

African horse sickness outbreaks in Namibia from 2006 to 2013: clinical, pathological and molecular findings

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Molecular characterization,
Namibia,
Pathology,
Serotyping.

Summary

African horse sickness (AHS) is a vector-borne viral disease of equids, endemic in Sub-Saharan Africa. This article reports the clinic-pathological and laboratory findings observed in the framework of passive surveillance during the AHS outbreaks which occurred in Namibia between 2006 and 2013. This study was conducted in the framework of the collaboration among the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (Teramo, Italy), the Namibian Ministry of Agriculture Water and Forestry, and the Namibian National Veterinary Association. A total of 92 horses were investigated, showing different clinical form of AHS: peracute/acute (n = 43), sub-acute (n = 21) and mild AHS fever (n = 19). Clinical data were not available for 9 horses, because they were found dead. Pathological findings have been recorded for 35 horses. At necropsy, pulmonary and subcutaneous oedema, haemorrhages and enlargement of lymph nodes were mainly observed. Diagnosis was confirmed by laboratory testing, AHS virus (AHSV) was isolated from 50 horses and the identified serotypes were: 1, 2, 4, 6, 7, 8, and 9. The phylogenetic analysis of the S10 genome sequences segregated the Namibian AHSV strains in the same clusters of those circulating in South Africa in recent years. The description of AHS clinical, pathological, and laboratory features of AHS provided in this article is of value for differential diagnosis and control of AHS, especially in areas currently free from this disease.

Episodi di peste equina in Namibia dal 2006 al 2013: rilievi clinici, patologici e molecolari

Parole chiave

Caratterizzazione molecolare,
Diagnostica di laboratorio,
Lesioni,
Peste equina,
Segmento genomico 10 (S10),
Serotipizzazione,
Sintomatologia.

Riassunto

La peste equina è una malattia degli equidi ad eziologia virale e trasmessa da insetti vettori, endemica nell'Africa sub-Sahariana. Si riportano i dati clinico-patologici e laboratoristici degli episodi di peste equina verificatisi in Namibia dal 2006 al 2013. Lo studio è frutto della collaborazione tra l'Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale", il Ministero dell'Agricoltura, delle Risorse Idriche e delle Foreste della Namibia e l'Associazione Nazionale dei Medici Veterinari della Namibia. In totale, sono stati esaminati 92 cavalli affetti da diverse forme di peste equina: peracuta/acute (n = 43), sub-acute (n = 21) ed oligosintomatica (n = 19). Nove cavalli sono stati rinvenuti già morti, senza che fosse possibile riportare alcun dato clinico. In 35 casi è stato possibile osservare e descrivere dettagliatamente le lesioni macroscopiche. In sede necroscopica, le principali lesioni sono state l'edema polmonare e sottocutaneo, le emorragie e l'aumento di volume dei linfonodi. Il sospetto diagnostico è stato costantemente confermato dalle indagini di laboratorio. Il virus della peste equina è stato isolato da 50 cavalli e la serotipizzazione ha dimostrato la presenza dei seguenti sierotipi: 1, 2, 4, 6, 7, 8 e 9. Le analisi filogenetiche del segmento S10 hanno permesso di collocare i ceppi virali namibiani all'interno degli stessi clusters circolanti in Sud Africa. La presente descrizione degli episodi di peste equina e delle loro caratteristiche cliniche, patologiche e laboratoristiche è utile per una rapida diagnosi differenziale ed un efficace controllo di tale malattia, soprattutto nelle regioni attualmente indenni.

Introduction

African horse sickness (AHS) is a non-contagious, insect-borne disease of equids caused by a double stranded RNA virus – namely, AHS virus (AHSV) – which belongs to the genus *Orbivirus* (family *Reoviridae*) and shares some morphological features with bluetongue and equine encephalosis viruses. The biting midge *Culicoides imicola* is considered the most important vector of AHSV in Africa (Mellor and Hamblin 2004).

Up to date, 9 AHSV serotypes (AHSV-1 to -9) have been identified by virus neutralization test. Some evidence exists about serological cross-reactions between serotypes, whereas no relationship has been demonstrated with other known orbiviruses (von Teichman *et al.* 2010). The AHSV genome consists of 10 double-stranded (ds)RNA segments, which encode 7 structural (VP1 to VP7) and 4 non-structural (NS1, NS2, NS3, and NS3A) proteins. The segment 10 (Seg-10) (755 -764 bp) is the smallest one and encodes NS3 and NS3A proteins (van Staden and Huismans 1991). The Seg-10 nucleotide-sequence analysis has been demonstrated to be a useful tool to investigate the genetic relationships among AHSV isolates (de Sá *et al.* 1994, Quan *et al.* 2008, Sailleau *et al.* 1997, van Niekerk *et al.* 2001).

Equids, including crossbreeds, are all susceptible to AHSV infection. Zebras rarely exhibit clinical signs, although they are likely to play a pivotal epidemiological role for AHS in Africa, thus being regarded as the natural vertebrate reservoir host of AHSV. However, AHSV persists also in African countries where zebra population is absent or negligible. Donkeys are also very resistant, most AHSV infections being sub-clinical, at least in Southern Africa (Coetzer and Guthrie 2004). On the contrary, high mortality rates are usually recorded in horses and occasionally in mules, which do not contribute to the 'persistence' of AHSV, thus being considered as 'indicator hosts' of AHS (Mellor and Hamblin 2004).

African Horse Sickness is endemic in Sub-Saharan Africa where all serotypes are present, while AHSV-9 has been involved in most of the epidemics outside Africa, with the only exception of AHS outbreaks caused by AHSV-4 in the Iberian peninsula (Mellor and Hamblin 2004). In Southern Africa, AHS occurs with a typically seasonal (December-May) and cyclical incidence, closely related to heavy rain and density of vectors (Scacchia *et al.* 2009).

Different AHSV serotypes are progressively colonizing Western African countries (from Nigeria to Mauritania), a finding which indicates that AHSV spreading capacity is greater than previously thought (Laaberki 1969, Mellor and Hamblin 2004,

Diouf *et al.* 2007). African Horse Sickness outbreaks have been occasionally reported outside the African continent: Middle East (1959-63), Spain (1966, 1987-90), Portugal (1989), Saudi Arabia, Yemen (1997), and Cape Verde Islands (1999) (Anwar and Qureshi 1972, Coetzer *et al.* 2004, Howell 1960, Howell 1963, Mellor and Hamblin 2004). Such epidemics mainly resulted from the movement of infected animals (de Sá *et al.* 1994, Mellor and Hamblin 2004), although the propagation of infected vectors by wind over long distances cannot be ruled out, as it is possible given the case concerning the bluetongue virus (Alba *et al.* 2004, Mellor and Hamblin 2004, Sellers *et al.* 1978).

This article describes the clinic-pathological and laboratory findings observed in the framework of passive surveillance during AHS outbreaks which occurred in Namibia between 2006 and 2013. The study was conducted within the framework of the policy of preparedness of the Italian National Reference Centre for Exotic Diseases at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise (IZSAM), and it was implemented in collaboration with the Namibian Ministry of Agriculture, Water and Forestry and the Namibian National Veterinary Association.

Materials and methods

Data collection: anamnesis, clinical signs and pathology

African Horse Sickness was suspected on the basis of anamnestic data (including the seasonality), clinical signs, and gross lesions. Whenever possible, the immune status – with respect to AHS vaccination – was recorded.

As far as symptoms are concerned, 4 AHS clinical forms are usually classified: pulmonary, cardiac, mixed, and horse sickness fever (Coetzer *et al.* 2004, Mellor and Hamblin 2004). However, such distinction is not easy to implement under field conditions. Therefore, AHS cases have been classified herein using the following criteria: (a) peracute/acute, with sudden clinical onset and fatal outcome within 2 days; (b) sub-acute, characterized by a slower progression of the disease and fatal outcome, if any, after more than 2 days; (c) AHS fever, with mild symptoms and steady recovery.

Blood samples in EDTA were collected from live animals during the febrile stage of infection. Dead horses were submitted for necropsy. Gross lesions were systematically recorded and several tissues (spleen, lung, cephalic, tracheo-bronchial, and mediastinal lymph nodes) sampled.

Laboratory tests

The diagnosis of AHS was always confirmed by laboratory testing. In detail, reverse transcriptase-polymerase chain reaction (RT-PCR) was firstly conducted either to demonstrate or to rule out the presence of AHSV in the aforementioned collected samples (Monaco *et al.* 2011, Stone-Marschat *et al.* 1994); AHSV serotype was determined by using a serotype-specific RT-PCR (Sailleau *et al.* 2000). Attempts to isolate AHSV were made from all RT-PCR positive samples, as previously described. The isolated strains were then serotyped by virus neutralization assay as previously described (OIE 2010).

Molecular characterisation

The Seg-10s of 5 AHSV isolates were partially sequenced as follows: confluent VERO cells were infected with AHSV field isolates and incubated at 37°C until the occurrence of the cytopathic effect. Total viral RNA was extracted from infected cells using the 'High Pure Viral Nucleic Acid' extraction kit (Roche Diagnostic, Basel, Switzerland) and amplified by RT-PCR as previously described (Monaco *et al.* 2011). The

amplicons were then purified, cloned (TOPO TA Cloning Kit for Sequencing - Life Technologies, Carlsbad, CA, USA) in competent cells (One Shot TOP10 cells - Life Technologies, Carlsbad, CA, USA) and a 665 nt fragment sequenced (ABI PRISM 377 DNA sequencer - PE Applied Biosystems, CA, USA). Raw sequence data were assembled using Contig Express (Vector NTI suite 9.1; Invitrogen) and the consensus sequence was aligned with homologous sequences of 114 AHSV strains available in GenBank (Table S1, available online).

Results

Anamnestic data

The large majority of AHS outbreaks occurred between January and April, during 2010 (12 cases) and 2011 (40 cases). A total of 92 AHS-affected horses from 41 farms located in different Namibian districts were investigated (Figure 1). Fifty-three of them were vaccinated against AHS. The affected animals were mainly Arab, English thoroughbred or saddle horses of both sexes, their age ranging from 2 months to 18 years.

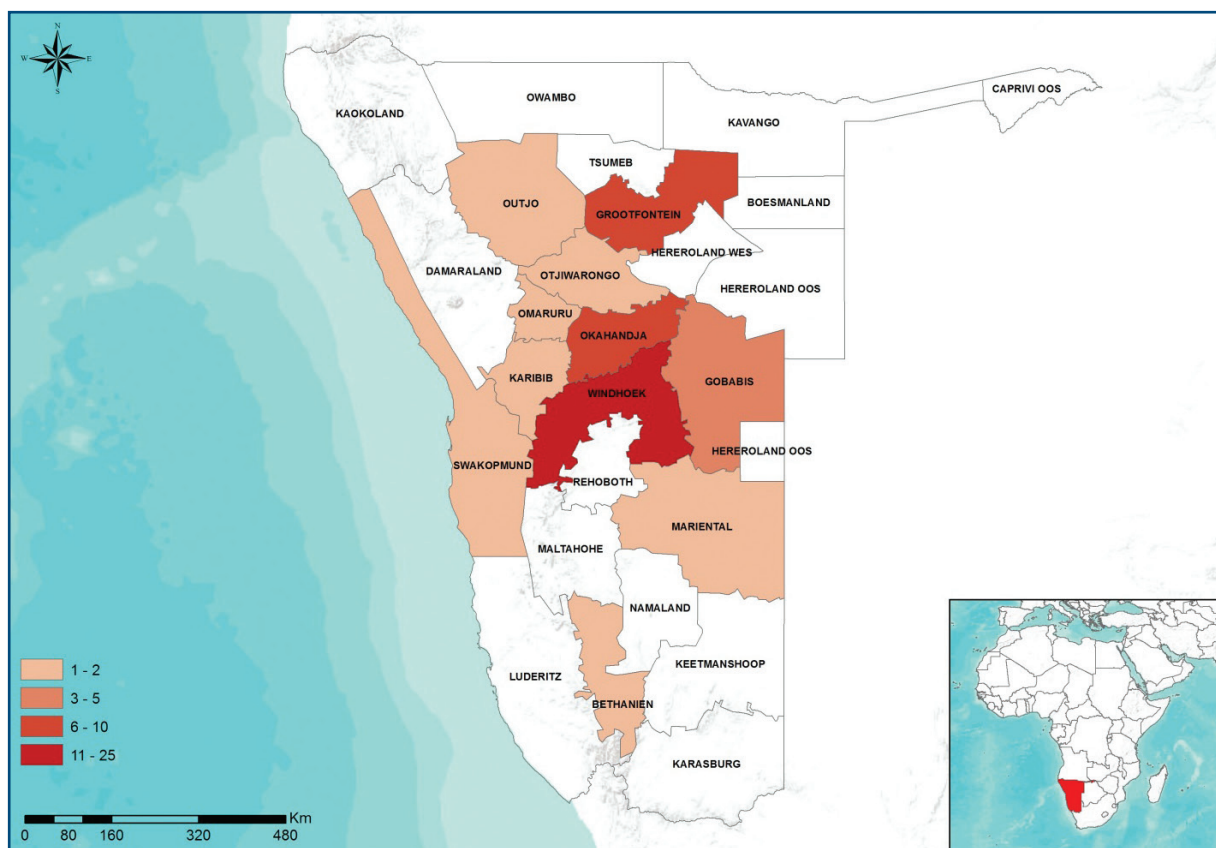


Figure 1. The Namibian districts where cases of African horse sickness (AHS) have been observed between 2006 and 2013. Different colours indicate the number of AHS cases observed in each district.

Table I. African horse sickness in Namibia between 2006 and 2013: clinical, pathological and laboratory findings. Clinic-pathological data are expressed as the percentage of animals showing specific symptoms and lesions.

	AHS clinical forms			
	Peracute/acute	Sub-acute	Mild AHS fever	Found dead
N° affected horses	43	21	19	9
Age	4 months-14 years	2 months-13 years	10 months-18 years	4-8 years
Aged > 2 years	18 (12 vaccinated)	17 (12 vaccinated)	15 (all vaccinated)	9
Aged ≤ 2 years	25 (9 vaccinated)	4 (1 vaccinated)	4 (all vaccinated)	0
Death	43	4 ≤ 2 year-old 2 > 2 year-old (irrespective of the immune status)	0	9
Clinical signs				
Fever	+++	+++	++++	na
Froth from the nares	+++	+++	-	na
Subcutaneous oedema	+++	++++	++++	na
Haemorrhagic conjunctivitis	+++	++	++++	na
Gross lesions				
Pulmonary oedema	+++	+++	na	+++
Subcutaneous oedema	+++	++++	na	+
Serosal petechiae	++	+++	na	++
Laboratory investigations				
RT-PCR positive horses	43	21	19	9
AHSV isolates	25	11	9	5
AHSV serotypes	1, 2, 4, 6, 8, 9	4, 8, 9	2, 4, 6, 9	6, 7, 8

+ = <25%; ++ = 25-50%; +++ = 50-75%; ++++ = 75-100%; na = not applicable.

Clinical signs

Clinical data were available for 83 animals. In addition, AHS was confirmed in 9 horses found dead without any clinical data (Table I).

In 43 animals aged from 4 months to 14 years, the observed clinical pattern could be included in the constantly fatal peracute/acute form. Of these animals, 21 were vaccinated against AHS, while this information was not available for 6 horses. Fever up to 41°C, subcutaneous oedema of the supraorbital fossae, head and neck, conjunctival petechiae, permanent recumbency, severe dyspnoea, and discharge of frothy fluid from the nares were usually observed (Figure 2).

In 21 horses aged from 2 months to 13 years, we observed fever (about 40°C), subcutaneous oedema of head, neck and chest; the sub-acute form was characterised by the presence of lingual and conjunctival petechiae (Figure 3). Irrespective of the immune status, the fatal outcome occurred in 6 horses (4 aged ≤ 2 years).

Mild AHS fever was observed in 19 horses aged between 10 months and 18 years (4 aged ≤ 2 years), all vaccinated against AHS and recovered after showing mild to moderate fever (39-40°C) and oedema of the supraorbital fossae.



Figure 2. Peracute/acute form of African horse sickness in a horse during an outbreak in Namibia between 2006 and 2013. Permanent recumbency and abundant discharge of frothy fluid from the nares.

Gross pathology

Necropsy was conducted on 35 out of 49 dead animals. Severe and diffuse pulmonary oedema, gelatinous exudate of the sub-pleural and interlobular connective, and abundant froth within the upper airways (Figure 4) were observed; hydrothorax were the main lesions noted in peracute/acute AHS affected horses. Tracheal and

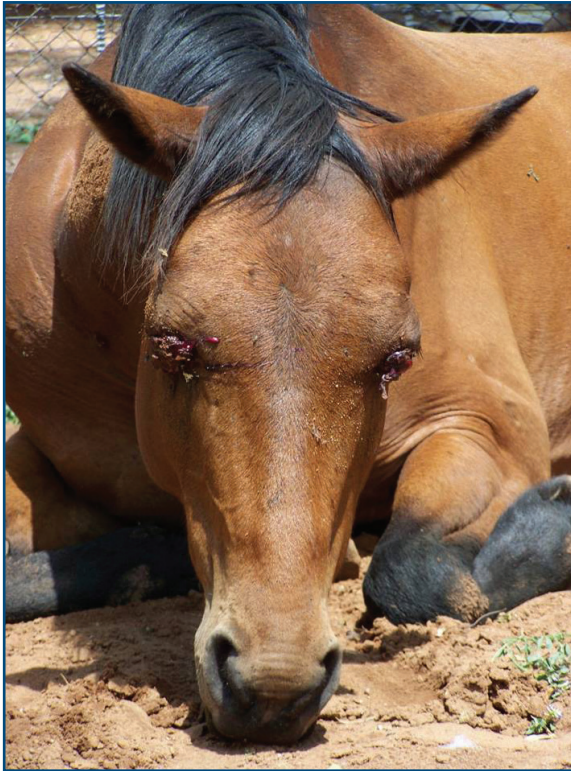


Figure 3. Sub-acute form of African horse sickness in a horse during an outbreak in Namibia between 2006 and 2013. Prominent head oedema and severe conjunctivitis. The present picture has been published in a previous paper (Scacchia et al. 2009).

sub-pleural haemorrhages were also commonly detected, along with ascites and hyperaemia of gastric mucosa.

The sub-acute form was mainly characterized by prominent subcutaneous, haemorrhagic-gelatinous oedema. Endocardial and/or epicardial haemorrhages (Figure 5) were also founded along to petechiae on the serosal surface of the large intestine. Typically, a distinct demarcation was noted between affected and unaffected tracts of the intestine. Haemorrhagic gastritis was also commonly recorded. Lymph nodes – with the only exception of the mesenteric ones – appeared enlarged because of oedema and haemorrhages.

Laboratory tests

The genome of the African Horse Sickness virus was detected in blood and/or tissue samples from all the 92 horses included in the study. However, it was possible to isolate AHSV only from 50 horses. Of these ones, 25 were affected with the peracute/acute form, 11 with the sub-acute form, 9 with the mild AHS fever, and 5 were found dead without clinical records. The virus was most frequently isolated from spleen and lymph nodes, and the isolated strains belonged to serotypes 1, 2, 4, 6, 7, 8 and 9 (Table II).



Figure 4. Peracute/acute of African horse sickness in a horse during an outbreak in Namibia between 2006 and 2013. Frothy fluid fills the tracheal lumen.

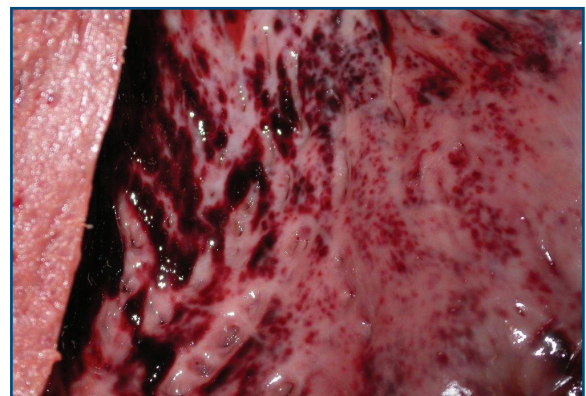


Figure 5. Sub-acute form of African horse sickness in a horse during an outbreak in Namibia between 2006 and 2013. Disseminated endocardial haemorrhages.

Table II. African horse sickness virus (AHSV) serotypes identified in Namibian districts between 2006 and 2013.

Namibian District	AHSV serotypes
Grootfontein	4
Windhoek	1, 2, 4, 6, 8, 9
Otjiwarongo	9
Bethanie	7
Okahandja	1, 4, 6, 9
Outjo	9
Gobabis	2, 6, 8
Windhoek	9
Kharibib	8
Omaruru	8
Swakopmund	2
Mariental	9

Molecular characterisation

The Seg-10s of 5 AHSV strains were partially sequenced (Table III). According to the Seg-10 phylogenetic analysis, AHSV strains are divided in

Table III. Details of the Namibian strains selected for Seg-10 partial sequencing between 2006 and 2013.

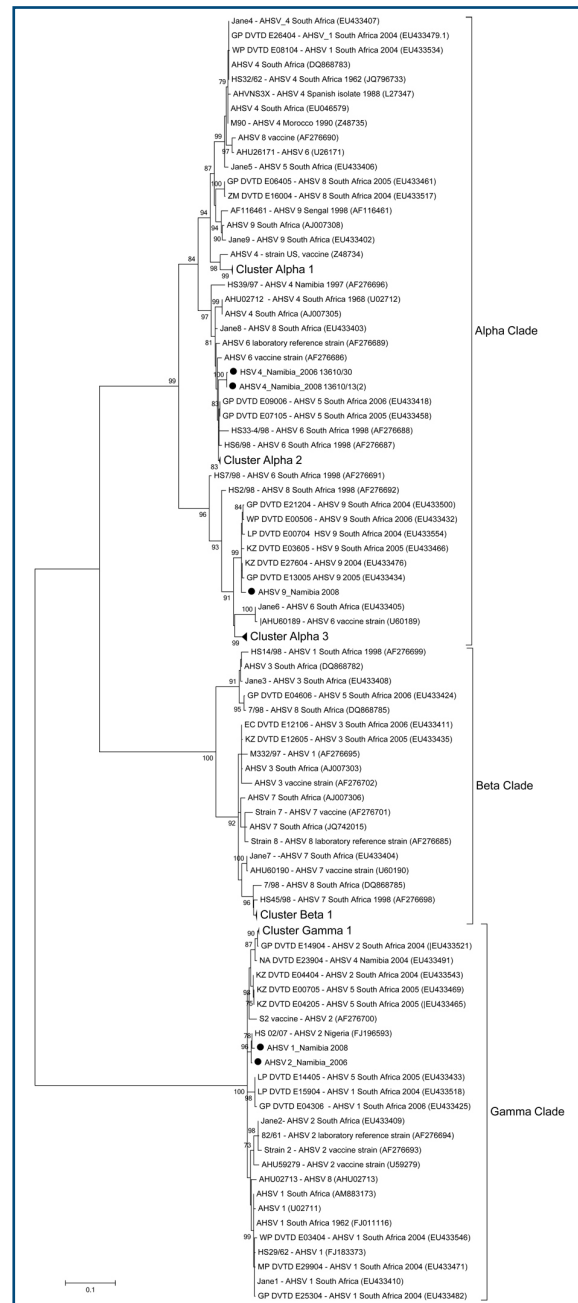
Virus	Serotype	Year of collection	Tissue	Municipality (District)
AHSV 1_Namibia_2008	1	2008	Spleen	Okahandja (Okahandja)
AHSV 2_Namibia_2006	2	2006	Lymph node	Witvlei (Gobabis)
AHSV 4_Namibia_2006	4	2006	Blood	Okahandja (Okahandja)
AHSV 4_Namibia_2008	4	2008	Spleen	Omitara (Windhoek)
AHSV 9_Namibia_2008	9	2008	Lymph node	Derm (Mariental)

3 phylogenetic clades, which have been indicated as α , β and γ (Quan *et al.* 2008, Sailleau *et al.* 1997, van Niekerk *et al.* 2001). The Namibian AHSV strains were closely related to the South African strains. The AHSV 4_Namibia_2006, the AHSV 4_Namibia_2008, and the AHSV 9_Namibia_2008 clustered in the α clade, whereas the AHSV 1_Namibia_2008 and the AHSV 2_Namibia_2006 clustered in the γ clade. A high level of sequence homology (close to 100%) was observed between AHSV 4_Namibia_2006 and AHSV 4_Namibia_2008 (α clade), as well as between AHSV 1_Namibia_2008 and AHSV 2_Namibia_2006 (γ clade). The seg-10 sequence of AHSV 9_Namibia_2008 was included in the α clade (Figure 6).

Discussion

African horse sickness represents a major health concern and negatively impacts the equine industry, mainly in those countries, such as Namibia, where high quality horses are bred and vaccination is not compulsory. A freeze-dried, polyvalent, live attenuated vaccine against AHS (Onderstepoort Biological Products, OBP) is currently used in Africa. Horses should be inoculated 3 times – at 6, 9, and 12 months of age – and then annually re-vaccinated, before the rainy season, to become immune to all the serotypes in the vaccine. As a result, the immune status of Namibian horses is likely to be variable, such speculation reasonably explaining the occurrence of severe, sometimes fatal AHS, in a number of immunized horses. Furthermore, a growing body of evidence indicates that prophylactic immunization against AHS is useful to prevent serious losses, but cannot fully protect horses from infection and disease under natural condition (Coetzer *et al.* 2004, Crafford *et al.* 2013, Weyer *et al.* 2013, Molini *et al.* forthcoming).

Furthermore, AHSV-5 is not included in OBP vaccine because of severe, sometimes fatal, adverse reactions reported in immunized animals. At the same time, AHSV-9 is not included in the OBP vaccine because considered of low virulence, epidemiologically irrelevant in Southern Africa, and antigenically cross-related with AHSV-6. However, AHSV-5 and AHSV-9 have been causing several AHS outbreaks in that region since 2006 (von Teichman

**Figure 6.** Phylogenetic tree. Genetic relationships among African horse sickness virus isolates.

The tree was constructed on the basis of a 665 bp fragment of the Seg-10. Black spots indicate the unique sequences obtained in the present study. The following data identify each strain: serotype, Country, year of collection, GenBank accession numbers. Analyses have been carried out by means of MEGA 5 software and maximum likelihood method (Tamura *et al.* 2011). Bootstrap support values >70 are shown (1,000 replicates). To facilitate the comprehension of data, highly similar sequences have been collapsed in clusters (Table S1, available online).

et al. 2010). Interestingly enough, AHSV-9 has been detected in peracute/acute, sub-acute, and mild AHS-affected horses under study. Therefore, our data show that AHSV-9 is relevant in Southern Africa (von Teichman *et al.* 2010), and/or that the vaccine does not confer protection against that serotype, in spite of the antigenic cross-relation with AHSV-6. As suggested by Howell (Howell 1960), AHSV-9 strains provided with different pathogenicity may be also present in that region.

Our results confirm RT-PCR as the first choice diagnostic tool. Spleen, tracheo-bronchial, and mediastinal lymph nodes are appropriate tissue samples for virus isolation, although it is worthwhile stressing that these samples could be negatively influenced by pH and temperature changes during transport to the laboratory (Coetzer *et al.* 2004)¹.

Although preliminary and partial, phylogenetic analyses suggest that a common viral population could circulate in Namibia and South Africa. In fact, the comparison of Seg-10 sequences did not generate geographically distinct virus lineages (topotypes), as observed in other *Orbiviruses* (Balasuriya *et al.* 2008). Up to date, almost exclusively Namibian and South Africa AHSV strains have been sequenced. Therefore, implementing the gene sequence database is of crucial relevance to define the existence of different AHSV topotypes.

AHSV 1_Namibia_2008 and AHSV 2_Namibia_2006 show an almost identical Seg-10, which is closely related with Seg-10 of several attenuated vaccine strains (Genbank accession: AF276693, AF276700 and U59279). Similarly, Seg-10s of AHSV 4_Namibia_2006

and AHSV 4_Namibia_2008 are closely related with an AHSV-6 vaccine strain (AF276686). These findings suggest that the reassortment of genomic segments may have occurred between field and vaccine viruses. In this respect, it would be worthy of interest to evaluate if AHSV circulates among immunized, sub-clinically AHS-affected horses in Namibia; in that case, the genetic constitution of such viruses should be carefully investigated.

A global approach is mandatory to effectively contrast trans boundary animal diseases. From that perspective, the cooperation network among IZSAM, on one side, and a number of Institutions and diagnostic laboratories located in the Mediterranean basin, Balkan area, Southern America, and Central-Southern Africa, on the other side, is a strategy of paramount relevance. In particular, IZSAM has been working in Namibia since 1996, to develop diagnostic tests and vaccines against emerging infectious diseases endemic in the tropics, such as AHS and Rift Valley fever (Caporale *et al.* 2009, Monaco *et al.* 2013).

AHS still represents a major concern for animal health, and the recent expansion of bluetongue virus northwards indicates that AHSV could enter into Europe (Alba *et al.* 2004, Mellor and Hamblin 2004, Sellers *et al.* 1978), thus further enhancing the need of valuable diagnostic tools and effective control strategies against vector-borne diseases. In this context, we consider of particular value the description of clinical, pathological, and laboratory findings provided in this article, which are crucial for a prompt differential diagnosis and control of AHS, especially in naïve areas.

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