 (7.50%, [2.82–12.51]), BTV-17 (23.90%, [17.35–29.35]), BTV-19 (10.20%, [4.62–15.56]), BTV-21 (30.66%, [25.00–36.00]), BTV-22 (12.14%, [5.91–18.55]), BTV-26 (57.00%, [51.41–63.59]). Table 1 shows the percentage of reactive and non-reactive animals and the respective neutralizing antibody titres for BTV serotypes (BTV-1, -2, -3, -4, -9, -12, -17, -19, -21, -22 and -26) in the seven regions of the State of São Paulo in 2011.

Results of the univariate analysis (Table 2) and the final logistic regression model (Table 3) indicated new cattle entering herd [2183 (odds ratio), 1619–2945 (95% confidence interval)] as a risk factor.

Discussion

Since the beginning of the 1980's, with the first report of the presence of BTV in Brazil, many seroprevalence studies have been carried out in the State of São Paulo, with reports of high prevalence of BTV-reactive cattle and sheep, proving that the virus is endemic and is widely distributed throughout the State of São Paulo (Grocock & Campbell 1982; Cunha 1990; Arita *et al.* 1992; Nogueira *et al.* 2009, 2016).

In the present study, a high seroprevalence of BTV-reactive bovines was detected, with the identification of antibodies specific for 11 serotypes (BTV-1, -2, -3, -4, -9, -12, -17, -19, -21, -22 and -26), indicating the circulation of several serotypes in the seven cattle-producing regions (Table 1). In a seroprevalence study conducted in the State of São Paulo, 100% of reactive bovines were detected by ELISA and 86% of bovines reactive to BTV-4 by means of VN. Although they detected only BTV-4, the authors suggested the presence other serotypes in the State (Nogueira *et al.* 2016). In 2011 and 2013, recurrent outbreaks of BT were reported in sheep in the State of Rio de Janeiro, where BTV-4 serotype was identified (Balara *et al.* 2014a,b). In this study, specific antibodies against BTV-1, 4 and 17 were detected. In 2014, BTV-1, 4 and 17 serotypes were identified in sheep with clinical signs (Guimarães *et al.* 2017), confirming the findings of this study.

In the analysis of the prevalence of BTV serotypes for the seven regions of the State of São Paulo, regions 1, 2 and 3 (Fig. 1) presented prevalence for all serotypes identified in this study. These regions are located in the western part of the state with intensive cattle production, and recognized as the main beef cattle area in the state, with large farms and intensive cattle marketing also associated with the presence of refrigerated slaughterhouses (IBGE, 2016). In these regions, the large presence of cattle is favourable for maintaining the BTV cycle, since bovines, due to their long-lasting viraemia, are sources

Table 1. Neutralizing antibody titers in bovine sera from the State of São Paulo, Brazil (2011) against BTV serotypes

Antibody Titer	BTV1	BTV2	BTV3	BTV4	BTV9	BTV12	BTV17	BTV19	BTV21	BTV22	BTV26
<1:10	(75.5) 1207	(67.2) 1074	(80.2) 1282	(74.1) 1184	(91.5) 1462	(91.5) 1462	(75.1) 1201	(87.7) 1403	(67.2) 1074	(86.1) 1376	(40.6) 650
1:10	(14.5) 233	(20.3) 324	(14.5) 231	(16.1) 258	(4.7) 75	(6.4) 102	(16.7) 267	(7.5) 120	(23.0) 368	(7.1) 114	(37.6) 602
1:20	(5.2) 83	(8.4) 134	(3.2) 52	(6.0) 96	(1.4) 23	(0.9) 11	(5.2) 84	(2.0) 33	(6.1) 98	(2.7) 44	(14.8) 238
1:40	(2.1) 33	(1.9) 30	(1.2) 19	(2.2) 34	(0.6) 9	(0.4) 6	(1.7) 28	(0.4) 7	(1.1) 18	(1.9) 31	(3.5) 56
1:80	(0.2) 4	(0.6) 10	(0.06) 1	(0.2) 4	(0.1) 2	(0.06) 1	(0.06) 1	(0.1) 3	(0.3) 1	(0.3) 2	(0.9) 9
1:160	(0.06) 1	0	0	(0.2) 4	0	0	(0.1) 2	0	0	0	(0.2) 4
1:320	0	0	0	0	0	0	0	0	(0.2) 4	0	0
1:640	0	0	0	0	0	0	0	0	0	(0.1) 3	(0.1) 2
1:1280	0	0	(0.06) 1	2	0	0	0	0	0	0	0
Toxic	(1.3) 20	(0.7) 10	(0.4) 7	(0.3) 4	(0.6) 10	(0.6) 9	(0.4) 7	(0.6) 11	(1.1) 19	(0.7) 12	(0.8) 14
Insufficient	(1.1) 17	(1.0) 16	(0.3) 5	(0.7) 12	(1.1) 17	(0.4) 7	(0.5) 8	(1.3) 21	(0.9) 15	(1.0) 16	(1.4) 23
Total	1598 (100%)										

Numbers in brackets are percentages.

Table 2. Univariate (chi-square) analysis of cattle herds reactive and non-reactive to BTV in the State of São Paulo, Brazil, in 2011, considering non-statistically significant variables ($P < 0.20$)

Variable	% (BTV reactive)	% (non-BTV reactive)	Total	<i>P</i>
Farm type				
Meat	13.0% (83)	87.0% (555)	638	0.95
Dairy	13.5% (76)	86.5% (486)	562	
Mixed	88.7% (353)	11.3% (45)	398	
Type of operation				
Extensive	12.8% (175)	87.2% (1195)	1370	0.48
Semi-extensive	12.6% (27)	87.4% (187)	214	
Confined	14.3% (2)	85.7% (12)	14	
Type of milking				
Mechanical	12.6% (79)	87.4% (546)	625	0.51
Manual	5.0% (1)	95.0% (19)	20	
Manual and mechanical	16.3% (15)	83.7% (77)	92	
—	13.6% (109)	86.4% (695)	804	
Artificial insemination (AI)				
Does not use AI	12.9% (192)	87.1% (1300)	1492	0.91
Uses AI and bulls	88.7% (63)	11.3% (8)	71	
Only uses AI	11.4% (4)	88.6% (31)	35	
Presence of sheep and goats				
Yes	11.3% (33)	88.7% (261)	294	0.40
No	13.1% (171)	86.9% (1133)	1304	
Presence of wild animals				
Yes	11.0% (75)	89.0% (607)	682	0.13
No	14.1% (129)	85.9% (787)	916	
Presence of deer				
Yes	10.0% (22)	90.0% (199)	221	0.51
No	13.2% (182)	86.8% (1195)	1377	
Abortions within the last 12 months				
No	13.0% (176)	86.9% (1177)	1353	0.35
Yes	9.2% (14)	90.8% (139)	153	
Not known	15.4% (14)	84.6% (77)	91	
Entry of new cattle into herd				
No	8.9% (85)	91.1% (871)	956	0.00
Yes	81.4% (522)	18.6% (119)	641	
Purchase of breeding stock				
No	11.2% (129)	88.8% (1032)	1161	0.016
Yes	17.2% (75)	82.8% (361)	436	
Sale of breeding stock				
No	12.7% (177)	87.3% (1219)	1396	0.81
Yes	13.4% (27)	86.6% (175)	202	
Slaughter of adults				
No	11.9% (133)	88.1% (989)	1122	0.19
Yes	14.9% (71)	85.1% (405)	476	
Pasture rental				
No	12.6% (167)	87.4% (1161)	1328	0.84
Yes	13.7% (37)	86.3% (233)	270	
Common grazing				
No	12.6% (173)	87.4% (1201)	1374	0.42
Yes	13.8% (31)	86.2% (193)	224	
Sharing rights of way with other farms				
No	13.0% (173)	87.0% (1167)	1340	0.31
Yes	12.0% (31)	88.0% (227)	258	

Table 2. Continued

Variable	% (BTV reactive)	% (non-BTV reactive)	Total	<i>P</i>
Flooded areas				
No	12.7% (140)	87.3% (967)	1107	0.85
Yes	13.1% (64)	86.9% (426)	490	
Concentrated cattle				
No	12.7% (187)	87.3% (1294)	1481	0.37
Yes	14.5% (17)	85.5% (100)	117	
Calving pens				
No	13.5% (169)	86.5% (1087)	1256	0.61
Yes	12.3% (35)	87.7% (250)	285	
Veterinary care				
No	11.4% (122)	88.6% (954)	1076	0.11
Yes	15.7% (82)	84.3% (440)	522	
Watering places shared with other farms				
No	12.4% (164)	87.6% (1162)	1326	0.22
Yes	14.7% (40)	85.3% (232)	272	
Farm type				
Rural	12.7% (179)	87.3% (1232)	1411	0.86
With some settlements	14.8% (13)	85.2% (75)	88	
Urban periphery	12.1% (12)	87.9% (87)	99	

Table 3. Final model of multivariate logistic regression of risk factors (Odds Ratio) for BTV in bovines of the State of São Paulo, Brazil, in the year of 2011

Variables	OR	CI (95%)	<i>P</i>
Entry of new cattle into herd	2183	[1619–2945]	0.00

of infection for the vector for a long period (Mayo *et al.* 2010). In a seroepidemiological survey in sheep in the city of Araçatuba (region 1), Nogueira *et al.* (2009) identified prevalences >60% by ELISA and IDGA, confirming that the animals in this region have antibodies against BTV, but asymptomatic.

The BTV-12 had already been identified in sheep with clinical signs in the State of Rio Grande do Sul (Antoniassi *et al.* 2010). BTV-3, -19 and -22 serotypes identified in this study had already been reported in 2016 in an ecological reserve in the State of Paraná, infecting pygmy brocket deers (*Mazama nana*) with clinical signs followed by death. This information reinforces the importance of carrying out seroepidemiological surveys and sanitary surveillance in rainforest areas where there is movement of

wild animals, mainly deer, susceptible to BTV, which can be infected because they are very close to areas used for cattle breeding. BTV-17 was identified for the first time in the State of São Paulo. In 2016, Matos *et al.* (2016) published for the first time in Brazil the complete sequence of BTV-17, isolated from sheep in the State of Rio Grande do Sul. Analysis of the nucleotide sequences indicated the occurrence of rearrangements of this strain with other BTV serotypes of the Americas. In addition to the serotypes mentioned above, the results of this study confirm the first occurrence of specific antibodies against the following serotypes in Brazil: BTV-2, BTV-9, BTV-21 and BTV-26. In an experimental study, Batten *et al.* (2014) described BTV-26 transmission by direct contact in goats, but more studies are needed to clarify the modes of transmission of this serotype in Brazil.

The State of São Paulo is located in a region with a tropical and subtropical climate, with high temperature and humidity throughout the year. These climatic characteristics associated with intensive cattle production in the region allow multiplication and constant maintenance of vector populations in the environment. The annual culicoides generations are influenced mainly by rain and temperature, which create vector breeding areas, and can increase transmission rates during rainy and hot periods (Mellor 2000). In China, in a BTV seroprevalence study in Asian sheep, bison and yaks (*Bos grunniens*), the authors observed that seasonality was a risk factor for BTV infection, with summer and fall at higher risk (Ma *et al.* 2017). Low prevalence of BTV antibodies in cattle and sheep has been observed in regions of Brazil where the climatic conditions are unfavourable to the development of vector insects (Alves *et al.* 2009; Souza *et al.* 2010). A large part of South America is located in an endemic area for the occurrence of BTV, and serological and molecular studies indicate that the virus is present in countries such as Argentina, Ecuador, French Guiana (Legisa *et al.* 2013; Lobato *et al.* 2015; Verdezoto *et al.* 2017).

'Entry of new cattle into herds' was identified as a risk factor ($P = 0.00$) associated with the prevalence of BTV in herds, indicating the importance of sanitary control of animals that may be in viraemic when

introduced into the herd, or come from disease-free areas and are introduced into herds in areas where the virus or certain serotypes are endemic. The animals were 24 months of age or older, and no clinical signs were observed. Some studies have suggested that seroprevalence of BT increases with the age of the animals, and is probably a reflection of the longer exposure to the vector and the virus, which can lead to multiple infections (Ward *et al.* 1994; González *et al.* 2000).

BTV is a notifiable disease, causing restrictions on semen trade and movement of live animals. This study increases our knowledge of the disease, both by identifying circulating serotypes and by determining their distribution in Brazil.

High seroprevalence of BTV was identified in the seven regions of the state of São Paulo, and knowledge of the prevailing serotypes will be decisive for the implementation of infection control strategies such as targeted vaccination. However, further multidisciplinary studies including entomological, climatic and epidemiological approaches are required, with a view to the effective implementation of active surveillance and health programs for BTV in Brazil. 'Entry of new cattle into herds' was identified as a risk factor associated with the prevalence of BTV in herds. To date, we described, for the first time in Brazil, serological evidence of the presence of serotypes BTV-2, BTV-9, BTV-21 and BTV-26.

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Conflict of interest

The authors certify that there is no conflict of interest with any financial organization.

Ethics statement

This study was approved by the Ethics Committee for Experimentation on Animals of Biological Institute of São Paulo and conducted in accordance with national and international guidelines on handling animals.

Contributions

Design of the experiments: Thaís Garcia da Silva, Giovanni Savini and Edviges Maristela Pituco. Samples collection: Fernando Gomes Buchala, Klaus Saldanha Hellwing, Adriana Hellmeister de Campos Nogueira Romaldini, Eliana De Stefano and Maira de Souza Nunes Martins. Experiments: Thaís Garcia da Silva, Michele dos Santos Lima, Massimo Spedicato, and Irene Carmine. Cell culture preparation: Liana Teodori, Alessandra Leone. Manuscript draft and Revision: Thaís Garcia da Silva, Michele dos Santos Lima and Edviges Maristela Pituco.

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