VETERINARY MICROBIOLOGY - SHORT COMMUNICATION

Outbreaks of Vesicular Stomatitis in Brazil caused by a distinct lineage of Alagoas vesiculovirus

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Abstract

This article describes the recurrence of outbreaks of Vesicular Stomatitis in the State of Maranhão, Brazil. The procedures for treating the outbreak of vesicular disease, sample collection, laboratory tests performed, and the results obtained were described. The clinical signs and observed injuries have been described. The sera showed antibodies that cross-react between the *Vesiculovirus* Indiana, Cocal, and Alagoas. The serological profile shows the presence of high antibody titers for Alagoas vesiculovirus in cattle, swine, and horses. Higher antibody titers indicate the viral serotype present in the outbreak. The genetic sequencing of the isolates confirmed the presence of Alagoas vesiculovirus, which grouped with the virus isolated in 2013 from cattle from the State of Maranhão.

Vesicular Stomatitis (VS) is a disease that affects cattle, buffaloes, small ruminants, pigs, and equines. It is mandatory to notify the disease in Brazil. The first isolation of a virus that causes Vesicular Stomatitis in Brazil was registered in 1964, in the State of Alagoas with the identification of Alagoas vesiculovirus (VSAV) from the epithelium of sick horses [1]. The viruses that cause VS belong to the family *Rhabdoviridae*, genus *Vesiculovirus* and are widely distributed in the Americas [2]. These viruses were previously divided into four serotypes, New Jersey, Indiana 1, Indiana 2, and Indiana 3. This classification changed to separate them into four species, New Jersey vesiculovirus (VSNJV), Indiana vesiculovirus (previously Indiana 1) (VSIV), Cocal vesiculovirus (Indiana 2) (COCV), and Alagoas vesiculovirus (Indiana

3) (VSAV) [2]. The old nomenclature is still used in some Brazilian studies.

The mechanisms involved in the transmission of VS are not yet fully understood. It is believed that the virus is transmitted by insect vectors, mainly blood-sucking mosquitoes of the genus *Lutzomyia* and blood-sucking flies of the *Simuliidae* family. Once introduced into a herd, stomatitis can be transmitted from one animal to another or through contaminated fomites such as water, food, and milking equipment [3].

Excoriations in the mouth, nasal mucosa, and foot epithelium are the gateway for the virus; the intradermal route constitutes another entrance for the virus in cattle and is important in the possible transmission by hematophagous insects; viremia has a short duration, with a feverish state between 24 and 48 h post-infection [4].

The economic importance of the disease is related to the fact that the affected animals show a decrease in the production of both milk and meat, in addition to presenting clinical signs similar to Foot-and-Mouth Disease (FMD) [5]. For this reason, trade and transit of animals are restricted in areas with suspected VS, until the definitive laboratory diagnosis is confirmed, which is done by ELISA, PCR, or viral neutralization [2, 6]. Monitoring and follow-up of the disease

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coverage area are extremely important, especially in terms of epidemiological focus and links.

VS is endemic in some regions of Brazil. From 2010 to 2014, 129 outbreaks were notified to the OIE in the states of Bahia, Ceará, Maranhão, Mato Grosso, Minas Gerais, Paraíba, Pernambuco, Piauí, Pará, Rio de Janeiro, Rio Grande do Norte, and Tocantins. Three outbreaks were recorded in 2010, 16 in 2011, one in 2012, 41 in 2013, and 68 in 2014 [7]. Previous studies demonstrated that VSAV (denominated Indiana 3 in these papers) is the dominant species in these regions. Some reports suggest that the virus has a broader viral activity in the north and the northeast than in the center-west and south regions [8–10].COCV was only found in states of the south region, but there are no recent records of its presence in livestock.

The affected animals present excessive salivation, the appearance of whitish vesicles, with a predilection on the upper surface of the tongue, surface of the lips and around the eyes, commissures of the mouth and gums (horses) and tongue, lips, gums, inner part of the mouth (palate hard), and, sometimes, muzzle and around the muzzle and nostril (bovine). As an important difference from FMD, horses are susceptible to VS. However, there are cases in which the disease has been identified in bovines and swine, not manifesting in equines [1].

The objective of this work was to describe the official diagnosis of VS during an outbreak of VS in the Brazilian Northeast. This study describes clinical signs that present and demonstrate the serological profile of the affected animals, and analyze phylogenetically the viral isolates from 2013 and 2016.

On January 7, 2016, the official veterinary service of Maranhão received a complaint by telephone, notifying the existence of cattle and horses showing tongue lesions with shedding and sialorrhea in the municipality of Porto Franco (Fazenda 1: 06° 20′ 18″ S; 47° 23′ 57″ W), location with the previous report of VSAV [11]. According to the notifier, the first cases had been observed about 7 days before. On the same day as the notification, the property was visited by the state health defense service, which took the necessary biosafety measures, carried out the investigation, inspection, clinical examination, and collection of animal samples with evidence of clinical signs of vesicular disease. The property had 808 cattle, 19 horses, and six pigs.

A vacuum tube system was used to collect blood and subsequently obtain serum from three horses and seven cattle by bleeding from the jugular vein. Oral epithelium samples were taken from five cattle using blunt-tipped scissors and forceps. The tissue fragments were deposited in wide-mouthed flasks (universal collector), sterile, adding Vallée liquid in sufficient quantity for the material to be submerged. The vesicular fluid was collected using a sterile insulin syringe and needle. No tissue samples were collected

from horses and pigs. The samples were placed in a thermal box with ice plates and sent to the official laboratory of the Ministry of Agriculture, Livestock, and Supply. On January 29, 2016, blood samples were collected from the three pigs that did not show clinical signs of vesicular disease.

After initial care, animal handling of the investigated property and the seven properties with an epidemiological link was preventively prevented. Active surveillance was maintained with clinical inspection of the herd at 3-day intervals on the investigated property. After 15 days, a paired serum collection was performed, as recommended [1] and following the procedures previously described. The animals of the property and the properties with epidemiological link (252 cattle, seven sheep, 84 pigs, and nine horses) were monitored, with an interval of 7 days. Veterinarians did the clinical inspection and evaluation of the mouth, paws, and roofs. Preventive and disease containment actions involved active surveillance and health education in all properties involved in the episode until 21 days after the last symptomatic animal was cured.

In April (Farm 2—6° 19′ 00″ S and 47° 11′ 55″ W with 619 cattle, 12 horses, and a mule) and in June 2016 (Farm 3—6° 21′ 30″ S and 47° 18′ 32″ W with 323 cattle, seven horses, and one mule), two other properties were observed, in the same municipality, with cattle with clinical signs of vesicular disease. The lesions observed in these animals were already in the healing phase and, therefore, only samples of serum and esophageal-pharyngeal fluid (LEF) were sent.

The diagnostic methods mentioned in this study follow the guidelines defined by the Plan of Action for Foot-and-Mouth Disease and that all of them were tested following the diagnostic protocols recommended by the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals of the World Organisation for Animal Health. The samples were subjected to the following tests: ELISA for detection of non-structural proteins for FMD virus, antibody detection test for Vesicular Stomatitis virus using the viral neutralization technique, virus isolation in cells, and molecular diagnosis of vesicular diseases (FMD virus, Vesicular Stomatitis Indiana Virus—VSIV, Vesicular New Jersey Virus—VSNJV, Alagoas Virus, Cocal Virus—COCV, Bovine Diarrhea Virus, Blue Tongue Virus, Herpes Bovine Virus, Vaccinia virus, and Parapoxvirus) as previously described [12].

The 3ABC ELISA for FMD diagnosis was performed according to the manufacturer's recommendations (Prionics). The method and interpretation of the results of the viral neutralization assay for VS were conducted as previously described [2]. Viral isolation was performed in monolayers of hamster kidney cells (BHK-21, ATCC) and porcine kidney cells (PK15, ATCC).

All VSAV positive samples were sequenced as previously described [6]. The amplicons were sent for Sanger

sequencing on the ABI 3500 equipment. The sequences were submitted to BLAST for identification [13] and the collection of sequences from previously published works [8, 14]. The MEGA X program [15] was used to reconstruct the phylogenetic tree using the Kimura 2-parameter model with gamma distribution and evolutionary inference from Maximum Likelihood with 1000 bootstrap replicates.

In 2013, an outbreak of Vesicular Stomatitis was reported in a bovine herd in the municipality of Porto Franco-MA [11]. Three years later, we reported the recurrence of outbreaks of the disease in three other properties in the same municipality in Maranhão, demonstrating that the virus persists in the region.

During clinical examination in animals from Farm 1, seven cattle with signs suggestive of vesicular disease were observed, being females, aged over 36 months, of mixed race, and Nellore. Clinical examination revealed sialorrhea, lesions on the gums, lips, tongue (shedding of epithelium) (Fig. 1C, D, and F) and oral mucosa (Fig. 1A), mild apathy, and moderate weight loss in most animals (lean body score). A cow showed marked glossitis with lesions, loss of epithelium (Fig. 1E), and cachexia. Body temperature ranged from 38.5 to 39.0 °C. Four cows had calves at their feet, but none of them showed clinical signs or injuries. One of the seven cattle with clinical signs died during the outbreak of the disease due to complications in the open wounds and the presence of myiasis. The animal did not eat well and therefore did not resist, demonstrating that the disease cannot be neglected. In the three symptomatic horses (one male and two females), it was possible to observe sialorrhea, wounds in the healing process (on the muzzle, lower and upper lips), in addition to erosions of rupture of vesicles on the tongue also at the beginning of the healing process (Fig. 1E).

Of the total of 1796 animals susceptible to VS, in the three properties, 23 showed clinical signs (1.3%). As observed by Arruda et al. (2015), clinical signs were more frequent in horses (7.5%) than in cattle (1.1%). Cattle and horses are more frequently affected by the clinical form, but there is a greater morbidity and it is more common among equine population, a characteristic described in previous work in the Brazilian Northeast [9]. The bovine and porcine serum samples were not reactive in ELISA 3ABC. Epithelium, vesicular fluid, and LEF samples were negative in RT-PCR for FMDV.

The results of viral neutralization tests showed reactivity of bovine and equine sera with VSIV, COCV, and VSAV. VSIV is considered exotic in Brazil, but cross-reactivity among VS viruses is known [1]. VSAV, VSNJV, COCV, and VSIV are different species, but they can produce similar humoral response that generate the cross-reactivity in the serologic tests. A previous study tests different isolates of these viruses and compared the genetic difference between them [14]. Results demonstrated the cross-reactivity but
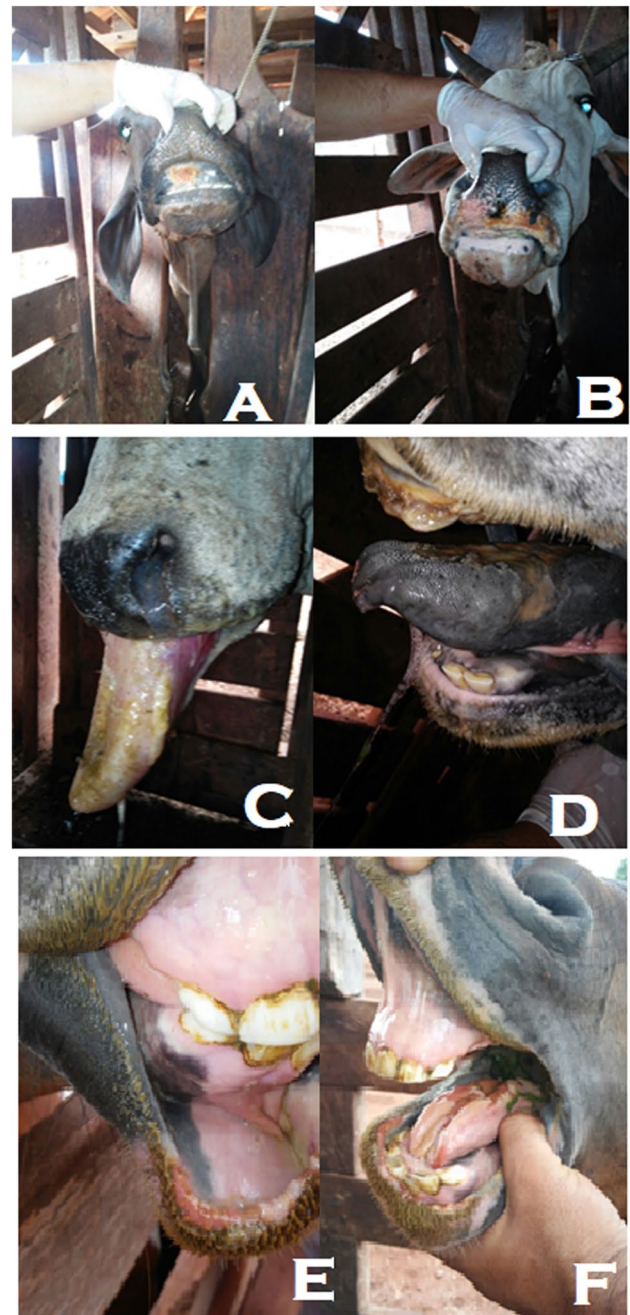

Fig. 1 Lesions found in animals in the foci surveyed in the present study. Lesions in the oral mucosa (**A**), and lips and snout (**B**) of cattle from Farm 1. Intact vesicles (**C**) and lesions (**D**) in the languages of cattle from Farm 1. Lesions in equine gums (**E**). Lesions in the gums, lips, and tongue (**F**) of horses from Farm 1, municipality of Porto Franco, Maranhão

isolates of the same species had higher and similar reactivity than isolates from different species.

As described above, the animals' serum was collected on three occasions called C1, C2, and C3. The antibody titers were higher, for most animals, for the VSAV, and at the time of collection C1 (Table 1). In cattle 1-Bov1 (bovine 1

Table 1 Antibody titers in the viral neutralization assay for different *Vesiculoviruses* in cattle in the municipality of Porto Franco-MA

Sample	Antibody titers								
	VSIV			COCV			VSAV		
Bovine	C1	C2	C3	C1	C2	C3	C1	C2	C3
1-Faz1	1.6	<1.3	1.6	<1.3	1.3	<1.3	<1.3	2.5	2.8
2-Faz1	<1.3	<1.3	1.3	1.6	2.5	2.2	4.0	3.1	3.1
3-Faz1	1.6	<1.3	1.6	1.6	1.9	2.2	3.1	2.8	3.1
4-Faz1	1.3	<1.3	1.3	<1.3	1.3	<1.3	1.3	3.1	2.8
5-Faz1	1.9	1.3	1.3	1.9	1.3	<1.3	3.4	3.4	2.8
6-Faz1	2.2	2.2	1.9	1.9	1.6	1.6	3.4	3.1	3.1
1-Faz2	1.3	1.3	*	2.2	1.9	*	4.3	3.7	*
2-Faz2	1.9	2.2	*	2.5	2.2	*	3.7	2.8	*
3-Faz2	1.3	1.3	*	1.9	1.9	*	3.1	3.1	*
4-Faz2	1.3	1.3	*	1.9	2.2	*	3.7	3.1	*
5-Faz2	1.6	1.3	*	2.5	2.5	*	3.4	3.1	*
6-Faz2	1.9	1.9	*	2.5	2.2	*	3.7	3.7	*
7-Faz2	1.9	2.5	*	2.5	2.5	*	3.1	4.3	*
8-Faz2	1.9	2.2	*	3.1	2.5	*	3.7	3.1	*
9-Faz2	2.2	1.9	*	2.2	1.9	*	3.4	3.1	*
10-Faz2	1.9	2.2	*	2.2	2.2	*	3.7	4	*
11-Faz2	1.9	<1.3	*	1.6	<1.3	*	3.1	2.8	*
1-Faz3	1.6	1.9	*	1.6	2.5	*	3.1	3.1	*
2-Faz3	2.5	1.9	*	2.8	2.8	*	4.6	4.0	*
Horse1	2.8	2.5	1.6	2.8	2.2	1.9	4.9	3.7	3.4
Horse2	2.8	2.5	1.3	1.9	1.9	1.3	4.3	3.7	3.1
Horse3	2.2	1.9	1.6	1.9	1.9	1.6	4.0	3.1	3.4
Pig1	NR	*	*	2.8	*	*	4.9	*	*
Pig2	NR	*	*	2.5	*	*	4.3	*	*
Pig3	NR	*	*	2.5	*	*	4.0	*	*

from Farm 1), 4-Bov1 (bovine 4 from Farm 1), and 7-Faz2 (bovine 7 from Farm 2), there was an increase in antibody titers between C1 and C2 indicating seroconversion. There was a decrease in the antibody titer in C2 and C3, that is, most animals were probably already in the convalescence phase.

The decrease in antibody titers in C2, for most animals, probably indicates a delay in the notification of diseases to the official veterinary service and reflects the need to expand health education actions to make farmers aware of the importance of vesicular diseases.

Pigs, collected only in C3, showed reactivity only for COCV and VSAV (Table 1). The infection probably was subclinical in pigs, considering that no clinical signs were observed in these animals or these signs went unnoticed by the owner. Not all pigs experimentally infected develop the disease [16]. In pigs, the peak of antibodies was reached 3 to 5 weeks after infection, after which the level of antibodies decreases, but remains at detectable levels for months.

VSAV has been reported in several Brazilian states [1, 8, 11]. The disease was previously reported in the region, including the state of Tocantins, near the border with Maranhão. These factors demonstrate that the VSAV persisted in the region before the outbreak occurred in the municipality of Porto Franco.

Viral detection was performed using viral isolation and by the real-time PCR technique in the bovine epithelium and vesicular fluid samples. Monolayers of cells inoculated with clinical specimens of cattle developed a cytopathic effect in the first passage, characterized by cell rounding, cell separation and lysis, and rapid dissemination in the culture. Monolayers were destroyed within 24 to 36 h after the initial visualization of the cytopathic effect. To confirm the identity of the virus isolated in cell cultures, clinical specimens were submitted to a real-time PCR technique capable of differentiating *Vesiculoviruses*. Positive samples were sequenced confirming that the VSAV was responsible for the outbreak.

The VSAV in the state of Maranhão is similar to the virus responsible for the 2013 outbreak, forming a different clade (samples LDDV-2014 and LDDV-2015). As in other studies [8, 14], isolates were genetically distant from the VSAV prototype isolated in 1968 (GenBank accession number EU373658.1) (Fig. 2). The genetic distance between the samples sequenced in this work and the virus

Fig. 2 Phylogenetic tree of VSAV genetic sequences built using the MEGA X program using the Kimura 2-parameter model with gamma distribution and evolutionary inference from Maximum Likelihood with 1000 bootstrap replicates
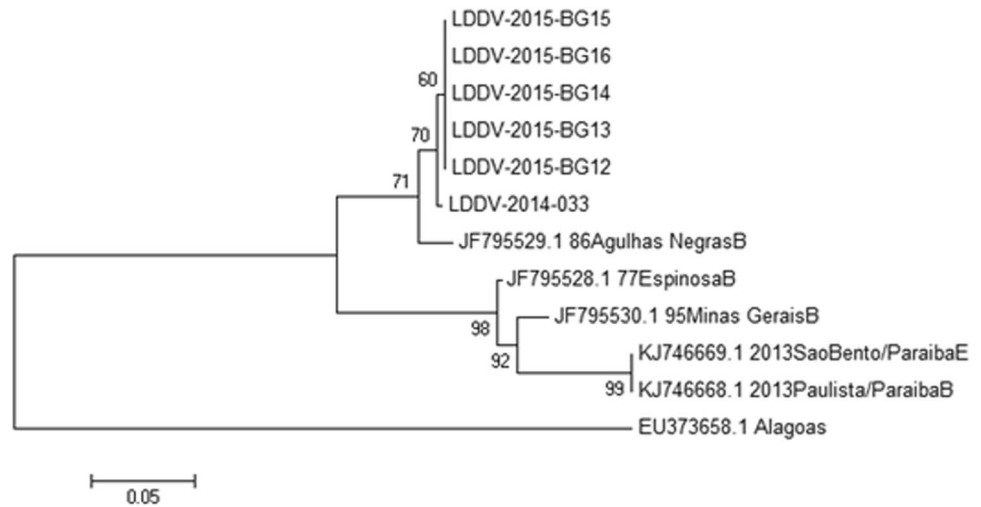
of 1968 was 0.49, a value greater than the distance of 0.17 that occurred in the state of Paraíba in 2013 previously reported in another study [8]. The results indicate a great variation of the VSAV strains circulating in Brazil, but more studies are needed to determine if this genetic difference has any impact in the disease progression. In the present research, we could not find any.

VS viruses can have different phylogenetic profiles according to geography. Previous studies with VSIV have shown different strains in different locations, without overlapping, with a genetic variation of up to 19% [17]. Ecological factors were more important for the separation of VSNJV strains than temporal factors, generating well-defined clades in the phylogenetic tree according to the regions analyzed [18]. The same seems to occur with the VSAV in Brazil. The outbreaks in the state of Maranhão and the state of Paraíba were limited and contained in those regions following a pattern typical of VS viruses. This factor may be important because it has already been shown that the evolution of VSNJV can imply distinct antigenic and genetic characteristics [19]. Such factors highlight the importance of continuous monitoring of the disease to assess not only the epidemiological characteristics but the influence of these changes in the molecular diagnosis.

The present work allowed us to identify a third strain of VSAV in Northeastern Brazil. The virus was isolated from cattle and horses and serologically identified in pigs, demonstrating the spread of the virus in different species and its continued presence in the region. Our results demonstrate the presence of one more variant, which is important for molecular diagnosis and can be associated with epidemiological and evolutionary studies of this virus.

Author contribution Anapolino Macedo de Oliveira, Mateus Laguardia-Nascimento, Mariana Lázaro Sales: acquisition, analysis, or interpretation of data.

Anapolino Macedo de Oliveira, Cristiano Melo, Anselmo Rivetti Vasconcelos Júnior, Marcelo Fernandes Camargos: approved the version to be published.

Anapolino Macedo de Oliveira, Cristiano Melo: agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Antônio Augusto Fonseca Júnior: acquisition, analysis, or interpretation of data, drafted the work, made substantial contributions to the conception or design of the work.

Declarations

Conflict of interest The authors declare no competing interests.

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