



## Detection of bluetongue virus in sheep by real-time RT-PCR in different production systems in San Martin, Peru



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### Abstract:

The present study aimed to determine the prevalence of Bluetongue Virus (BTV) in sheep, by the real-time Reverse Transcription-polymerase chain reaction (RT-PCR) technique. Three hundred sixty-six sheep from the ten provinces of the Peru region were evaluated. The methodology used was the collection of blood samples from the jugular vein of the sheep, then the process of extraction and purification of RNA was carried out with the QIAmp® kit, then the reverse transcription to obtain the cDNA, and finally perform the real-time RT-PCR, for which the SuperScript III platinum One-step qRT-PCR kit was used, with the primers and probes being directed to segment 10 of the NS3 gene of BTV. The results of the real-time RT-PCR test revealed two positive sheep with a value of cycle threshold (Ct) of 35.21 and 35.57, with a prevalence of 0.54 % of BTV-positive sheep in the extensive production system, with environmental conditions that favor the development of the *Culicoides* vector. It is concluded that, by means of the real-time RT-PCR technique, the presence of BTV in this region of Peru is confirmed, which makes future studies necessary to determine the detection of other potential

serotypes of BTV in the Peruvian Amazon in order to improve the control strategies of the disease.

**Key words:** RNA, Gene, Molecular diagnosis, Ruminants, Amazonia.

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## Introduction

Bluetongue virus [BTV; in Spanish: *virus de la lengua azul* (VLA)], genus *Orbivirus*<sup>(1)</sup>, without envelope, the genome has 10 segments of double-stranded RNA, seven structural and five non-structural proteins<sup>(2)</sup>. VP7 is a viral capsid protein<sup>(3)</sup>. VP2 of the surface of the virion<sup>(4)</sup>. In addition, serotypes 9, 13, 18<sup>(5)</sup> and 27<sup>(6)</sup>, with serotype 28 being recently identified<sup>(7)</sup>.

Bluetongue is a disease, non-contagious and transmitted through the bite of *Culicoides* insects<sup>(8)</sup>. Of the 1,357 species of *Culicoides* in the world<sup>(9)</sup>, only about 30 have been reported as vectors<sup>(10)</sup>. Vectors, when feeding on an animal with BTV, will be infected all their lives<sup>(11)</sup>, they can infect animals with variations in clinical signs<sup>(12)</sup>.

Sheep manifest seromucous nasal discharge, facial edema, thrombo-hemorrhagic fever, coronitis and ulceration in the lips and hard palate<sup>(13)</sup>; the viremia is usually detected between 3 and 5 days after infection, reaching febrile peaks on day 7<sup>(14)</sup>. However, these clinical signs (e.g., fever in sheep and cattle, salivation in cattle, facial edema in sheep) were observed in infected and non-infected herds, these signs are not indicative of the disease<sup>(15)</sup>. Likewise, macroscopic lesions are pulmonary edema, cranio-ventral pulmonary consolidation, swollen and cyanotic tongue; and hemorrhagic lesions in rumen mucosa<sup>(16)</sup>. Cattle, goats, buffaloes and other wild ruminants act as reservoirs of the virus, being animals that may not show clinical signs<sup>(17,18,19)</sup>, however, there are reports of mortality in cattle with BTV serotype 8<sup>(20)</sup>; other clinical signs such as amaurosis, which is the inability to stand and the absence of the sucking reflex in infected calves<sup>(21)</sup>. Therefore, the different serotypes of BTV in small and large ruminants indicate its enzootic expansion<sup>(22)</sup>. Likewise, the transmission of the virus, host-vector is complex, with a variety of ecological drivers<sup>(23)</sup>.

The epidemiology of BTV is listed in the OIE Terrestrial Animal Health Code<sup>(24)</sup>. It has generated outbreaks in Israel in 2006, generating losses of \$ 2.5 million<sup>(25)</sup>, as well as an outbreak in Western Europe<sup>(26)</sup>. Thus, it has had an annual cost of \$ 3 billion, it has become one of the diseases of economic importance<sup>(27)</sup>.

Currently, this disease is found worldwide, except in Antarctica<sup>(28)</sup>. It has been reported in Brazil in sheep<sup>(16)</sup>; in Sudan, in reproductive disorders in cattle<sup>(29)</sup>; in Japan<sup>(30)</sup> and India, in a transplacental transmission of BTV-1, in the middle stage of gestation in sheep<sup>(31)</sup>. In Australia, BTV originated from Southeast Asia<sup>(32)</sup>. While in the United Kingdom, wild animals in zoos are susceptible to arboviruses, acting as native hosts of *Culicoides*<sup>(33)</sup>.

In Peru, BTV has been reported in sheep, camelids and wild animals in tropical areas<sup>(34,35,36)</sup>. Preliminary studies were conducted in 1984 and 1987, finding 88, 41 and 56 % of sheep from three regions (north, central and south)<sup>(37)</sup> that were seropositive for BTV and 21 % of seroreactor alpacas from the southern region<sup>(38)</sup>. In studies in *Tayassu tajacu* in Madre de Dios, 7.5 % of samples with BTV-specific antibodies are reported and 29.2 % had antibodies to the BTV serogroup<sup>(34)</sup>. Recently, Navarro *et al*<sup>(35)</sup> confirmed the presence of the virus in sheep of extensive farming, in addition to identifying the *Culicoides* that are vectors of the disease.

However, the San Martín region, in its tropical climate, is an optimal environment for the vector and facilitates its infection routes in domestic and wild hosts; mentioned by Felipe-Bauer<sup>(39)</sup>, who reported *Culicoides* species.

In relation to the diagnosis, the seroneutralization test is specific, but its disadvantage is the low sensitivity and cost<sup>(40)</sup>. However, competitive ELISA is suggested in llamas, wild ruminants<sup>(41)</sup>, sheep and goats<sup>(42)</sup>. Currently, the tests recommended by the OIE for BTV are RT-PCR, agar gel immunodiffusion assay and competitive ELISA<sup>(43,44)</sup>.

With the real-time RT-PCR being for the rapid detection of BTV directed to Seg-1/VP1<sup>(45,46)</sup>, Seg-2/VP2 and Seg-10/NS3<sup>(47)</sup>, it is a widely used method<sup>(48)</sup>, in the detection of all BTV serotypes<sup>(49)</sup>, based on the TaqMan fluorescence probe<sup>(50,51,52,53)</sup> to detect BTV in samples of infected sheep<sup>(54)</sup>. Therefore, the objective of the study was to detect BTV in sheep by real-time RT-PCR, considering the influence of environmental characteristics and presence of the disease.

## Material and methods

### Place of study

The study was conducted in the San Martín region, Peru. Blood samples were collected from sheep in stable health condition, in the 10 provinces of the San Martín region (Table 1), in the period from August to December 2018.

**Table 1:** Number of sheep sampled by province suitable for the development of the vector

Provinces	No. of samples
Moyobamba	9
Rioja	11
El Dorado	35
Lamas	41
Bellavista	74
Picota	41
Mariscal Cáceres	15
Huallaga	24
Tocache	30
San Martín	86
Total	366

### Sample collection

Three hundred sixty-six blood samples were collected from the jugular vein of the randomly selected sheep, which were from 42 farms. For the collection, vacuum vacutainer needles and tubes with EDTA anticoagulant were used. The samples were transported in a refrigerated container with cooling gel for their processing in the Laboratory of Molecular Biology and Genetics of the Professional School of Agronomy - Faculty of Agricultural Sciences, National University of San Martín.

According to the 2012 Census<sup>(55)</sup>, the population is 7,656 sheep in the entire department of San Martín. The sample was calculated based on this population:

$$n = \frac{Z^2 pq N}{E^2(n-1) + Z^2 pq}$$

Where:  $n$  is the sample size;  $Z$  is the confidence level 95% = 1.96;  $p$  is the probability of success 50%/100 = 0.5;  $q$  is the probability of failure 50%/100 = 0.5;  $E$  is the error level 5%/100 = 0.05;  $N$  is the population size = 7656.

$$n = \frac{(1.96)^2 (0.5) (0.5) (7656)}{(0.05)^2 (7656-1) + (1.96)^2 (0.5) (0.5)}$$

$$n = 366$$

Sample subpopulation formula.

$$nh = \frac{Nh}{N} (n)$$

Nh= subpopulation or group; N= total population; n= total sample; nh= sample of the groups.

### **RNA extraction**

For the processing of the blood sample, it was centrifuged at 800 xg for 10 min, then the plasma was extracted, and sterile PBS was added, the tube was inverted several times to mix, centrifuge again at 800 xg for 10 min to separate the red blood cells from the PBS. The QIAmp® kit, Qiagen brand, was used for RNA extraction. The RNA extraction step was performed according to the procedures specified by the manufacturer. The final product obtained is RNA in Buffer, to finally continue with the real-time RT-PCR, which targets segment 10 of BTV (NS3 Gene).

### **Real-time Reverse transcription - Polymerase chain reaction**

Real-time RT-PCR is a test of choice for diagnosis. The method described here is recommended by the OIE and Hofmann *et al*<sup>(56)</sup>, to detect segment 10 of the NS3 Gene of BTV. To obtain the cDNA by real-time reverse transcription and PCR, the SuperScript III platinum One-step qRT-PCR kit, Invitrogen brand, was used, being the sequences of the primers for the detection of segment 10 of the NS3 gene of BTV. The primary solutions of the primer were diluted to a concentration of 20 pmol/μl, the nucleotide sequences of the primers: VLA\_IVI\_F 5'-TGG-AYA-AAG-CRA-TGT-CAA-A-3', VLA\_IVI\_R 5'-ACR-TCA-TCA-CGA-AAC-GCT-TC-3'<sup>(57)</sup>. The probe solution for the NS3 gene of BTV was diluted to a concentration of 5 pmol/μl, the sequence of the probe: VLA\_IVI\_P 5'FAM-ARG-CTG-CAT-TCG-CAT-CGT-ACG-C-3' BHQ1.

Zero point five microliters of each primary primer at a concentration of 20 pmol/μL were added to each well, the plate must be kept on ice. Then 2 μL of RNA samples, both from the target sample and from the positive and negative controls, are added to the corresponding wells of the plate following the distribution. The denaturing temperature was 95 °C for 5 min, and they were kept on ice for another 3 min. A volume of the primary mixture<sup>(57)</sup> of the RT-PCR was prepared, following the manufacturer's instructions. The probe was included in the primary mixture to obtain a final concentration of 0.2 pmol/μL per sample. Twenty microliters of primary mixture were distributed in each well of the plate located in the real-time thermocycler programmed for reverse transcription and amplification, detection by fluorescence of cDNA.

### **Temperature conditions**

The reaction was carried out in a light cycler 480 System Roche Applied Biosystems. Following the following thermal profile: Reverse transcription 48 °C for 30 min, reverse

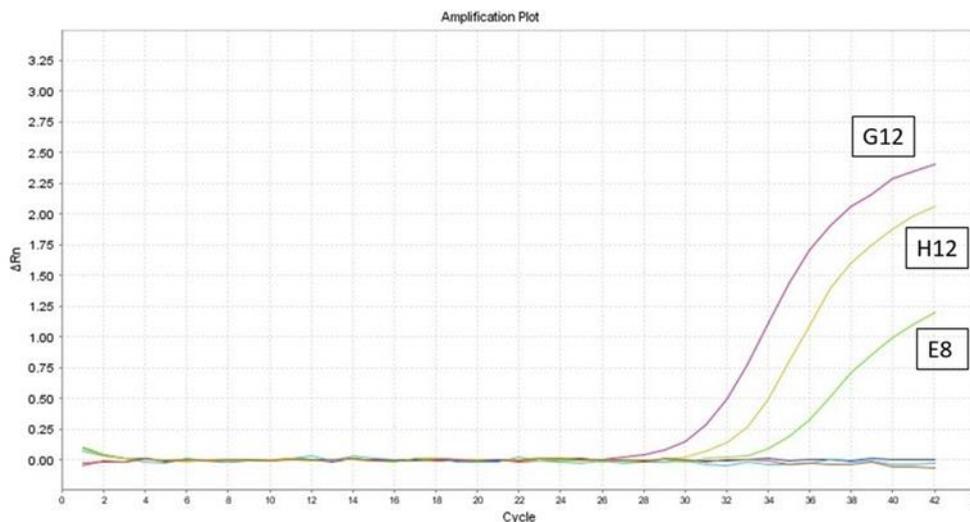
transcriptase inactivation or initial denaturation 95 °C for 2 min, followed by 50 cycles, amplification 95 °C for 15 sec, 56 °C for 30 sec, 72 °C for 30 sec<sup>(57)</sup>.

## Results and discussion

### Real-time RT-PCR analysis

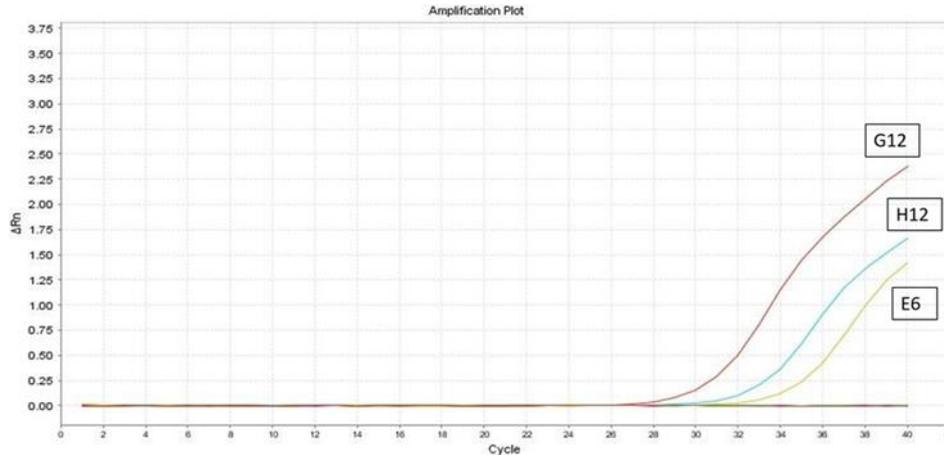
Real-time RT-PCR results indicated that 0.54 % (2/366) of the samples were BTV-positive. For the first individual, with code (E8), positive for BTV, it had a cycle threshold (Ct) value of about 35.21, with a dissociation temperature<sup>TM</sup> value of 84 °C for BTV. For the sample with code (G12) – C+VLA 1/10, it had a Ct of 28.22 and 31.63 for the sample with code (H12) – C+VLA 1/100. No amplification was observed in the negative control. The high viral load and amplification of a specific product were evidenced (Figure 1).

**Figure 1:** Results of the real-time RT-PCR test for the first positive individual (E8), in the amplification curve, it is observed with a Ct value of 35.21. Opticon Monitor software v.3.0



For the second individual (E6) positive for BTV, it had a Ct value of about 35.57, with a dissociation temperature<sup>TM</sup> value of 84 °C. For the sample (G12)- C+VLA 1/10, it had a Ct of 28.81 and 32.72 for the sample (H12)- C+VLA 1/100. No amplification was observed in the negative control. The high viral load and amplification of a specific product were evidenced (Figure 2).

**Figure 2:** Results of the real-time RT-PCR test for the second positive individual (E6), in the amplification curve, a Ct value of 35.57 is observed. Opticon Monitor Software v.3.0



A panoramic review in South America, using serological studies for the detection of antibodies conducted on cattle, goats, sheep and buffaloes, indicates varied minimum and maximum prevalences in the following countries: in Argentina (0-95 %) <sup>(58)</sup>, Brazil (1.22-89.69 %) <sup>(59,60)</sup>, Chile (0-19.6 %) <sup>(61,62)</sup>, Colombia (51.8-56 %) <sup>(63,64)</sup>, Ecuador (10 %) <sup>(65)</sup>, Guyana (0-56 %) <sup>(66)</sup>, Suriname (82-91 %) <sup>(66)</sup> and Venezuela (74.8-94.7 %) <sup>(67)</sup>, in Peru, preliminary studies in 1984 and 1987 reported a seroprevalence of 87.5, 41 and 55.5 % of BTV in sheep from the north, center and south of the country's highlands <sup>(37)</sup>. In the present research, the viral genome of BTV was detected, with the prevalence being 0.54 % in sheep sera by real-time RT-PCR, which confirms the epidemiological presence of this virus in this region. The areas where the positive animals were detected are very close to water tributaries, such as the Sisa River and the Ishangayacu Ravine. With environmental sensitivities being as a key component of the capacity of the vector <sup>(68)</sup>.

It is also possible that BTV infection in the sampled sheep was subclinical, as the animals were under normal conditions. This is explained, according to Maclachlan *et al* <sup>(69)</sup>, because the development of the clinical signs of the disease depends on whether the infection is endemic or not; as a consequence, animals have antibodies, but rarely show clinical signs. This is possibly because in sheep the period of viremia rarely persists for more than 14 d, unlike in cattle, whose viremia can be up to 90 to 120 d.

As mentioned by Navarro <sup>(36)</sup>, Peru is one of the countries that is predisposed to present BTV disease, since it has several ecosystems conducive to the development of *Culicoides*. It is considered necessary to determine the seroprevalence of BTV in livestock areas, identify the different serotypes, map the location of the different species of *Culicoides* spp., in the different geographical areas and altitudes, and determine the endemic areas.

## Environment of BTV-positive sheep

An assessment of the surroundings of the farms was carried out to observe the habitat of the *Culicoides* vector. It was found that BTV-positive animals are near water tributaries, which contributes to the development of the vector (Table 2). On the other hand, the positive sheep were of the Pelibuey breed, however, the predisposition due to breed is not determinant. In addition, extensive farming of sheep allows their farming together with cattle, chickens, dogs and horses. In the surveys conducted, the owners of extensive, intensive and semi-intensive farms do not deworm their animals, nor do they fumigate for the presence of flies or mosquitoes.

**Table 2:** Environment of BTV-positive sheep

Province	District	Location of the farm	Farming system	Environment of the farm	Breed	Body condition
Bellavista	San Pablo	Angélica Sector	Extensive	Left bank of the Sisa River	Pelibuey	3
Bellavista	San Pablo	Hamlet of San Ignacio	Extensive	Left bank of the Ishangayacu Ravine	Pelibuey	2.5

Epidemiological studies indicate that BTV exists in a large area in the world, between 40° north latitude and 35° south latitude, with tropical, subtropical and temperate ecosystems<sup>(70)</sup>, these characteristics coincide with the San Martín region.

The first studies of the presence of the BTV-transmitting vector in this region were conducted by Felipe-Bauer *et al*<sup>(39)</sup>, who identified five species of *Culicoides* that are vectors of the virus. Similarly, Navarro *et al*<sup>(35)</sup>, of the 7,930 mosquitoes captured, 94.8 % were identified as *Culicoides insignis*, and the presence of BTV in sheep in the Pucallpa region is also confirmed<sup>(36)</sup>.

On the other hand, the *Culicoides* vector usually develops in areas where there are certain types of drivers such as land use, trade, animal husbandry and the presence of wild animals as a reservoir of BTV; the latter is reinforced by the work carried out by Rivera *et al*<sup>(34)</sup>, who found 7.5 % of white-lipped peccaries (*Tayassu pecari*) positive for BTV in the Madre de Dios region.

The present research work is the first to be developed in the entire San Martín region, where the results using real-time RT-PCR show the presence of BTV. Felipe-Bauer *et al*<sup>(39)</sup> mention having found the *Culicoides* vector of the virus. Therefore, it is required to detect other existing serotypes of BTV in domestic and wild animals of the region that are susceptible or reservoirs of the disease.

## Farming systems

Regarding the farming system, most producers opt for an extensive sheep farming in the San Martín Region (Table 3).

**Table 3:** Production systems

Provinces	Farming systems		
	Intensive	Extensive	Semi Intensive
Moyobamba	0	9	0
Rioja	0	11	0
El Dorado	0	35	0
Lamas	0	27	14
Bellavista	0	74	0
Picota	0	29	12
Mariscal Cáceres	0	15	0
Huallaga	0	19	5
Tocache	0	20	10
San Martín	33	15	38
		Total	366

## Conclusions and implications

It is concluded that BTV has a low prevalence in sheep in the San Martín Region of Peru, however, future studies are needed to determine morbidity and the detection of other potential BTV serotypes in the country, to better elucidate the management of vectors and control strategies of the disease.

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## Literature cited:

1. Batten CA, Henstock MR, Steedman HM, Waddington S, Edwards L, Oura CA. Bluetongue virus serotype 26: infection kinetics, pathogenesis and possible contact transmission in goats. *Vet Microbiol* 2013;162(1):62–67. <https://doi.org/10.1016/j.vetmic.2012.08.014>.
2. Patel A, Roy P. The molecular biology of Bluetongue virus replication. *Virus Res* 2014;(182):5-20. doi: 10.1016/j.virusres.2013.12.017.

3. Roy P. Bluetongue virus proteins and particles and their role in virus entry, assembly, and release. *Adv Virus Res* 2005;64:69–123. <https://doi.org/10.1111/tbed.12625>.
4. Wilson WC, Hindson BJ, O'Hearn ES, Hall S, Tellgren-Roth C, Torres C, *et al.* A multiplex real-time reverse transcription polymerase chain reaction assay for detection and differentiation of Bluetongue virus and Epizootic hemorrhagic disease virus serogroups. *J Vet Diagn Invest* 2009;21(6):760-70. doi: 10.1177/104063870902100602. PMID: 19901276.
5. Verdezoto J, Breard E, Viarouge C, Quenault H, Lucas P, Sailleau C, *et al.* Novel serotype of bluetongue virus in South America and first report of epizootic haemorrhagic disease virus in Ecuador. *Transbound Emerg Dis* 2018;65(1):244-247. doi: 10.1111/tbed.12625. Epub 2017 Feb 26. PMID: 28239988.
6. Schulz C, Bréard E, Sailleau C, Jenckel M, Viarouge C, Vitour D, *et al.* Bluetongue virus serotype 27: detection and characterization of two novel variants in Corsica, France. *J Gen Virol* 2016;97(9):2073-2083. doi: 10.1099/jgv.0.000557. Epub 2016 Jul 19. PMID: 27435041.
7. Bumbarov V, Golender N, Jenckel M, Wernike K, Beer M, Khinich E, Zalesky O, Erster O. Characterization of bluetongue virus serotype 28. *Transbound Emerg Dis* 2020;67(1):171-182. doi: 10.1111/tbed.13338. Epub 2019;31469936.
8. Purse BV, Brown HE, Harrup L, Mertens PP, Rogers DJ. Invasion of bluetongue and other orbivirus infections into Europe: the role of biological and climatic processes. *Rev Sci Tech* 2008;27(2):427-42. PMID: 18819670.
9. Borkent A, Willis WW. World species of biting midges (Diptera: Ceratopogonidae). Vol. 233. New York, USA: American Museum of Natural History. 1997.
10. Linley JR. Autogeny in the Ceratopogonidae: literature and notes. *Fla. Entomol* 1983;(66):228–234.
11. Maclachlan NJ, Zientara S, Wilson WC, Richt JA, Savini G. Bluetongue and epizootic hemorrhagic disease viruses: recent developments with these globally re-emerging arboviral infections of ruminants. *Curr Opin Virol* 2019;34:56-62. doi: 10.1016/j.coviro.2018.12.005. Epub 2019 Jan 14. PMID: 30654271.
12. Labadie T, Sullivan E, Roy P. Multiple routes of bluetongue virus egress. *Microorganisms*. 2020;27;8(7):965. doi: 10.3390/microorganisms8070965. PMID: 32605099; PMCID: PMC7409164.
13. Schwartz-Cornil I, Mertens PP, Contreras V, Hemati B, Pascale F, Bréard E, Mellor PS, MacLachlan NJ, Zientara S. Bluetongue virus: virology, pathogenesis and immunity. *Vet Res* 2008;39(5):46. doi: 10.1051/vetres:2008023.

14. Foster NM, Luedke AJ, Parsonson IM, Walton TE. Temporal relationships of viremia, interferon activity, and antibody responses of sheep infected with several bluetongue virus strains. *Am J Vet Res* 1991;52(2):192–196. <https://pubmed.ncbi.nlm.nih.gov/1707246/>. Accessed Feb 17, 2020.
15. Elbers ARE, Backx A, Ekker HM, van der Spek AN, van Rijn PA. Performance of clinical signs to detect bluetongue virus serotype 8 outbreaks in cattle and sheep during the 2006-epidemic in The Netherlands. *Vet Microbiol*;2021;129(1–2):156–162. <https://pubmed.ncbi.nlm.nih.gov/18164148/>.
16. Bianchi RM, Panziera, Welden FTC, Almeida GL, Cargnelutti JF, Flores EF, Kommers GD, Figuera RA. Clinical, pathological and epidemiological aspects of outbreaks of bluetongue disease in sheep in the central region of Rio Grande do Sul. *Pesq. Vet Bras* 2017;37(12):1443-1452. <https://doi.org/10.1590/S0100-736X2017001200014>.
17. Wilson AJ, Mellor PS. Bluetongue in Europe: past, present and future. *Philos Trans R Soc Lond B Biol Sci* 2009; 27;364(1530):2669-81. doi: 10.1098/rstb.2009.0091. PMID: 19687037; PMCID: PMC2865089.
18. Lorca-Oró C, López-Olvera JR, Ruiz-Fons F, Acevedo P, García-Bocanegra I, Oleaga Á, Gortázar C, Pujols J. Long-term dynamics of bluetongue virus in wild ruminants: relationship with outbreaks in livestock in Spain, 2006-2011. *PLoS One*. 2014;18;9(6):e100027. doi: 10.1371/journal.pone.0100027. PMID: 24940879; PMCID: PMC4062458.
19. Lager IA. Bluetongue virus in South America: overview of viruses, vectors, surveillance and unique features. *Vet Ital* 2004;40(3):89-93. PMID: 20419641.
20. Santman-Berends IM, van Schaik G, Bartels CJ, Stegeman JA, Vellema P. Mortality attributable to bluetongue virus serotype 8 infection in Dutch dairy cows. *Vet Microbiol* 2011;24;148(2-4):183-188. doi: 10.1016/j.vetmic.2010.09.010. Epub 2010 Sep 16. PMID: 20889271.
21. Vinomack C, Rivière J, Bréard E, Viarouge C, Postic L, Zientara S, *et al*. Clinical cases of Bluetongue serotype 8 in calves in France in the 2018-2019 winter. *Transbound Emerg Dis* 2020;67(3):1401-1405. doi: 10.1111/tbed.13466. Epub 2020 Jan 8. PMID: 31883429.
22. Maan S, Tiwari A, Chaudhary D, Dalal A, Bansal N, Kumar V, *et al*. A comprehensive study on seroprevalence of bluetongue virus in Haryana state of India. *Vet World* 2017;10(12):1464-1470. doi: 10.14202/vetworld.2017.1464-1470. Epub 2017 Dec 13. PMID: 29391687; PMCID: PMC5771171.

23. Mayo C, McDermott E, Kopanke J, Stenglein M, Lee J, Mathiason C, Carpenter M, Reed K, Perkins TA. Ecological dynamics impacting bluetongue virus transmission in North America. *Front Vet Sci* 2020;17;7:186. doi: 10.3389/fvets.2020.00186. PMID: 32426376; PMCID: PMC7212442.
24. OIE. Listed diseases, infections and infestations 2021. <https://www.oie.int/es/enfermedad/lengua-azul/>. Accessed Jun 13, 2021.
25. Kedmi M, Van Straten M, Ezra E, Galon N, Klement E. Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds. *J Dairy Sci* 2010;93(6):2486-2495. doi:10.3168/jds.2009-2850.
26. Velthuis AG, Saatkamp HW, Mourits MC, de Koeijer AA, Elbers AR. Financial consequences of the Dutch bluetongue serotype 8 epidemics of 2006 and 2007. *Prev Vet Med* 2010;1;93(4):294-304. doi: 10.1016/j.prevetmed.2009.11.007. Epub 2009 Dec 3. PMID: 19962204.
27. Rushton J, Lyons N. Economic impact of Bluetongue: a review of the effects on production. *Vet Ital* 2015;51(4):401-406. doi: 10.12834/VetIt.646.3183.1. PMID: 26741252.
28. Van der Sluijs MT, de Smit AJ, Moormann RJ. Vector independent transmission of the vector-borne bluetongue virus. *Crit Rev Microbiol* 2016;42(1):57-64. doi: 10.3109/1040841X.2013.879850. Epub 2014 Mar 19. PMID: 24645633.
29. Elhassan AM, Babiker AM, Ahmed ME, El Hussein AM. Coinfections of Sudanese dairy cattle with bovine herpes virus 1, bovine viral diarrhoea virus, bluetongue virus and bovine herpes virus 4 and their relation to reproductive disorders. *J Advanced Vet Anim Res* 2017;3(4):332-337. <http://doi.org/10.5455/javar.2016.c169>.
30. Kato T, Shirafuji H, Tanaka S, Sato M, Yamakawa M, Tsuda T, Yanase T. Bovine arboviruses in *Culicoides* biting midges and sentinel cattle in Southern Japan from 2003 to 2013. *Transbound Emerg Dis* 2016;63(6):e160-e172. doi: 10.1111/tbed.12324. Epub 2015 Jan 19. PMID: 25597441.
31. Saminathan M, Singh KP, Khorajiya JH, Dinesh M, Vineetha S, Maity M, *et al*. An updated review on bluetongue virus: epidemiology, pathobiology, and advances in diagnosis and control with special reference to India. *Vet Q* 2020;40(1):258-321. doi: 10.1080/01652176.2020.1831708. PMID: 33003985; PMCID: PMC7655031.
32. Kedmi M, Van Straten M, Ezra E, Galon N, Klement E. Assessment of the productivity effects associated with epizootic hemorrhagic disease in dairy herds. *J Dairy Sci* 2010;93(6):2486-2495. doi:10.3168/jds.2009-2850.

33. England ME, Pearce-Kelly P, Brugman VA, King S, Gubbins S, Sach F, *et al.* *Culicoides* species composition and molecular identification of host blood meals at two zoos in the UK. *Parasit Vectors* 2020;16;13(1):139. doi: 10.1186/s13071-020-04018-0. PMID: 32178710; PMCID: PMC7076997.
34. Rivera H, Cárdenas L, Ramírez M, Manchego A, More J, Zúñiga A, *et al.* Orbivirus infection in white-lipped peccaries (*Tayassu pecari*) from Madre de Dios region, Perú. *Rev Inv Vet Perú* 2013;24(4):544–550. <https://doi.org/10.15381/rivep.v24i4.2738>.
35. Navarro D. Identificación de *Culicoides* spp, como vectores del virus Lengua Azul en áreas de ovinos seropositivos de Pucallpa, Ucayali [tesis maestría]. Perú: Universidad Nacional Mayor de San Marcos; 2017.
36. Navarro MD, Rojas M, Jurado PJ, Manchego SA, Ramírez VM, Castillo EA, *et al.* Molecular detection of Bluetongue virus in *Culicoides insignis* and sheep of Pucallpa, Perú. *Rev Investig Vet Perú* 2019;30(1):465–476. <http://dx.doi.org/10.15381/rivep.v30i1.15690>.
37. Rosadio RH, Evermann JF, De Martini JC. A preliminary serological survey of viral antibodies in peruvian sheep. *Vet Microbiol* 1984;10(1):91–96. doi: 10.1016/0378-1135(84)90059-2.
38. Rivera H, Madewell BR, Ameghino E. Serologic survey of viral antibodies in the Peruvian alpaca (*Lama pacos*). *Am J Vet Res* 1987;48(2):189-191. PMID: 3826854.
39. Felipe-Bauer ML, Cáceres A, Silva CS, da Valderrama-Bazan W, Gonzales-Pérez A, Costa JM. New records of *Culicoides latreille* (Diptera: Ceratopogonidae) from Peruvian Amazonian region. *Biota Neotropica* 2008;8(2):33–38. <https://doi.org/10.1590/s1676-06032008000200002>.
40. Singer RS, Boyce WM, Gardner IA, Johnson WO, Fisher AS. Evaluation of bluetongue virus diagnostic tests in free-ranging bighorn sheep. *Prev Vet Med* 1998;35(4):265-282. doi:10.1016/s0167-5877(98)00067-1.
41. Afshar A, Heckert RA, Dulac GC, Trotter HC, Myers DJ. Application of a competitive ELISA for the detection of bluetongue virus antibodies in llamas and wild ruminants. *J Wild Dis* 1995;31(3):327-30. doi: 10.7589/0090-3558-31.3.327. PMID: 8592352.
42. Elmahi MM, Karrar ARE, Elhassan AM, Hussien MO, Enan KA, Mansour MA, El Hussein ARM. Serological investigations of Bluetongue Virus (BTV) among sheep and goats in Kassala State, Eastern Sudan. *Vet Med Int* 2020;2020:8863971. doi: 10.1155/2020/8863971. PMID: 33062245; PMCID: PMC7547342.

43. Saminathan M, Singh KP, Vineetha S, Maity M, Biswas SK, Manjunathareddy GB, *et al.* Virological, immunological and pathological findings of transplacentally transmitted bluetongue virus serotype 1 in IFNAR1-blocked mice during early and mid-gestation. *Sci Rep* 2020;10(1):2164. doi: 10.1038/s41598-020-58268-0.
44. Díaz-Cao JM, Lorca-Oró C, Pujols J, Cano-Terriza D, de Los Ángeles Risalde M, Jiménez-Ruiz S, Caballero-Gómez J, García-Bocanegra I. Evaluation of two enzyme-linked immunosorbent assays for diagnosis of bluetongue virus in wild ruminants. *Comp Immunol Microbiol Infect Dis* 2020;70:101461. doi: 10.1016/j.cimid.2020.101461. Epub 2020 Feb 29. PMID: 32151837.
45. Shaw AE, Monaghan P, Alpar HO, Anthony S, Darpel KE, Batten CA, *et al.* Development and initial evaluation of a real-time RT-PCR assay to detect bluetongue virus genome segment 1. *J Virol Meth* 2007;145(2):115-126. <https://doi.org/10.1016/j.jviromet.2007.05.014>.
46. Toussaint JF, Sailleau C, Breard E, Zientara S, De Clercq K. Bluetongue virus detection by two real-time RT-qPCRs targeting two different genomic segments. *J Virol Meth* 2007;140(1):115- 123. <https://doi.org/10.1016/j.jviromet.2006.11.007>.
47. Van Rijn PA, Geurts Y, Van der Spek AN, Veldman D, Van Gennip RG. Bluetongue virus serotype 6 in Europe in 2008-emergence and disappearance of an unexpected non-virulent BTV. *Vet Microbiol* 2012;158(1):23-32. doi: 10.1016/j.vetmic.2012.01.022.
48. Rojas JM, Rodríguez-Martín D, Martín V, Sevilla N. Diagnosing bluetongue virus in domestic ruminants: current perspectives. *Vet Med* 2019;(10):17–27. <https://doi.org/10.2147/VMRR.S163804>.
49. Mulholland C, McMenemy MJ, Hoffmann B, Earley B, Markey B, Cassidy J, Allan G, *et al.* The development of a real-time reverse transcription-polymerase chain reaction (rRT-PCR) assay using TaqMan technology for the pan detection of bluetongue virus (BTV). *J Virol Meth* 2017;(245):35–39. <https://doi.org/10.1016/j.jviromet.2017.03.009>.
50. MacLachlan NJ. Bluetongue: pathogenesis and duration of viraemia. *Vet Ital* 2004;40(4):462–467. PMID: 20422570.
51. Mertens PPC, Maan NS, Prasad G, Samuel, AR, Shaw AE, Potgieter AC, Anthony SJ, Maan S. Design of primers and use of RT-PCR assays for typing European bluetongue virus isolates: differentiation of field and vaccine strains. *J Gen Virol* 2007;88(10):2811-2823. doi: 10.1099/vir.0.83023-0.

52. Hoffmann B, Beer M, Reid SM, Mertens P, Oura CA, Van Rijn PA, *et al.* A review of RT-PCR technologies used in veterinary virology and disease control: sensitive and specific diagnosis of five livestock diseases notifiable to the World Organisation for Animal Health. *Vet Microbiol* 2009;139(1-2):1-23. <https://doi.org/10.1016/j.vetmic.2009.04.034>.
53. Maan NS, Maan S, Belaganahalli MN, Ostlund EN, Johnson DJ, Nomikou K, Mertens PP. Identification and differentiation of the twenty-six bluetongue virus serotypes by RT-PCR amplification of the serotype-specific genome segment 2. *PLoS One* 2012;7(2):e32601. doi: 10.1371/journal.pone.0032601. Epub 2012 Feb 28. PMID: 22389711; PMCID: PMC3289656.
54. Lakshmi I, Putty K, Raut SS, Patil SR, Rao PP, Bhagyalakshmi B, *et al.* Standardization and application of real-time polymerase chain reaction for rapid detection of bluetongue virus. *Vet World* 2018;11(4):452–458. <https://doi.org/10.14202/vetworld.2018.452-458>.
55. INEI. Resultados Definitivos. IV Censo Nacional Agropecuario [Internet]. IV Censo Nacional Agropecuario. 2012: <https://sigrid.cenepred.gob.pe/sigridv3/documento/906>. Consultado 21 May, 2021.
56. Hofmann MA, Renzullo S, Mader M, Chaignat V, Worwa G, Thuer B. Genetic characterization of toggenburg orbivirus, a new bluetongue virus, from goats, Switzerland. *Emerg Infect Dis* 2008;14(12):1855-61. doi: 10.3201/eid1412.080818. PMID: 19046507; PMCID: PMC2634640.
57. Manual Terrestre de la OIE-2014. Lengua azul: Infección por el virus de la Lengua Azul. Capítulo 2.1.3.
58. Lager IA. Bluetongue virus in South America: overview of viruses, vectors, surveillance and unique features. *Vet Ital* 2004;40(3):89-93. PMID: 20419641.
59. Cunha RG, de Souza DM, Texeira AC. Anticorpos precipitantes para o virus da lengua azul em soros de bovinos do estado do Rio de Janeiro. *Biológico, São Paulo*. 1982;48(4):99-103.
60. Melo CB, Oliveira AM, Castro RS, Lobato ZIP, Leite RC. Precipitating antibodies against the bluetongue virus in bovines from Sergipe, Brazil. *Cienc Vet Trop Recife* 1999;2(2):125-127.
61. Fundación Vida Silvestre Argentina (FVSA). Operativo de captura de venados de las pampas en campos del Tuyú. FVSA, Buenos Aires. 2004; [vidasilvestre.org.ar/pastizales/2\\_6.asp](http://vidasilvestre.org.ar/pastizales/2_6.asp). Consultado 9 Jul, 2004.
62. Tamayo R, Schoebitz R, Alonso O, Wenzel J. First report of bluetongue antibody in Chile. *Progr Clin Biol Res* 1983;178:555-558.

63. Homan EJ, Lorbacher de Ruiz H, Donato AP, Taylor WP, Yuill TM. A preliminary survey of the epidemiology of bluetongue in Costa Rica and Northern Colombia. *J Hyg Camb* 1985;94:357- 363.
64. Homan EJ, Taylor WP, Lorbacher de Ruiz H, Yuill TM. Bluetongue virus and epizootic haemorrhagic disease of deer virus serotypes in northern Colombian cattle. *J Hyg Camb* 1985;95:165-172.
65. Lopez WA, Nicoletti P, Gibbs EPJ. Antibody to bluetongue virus in cattle in Ecuador. *Trop Anim Hlth Prod* 1985;17:82.
66. Gibbs J, Greiner E, Alexander F, King H, Roach C. Serological survey of ruminant livestock in some countries of the Caribbean region and South America for antibody to bluetongue virus. *Vet Rec* 1984;114(26):635-638.
67. Gonzalez MC, Perez N, Siger J. Serologic evidence of bluetongue virus in bovines from Aragua State, Venezuela. *Rev Fac Cienc Vet UCV*2000;41(1-3):3-12.
68. Purse BV, Carpenter S, Venter GJ, Bellis G, Mullens BA. Binomics of temperate and tropical *Culicoides* Midges: Knowledge gaps and consequences for transmission of *Culicoides* -Borne viruses. *Annu Rev Entomol* 2014;(60):373–392. <http://doi.org/10.1146/annurev-ento-010814-020614>.
69. Maclachlan NJ, Drew CP, Darpel KE, Worwa G. The pathology and pathogenesis of bluetongue. *J Comp Pathol* 2009;141(1):1-16. doi: 10.1016/j.jcpa.2009.04.003. Epub 2009 May 23. PMID: 19476953.
70. Mertens PP, Diprose J, Maan S, Singh KP, Attoui H, Samuel AR. Bluetongue virus replication, molecular and structural biology. *Vet Ital* 2004;40(4):426-37. PMID: 20422565.