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Immunization of horses with a polyvalent live-attenuated African horse sickness vaccine: Serological response and disease occurrence under field conditions

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ABSTRACT

African horse sickness (AHS) is a non-contagious, insect-borne disease of equids caused by a RNA virus (AHSV), which belongs to the genus *Orbivirus*, family *Reoviridae*. The disease is endemic in sub-Saharan and western Africa, where prevention strictly depends upon vaccination. The present paper aims at evaluating the serological response and the occurrence of AHS in horses bred under field condition and regularly immunized using the commercially available live attenuated vaccine (LAV) produced by Onderstepoort Biological Products.

The study was carried out in a farm located in the district of Windhoek (Namibia), where the disease is endemic. A total of 72 cross-breed horses, out of the 150 housed on the farm, were subdivided in six age groups, from 2 to 7 years-old. Each group consisted of 12 heads which were born during the same breeding season and had undergone from four to nine vaccination courses. AHSV specific immune response was evaluated by serum-virus neutralization test. Data about the clinical occurrence of the AHS from 2006 to 2011 were made available. The immune response, in terms of number of seropositive horses and serum neutralizing titers, was quite variable among horses and against different serotypes. Neutralizing antibodies against all serotypes were recorded in all the horses only after eight vaccination courses at 6 years of age onwards. Immune response to AHSV-5 and 9, which are not included in the LAV formulation, were also established. A severe AHS epidemic occurred in Namibia in 2011. On the farm under study, a total of 32 animals were clinically affected, 12 died, 11 of them were 2 year-old or younger.

Our data confirm that vaccination with LAV is a useful tool to reduce the severity of the disease in endemic areas. However, clinical and sometimes fatal AHS can still affect young vaccinated horses, thus highlighting the necessity to better understand the immune response to AHSV and to dispose of more effective vaccines.

Introduction

African horse sickness (AHS) is a non-contagious insect-borne disease of equids caused by a double stranded RNA virus (AHS virus, AHSV) which belongs to the genus *Orbivirus*, family *Reoviridae,* along with bluetongue and equine encephalosis viruses. In naïve horse populations AHS is often fatal with a mortality rate that may exceed 95%. High mortality rates may occasionally be recorded also in mules, whereas infection is usually sub-clinical or asymptomatic in African donkeys, differently from what observed in the European and Asiatic ones. Zebras rarely exhibit clinical signs but play a relevant epidemiological role, thus being regarded as the natural vertebrate reservoir of AHSV. However, AHS is also endemic in African countries where zebra population is absent or negligible as in Nigeria, indicating that other unknown reservoir might exist [1,2].

AHSV genome consists of 10 RNA segments, which codify for 11 viral proteins. The structural protein VP2, located within the outer capsid of the viral particle, represents the major neutralizing determinant of the virus [3]. Up to date, nine AHSV serotypes (1–9) have been identified by virus neutralization test. *In vitro*, some evidence exists of serological cross-reactions among serotypes 6 and 9, 5 and

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8, 1 and 2, whereas no antigenic relationship has been demonstrated with other known orbiviruses [1,2].

AHS is endemic in large areas of sub-Saharan Africa where all serotypes are present. The disease has periodically occurred into northern African countries, Iberian peninsula and Middle East up to Turkey and India. AHSV-9 has been involved in the epidemics in the Middle East (1959–63), Saudi Arabia, Yemen (1997), Cape Verde Islands (1999), northern Africa and Spain (1966); in the latter country, a subsequent outbreak (1987), was caused by AHSV-4. Such epidemics mainly resulted from the movement of infected animals, although the spreading of infected vectors by wind, over long distances, cannot be ruled out [2].

In Africa, biting midges *Culicoides imicola* and *Culicoides bolitinos* are considered the most important vectors of AHSV. As a consequence, AHS shows a typical seasonal occurrence, closely related to rain and high environmental temperatures that favor breeding of the vectors [1,2,4].

Colonization of different AHSV serotypes into western African countries, from Nigeria to Mauritania, indicates that AHSV spreading capacity is greater than previously thought [2,5].

In endemic areas, AHS prevention strictly depends upon vaccination, that has undoubtedly provided protection from this devastating disease. The use of a polyvalent vaccine seems mandatory in southern Africa, where all AHSV serotypes circulate with different time distribution and prevalence [1,2,6,7]. A freeze-dried, polyvalent live attenuated vaccine (LAV) produced by Onderstepoort Biological Products (OBP) is currently used in southern Africa. The vaccine is formulated in two components, which have to be administered two or three weeks apart: (1) trivalent, containing AHSV-1, 3 and 4; (2) tetravalent, containing AHSV-2, 6, 7 and 8. The OBP-LAV does not include AHSV-5, due to residual virulence recorded in the field in 1993 and AHSV-9, considered epidemiologically irrelevant in southern Africa. Furthermore, AHSV-6 should have provided some cross protection against AHSV-9, due their serological cross-reaction. However, AHSV-5 and 9 both dominated the 2006 AHS season, particularly in the Western Cape Province [8].

It is widely accepted that several vaccination courses are needed to immunize horses with OBP-LAV. According to the manufacturer, young horses should be treated at 6, 9 and 12 months of age and thereafter yearly, before the AHS rainy season [8]. However, only few data exist about the immune response and the efficacy of OBP LAV under field conditions [9,10].

On the basis of what above, the present study aimed at investigating the serological response and the occurrence of AHS in horses from 2 to 7 year-old, which had undergone from four to nine vaccination courses with the OBP-LAV. Horses were housed in a strictly controlled farm, supervised by a veterinarian, in the Windhoek district (Namibia), where the disease is known to be endemic. The investigation, implemented in collaboration with the Namibian Ministry of Agriculture, Water and Forestry, was carried out within the framework of the policy of preparedness of the Italian National Reference Centre for Exotic Diseases (Istituto Zooprofilattico Sperimentale dell'Abruzzo e Molise "G. Caporale", Teramo, Italy).

Materials and methods

Horses

The present study has been carried out on a farm located in the district of Windhoek, approximately 82 km far from the Namibian Capital. AHS is known to be endemic in Namibia, including the district of Windhoek where different AHSV serotypes circulate [6,7]. The farm housed 150 cross-breed horses, that number was kept constant due to a specific replacement herd plan. All animals were regularly vaccinated using the OBP-LAV. More in detail, young horses were immunized at 6, 9 and 12 months of age, and then annually re-vaccinated before the rainy season, between August and October. No relevant side-effect to OBP-LAV was ever reported.

A total of 72 horses, out of the 150 were investigated. They were selected in order to obtain six groups of 12 animals from 2 to 7 years of age. Younger animals, aged 6–12 months, were not available at the time of blood sampling, which was carried out in November 2011, about 1 month after vaccination.

Serology

AHSV serotype-specific immune response was evaluated by serum-virus neutralization test (SN) [11]. Details in Appendix A.

Outbreaks of AHS on the farm: Anamnestic data and laboratory tests

Clinical and pathological data about AHS outbreaks, which occurred on that farm between 2006 and 2011, had been recorded at the time and made available.

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

Reverse transcriptase-polymerase chain reaction (RT-PCR) had been firstly carried out to demonstrate or, alternatively, to rule out the presence of AHSV; virus isolation and typing had been performed according to previously described methods [12].

Statistical analysis

Probit analysis was applied to serological data. Nine probit analysis, one for each serotype were performed [13,14]. Details in Appendix A.

Results

Serology

Serological results are summarized in Table 1. The immune response, in terms of number of seropositive horses and SN titers, were quite variable among horses and against the different AHSV serotypes. In particular, at 2 years of age, after four vaccination cycles, a high number of horses seroconverted against serotypes 6 and 7 whereas, at that time point, only a single horse showed low immune response to AHSV-2. Antibody response in all horses to all serotypes was recorded in the 6 and 7 year-old animal group and varied between 1/10 and 1/640. Immune response to AHSV-5 and 9 were also recorded.

The probit analysis proved statistically significant (χ^2 test p value <0.001) and provided useful information about the timing of serological immune response (Fig. 1). The probit model allowed identifying a 95% confidence interval (C.I.) of the time (i.e. age) during which we can be confident to record a serological immune response *versus* a specific AHSV serotype in 95% of horses. The lowest values were obtained for AHSV-6 (C.I. 4.1–8.0 years) and AHSV-1 (C.I. 4.8–8.7 years); on the contrary, the highest values were found for serotypes 2 (C.I. 6.6–11.2 years) and 9 (C.I. 5.6–12.9 years).

Table 1
Serotype-specific immune response in vaccinated horses of different age.

Age (years)/vaccination courses	2/4	3/5	4/6	5/7	6/8	7/9
AHSV serotypes	Positive animals/total animals					
1	4/12	5/12	8/12	11/12	12/12	12/12
SN titres Min–Max*	80–160	20–320	10–160	20–160	40–320	40–640
2	1/12	1/12	1/12	4/12	12/12	12/12
SN titres Min–Max*	10	10	20	10–160	10–160	10–640
3	6/12	6/12	6/12	12/12	12/12	12/12
SN titres Min–Max*	10–40	10–80	10–80	10–320	10–320	20–320
4	5/12	6/12	7/12	10/12	12/12	12/12
SN titres Min–Max*	10–320	40–640	10–640	20–160	10–640	80–640
5	5/12	6/12	8/12	9/12	12/12	12/12
SN titres Min–Max*	10–40	10–40	10–640	20–640	10–160	20–640
6	8/12	8/12	8/12	12/12	12/12	12/12
SN titres Min–Max*	10 – 160	10 – 160	10–80	10–160	10–320	40–640
7	7/12	7/12	7/12	10/12	12/12	12/12
SN titres Min–Max*	10–40	10–40	10–320	10–160	10–160	10–640
8	4/12	4/12	7/12	9/12	12/12	12/12
SN titres Min–Max*	10–80	10	10–640	10–640	10–640	10–640
9	6/12	6/12	6/12	9/12	12/12	12/12
SN titres Min–Max*	10–640	10–320	40–160	10–640	10–160	10–640

* Reciprocal of SN titres in horses immunized with OBP polyvalent live attenuated vaccine.

AHS outbreaks on the farm under study

Table 2 summarizes data about the occurrence of AHS from 2006 to 2011. No clinical case occurred in 2006 and 2007. In 2008, only two horses (2 and 4 years-old respectively), showed mild AHS clinical signs, both recovered; AHSV 4 was isolated. During the following 2 years, 2009–2010, no clinical case was recorded. On the contrary, AHS showed a severe epidemic course in 2011 concomitantly with heavy rain and flooding. A total of 32 horses became clinically affected, with twelve fatal outcomes, 11 were ⩽2 year-old or younger, one was aged 4 year.

The remaining recovering horses showed moderate fever (39.5–40 °C), subcutaneous edema at level of the supraorbital fossae, and conjunctival petechiae. Fatal cases of AHS were clinically characterized by prominent subcutaneous edema of head and neck, conjunctival petechiae, permanent recumbency, severe dyspnea, and discharge of frothy fluid from the nares.

At necropsy, the pulmonary form of AHS was observed in foals aged 9–12 months old, while pathological changes of the pulmonary and cardiac forms overlapped in the older ones.

Discussion

AHS is an extremely serious notifiable trans boundary infectious disease affecting the respiratory and cardiovascular systems and causing high mortality in horses, with a negative impact on the equine industries of countries, such as South Africa and Namibia, where the disease is endemic. Previous surveys demonstrated that several AHSV serotypes (AHSV 1, 2, 4, 6, 7, 8 and 9) circulate in Namibia. Under such circumstances, immunization with a polyvalent vaccine is considered the most, if not the only effective control method [7,8].

Experimental studies demonstrated that serological response to all AHSV serotypes is evident after the second OBP-LAV immunization course and that the immune response is not enhanced in horses given monovalent LAV, a finding that argues against the antigenic competition among multiple AHSV serotypes. In addition, it has been recently shown that colostrum antibodies could also influence timing of first vaccination course [9,10,15].

In our study no data were available on the immune status of colts. Interestingly, our results indicate that a higher number of OBP-LAV administrations are required to reach a detectable serological immune response under field conditions. In accordance with von Teichman et al. [8], our data show that the serotype-specific immune response is quite variable among horses, as well as against the different virus serotypes. Such findings might be due to a number of still unknown host and/or vaccine related factors.

In vitro, serological cross-reactions have been demonstrated between serotype 6 and serotype 9, as well as between serotype 5 and serotype 8. As also previously reported [8], such cross-reactions could explain the detection of neutralizing antibodies against the AHSV-5 and 9,wich are not included in the OBP-LAV. Actually, the AHSV-9 specific immune response reported in the present study might also result from natural and undetected infections, since the serotype is known to circulate in the district of Windhoek. On the contrary, that scenario seems questionable for AHSV-5, the presence of which was not been reported in Namibia during the years 2006–2011 [6,7].

As recently reported by Weyer [9], our data confirm that immunized horses can be affected by AHS. Although the LAV manufacturer relies on minimum protective SN titers of 1:16, the real protective SN titer is still unknown and likely variable. In addition, serum negative immunized horses could be also protected at challenge, a finding which might further remark the importance of the cell-mediated immune response [9]. However, our data suggest that seroconversion is an appropriate index of protection to clinical disease. In fact, in the present study, clinical AHS affected horses 4 years old or younger and fatal outcomes almost exclusively occurred in younger horses up to 2 years of age, which according to the results shown could have been seronegative for some AHSV serotypes. Remarkably, environmental conditions (heavy rain) increasing the density of vectors and the viral load could be regarded as additional factors, capable to affect the efficacy of the vaccine.

Taken together our data emphasize that the immune response to AHSV is still poorly understood and that further studies are needed to better clarify the interactions between the host's immune system, on one side, and the virus, on the other. OBP-LAV still plays a crucial role in endemic areas, concerns still exist about its safety, since attenuated virus recombination with wild types AHSV might result in more virulent strains. As a consequence, the prompt availability of effective inactivated and new

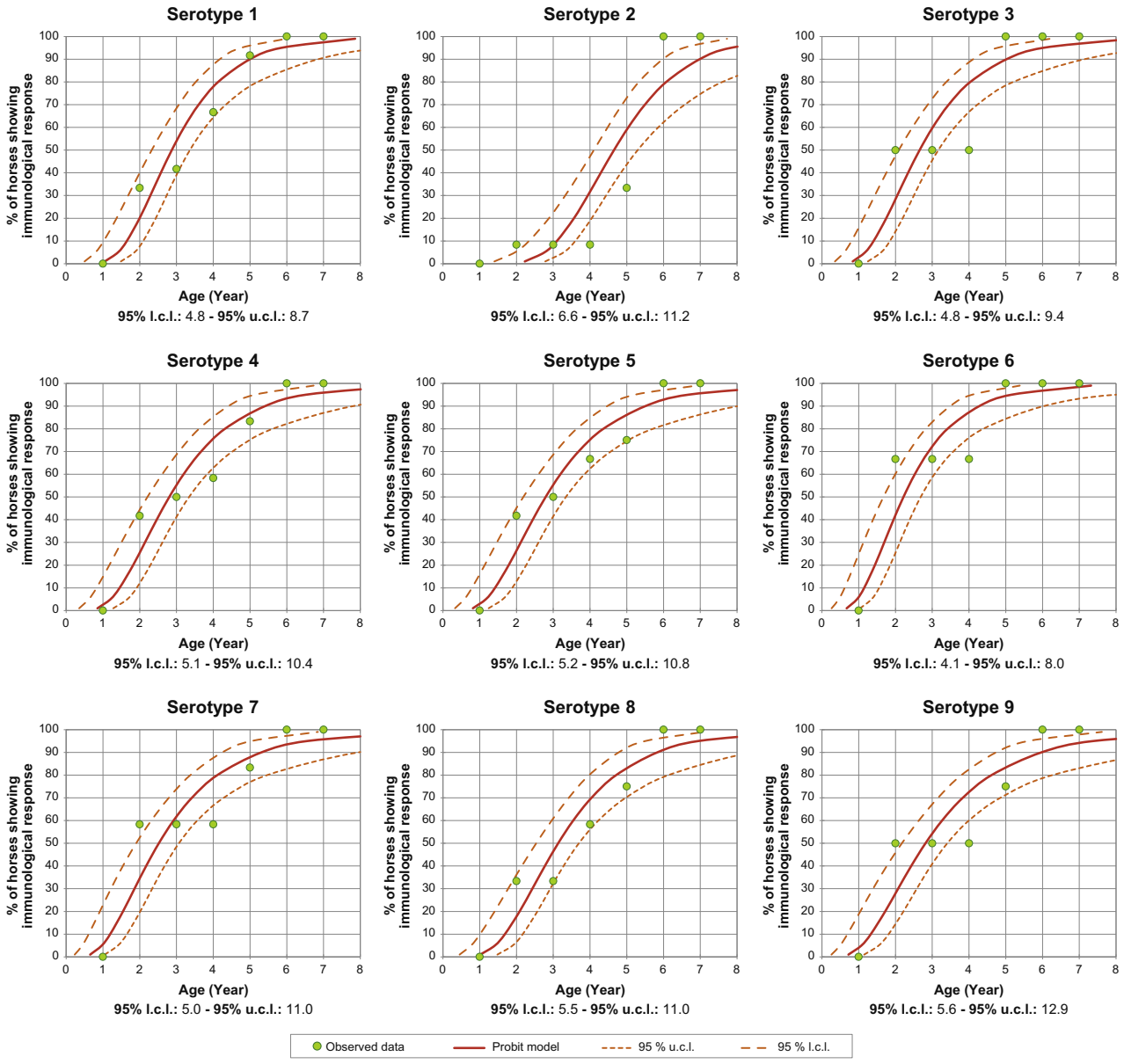

Fig. 1. Serotype-specific probit analysis allows to determine a continuous 95% confidence interval of the probability of achieving a positive result at any given input time within the time range of the experiment. Upper and lower intervals expressed in years, for each seroptype, are shown.

Table 2
AHS cases recorded in the farm during the rainy seasons 2006–2011.

Year	Age of AHS affected horses	Number of AHS-affected horses	Dead	Recovered	Vaccination courses	AHSV serotype	Annual rainfall (mm) Windhoek district
2006	//	0	//	//	//	//	Incomplete data 688
2007	//	0	//	//	//	//	Not available
2008	2 years	1	0	1	5	4	574
	4 years	1	0	1	7	4	
2009	//	0	//	//	//	//	518
2010	//	0	//	//	//	//	582
2011	6 months	4	4	0	1	4 (2 horses) and 6 (2 horses)	1221
	9 months	3	3	0	2	4	
	1 year	5	2	3	3	4	
	2 years	12	2	10	4	6	
	3 years	2	0	2	5	n.d.	
	4 years	6	1	5	6	4	

generation vaccines appears highly desirable. Up to date, the latter confirmed to prevent AHS under experimental conditions, but have not been pursued further [3,16–21].

Conclusions

In conclusion, our study indicate that the regular and correct use of OBP-LAV is useful to reduce the severity of the disease. However, the same results confirm that vaccinated horses can be affected by AHS under field conditions, thus highlighting the necessity to better understand the immune response, and to search for alternative, more effective and safer vaccine.

Conflict of interest

None declared.

Appendix A

Serum–virus neutralization test

Collected sera were stored at −20 °C and inactivated at 56 °C for 30 min before testing. VERO cell (ECACC) at a concentration of 100,000 cells/ml were used. AHSV serotypes were made available from the reference antigens, *Bob Swanepoel* collection. Sera were diluted from 1:10 to 1:1280 and then incubated for 60 min with $100TCID_{50}$ of AHSV; the virus-serum mixtures were added in duplicate to 92 well plates with confluent cell monolayers. The specific cytopathic effect was evaluated under a light microscope after five days of incubation at 37 °C in 5% CO_2. Neutralizing titer was defined as the reciprocal of the highest dilution of serum, neutralizing $100TCID_{50}$ of AHSV in $\geqslant$75% of the wells.

Statistical analysis

A model of non-linear regression (probit analysis) was applied to serological data by means of a commercial software suite (XLSTAT Version 2013.2.04 – Copyright Addinsoft, 1995–2013). The qualitative response (positive or negative) was the depending variable, while the time was the independent quantitative variable. Probit analysis allows to determine a continuous 95% confidence interval of the probability to achieve a positive result at any given input time, within the time range of the experiment. Nine probit analyses, one for each serotype, were performed [13,14].

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