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Absence of IgG antibodies against Schmallenberg virus in ruminant sera (bovine, goat and sheep) collected in Costa Rica between 2012 and 2014.

Ausencia de Anticuerpos IgG al virus de Schmallenberg en sueros de rumiantes (bovino, caprinos y ovinos) colectados en Costa Rica entre 2012 y 2014.

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Abstract

Schmallenberg virus affects ruminants, which causes significant economic losses. The virus is transmitted through vectors of the genus *Culicoides*; however, other studies do not rule out the possibility of sexual transmission due to its presence in semen. For this reason, the National Service of Animal Health of Costa Rica (SENASA) imposed restrictions on the import of semen from animals from the European Union in 2013. Consequently, SENASA conducted a study to determine the presence or absence of antibodies against this virus in bovine, ovine and caprine samples. As a result, no antibodies against this virus were detected in the 748 samples tested. It was concluded that Schmallenberg virus was not circulating in Costa Rican ruminants during the tested period.

Keywords: Ruminants, SENASA, Schmallenberg virus, Costa Rica, serology.

Resumen

El virus de Schmallenberg afecta a rumiantes produciendo pérdidas económicas importantes, el cual se transmite a través de artrópodos del género *Culicoides*, sin embargo, no se descarta la posibilidad de transmisión sexual debido a la presencia del virus en semen, razón por la cual, el Servicio Nacional de Salud Animal de Costa Rica (SENASA), estableció restricciones a la importación de semen de rumiantes procedentes de la Unión Europea en el 2013. En consecuencia, el SENASA realizó un estudio para determinar la presencia o ausencia de anticuerpos contra este virus en muestras de bovinos, ovinos y caprinos. Como resultado de este estudio no se encontró la presencia anticuerpos contra el virus de Schmallenberg en ninguna de las 748 muestras evaluadas, por lo que se concluye que este virus no está circulando en los rumiantes de Costa Rica.

Palabras claves: Rumiantes, SENASA, virus Schmallenberg, Costa Rica, serología.

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Introduction

Reports of emerging and re-remerging viral diseases have increased over the last decade. Some of these etiological agents produce diseases that concern health authorities and professionals worldwide because of the unpredictable effects and impact of these agents (Ruiz-Fons 2012).

Diseases transmitted by arthropods that affect human and animal populations are causing serious local and international health problems, especially in underdeveloped countries. Schmallenberg virus (SBV) is a clear example of these emerging diseases (Alarcón-Elbal & Lucientes 2012). This epizootic disease began in the summer of 2011 in northwestern Germany and the eastern region of the Netherlands. In October 2011, the virus was identified by researchers of the Friedrich Loeffler Institut (FLI), Federal Research Institute for Animal Health in Germany (Bilk *et al.* 2012). Schmallenberg disease was characterized by clinical signs such as fever, diarrhea, and decreased milk production (Gariglinany *et al.* 2012). The acute disease in pregnant females was followed by an epidemic of stillbirths and congenital malformations, suggesting an infection in the uterus that affects the fetus in the different stages of the gestational process. In sheep and goats, this virus causes congenital malformations in the fetuses of infected females (Doceul *et al.* 2013).

The disease was rapidly spread to several European countries by midges of the genus *Culicoides* as *C. dewulfi, C. chiopterus* (de Regge *et al.* 2012) *and C. obsoletus*. It has been demonstrated that the virus can replicate in the latter (Montgomery, Tack & Obonyo 2012). In January 2012, the virus was already present in six departments of northern France, and by March 23 of the same year SBV was confirmed in 894 farms (Ministère de L'Agriculture de L'Alimentation de la Pêche de la Ruralité et de L'Aménagement du Territoire 2012). By the end of April 2012, SBV was reported in 3628 herds in Europe (ProMed-mail 2012).

Several studies have demonstrated that natural or experimentally induced antibodies can survive for over a year in ruminants, and this humoral immunity protects the animal against clinical signs, RNAemia and virus replication in target organs upon reinfection for at least 15 months (Poskin *et al.* 2015). Studies have stablished that venereal transmission of SBV is possible (Ponsart *et al.* 2014). Analysis done in a total of 766 semen batches from 11 of 95 SBV infected bulls, determined by PCR the presence of the virus in 29 semen samples (Cq-values 26-37) intermittent virus excretion was also observed in 2 of the infected bulls (Hoffmann, Schulz & Beer 2013, Schulz *et al.* 2014).

Due to the presence of this new virus and the possibility of semen transmission, the National Service of Animal Health (SENASA) imposed restrictions on the import of genetic material (semen and embryos) in 2013. As a response to this restriction, the European Union requested to the government of Costa Rica and other Central American countries to demonstrate and confirm the absence of Schmallenberg virus in the region.

SBV has shown to be potentially dangerous, not only for the economic losses that it generates, but also because its segmented genome can open the possibility of a recombination with native viruses of the same family (Pepin *et al.* 2010).

No previous studies had been conducted demonstrating the presence or absence of Schmallenberg virus in Costa Rica; consequently, it was of the utmost importance to officially determine the status of this virus in the national herd. For these reasons, the objectives of the present study were to provide scientific evidence to determine whether or not ruminants in Costa Rica have been in contact with

this virus, as well as to establish a possible prevalence based on the presence of antibodies against this virus. The data provided in this study is crucial for the local government to obtain scientific evidence to decide whether or not to maintain the ban in the import of genetic material from member countries of the European Union.

Methodology

Samples were obtained from the National Bank of Ruminant Sera for the Analysis of Brucellosis collected by officials of SENASA (Hernández-Mora *et al.* 2017). Farms were selected from 50,014 cattle farms registered in the Integrated Database of Agricultural Establishments (*Sistema Integrado de Registro de Establecimientos Agropecuarios*-SIREA, SENASA).

At the time of the analysis, meat farms represented 68% of total bovine farms in the country. These establishment were distributed mostly along the Chorotega, Brunca, and Huetar Norte regions. Dairy farms constituted 13% of the bovine industry located in Huetar Norte, the Metropolitan area and the Central Western region. Double purpose farms included 19% of total establishments, which were located mostly in the Huetar Norte region (47% of the farms) followed by the Chorotega and Brunca regions (33% of the farms).

The number of farms was estimated considering a 20% brucellosis prevalence, a 5% error, and a 95% confidence level, while the number of cattle in each farm was estimated assuming a 50% prevalence, a 95% confidence level, and a 10% error. Samples for the Brucellosis study were collected between September and December 2012, after the Schmallenberg virus appeared in Europe. It should be noted that bovine samples were taken before SENASA's restrictions on the import of genetic material.

Size of the SBV sample analyzed.

The bank of brucellosis sera included 13110 bovines, 424 caprines and 510 ovines. These serum samples were stored at -20°C with glycerin in a 1:1 ratio as well as duplicate serum samples without glycerol. Each sample is recorded in the SIREA's database including information such as farm location and type of production.

The sample size of the Schmallenberg study was calculated considering the possibility to detect at least one positive animal in the brucellosis sera population. The parameters used in this calculation were: 95% confidence level, size of the unknown population, and 0.4% expected prevalence. Under these conditions the sample size obtained was 748 individuals, WinEpi, 2006) (Thrusfield *et al.* 2001; De Blas, Ruiz-Zarzuela & Vallejo, 2006). Samples selected included 433 bovines (57.8%), 174 ovines (23.2%), and 141 caprines (18.8%). For subsampling, the 748 samples required were selected using random numbers, since all samples in the sera bank are identified by consecutive numbers. Once selected, a 300 µl aliquot was taken from the glycerol-preserved samples to avoid non-sampled contamination and degradation of the potential antibodies present in the sample during the thawing process.

Analysis of animals with symptomatology.

In addition to the prior information, 76 samples of farm animals with a history of abortions were analyzed. These samples were collected between January and June 2016 and were stored as pure serum at -20°C.

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ELISA Test.

The research was conducted in the Virology Lab (LSE) of SENASA's National Laboratory of Veterinary Services (LANASEVE). The study was focused on determining antibodies directed against the Schmallenberg nucleoprotein virus in bovine, ovine and caprine sera. Samples were analyzed with a multi-species competitive Enzyme-Linked Immunosorbent Assay (ELISA) made by ID.vet Innovative Diagnostics (Grabels, France) (Wensman *et al.* 2013; Schulz *et al.* 2014). ELISA was used following the manufacturer's instructions, with the exception that samples used were prediluted in glycerol, as mentioned above.

Results and Discussion

After the 748 selected samples were processed, 6 resulted positive, 2 suspicious and 740 negative. Duplicates of positive and suspect samples were reanalyzed using the pure serum sample to eliminate any effect that the cryoprotectant (glycerol) could have on ELISA's performance. Neither positive nor suspect samples resulted positive once the pure glycerol-free sera were reevaluated.

Although glycerol to a final concentration of 25-50% helps to stabilize proteins (antibodies) at -20°C by preventing the formation of ice crystals that could destroy protein structure, its presence led to false positives in eight samples in this assay. This interference was demonstrated when the same sera were processed without the cryoprotectant. Glycerol has a viscosity of 14.9 poises and, in this particular method, a large amount sampled (50 µL) was dispensed directly into the wells, without any diluent, which could facilitate for some unclear reason the mix of the conjugate with glycerol in the samples, avoiding the development of color with the substrate. Finally, no antibodies against Schmallenberg virus were found in the 76 samples collected from animals with a history of abortion between January and June 2016.

Since none of the samples presented antibodies against SBV it is fundamental to demonstrate that the subsample taken from the Brucellosis sera bank was representative of the total Costa Rican ruminant herd and that the sample size is large enough to support the results.

Sample size must be adequate to reduce the cost of excessive sampling and avoid type II errors, not to reject a null hypothesis when it is false (Kim & Seo 2013). Our Ho is: Schmallenberg virus is present in Costa Rica; however, when the null hypothesis is false and it is accepted, a type II error named β is made. This error depends on the power of the test (1-β). The risk of making a type II error decreases when the test has enough power. It can be done by confirming that the sample size is large enough to detect the presence of antibodies against Schmallenberg virus when it is truly circulating in the herd. The statistical power is acceptable when it is 0.8, then the error is 20%. In this study, the power of the test was 0.8 by default for 748 samples; in other words, the size of the samples was broad enough to accept the null hypothesis.

Another factor that could affect the size sample is the sensitivity and specificity of the screening test. For instance, if the sensitivity of a test is low a bigger sample would be required. According to the ELISA manufacturer, the specificity of the cELISA is 100%, while sensitivity is 96.47%, if we use the EpiTools software (Cameron & Baldock 1998) to determine the size sample considering the sensitivity and specificity of the method described above, and with a population size of 13110, a SVB prevalence of 0.05, and a type I and II error of 0.05, the total animals to be sampled would be 61 bovines. Even though the bovine population is increased, the number of animals to be sampled is the same. According to

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the EpiTools epidemiological calculator, the probability that antibodies against SBV are present at a prevalence of 0.05 is 0.0498 if no reactors are found in 61 samples. It can be inferred from this data that 433 animals are adequate to reject the null hypothesis and conclude that the population is free from the disease (at the expected minimum prevalence of 5%) at a confidence level equivalent to 1.

In addition to sample size, the geographical distribution of the samples is another element to be considered in the results obtained. The following characteristics which were considered in the brucellosis study, such as: Animal species, economic zone and type of commercial production, were also represented in the SBV study, the socioeconomic area with the largest number of animals in both studies was the Huetar Norte region (36% of 433), and since it is a double purpose area it is also the largest productive type (19% of sampled animals). Samples of goat and sheep species come from double purpose establishments since it is the only type of production reported by producers.

As it can be seen in figures 1 to 3, samples tested came from all over the country, which guaranteed that all regions were represented in the study, supporting the veracity of the results obtained.

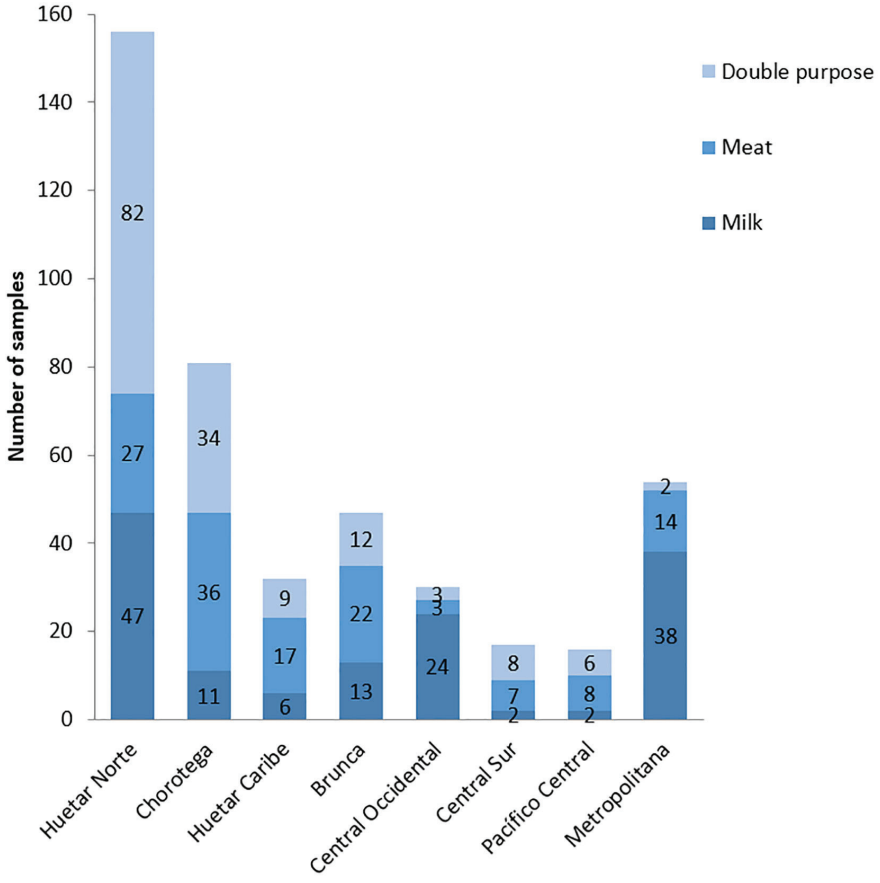

Figure 1. Samples taken from the Brucellosis bovine sera bank categorized by region and type of production.

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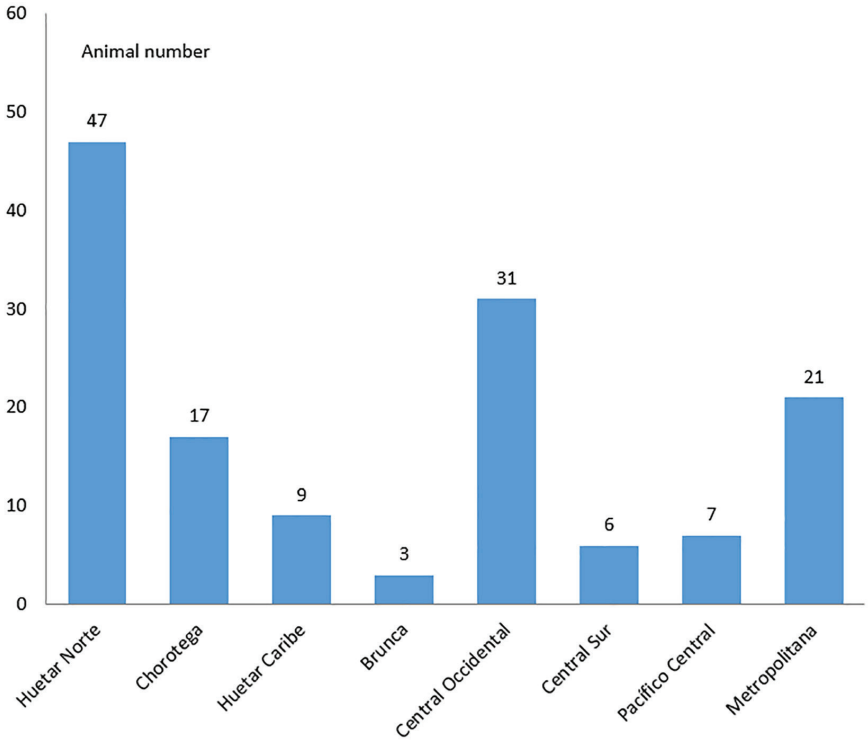

Figure 2. Distribution of double purpose goat serum samples by region.

Bluetongue virus (BTV) is an endemic virus in Costa Rica also transmitted by *Culicoides* (Homan *et al.* 1985). It has been demonstrated that BTV and SVB share *Culicoides* vectors in Europe (Elbers *et al.* 2013; Koenraadt *et al.* 2014).

As a result of a study conducted on the epidemiology of the bluetongue virus in the Caribbean and Central America, a total of 42 species of *Culicoides* were determined. From these arthropods, 18 species were in the country, *C. insignis* being the most common species. It was demonstrated in that study that most of these *Culicoides* are able to transmit BTV (Greiner *et al.* 1993). Another study on sheep confirmed the presence of BTV antibodies in 290 out of 370 sera analyzed in all Costa Rican regions except for the Brunca Region (Villagra-Blanco, Dolz & Montero-Caballero 2008).

Although the *Culicoides* species transmitting BTV described in Costa Rica are not the same as the species transmitting SBV reported in Europe, it is possible that SBV in Costa Rica could share some *Culicoides* vectors with BTV, as they do in Europe. However, this is just a hypothesis. What is for sure is that *Culicoides* occur in varying numbers virtually in every region of Costa Rica, from the coast to the highest elevations, (InBIO) and no SBV antibodies have been found in the analyzed samples.

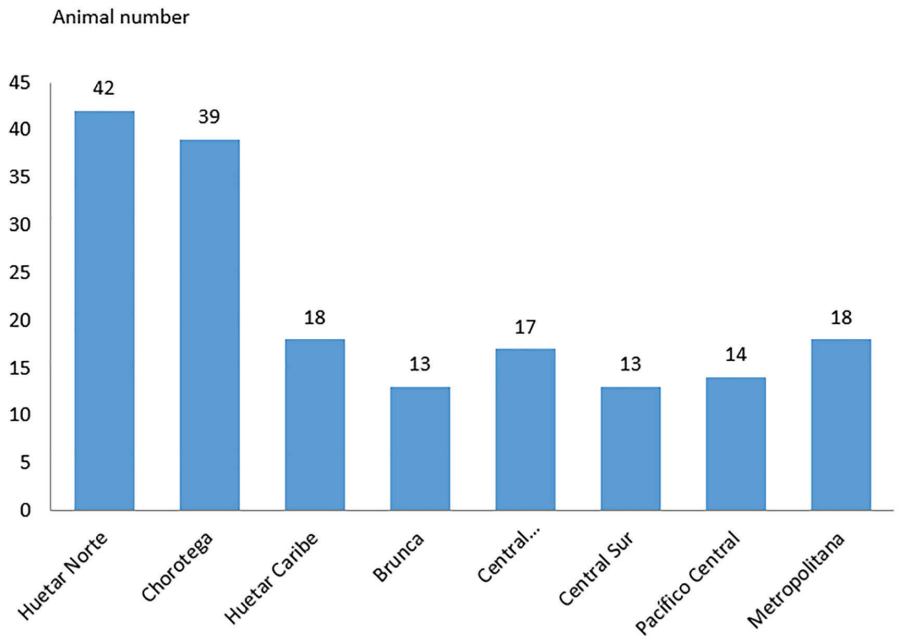

Figure 3. Distribution of the double purpose sheep serum samples selected in this study by region.

Finally, the concern regarding the conservation of samples and the possible degradation of antibodies was underestimated since the antibodies against brucellosis detected in this sample bank were still positive when reevaluated in 2016.

Assuming that Costa Rica is free of SBV, the import of animals or semen from endemic countries is a risk. Testing animals for trading purposes is complicated considering that serological tests based on IgG antibodies do not indicate when an animal is infected, making it impossible to know if the animal will give birth to an infected fetus (Tarlinton *et al.* 2012). In the case of semen, a study pointed out an intermittent virus excretion in semen batches (Hoffmann, Schulz & Beer 2013) transmission of Schmallenberg virus (SBV), while another study regarding a European interlaboratory comparison of Schmallenberg virus diagnosed in PCR established that SBV RNA detection was robust and produced positive results for all SBV RNA–positive specimens in most of the samples with SBV RNA loads at the detection limit in all matrices, except semen. The foregoing is due to the inhibitory effects of semen components on PCR results, which was found critical for reliable detection of SBV RNA in bovine semen (Schulz *et al.* 2015).

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As of 2015, "the dynamics, clinical importance, and duration of SBV RNA excretion in semen from bulls after SBV infection remain unknown" (Schulz *et al.* 2015); however, considering the scientific evidence about the presence of this virus in semen in bulls in the EU nations (Hoffmann, Schulz & Beer 2013; Schulz *et al.* 2015), many other countries outside the EU have imposed restrictions on the import of live animals and products from the EU such as semen and embryos (Doceul *et al.* 2013; Hoffmann, Schulz & Beer 2013).

In conclusion, no antibodies against Schmallenberg virus were found in ruminants sampled in Costa Rica. Therefore, the disease is not prevalent in the country. Restrictive measures over the genetic material imported from Europe must be maintained. SENASA has the obligation to safeguard the health of the national herd and the livestock production from threats. Consequently, more epidemiological studies should be conducted to continue the surveillance of this and other emerging diseases in our country.

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