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Virus De La Lengua Azul Y Virus De La Enfermedad Epizoótica Hemorrágica Co-Infectando Ganado Del Ecuador

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RESUMEN

EL virus de la lengua azul (BTV) y el virus de la enfermedad epizoótica hemorrágica (EHDV) son *Orbivirus* relacionados estrechamente que afectan principalmente rumiantes domésticos y salvajes causando pérdidas económicas debido principalmente a defectos reproductivos; estos virus son transmitidos por *Culicoides*. La única evidencia de estos virus en Ecuador es la presencia de anticuerpos contra BTV en rumiantes domésticos. En este estudio n = 295 muestras de sangre de ganado, sin síntomas típicos de la infección, fueron colectadas en granjas y mataderos de ganado tres localidades. Ensayos serológicos muestran prevalencia de 98,9% para BTV y 81,3% para EHDV. Además, el cultivo viral nos permitió confirmar la infección e identificar los serotipos circulantes (EHDV serotipos 1 y 6; BTV serotipos 9, 13, 18 y 22). Este estudio constituye el primer informe de EHDV y BTV en el Ecuador.

Palabras clave: *Culicoides* - *Orbivirus* - arbovirus - virus de la lengua azul - virus de la enfermedad epizoótica hemorrágica - fiebre catarral ovina - bovinos.

Bluetongue Virus and Epizootic Hemorrhagic Disease Virus co-infecting Cattle from Ecuador

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ABSTRACT

Bluetongue virus (BTV) and Epizootic hemorrhagic disease virus (EDVH) are closely related *Orbivirus* which affect mainly domestic and wild ruminants and cause economic losses mainly due to reproductive failure; these viruses are transmitted by *Culicoides* biting midges. The only evidence of these viruses in Ecuador is the presence of antibodies against BTV in domestic ruminants. In this study n=295 blood samples from cattle without typical symptoms of infection were collected in farms and slaughterhouses from three livestock localities. Serological assays show prevalence of 98.9% for BTV and 81.3% for EHDV. Additionally, viral culture allowed us to confirm infection and to identify circulating serotypes (EHDV serotypes 1 and 6; BTV serotypes 9, 13, 18 and 22). This study constitutes the first report of EHDV and BTV in Ecuador.

Key words: *Culicoides* - *Orbivirus* - arbovirus - Bluetongue virus - Epizootic hemorrhagic disease virus - catarrhal ovine fever - cattle

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INTRODUCTION

The Reoviridae family (derived from ***r****espiratory* ***e****nteric* ***o****rphan viruses*) contains 30 genera and 87 species; the genus *Orbivirus* includes 22 species [1], which can be transmitted by the bite of arthropod (ticks and other hematophagus insect vectors) and replicates within a wide variety of vertebrate hosts (like cattle, goats, sheep, white-tailed deer, equids, camelids). Bluetongue virus (BTV) and Epizootic hemorrhagic disease virus (EHDV) are associated with economical loss in animal production [2].

Bluetongue is vector borne disease which affects domestic and wild ruminants [3, 4, 5]. Most cases in sheep and cattle are asymptomatic then animals can become sources of infection [6]. Epizooties may occur causing sheep deaths; the symptoms of this illness may include lesions in muzzle, cyanosis (observed in the tongue), pneumonia and massive internal hemorrhage [7]. In pregnant ruminants this infection may cause underweight calves, congenital deformities, stillbirths and abortions. The global distribution of BTV is well defined, there are places in the world that remain disease free. The transmission of BTV is mainly by the bite of certain species of infected female blood-sucking midges belonging to the genus *Culicoides* (Diptera: Ceratopogonidae) [8]. The expansion of bluetongue disease is attributed principally to windborne infected *Culicoides* females coming from enzootic areas favored by global warming [9, 10]. Another way of BTV dispersion is the movement of viremic animals from an infected area to a disease-free one [11]. BTV could be found in certain livestock products, such as semen [12]. Bluetongue disease has been declared a notifiable disease by Office Internationale des Epizooties (OIE) and imposes restriction in the movement of animals and livestock products such as semen [13].

The BTV genome is composed of ten linear segments (Seg-1 to Seg-10) of double-stranded RNA (dsRNA) which encode the viral proteins. Seven of these proteins (named VP1

to VP7) are structural components of the viral icosahedral capsid, all the rest are non-structural proteins (named NS1, NS2, NS3/NS3a, NS4) [14, 15]. The segment 2 and segment 6 encode respectably the VP2 and VP5 proteins which constitute the outer capsid, and are primarily involved in cell-attachment and entry during the early stages of infection [16]. The VP2 protein is the main component of the outer capsid, exposed on the surface of virion and it is the primary determinant of the serotype-specific antigen. At this moment 27 serotypes of BTV have been identified (BTV-1 to BTV-27) with variable levels of virulence [17, 18, 19]. The diversity of serotypes complicates immunization; the immune response to one serotype do not protects against infections by another serotype [20, 21].

On the other hand, Epizootic hemorrhagic disease (EHD) is an infectious non-contagious vector borne viral disease of ruminants caused by Epizootic hemorrhagic disease virus (EHDV), which shares several morphological and structural characteristics with BTV. Although sheep are susceptible to EHDV clinical signs of the disease has not been observed in the most cases but in wild ruminants causes clinical signs mainly in certain species of deer [22]. EDVH can be devastating for white-tailed deer (*Odocoileus virginianus*) which develop the most severe clinical signs [23]. In cattle several strains, such as EHDV-2, 6 and 7, cause severe disease with clinical signs including fever, anorexia, diminished milk production, ulcerative oral lesions, excessive nasal discharges and ocular secretion, reproductive disorders (abortion, fetal malformations and stillbirth) [24, 25, 26, 27]. EHDV is distributed in geographical regions located between the 35°S and 49°N latitudes overlapping with the presence of competent EHDV vectors implicated in the transmission (*Culicoides sonorensis, C. imicola, C. brevitarsis, C. schultzei*) [28].

Currently, seven distinct serotypes of EHDV have been identified (EHDV-1, 2, 4, 5, 6, 7 and 8; the serotype earlier designated as EHDV-3 actually is contained within EHDV

serotype 1) [4, 29]. The protein coding organization for BTV and EHDV are the same, comprising of ten genome segments which encode structural proteins (VP1-VP7) and nonstructural proteins (NS1-NS4, NS3a), being the most variable segment 2 the target used for serotyping [30]. The reports of EHD in cattle from EHDV-free zones caused the insertion of EHD in the OIE list of notifiable diseases [13].

The presumptive diagnostics of BTV and EHDV are based in the clinical symptoms, detection of specific antibodies in serum and the presence of RNA by molecular techniques such as RT-PCR [31, 32]. In order to confirm the infection is required to isolate the virus by inoculation in chicken embryonic eggs before cell culture, taking a long time.

In South America the prevalence of orbiviruses in ruminants could be high but the information of the current circulating serotypes is very limited. BTV has been isolated in Brazil (BTV - 4, 12), Argentina (BTV-4) and French Guiana (BTV-1, 2, 6, 10, 12, 13, 17, 24) [33, 34, 35, 36]. In Ecuador the circulating serotypes of BTV remain unknown [37, 38, 39]. Epizootic hemorrhagic disease has not ever been reported in Ecuador even at optimal climatic conditions for the development of the vector. However, serological evidence of BTV in domestic ruminants has reported in Ecuador [40, 41]. Bluetongue detected by competition ELISA (cELISA) in 7 bovine sampled from the Amazonian Province of Napo was recently reported at OIE from Ecuador by AGROCALIDAD [42].

C. insignis is considered possible vector of BTV and EHDV in South America which has been recorded in some places of the country [43]. The exposure of cattle to the vector increases the risk of infection [44], therefore the vector competence in enzootic places requests to be tested [45]. The aim of this study was to confirm the presence and characterize the serotypes of BTV and EHDV in Ecuadorian livestock.

MATERIALS AND METHODS

Blood sampling collection from domestic ruminants

The sampling collection was carried out on cattle during April 2015. A total of n=295 blood samples from domestic bovines were collected. The samples were obtain from one lower Andean region farm (n=26), another one Amazon farm (n=35), and the rest of samples (n=234) came from a lower Andean region slaughterhouse (Fig. 4). All the animals presented no signs of infection nor were they vaccinated against BTV or EHDV.

Four mL of blood sample was collected from each animal in tubes with EDTA (stored at 4 °C) for RT-PCR and virus isolation. Another 10 ml blood sample was centrifuged to obtain sera (stored at -20 °C) to carry out serologic analysis.

Serological detection

Two commercial diagnostic kits were used to detect BTV and EHDV antibodies in sera: The first one detected anti-VP7 BTV antibodies by competition ELISA (ID Screen® Bluetongue Competition) [46] and the other detected EHDV antibodies (LSIVet™ Ruminant EHDV Serum ELISA Kit). The samples and solutions were prepared following the manufacturer's instructions.

RNA isolation

Total RNA was extracted from 100 µL of blood plasma using a QIAcube robot (QIAamp Viral kit, QIAGEN) according to the manufacturer's instructions; RNA was diluted in 50 µL of ultrapure water [32].

RT-qPCR of BTV and EHDV

In order to detect BTV in samples, the genomic segment 10 (NS3 protein) was amplified [47]. Denaturation step of RNA was performed in DMSO (10%) for 3 min at 95 °C followed by

rapid cooling. To perform the reverse transcription and amplification was used a commercial real-time RT-PCR kit (ADI-352, AES) at 5 µL of denaturalized RNA in 20 µL of RT-qPCR mix according to the manufacturer's instructions. On the other hand, the presence of EHDV was investigated by a real-time RT-PCR commercial kit (Kit TaqVet Epizootic Hemorrhagic Disease Virus – Duplex, LSI, France). Denaturation of total RNA was performed as described above. The amplification cycles were programmed as following: 45 °C for 10 minutes, 95 °C for 10 minutes, 40 cycles of 15 seconds at 95 °C with 1 minute at 60 °C [48].

Virus isolation

To isolate virus by cell culture, 29 blood samples with CT value <35.7 in RTq-PCR were selected. To a monolayer of KC cells (a *cell-line* derived from *C. sonorensis*) was added 0.5 mL of a solution of washed and lysed red blood cells at 10^{-1} dilution. After incubate for 30 min at room temperature, Schneider's medium (supplemented with 10% FBS) was added. The inoculated flasks were incubated for 7 days at 28 °C, and then the KC cells were freeze and thawed and analyzed by RT-PCR [49].

Around 100–200 µL of the previous dilution were inoculated intravascular performed in triplicate into chicken embryos [50]. Then eggs were incubated for 5 days at 35 °C and observed day after day using a cold lamp, the embryos that died among 2-5 days post infection were homogenized using a pestle in Eagle's MEM (supplemented with streptomycin 100 mg/mL and penicillin 100 UI/mL) [49]. The suspensions were centrifuged at 2000 x g for 10 minutes at 4 °C.

RESULTS

A group of 182 cattle samples were chosen for specific serological assays (cELISA) 98.9% of which were positive for BTV, 81.3% were positive for EHDV and 80.2% for both viruses [

Table 4]. Neither EHDV nor BTV antibody were detected in animals from Galapagos Islands (data not shown). In order to detect the viral genome, 295 samples were tested by RT-PCR, 24.1% (n=71) were positive for RNA-BTV and 10.2% (n=30) was positive for RNA-EHDV. Additionally, 7.1% (n=21) corresponded to co-infections of BTV and EHDV [Table 5]. These findings showed that orbiviruses are present as mono and co-infections in the cattle [Table 6]. The sequences analysis of segment 2 of BTV demonstrated the presence of 4 serotypes (9, 13, 18, 22) [Fig. 5]. Regarding the sequences of segment 2 from EHDV, serotypes 1 and 6 were identified [Fig. 6].

DISCUSSION

The results of serologic assays show that cattle in Ecuador have been exposed to orbiviruses as 98.9% and 81.3% of the samples were positive for BTV and EDVH respectively, that animals were not vaccinated for *Orbivirus*. Serologic assays from cattle of Galapagos Islands were negative for BTV and EHDV (data not shown); as same as samples of sheep and alpacas from one Andean farm were negative (not shown data). The presence of BTV or/and EHDV in cattle was confirmed by isolation of the viral agents from blood samples. The nucleotide sequences allowed us to identify viral serotypes 1 and 6 for EHDV and serotypes 9, 13, 18, 22 for BTV. To our knowledge this is the first report of isolation of BTV and EHDV in Ecuador. EHDV serotypes 1 and 6 have been reported previously in cattle from French Guiana without clinical signs [36]. Several strains of EHDV-6 lead to severe disease in cattle in Turkey [51]; and in 2009 in Reunion Island [2]. This study also shows high rate of BTV and EHDV infection in Ecuadorian cattle.

In Ecuador there are multiple etiologic agents that cause abortion in many cases the cause of it is unknown, for Ecuadorian cattle herd in Andean region: in 2004 has average rates of

6.21% abortion and 11.66% fetal resorption [52]; in 2013 abortion cases of 21% were found [53]. Abortion and other reproductive failures may be associated to mothers that are apparently asymptomatic [54]. This study suggest that the introduction of vaccines for cattle may be necessary

The simultaneous presence of several strains of orbiviruses (BTV/BTV and/or EHDV/EHDV) in the same animal could increase the possibility of reassortment mechanisms that may lead to the occurrence of new viral strains which can get a virulent phenotype due to the capacity of the virus to exchange genome segments during mixed infections [55, 56, 57].

In the neighbor countries as Colombia and Peru the Bluetongue disease is less frequent in domestic ruminants and it is not known what happens on wild animals, however seroposivity for BTV has been found in some animals such as peccaries, rams or alpacas [58, 59, 60].

Global warming may have a considerable effect on vector distribution, such as *Culicoides* spp, therefore entomological studies of the distribution and seasonal activity of *Culicoides* biting midges in Ecuador is necessary to localize the main biological vector species in the enzootic places. Furthermore, it must be research vectorial competence to recognize possible vectors such as *Culicoides pusillus, C. furens, C. filarifer* and *C. trilineatus* which might act as vectors of numerous BTV serotypes [61].

In conclusion, this study shows high prevalence of BTV and EHDV in cattle from Ecuador, in places from Tandapi, Cotundo and Santo Domingo's slaughterhouse. *Orbivirus* in cattle in Ecuador are circulating as mono and coinfection. These findings, confirm a high viral circulation of these two orbiviruses in cattle.

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FIGURES AND TABLES

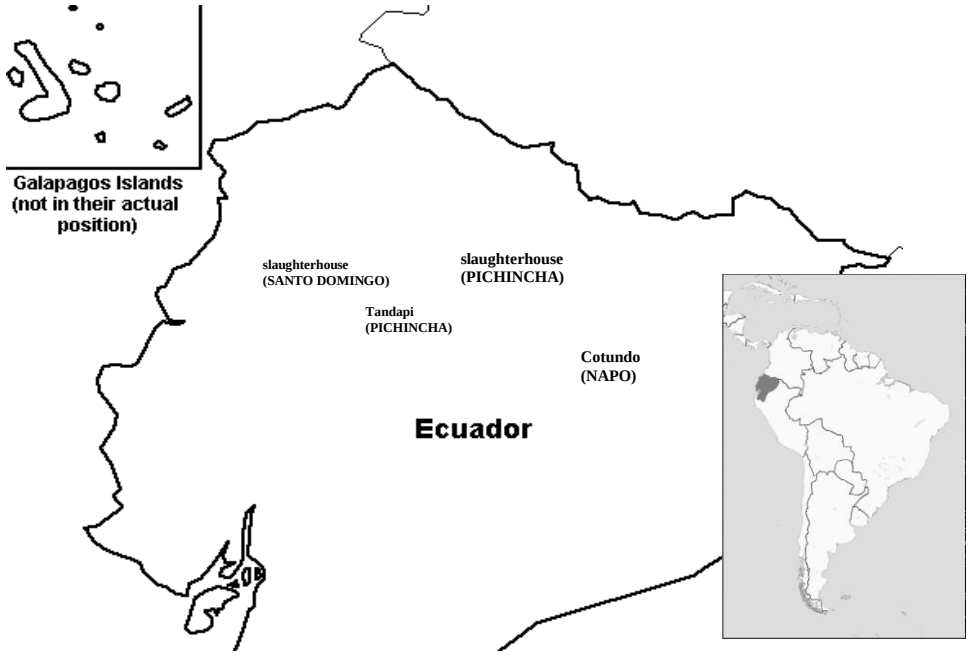

Fig. 4 Places of cattle blood sampling in 2015

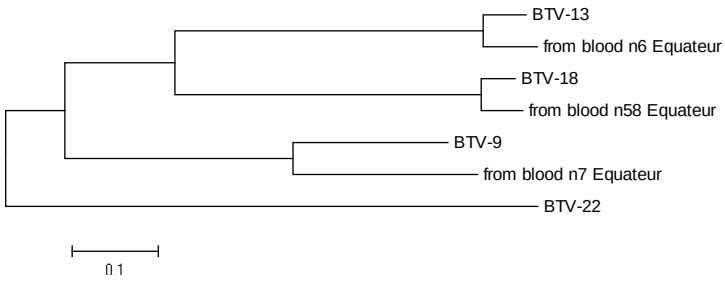

Fig. 5 Evolutionary relationships of BTV taxa in base of sequence of segment 2

The evolutionary history was inferred using the Neighbor-Joining method among sequences available on genbank.

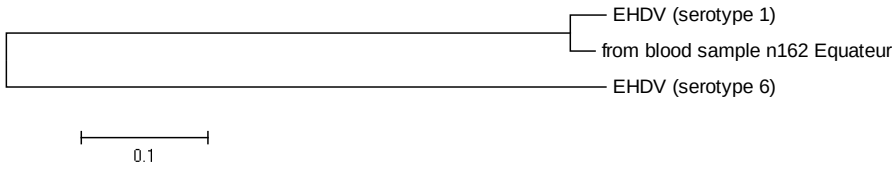

Fig. 6 Evolutionary relationships of EHDV taxa in base of sequence of segment 2

The evolutionary history was inferred using the Neighbor-Joining method among sequences available on genbank.

Table 4 Prevalence of *Orbivirus* BTV and EHDV in Tandapi, Cotundo and Sto Domingo's slaughterhouse by serological analysis

Place of Sampling (n=182)	Seropositivity			Prevalence		
	BTV	EHDV	BTV and EHDV	BTV	EHDV	BTV and EHDV
Tandapi (n=26)	26	18	18	100%	69.2%	69.1%
Cotundo (n=35)	35	30	30	100%	85.7%	85.7%
Sto. Domingo's slaughterhouse (n=121)	119	100	98	98.3%	82.6%	81.0%
				Total Prevalence		
				98.9%	81.3%	80.2%

Table 5 Prevalence of *Orbivirus* BTV and EHDV in Tandapi, Cotundo and Sto Domingo's slaughterhouse by molecular analysis

Place of Sampling (n=295)	qPCR positives			Prevalence		
	BTV	EHDV	BTV and EHDV	BTV	EHDV	BTV and EHDV
Tandapi (n=26)	21	3	3	80.8%	11.5%	11.5%
Cotundo (n=35)	20	14	12	57.1%	40.0%	34.3%
Sto. Domingo's slaughterhouse (n=234)	30	13	6	12.8%	5.6%	2.6%
				Total Prevalence		
				24.1%	10.2%	7.1%

Table 6 Viral serotypes isolated from individual blood samples, displayed in cattle like single and co-infection from VP2 gene sequence.

Place of Sampling	Sample identification	Analysis performed				Virus serotype (VP2 sequence)
		cELISA BTV	RT-qPCR BTV	cELISA EHDV	RT-qPCR EHDV	
Tandapi (n=7)	**5**	+	+	-	-	-
	6	+	+	-	-	BTV-13
	7	+	+	-	-	BTV-9
	8	+	+	+	+	-
	10	+	+	+	+	-
	16	+	+	-	-	BTV-22
	25	+	+	+	+	-
Cotundo (n=7)	**29**	+	+	+	+	-
	47	+	+	+	+	-
	49	+	+	+	+	EHDV-1, -6
	50	+	+	+	+	-
	51	+	+	+	+	-
	52	+	+	+	-	-
	58	+	+	+	+	BTV-18, EHDV-6
Sto. Domingo's slaughterhouse (n=15)	**87**	+	+	+	+	-
	107	+	+	+	-	-
	115	+	+	+	-	-
	118	+	-	-	+	-
	129	+	+	-	+	-
	138	+	+	+	-	-
	148	+	+	+	-	-
	162	+	+	+	+	EHDV-1
	169	+	+	+	-	-
	178	+	+	+	+	-
	188	nd	+	nd	+	-
	199	nd	+	nd	-	-
	212	nd	+	nd	-	-
	224	nd	-	nd	+	-
	246	nd	+	nd	-	-

nd = non data

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