Sudan University of Science and Technology
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Epidemiological Studies on African Horse Sickness Virus Infection in Sudan

دراسات وبائية عن مرض طاعون الخيل الأفريقي في السودان

A thesis Submitted to College of Graduate Studies, Sudan University of Science and Technology in fulfillment of the requirements for the Degree Doctor of Philosophy in Preventive Veterinary Medicine

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DEDICATION

I would like to dedicate this work to my wife, my family and my friends who encouraged and supported me to complete this work. Everyone who made this work see the light.

With my love to all

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LIST OF ABBREVIATION

Ab: Antibody

Ag: Antigen

AHSV: African Horse Sickness Virus

BP: Blocking percentage

BTV: Blue tongue virus

CI: Confidence interval

dsRNA: double-stranded RNA viruses

EDTA: ethylene-diamine-tetra-acetic

EHDV: Epizootic hemorrhagic disease virus

ELISA: Enzyme linked immune serobent assay

IU: International unit

µ: Micron

M: Molarality

MEM: Minimum essential medium

Ml: Millilitre

MS: Monkey Stable

NS: Normal saline

Nt: Neutralization test

PBS: Phosphate buffer saline

PCR: Polymerase chain reaction

PD: Phosphate diluents

PLAV: Polyvalent live attenuated vaccine

OD: Optical density

OIE: World Organization for Animal Health

OPG: Edington's solution (Oxalate 0.5%, phenol 0.5% and glycerol 50 %)

OR: Odd ratio

RPM: Revolution per minute

RT-PCR: Reverse transcriptase polymerase chain reaction

SNT: Serum neutralization test.

ABSTRACT

African horse thickness disease is one of the most prevalent viral diseases that threaten the equine breading in Sudan. A cross-sectional study was conducted in four states (Northern, River Nile, Khartoum and Southern Darfur) from September 2016 to October 2017 to determine the sero-prevalence and to investigate the risk factors which associated with African Horse Sickness (AHS) in Sudan. A total of 920 equines (590 horses and 330 donkeys) were randomly selected and sampled. Competitive Enzyme Linked Immuno-Sorbent Assay (c-ELISA) was employed to detect antibodies to AHS virus. The overall sero-prevalence was 72.2 %, while it was 80% in horses and 58.2% in donkeys. The univariate analysis of associations of potential risk factors with sero-prevalence of AHS showed statistically significant ($p \leq 0.05$) results with state ($\chi^2 = 47.434$, $p<0.001$), species ($\chi^2 = 50.163$, $p<0.001$), sex ($\chi^2 = 26.206$, $p<0.001$), housing ($\chi^2 = 26.477$, $p<0.001$), vaccination ($\chi^2 = 44.466$, $p<0.001$), breed ($\chi^2 = 57.256$, $p<0.001$), water bodies ($\chi^2 = 26.271$, $p<0.001$), Cullicoides ($\chi^2 = 42.658$, $p<0.001$), ticks ($\chi^2 = 23.608$, $p<0.001$), activity of animal ($\chi^2 = 41.435$, $p<0.001$), awareness of owner ($\chi^2 = 25.639$, $p<0.001$),age ($\chi^2 = 20.186$,$p<0.001$), health score($\chi^2 = 12.038$, $p<0.001$), pregnancy ($\chi^2 = 3.249$,$p = 0.0355$),and infection with other disease ($\chi^2 = 14.637$,$p<0.001$). However, the risk factors of presence of other animals and pervious infection with AHS did not show statistically significant ($p>0.05$) associations. Furthermore, in the multivariate analysis only state (OR = 4.909, p = 0.017), breed (OR = 2.532, p =

0.004), species (OR = 3.776, p = 0.017), water bodies (OR = 2.172, p = 0.033), and vaccination (OR =17.298, p< 0.001) were found to be statistically significantly≤ (p0.05) associated with sero-prevalence of AHS. The other study was carried out during fall 2018 and 2019 to estimate the prevalence of the disease in horses, to detect the virus in *Cullicoides* (Diptera: Ceratopogonidae) pools and assess the contribution of major risk factors for the occurrence of the disease in horses. Whole blood samples (184) were collected randomly from horses from two governorates in Khartoum state and three provinces in Southern Darfur state. In addition, 1916 insects in 18 pools, the pool range 70- 150 non-engorged female different species of *Cullicoides* biting midges were collected from Khartoum governorate and tested for the presence of ribonucleic acid of African horse sickness virus (AHSV) using one-step reverse transcriptase polymerase chain reaction technique. The results revealed that the overall prevalence of AHSV in horses was 38.6% and from the 18 pools of *Cullicoides* the virus was detected in 6 pools (33.3%). Furthermore, in the univariate analysis, risk factors such as locality (p = 0.00), age (p = 0.01) and sex (p = 0.01) were significantly associated with the prevalence of AHSV in horses. However, the breed of horses did not show statistically significant associations (p>0.05). In the multivariate analysis, locality (OR = 28.2, p = 0.00) was found to be the most statistically significant risk factor for the occurrence of AHSV. The results of this study highlight that the overall

prevalence of AHSV was high in the areas surveyed. There were statistical significant differences of the prevalence of AHSV between the various sites in Khartoum state and South Darfur state ($p \leq 0.05$).

ملخص الأطروحة

يعتبر مرض طاعون الخيل الأفريقي من أكثر الأمراض الفيروسية انتشاراً وتهديداً لتربية الفصيلة الخيلية بالسودان. تم إجراء دراسة القطاع العرضي في أربعة ولايات (الولاية الشمالية و ولاية نهر النيل و ولاية الخرطوم و ولاية جنوب دارفور) في الفترة من سبتمبر 2016م إلى أكتوبر 2017م لتقدير معدل انتشار الاجسام المناعية للمرض والتقصي عن عوامل الخطر المهيئة للاصابة بالمرض. تم اختيار ما مجموعه 920 من الفصيلة الخيليةً (590 خيل و 330 حمير) بشكل عشوائي وتم أخذ عينات الدم منها وفصل المصل. تم استخدام فحص المناعي المرتبط بالإنزيم المناعي (c-ELISA) للكشف عن الأجسام المضادة لفيروس المرض. بلغ معدل الانتشار المصلي الإجمالي 72.2٪ ، بينما كان معدل الانتشار 80٪ في الخيول و 58.2٪ في الحمير. وأظهر التحليل أحادي المتغير من عوامل الخطر المحتملة مع انتشار الأجسام المضادة لفيروس المرض نتائج ذات دلالة إحصائية ($P \leq 0.05$) للاصابة بالمرض في الولايات ($\chi^2 = 47.434$ ، P <0.001) ، والاصابة بين أنواع الفصيلة الخيلية ($\chi^2 = 50.163$ ، P <0.001) ، وجنس الحيوان ($\chi^2 =$ 26.206، p <0.001)، طريقة الايواء ($\chi^2 = 26.477$ ، p <0.001)، التطعيم ضد المرض ($\chi^2 =$ 44.466، p <0.001)، سلالة الحيوان ($\chi^2 = 57.256$ ، p <0.001)، وجود المسطحات المائية ($\chi^2 =$ 26.271، p <0.001) وجود الهاموش (p <0.001 ، $\chi^2 = 42.658$)، ، وجود القراد في الحيوان ($\chi^2 =$ 23.608، p <0.001) ، نشاط الحيوان ($\chi^2 = 41.435$ ، p <0.001) ، إدراك المالك بالمرض ($\chi^2 =$ 25.639، p <0.001) ، عمر الحيوان($\chi^2 = 20.186$ ، p <0.001) ، الحالة الصحية للحيوان ($\chi^2 =$ 12.038، p <0.001) ، الحمل في الإناث ($\chi^2 = 3.249$ ، p = 0.0355) ، والإصابة بمرض آخر ($\chi^2 =$ 14.637، p <0.001). ومع ذلك ، فإن عوامل الخطر مثل وجود الحيوانات الأخرى والعدوى السابقة بالمرض لم تظهر الجمعيات ذات دلالة إحصائية (P> 0.05). علاوة على ذلك ، في التحليل متعدد المتغيرات فقط الاصابة بالمرض بالولايات (OR = 4.909، p = 0.017) ، السلالات (OR = 2.532، p = 0.004) ، الأنواع (OR = 3.776، p = 0.017) ، المسطحات المائية (OR = 2.172، p = 0.033) ، وقد وجد أن التطعيم (OR = 17.298، P <0.001) له دلالة إحصائية ($p \leq 0.05$) المرتبطة بالتردد المصلي لـ AHS. وأجريت الدراسة الأخرى خلال خريف 2018 و 2019 لتقدير معدل انتشار المرض في

الخيول ، و للكشف عن الفيروس في حشرة الهاموش (Cullicoides (Diptera: Ceratopogonidae) وتحديد عوامل الخطر الرئيسية في حدوث المرض في الخيول. تم جمع عدد (184) عينة دم بطريقة عشوائياً من الخيول من محافظتين بولاية الخرطوم وثلاث محليات بولاية جنوب دارفور. بالإضافة إلى ذلك ، تم جمع عدد (1916) حشرة الهاموش في 18 مجموعة ، يتراوح حجم المجموعة بين 70 الى 150 من الإناث غير المحتقنة من مختلف الانواع . تم جمع الهاموش من محافظة الخرطوم و ذلك لاختبار وجود حمض الريبونوكليك لفيروس مرض الحصان الأفريقي (AHSV) باستخدام تقنية تفاعل البوليميراز المتسلسل العكسي. أوضحت النتائج أن معدل انتشار فيروس المرض (AHSV) في الخيول كان 38.6٪ ومن مجموع 18 مجموعة من الهاموش تم اكتشاف الفيروس في 6 مجموعات (33.3٪). علاوة على ذلك ، عند إجراء التحليل أحادي المتغير ، ارتبطت عوامل الخطر مثل موقع الخيول ($p = 0.00$) وعمر الخيل ($p = 0.01$) وجنسها ($p = 0.01$) بشكل كبير مع انتشار فيروس المرض (AHSV) في الخيول. إلا أن سلالة الخيول لم تظهر ارتباطا ذات دلالة إحصائية مع انتشار فيروس المرض ($P> 0.05$). في التحليل متعدد المتغيرات الذي استخدم فيه معامل الانحدار، تم اثبات أن موقع الخيول ($OR = 28.2$، $p = 0.00$) ليكون عامل الخطر الأكثر أهمية من الناحية الإحصائية لإنتشار فيروس المرض (AHSV). تسلط نتائج هذه الدراسة الضوء على أن معدل الانتشار لفيروس المرض (AHSV) كان مرتفعًا في بعض المناطق التي تم مسحها. توجد فروق ذات دلالة إحصائية في انتشار فيروس المرض بين المواقع المختلفة بولاية الخرطوم وولاية جنوب دارفور ($p \leq 0.05$).

List of Tables and Figure

LIST OF CONTENT **Page**

CHAPTER THREE

CHAPTER ONE

1.1 Introduction

African horse sickness is a fatal vector-borne viral disease of horses. It is generally a sub-clinical disease in other Equidae such as mules and donkeys, while zebra is considered a reservoir for the disease. Some studies pointed to the importance of Zebra in continuing the emergence of the disease in Africa (*Radostits et al., 2007*). AHS was first recognized in South Africa, with the first major outbreak recorded in 1719, when more than 1700 animals died. Although the outbreak of epidemic in sub-Saharan Africa equines, it has also been recorded in North Africa, Middle East, near east and southern Europe (MacLachlan and Guthrie 2010). Both AHS and bluetongue viruses are transmitted by the biting midges *Culicoides* (Diptera: Ceratopogonidae), of which *Culicoides imicola* represents a major role in the transmission of the disease. Although *Culicoides imicola* is the field vector of AHS virus, *Culicoides bolitinos* has been proven to play an important role in the transmission of AHS virus in the cool upper highland territories (Meiswinkel and Paweska 2003). The highest incidence of the disease usually occurs in the late summer and early autumn in years, when the climatic conditions favor an abundance of *Culicoides* midges (Coetzer and Guthrie 2004). In addition to equids , goats, cattle and Dorcas gazelle can become infected (Elghazali and Ali, 2013). Dogs may be infected due to consuming horsemeat of the horses that have died in result of the disease (*Van Rensburg et al., 1981*). However, recently some reports in South Africa articulated that dogs were infected by exposure to Culicoides bites (*Van Sittert et al., 2013* and *Nicolize et al., 2018*). Given the potential of this virus to cause wide spread death and debilitating disease in naïve equid populations, it is listed as notifiable equine disease by the World Organization for Animal Health (OIE), which makes outbreaks of the disease compulsorily reportable to the OIE.

Such occurrences can result in serious consequences for international animal trade and animal products for the affected country (OIE 2019). It is currently predicted, that a widespread and outbreak of this disease would have a devastating effect on the horse industry of any country affected. The distribution of AHSV seems to be governed by a number of factors, including the efficiency of control measures, the presence or absence of a long term vertebrate reservoir. Most importantly, the seasonal incidence of the major vector which is controlled by the climate. However, with the advent of climate change the major vector, *C. imicola,* has now significantly extended its range northwards**,** to include most of Portugal, Spain, Italy and Greece**,** and has even been recorded by southern Switzerland. Furthermore, in many of these new locations the insect is present and active throughout the entire year (Mellor and Hamblin 2004). It was shown by Wellby *et al*.; (1996) that the virogenesis and infection rates of the *Culicoides* vectors were directly proportional to an increase in temperature, but that the survival rates of *Culicoides* were inversely related to temperature. This proposes that transmission should be possible only at certain times of the year, giving the disease the same seasonality as the vector species. Although AHS is clinically known to have existed in Sudan since 1903 (Anon, 1903), the first virological confirmation was made in 1957 (Anon 1957). The samples from infected horses were tested by Onderstepoort laboratory in South Africa, and found to contain AHS virus type 3. Later, an AHS virus isolate was identified as serotype 9 by the Razi Institute, Iran (Eisa M 1974). In later years, only serotype 9 has been isolated recurrently and serotyped locally from several disease outbreaks in the country (*Hajer et al; 1980*).

1.2. Objectives:

The present study was conducted to fill the information gap regarding with the following objectives:

1. Determine the sero-prevalence of African Horse Sickness in equines in different agro-ecology of Sudan.
2. To detect the African horse sickness virus in horses whole blood.
3. To detect the African horse sickness virus in Culicoides spp.
4. To investigate the potential risk factors associated with the disease in Sudan.
5. To assess the owner's awareness about the disease and the vaccination against it.

CHAPTER TWO
LITERATURE REVIEW

2.1. Introduction

African horse sickness (AHS) is a viral disease of equidae (Horses, Ponies, zebras and donkeys)**.** It is transmitted by hematophagous *Culicoides midges* (Diptera, Ceratopogonidae)**.** It was formerly classified as an A-list infectious disease by the Office International des Epizooties (OIE) with important economic consequence for the horse trade (*Wilson et al., 2009* and *De waal et al., 2016*). The disease was also defined as an infectious but not contagious disease of equine, (Sailleau *et al.,* 2000). It is characterized by mild to high fever, respiratory symptoms, severe weight loss, lethargy, rough hair, apathy which can eventually lead to death (*Calvo-Pinilla et al., 2015* and *Bekker et al., 2014*). The morbidity and mortality rate is very high in infected animals and reaches up to 95% in horses (*De waal et al., 2016, Zwart et al., 2015* and *Fall et al., 2015*). The disease is endemic in sub-Saharan Africa. There were sporadic outbreaks in North Africa, Spain 1969, Portugal 1987 and the Middle East with few outbreaks recorded in India and Pakistan (*Potgieter et al*., 2015 and *Alberca et al., 2014*). In the last decade the disease has emerged in different African countries such as the (AHSV-2) in Senegal**,** Nigeria 2007, Ethiopia 2008**,** and Ghana 2010. (AHSV-4) in Kenya 2007 and (AHSV-7) in Senegal 2007 (*Fall et al., 2015* and *Bachanek-Bankowska et al., 2014*) as well as in Sudan that has frequently ± reported AHS disease in imported race and breeding horses (Elghazali and Ali 2013). More recently outbreaks were recorded in South Africa 2013 and 2014 (*Fall et al., 2015* and *Bachanek-Bankowska et al., 2014*)**.** The recent effects of climate change further increase the risk of virus introduction into Europe, where the insect vector also occurs (*Zwart et al., 2015* and *Fall et al., 2015)*.

2.2. History:

African horse sickness (AHS) was first identified as a disease in horses in South Africa in 1891**.** The viral nature of the etiological agent, African Horse Sickness Virus (AHSV), was established in 1900 (Verwoerd. 2012). It is endemic in most sub-Saharan African countries, however occasional outbreaks have occurred in northern Africa (1965, 1989–1990, 2007–2010), the Middle East (1959–1961), and in Europe (Spain: 1966, 1987–1990, and Portugal: 1989) (Sanchez-Vizcaíno, 2004). Historically, the disease was reported for the first time in eastern Sudan, when the death of horses ascribed to African horse sickness in Kassala province, and spreading to Blue Nile province in the same year (Anon, 1903). However, only serotype nine was isolated (Eisa, 1974; *Hajer et al., 1980*) indicating that serotype 9 might be the prevalent serotype of AHSV in Sudan.

2.3.1. A etiology

AHSV containing nine different serotypes belongs to the genus Orbivirus of the Reoviridae family. The virus is similar in morphology and shares many biochemical properties with orbiviruses, such as equine encephalosis virus, bluetongue virus and epizootic haemorrhagic disease virus (Coetzer and Guthrie, 2004). It is a double-stranded RNA virus (dsRNA) with ten parts of the genome encoding seven structural proteins (VP1-VP7) and four non-structural proteins (NS1, NS2, NS3, NS3A) (*Wilson et al., 2009*; *Fowler et al., 2016*). There are two major outer capsid structural proteins in the African horse sickness virus; (VP2) and (VP5) (*Potgieter et al., 2015* and *De la Poza et al., 2013*). (VP2) is the major protective antigen of (AHSV) that is responsible for the serotype formation. The majority of neutralizing epitopes are located on (VP2) which can be considered the main target of immune response to the virus (*Fall et al., 2015, Alberca et al., 2014*, *De la Poza et al., 2013* and *Calvo-Pinilla et al., 2015)*. The structure of the AHSV virion is very similar to that of BTV; virions are triple-layered icosahedrally

symmetric particles. Major structural proteins VP2 and VP5 comprise the outermost capsid layer of the virion, enclosing the double-layered core particle (*Forzan et al., 2007* and *Stassen et al., 2011*). The core surface layer is composed of VP7 trimmers, while the innermost shell is a lattice of VP3 dimers. Inside this shell, the ten genome segments are arranged in three layers of RNA that surround the transcription complexes (*Manole et al., 2012* and *Gouet et al., 1999*) consisting of the three minor proteins VP1 (polymerase), VP4 (capping enzyme) and VP6 (helicase) (*Boyce et al., 2004,* de Waal and Huismans 2005). Recently, an additional protein was predicted to be encoded by genome segment 9 (Seg-9), which also encodes VP6, of most orbiviruses. This has since been confirmed in bluetongue virus and Great Island virus, and the non-structural protein was named NS4 and in silico analysis of AHSV Seg-9 sequences revealed the existence of two main types of AHSV NS4, designated NS4-I and NS4-II, with different lengths and amino acid sequences (*Zwart et al., 2015*). Viral replication and assembly of new core particles occur in cytoplasmic virus inclusion bodies (VIB) that are formed by non-structural protein NS2 (*Kar et al., 2007* and *Uitenweerde et al., 1995*). The trafficking and release of mature virus particles from the cell is mediated by NS3, the only membrane-associated viral protein (*Hyatt et al., 1991* and *van Gennip et al., 2014*). NS1 may also be involved in virus egress and morphogenesis (*Owens et al., 2004*) and it was recently shown that NS1 preferentially up-regulates viral protein expression during infection (*Boyce et al., 2012*). Recently, Firth (2008) showed that most orbi viruses are expected to have an additional open reading frame (ORF) within the genome segment encoding the minor structural protein VP6.

2. 3. 2. Persistence of the virus

According to Martinez-Torrecuadrada et al. (2001); Grubman and Lewis (1992) and Murphy et al. (1999), AHS virus is very stable outside the host and the virus

has the following properties.**:** An optional pH for survival of 7.0 - 8.5; the virus is sensitive to acid pH values but is relatively resistant to alkaline conditions, ether and other lipid solvents. Relatively heat stable and can be stored for at least six months at 4°C. It isn't destroyed by putrefaction and may retain infectivity in putrid blood for more than two years and doesn't contain lipid and is resistant to detergents. Generally, the virus can be detected in blood for 4 days before and 2 days after clinical signs are first observed. Persistence of the viraemic state for up to 49 days has been observed in vaccinated horses (*Ferhandey et al., 2007*). It can survive in frozen but not salted meat. At pH value below 6.0, that is, at the sort of pH usually found in meat that has rigor mortis, the virus of AHS is inactivated quickly. It is generally accepted that vectors that become infected with an Orbivirus remain so far life. AHS virus multiplies and reaches a titer in *Cullicoides imicola* on the fifth day after ingestion of infected blood. This midge has been able to transmit infection to other horses 7 - 13 days after feeding on an infected horse. The shortest period between a midge biting an infected horse and the disease being seen later in another horse bitten by that midge could be from 12 to 16 days (Meiswinkel and Pawerska, 2003).

2. 4. **HOST RANGE**

African horse sickness (AHS) is a fatal vector-borne viral disease of horses, and generally sub-clinical disease in other Equidae such as mules and donkeys (*Radostits et al., 2007*). Zebras can become infected but are usually resistant to the disease and have been identified as asymptomatic maintenance hosts of AHSV (*Barnard et al., 1994*). Dogs may be infected due to consuming horsemeat from horses that have died by the disease (*Van Rensburg et al., 1981*). However, recent reports in South Africa showed that dogs got the infection by exposure to *Culicoides* attack (*Van Sittert et al., 2013*; *Nicolize et al., 2018*). Infection in camels is rare and apparent with no available details on the level and duration of

viraemia, if any. Antibodies (Abs) to AHSV were detected in goats, cattle and Dorcas gazelle (Elghazali and Ali 2013).

2.5. Pathogenesis

There are number of factors that decide the outcome in the horse that is bitten by a midge infected by AHS virus, including the virulence of the individual virus serotype and the immune status of the horse (*Radostitis et al., 2007*). After the virus is inoculated into the body, it is carried to the regional lymph nodes where it finds conditions favorable to its multiplication. Virus is released into the blood whereby it finds itself infecting the target organs, namely the lungs and other lymphoid tissues of the body. The viraemia is associated with the red blood cells and lasts for about four to eight days. By the third day after inoculation, the virus may be found in organs such as the spleen, lungs and pharynx, as well as most lymph nodes. The heart is not primary site for virus replication (Meiswinkel and Pawerska, 2003; *Ferhandey et al., 2007*).

2.6. Clinical signs:

There are four different forms of African horse sickness. Symptomatic infections occur most often in horses and mules. The pulmonary and mixed forms usually predominate in susceptible populations of horses. The pulmonary form of African horse sickness, also called “dun kop”, is characterized by an incubation period of 3 - 5 days. Acute fever of 40 - 42°C (104 - 107°F) for 1 - 2 days is followed by the sudden onset of severs respiratory distress. Dyspnea usually progresses rapidly, and the animal often dies within a few hours after the respiratory signs appear. Recovery is rare and death which may reach up to 95% is due to anoxia (*Radostitis et al., 2007*; Kahn and Line, 2005). The cardiac form of AHS is a sub-acute disease with a longer incubation period (1 - 2 weeks) and a more protracted course than the acute respiratory form. The fever (39 - 41°C) last less than two week and is followed by swelling of the supra orbital fossa, which is pathognomonic. Swelling

usually extends to the eyelids, facial tissues, neck, thorax, brisket and shoulders. Death (50 - 70%) usually occurs within one week from cardiac failure. It is important to note that no edema of the lower legs is observed. If the animal recovers, the swellings gradually subside over the next 3 to 8 days (*Coetzer et al., 2004*; Sinklair, 2006). In the mixed form of African horse sickness, symptoms of both the pulmonary and cardiac forms are seen. In most cases, the cardiac form is subclinical and is followed by severe respiratory distress, occasionally; mild respiratory signs may be followed by edema and death from cardiac failure (*Radostitis et al., 2007*; *Coetzer et al., 2004*). The mixed pulmonary and cardiac form of African horse sickness is rarely diagnosed clinically, which is usually found in outbreaks, and is the most commonly diagnosed form at post-mortem (Kahn and Line, 2005). The fever form of AHS is a mild to subclinical infection; the incubation period varies from 4 to 14 days. The characteristic fever of 39-40°C usually lasts for 3 to 8 days; morning remissions and afternoon exacerbations are often seen, and may be the only clinical sign observed. Other symptoms are generally mild and may include mild anorexia or depression, edema of the supraorbital fossae, congested mucous membranes and an increased heart rate. Almost all animals affected with this form recover (*Coetzer et al., 2004*; Sinklair, 2006; *Binepal et al., 1992*).

2.7. EPIDEMIOLOGY

2.7.1. Distribution of AHS in sub-Saharan Africa

At present AHSV is endemic in tropical and sub-tropical areas of Africa south of the Sahara occupying a broad band stretching from Senegal in the west to Ethiopia and Somalia in the east, and extending as far south as northern South Africa (Howell 1963). The Sahara desert however, seems to provide an effective geographical barrier which has prevented the virus from establishing itself permanently in northern Africa or beyond. The virus may also be endemic in one

place outside Africa, in Yemen in the Arabian Peninsula, although it's long-term status in this area is so far uncertain (*Sailleau et al., 2000*).

2.7.2. AHSV outside sub-Saharan Africa

AHSV has been responsible for epizootic outbreaks outside of its endemic area in regions previously free of the disease, namely in the Middle East and Asia between 1959 and 1960, in the Maghreb and the Iberian peninsula between 1965–1966, on the Iberian peninsula between 1987 and 1990 (Mellor and Hamblin 2004). In Yemen in 1997 (OIE 2009), and in the Cape Verde Islands in 1999 (OIE 2009). However, in the period 1959 to 1961 AHSV-9 expanded out of Africa and spread in a broad swath across Saudi Arabia, Syria, Lebanon, Jordan, Iraq, Turkey, Cyprus, Iran, Afghanistan, Pakistan and India (Howell 1963; Mirchamsy and Hazrati 1973). Nevertheless, by the end of 1961 in the face of a massive vaccination campaign and the death of over 300 000 equidae the disease in Asia ended (*Awad et al., 1981*). It is now known that AHSV is able to overwinter in southern Spain and Portugal and in Morocco because an efficient vector species of *Culicoides* is present in these regions and because the climate is sufficiently mild for the adults of this vector to be active throughout the year (Caracappa *et al., 2003*).

2.7.3 AHSV in Sudan

Historically, the disease was reported for the first time in eastern Sudan, when the death of horses ascribed to African horse sickness in Kassala province, and spreading to Blue Nile province in the same year (Anon, 1903). However, only serotype nine was isolated (Eisa, 1974; *Hajer et al., 1980*) indicating that serotype 9 might be the prevalent serotype of AHSV in Sudan. There were some serological studies conducted to investigate the disease in horses and donkeys in Sudan (*Abu*

Elzein et al. 1989; Ihsan 2004; Elghazali and Ali, 2013; *Karamalla, et al., 2018*). Recently the RT-PCR procedure has been used as a means of a highly sensitive technique, less expensive and conducted in less time compared to the isolation of the virus (*Aradaib et al., 2006*; Aradaib, 2009).

2.7.4 Vectors, transmission and climate

The transmission of the virus is biologically exclusively by *Culicoides* (Diptera: Ceratopogonidae), of which *Culicoides imicola* represents a major role in the transmission of the disease. The females need an adequate dose of animal blood to be able to lay out eggs, causing the spread of the disease among the vulnerable horses (Borkent, 2005; *Meiswinkel et al., 2004*). Some reports explained that the virus may present in the adult phase of *Culicoides* throughout the year. This may lead to some outbreaks when the herd immunity declines and the population of eguidae becomes under the appropriate risk factors for the disease (*Venter et al., 2014*). Two Culicoides species have been implicated in the transmission of BTV and AHSV in Africa, namely C. imicola and C. bolitinos. Culicoides (Avaritia) imicola Kieffer is one of the most widely distributed members of Culicoides in the world, extending over the African continent into southern Europe and eastwards to south China (Veronesi et al., 2009). In Sudan, *Culicoides imicola* was previously collected, viruses of bluetongue disease, epizootic haemorrhagic disease and

palyam disease were isolated from them and some other species of *Culicoides* (Mohammed and Mellor, 1990).

Prevalence and risk factors

The spread of the disease is influenced by climatic conditions which favor the survival of vector insects**,** including warm, moist weather and high rainfall, as well as spread by wind dispersal. Virus replication within the insect vector requires a sustained minimum temperature. This explains why the disease does not persist in countries with colder climates (*Mellor et al., 1998*). A cross sectional study was conducted on equine in southern Ethiopia. The apparent sero prevalence was found to be 24.60% in donkey, 20.34% in horses and 20% in mules. The overall sero prevalence of AHS virus was found to be 21.45%. There was no significant variation between the horse, donkey and mules in the sero positivity ($p>0.05$). The sero prevalence was observed in different study area, confirming the existence of agro-ecology variation in the occurrence of AHS, thus higher sero prevalence of AHS was documented in midland (31.38%) followed by highland (15.06%). Significant variation was not observed in sero prevalence among age groups and sex of equine. All age groups as well as male and female of equine population were equally affected. Most equine owners did not know about Culucoides vector and mode of transmission (*Tesfaye et al., 2012*). A study was performed to determine the prevalence of type specific neutralizing antibodies to African horse sickness virus (AHSV) in equidae and some other animal species in Khartoum State, Sudan. Precipitating antibodies to AHSV were detected only in horses, donkeys, goats, cattle and Dorcas gazelle, in a rate of 78.9%, 76.7%, 20%, 15% and 11.1%, respectively (Elghazali and Ali 2013). In central high land of Ethiopia Ende et al (2013) found that the apparent prevalence of AHSV was found to be 46 % in horses, 61.1 % in mules and 36.4 % in donkeys. The overall apparent sero-

prevalence of AHSV in three species of equine was found to be 46.2 %. There were no significant variations (P> 0.05) among age groups and sexes for sero-prevalence of AHSV. A study of the prevalence of African horse sickness in horses was conducted, using records of two private equine practices in Zimbabwe. Results indicated a higher prevalence of the disease in the late rainy season (68.9%). Age of the horse was found to be a significant risk factor, with foals or yearlings appearing to be 1.80 times more likely to contract the disease compared with horses older than two years. The case fatality rate in foals or yearlings was also higher than in older age groups, but this difference was not significant. The vaccination status was an important risk factor, with vaccinated horses 0.12 times less likely to die by the disease compared with unvaccinated horses. Young, unvaccinated horses therefore seem to be the most susceptible to the disease and have greater chances of fatality (*Gordon et al., 2013*). A sero-epidemiological survey was done in selected areas of central Ethiopia and prevalence rate of 10.4, 29.7 and 10.37% from horses, donkey and mule respectively, with over all sero prevalence rate 23% was recorded by Kassa (2006). Studies conducted by Keith (2005) and Kassa (2006) in Ethiopia indicated that AHS exist in almost all age groups and all agro-ecological zones of the surveyed areas.

2.8. Diagnosis

2.8.1. Clinical diagnosis

African horse sickness should be suspected in animals with typical symptoms of the cardiac, pulmonary or mixed forms of the disease. The supra orbital swellings are particularly characteristic of the disease. The horse sickness form can be difficult to diagnose (*Ferhandy et al., 2007*; European Commission, 2002).

2.8.2 Laboratory diagnosis

2.8.2.1. Virus isolation

Samples suitable for virus isolation are heparinized blood samples which are taken in acute phase from sick animals, and organs such as spleen, lungs, lymph nodes and heart from dead animals (Blackurn and Swanepoel, 1988). Samples should be kept at 4°C during transportation and short-term storage prior to processing.

2.8.2.2. Tissue culture

Cultivation of AHS virus in sheets of Monkey Stable (MS), baby hamster kidney (BHK-21) and African green monkey kidney (Vero) cell lines are used for virus isolation (Hazrati and Ozawa, 1965). When both virulent and attenuated neuro tropic strains of AHSV inoculated in MS cell line, the virus showed cytopathic effect in 2-3 days. Following the virus passages, the incubation period become shorter, with only eight hours latent period before the virus production is demonstrable. Plaques are formed after 10 days post inoculation, (P.I) ranging in size from 1-3 mm in diameter (Ozawa and Hazrati, 1964). Usually the cell cultures showed CPE in 2-5 days after inoculation, including cells rounding and detaching from the surface. Three serial passages are completed on any simple specimen before the isolation attempt is considered negative (Carol House, 1992).

2.8.2.3. Newborn Mice:

AHS virus isolation can be attempted by intra cerebral inoculation into suckling mice (1-3 day –old-BALB/ C, CD-1 Swiss). After 2-3 days mice developed nervous signs if the specimen is positive. The brains of the mice are collected and homogenized as suspension to make two or three more passages by re inoculation intera cerebrally into newborn mice before the specimen is considered to be positive (Alexander, 1935).

2.8.2.4. Chicken embryos

The propagation of the neuro tropic and viscera tropic AHSV strains by yolk sac inoculation of fertile eggs is also possible. Embryo's mortality was low in the first passage and increased to 100% by the 6th passage (Goldsmith, 1967).

2.8.2.5. Histopathology

Histopathological study on the tongue, trachea, lungs, heart and liver collected at autopsy from naturally infected animals showed degenerative changes of epithelium cells, severe haemorrhages, oedematous changes, congestion and swelling (Gorhe *et al*., 1962). Horses infected by the AHSV, showed ultrastructural changes in endothelial cells of capillaries in the myocardium, lungs spleen and liver. Alterations were also detected in the endothelial cells of the vessels, including hypertrophy, degenerative changes, appearance of cytoplasmic projections and changes in permeability. Alteration in the intercellular junctions, loss of endothelium, sub endothelial deposition of cell debris and fibrin and vascular repair. In addition, oedema**,** haemorrhages and microthromboses were also seen (Gomez-Villamandos *et al*., 1999).

2.8.2.6. Electron Microscopy

Ozawa *et al*. (1966) described the electron microscopic (EM) technique for the diagnosis of AHSV. They used this technique to identify the virus particles present in infected materials when inoculated in monkey kidney cells. The E.M showed the cubic symmetry of the virions and many capsomeres could be discerned (Hajer *et al.*, 1980).

2.8.2.7. Reverse transcription Polymerase Chain Reaction (Rt. PCR):

RT-PCR is a highly sensitive technique that provides a rapid identification of AHS viral nucleic acid in blood and other tissues of infected animals. This technique has greatly improved the laboratory diagnosis of AHS by increasing the sensitivity of detection and shortening the time required for the diagnosis. The RT-PCR

procedure will detect virus-specific nucleic acid after the virus is no longer viable and capable of establishing a new infection in either insects or mammalian cells. Therefore, positive results do not necessarily indicate the presence of infectious virus (OIE 2019). Several agarose gel-based RT-PCR assays for the specific detection of AHSV RNA have been described targeted at viral segments 3, 7 or 8 (Aradaib, 2009; Bremer *et al*., 1998; Zientara *et al*., 1994). The most widely used method employs primers corresponding to the 5‟ end (nucleotides 1–21) and 3‟ end (nucleotides 1160–1179) of RNA segment 7 (coding for VP7) amplifying the complete viral segment (Zientara *et al.*, 1994). Real-time RT-PCR methods for the highly sensitive and specific detection of AHSV RNA have been developed based on the use of a pair of primers and a labeled probe from conserved sequences of viral segments 3, 5 or 7 (Agüero *et al*., 2008; Bachanek-Bankowska *et al.*, 2014; Guthrie *et al.* 2013). A duplex real-time RT-PCR has also been described that targets segments 7 and 8 of the genome (coding for NS1 and NS2 respectively) (Quan *et al*., 2010). Although both gel-based and real-time RT-PCR procedures can detect reference strains from the nine virus serotypes, real-time RT-PCR provides advantages over agarose gel-based RT-PCR methods, with its faster analysis time, higher sensitivity, and suitability for high-throughput automation. Nevertheless, gel-based RT-PCR methods, particularly those amplifying long RNA fragments can be very useful in the further genetic characterization of the virus by sequencing of the amplicons. In addition, it may be beneficial in laboratories without the capacity to perform real-time RT-PCR (Zientara *et al*., 1994).

2.8.3. Serological tests:

Indirect and competitive blocking ELISAs**,** using either soluble AHSV antigen**,** or a recombinant protein VP7 Maree & Paweska, 2005)**,** have proved to be good methods for the detection of anti-AHSV group-reactive antibodies, especially for large-scale investigations (Rubio *et al*., 1998). Both of these tests have been

recognized by the European Commission (2002). The competitive blocking ELISA can also be used for testing wildlife as species-specific anti-globulin is not required with this method. An immunoblotting test has also been adapted for anti-AHS antibody determination (Laviada *et al*., 1992), which is especially suitable for small numbers of sera. The complement fixation (CF) test has been widely used, but some sera are anti-complementary, particularly donkey and zebra sera (OIE 2017).

2.8.3.1. Serum Neutralization Test (SNT)

Ozawa *et al*. (1966) established the tube technique of serum neutralization test in the diagnosis of AHS virus using MS cell cultures as host cell system. The serum neutralization test was also carried out with Vero cell cultures in 96 well flat tissue culture microtitre plates**,** to detect antibodies in sera of equidae (Binepal *et al.,* 1992). The test can also be used for serotyping the virus in tissue culture (Hazrati & Ozawa, 1965, Blackburn and Swanepoel, 1988).

2.8.3.2. Blocking Enzyme-Linked Immunosorbent Assay

The competitive blocking ELISA technique detects specific antibodies against AHSV, present in any equine species. VP7 is the main antigenic protein within the molecular structure of AHSV**,** it is conserved across the nine AHSV serotypes. An MAb directed against VP7 is used in this test, allowing high sensitivity and specificity. Moreover, other species of equidae (e.g. donkeys, zebra, etc.) can be tested**,** thus preventing the problem of specificity experienced occasionally using the indirect ELISAs. VP7 recombinant antigen is non-infectious, which provides a high level of security (European Commission, 2002). The principle of this test is**:** blocking the specific reaction between the recombinant VP7 protein**,** which is absorbed on an ELISA plate and a conjugated MAb against VP7. AHSV antibodies present in a suspect serum sample will block this reaction. The decrease in the

amount of color is an evidence of the existence of AHSV antibodies in the serum sample (Paweska *et al.*, 2000).

2.8.3.3. Indirect Enzyme-Linked Immunosorbent Assay

The recombinant VP7 protein has been used as antigen[2] for AHSV antibody determination with a high degree of sensitivity and specificity (Laviada *et al.*, 1992; Maree & Paweska, 2005). Other advantages of this antigen are its stability and its lack of infectivity. The conjugate used in this method is a horseradish peroxidase anti-horse gamma-globulin reacting with horse, mules and donkeys. The method described by Maree & Paweska (2005) uses protein G as conjugate that also reacts with zebra serum. A group-specific sandwich ELISA (S-ELISA) was also developed recently to confirm the orbivirus identity (Crafford *et al*., 2003).

2.8.3.4. Haemagglutination test (HA)

Tokuhisa *et al*. (1982) proved that concentrated culture fluid of AHSV agglutinated erythrocytes from cattle, horse, sheep, goats, guinea pigs, rabbits and poultry at 4^{o}C, room temperature and 37^{o}C. Optimal titres obtained when the virus is diluted in normal saline of pH ranging between 6.0 and 7.5. The HAI test was also used as a specific test for diagnosis of AHS virus. (Tewari *et al.* (1972).

2.8.3.5. Passive Haemagglutination (PHA)

AHS virus partially purified soluble antigen, used in passive haemagglutination for detection of AHS antibodies gave a positive reaction with tanned sensitized horse RBCs (Soliman and Salama, 1979).

2.8.3.6. Fluorescent Antibody Technique (FAT)

Direct and indirect fluorescent antibody techniques were successfully applied to the identification of virus of AHS isolates in tissue culture and for the screening of sera for antibody detection. The test was proved to be group specific test (Davies and Lund, 1974).

2.8.3.7. Immuno blotting:

Viral proteins separated by electrophoresis and transferred to nitrocellulose paper had been used for the determination of anti-AHS virus antibodies. The test is especially suitable for small numbers of sera (Paweska *et al*., 2000). Immunoblotting procedure had been developed for the detection of (AHSV) and identified antibodies specific for viral proteins (VP) and non-structural proteins (NS) (Bougrine *et al*.,1998).

2.8.3.8. Agar Gel Precipitation Test (AGPT):

Hug and Ansari (1961) described the successful establishment and application of AGPT in the diagnosis of AHS viral infection. The AGPT was described as group specific test for AHS when the virus was isolated in suckling mice, (Hajer *et al*., 1980). Hazarati *et al*. (1968) stated that two precipitin lines were found when AHSV antigen and positive serum diffused towards each other through an agar medium. Immuno difusion test could be used as screening test as it detects all the nine types of the virus; its effectiveness depends on the quality and standardization of the reagents (Erasmus, 1989).

2.8.3.9. Complement fixation test (CFT)

The CF test has been used extensively in the past (MacIntosh 1956), but currently its use is decreasing and has been replaced in many laboratories by ELISA as a screening technique. This progressive replacement is because of the higher sensitivity and degree of standardization of ELISA as well as a significant number of sera with anti-complementary activity. Nevertheless**,** the CF test is a useful tool in endemic areas for the demonstration and titration of the group-specific IgM antibodies against AHSV notably following a recent infection or vaccination (OIE 2017).

2.8.4. Differential Diagnosis

The clinical signs of AHS particularly when not fully developed may be confused with other infections notably Equine Encephalosis and Equine Viral Arteritis (EVA). These diseases occur under the same epizootiological conditions as AHS. Horses suffering from equine encephalitis usually do not have characteristic lung oedema or subcutaneous oedema, the mortality rate is considerably lower than in AHS. Severe cases of EVA may readily be confused with AHS, the existence of ventral oedema in EVA of the lower limbs, and the much lower mortality rate should allow differentiation (Erasmus, 2003). In countries where piroplasmosis occurs, the early stage of this disease before blood parasites can be demonstrated and anemia develops may be confused with AHS, also trypanosomasis shares this sign with the disease (Paweska *et al*., 2000). The necropsy lesions of AHS can be confused with those of Purpura Heamorrhagica, in which the haemorrhages and edema seem to be more severe and widely spread than in AHS and usually involve the limbs and lower abdomen. The highly sporadic occurrence of purpura also differentiates between the two diseases. (Erasmus 2003). Haemorrhages and Sudden death in Anthrax can be confused with AHS (Paweska *et al*., 2000).

2.9. Prevention and control

There is no specific treatment for animals suffering from AHS apart from rest and good husbandry. Complicating and secondary infections should be treated appropriately during the recovery period. AHSV is non-contagious and can only be spread via the bites of infected vector species of *Culicoides*. Control may therefore be effected by:

1. Introducing animal movement restrictions to prevent infected animals initiating new foci of infection.

2. Slaughter of viraemic animals, in certain circumstances (e.g. for welfare reasons or very early in an epidemic) to prevent them acting as a source of virus for vector insects.
3. Husbandry modification.
4. Vector control.
5. Vaccination.

While the first two measures are largely self-explanatory, the part played by husbandry modification, vector control and vaccination in AHS control may require additional comment.

2.9.1. Husbandry modification

Habitat modification methods involve either the removal or alteration of habitats utilized by *Culicoides*, in order to reduce or eradicate larval populations. *Culicoides* larvae are generally semi-aquatic and have limited ability to survive periods of desiccation. Hence, targeting the development sites is more straightforward within relatively dry climates (Purse *et al.* 2015). More recent studies have manipulated these habitats via removal of lagoons suitable for *C. sonorensis* development and examined the impact on adult populations (Mayo *et al.* 2014). In addition to the uncertainty surrounding their impact on arbovirus transmission, the lack of data concerning habitat usage by juvenile *Culicoides* is also a major challenge for implementing habitat modification techniques, with only 13% and 17% of *Culicoides* species known as larvae or pupae, respectively (Borkent 2016).

2.9.2. Vector control

Vector control techniques are generally applied under two scenarios. Firstly,**:** safe and efficacious vaccines for circulating arboviruses are not available. In this scenario, movement restrictions and vector control become the only available methods to reduce transmission and spread. In epidemic regions, vaccines to exotic

strains and species of arbovirus may not be available as the new vaccines require time to be developed, to be licensed and deployed (Carpenter *et al.* 2009). Secondly: In this scenario, safe and efficacious vaccines are available, but are not economically viable to deploy. This scenario may result from trade issues regarding the use of vaccines, or more simply from farmers not being able to afford to purchase them. In subsistence, farming in particular the use of vector control plays a major role and is often based around traditional methods that are inexpensive to implement but entirely unquantified in effect (*Harrup et al.2016*). Methods currently available for vector control can be divided into 4 broad categories (1) mechanical, (2) chemical, (3) biological, and (4) genetic.

2. 9. 2. 1 Mechanical control methods:

Habitat modification methods involve either the removal or alteration of habitats utilized by *Culicoides*, in order to reduce or eradicate larval populations. *Culicoides* larvae are generally semi-aquatic and have limited ability to survive periods of desiccation. Hence, targeting the development sites is more straightforward within relatively dry climates (Purse *et al.* 2015). The majority of *Culicoides* are, however, habitat generalists utilising a diverse range of substrates this results in limitations in the feasibility of attempting large-scale modifications in terms of economics and environmental impact. Difficulties also arise in regions where multiple vector species are active, for this broadens the range of habitats that require treatment. More recent studies have manipulated these habitats via removal of lagoons suitable for *C. sonorensis* development and examined the impact on adult populations (Mayo *et al.* 2014).

2. 9. 2. 2. Chemical control methods:

Topical repellents are frequently used by horse owners. However, there is a little quantitative evidence of their efficiency in reducing *Culicoides* biting rates or the

occurrence of equine seasonal recurrent dermatitis, and there is no evidence of their impact on reducing the incidence of equine arbovirus transmission (Carpenter *et al.* 2008). The use of adulticides has two potential impacts on arbovirus transmission. It can reduce successful blood-feeding rates of *Culicoides* through contact irritation, and it can lower the survival rates of *Culicoides*, which have been exposed (*Harrup et al.2016*). There has been a little advance in the potential to use ivermectin or other avermectins for *Culicoides* vector control, either through toxicity to blood-feeding adults or via a residual effect on larvae developing in dung from treated livestock. Adult mortality and other sub-lethal effects including reduced ovarian development, decreased fecundity, and reduced larval survival of subsequent generations have previously been reported in *C. brevitarsis*, which had fed upon cattle treated with ivermectin (*Standfast et al. 1984*, *Standfast et al. 1985*).

2. 9. 2. 3. Biological control methods

Research on *Culicoides* in this subject area during the last 10 years has been confined to a handful of laboratory-studies showing the larvicidal effects of insect pathogenic fungi. In a series of laboratory and semi-field based experiments in the UK, insect-pathogenic fungi from 4 genera [*Metarhizium anisopliae* (Metchnikoff) Sorokin, *Beauveria bassiana* (Balsamo) Vuillemin, *Paecilomyces fumosoroseus* (Wize), and *Verticillium longisporum* (Starke) Karapapa, Bainbr and Heale] were found to kill colony-reared *C. nubeculosus* larvae (Ansari *et al.* 2010) and reduce adult survival (Ansari *et al.* 2011) in laboratory-studies. The use of endosymbionts to reduce pathogen transmission and adult longevity has gained increasing attention in recent years, and a strain of the endosymbiont bacterium *Wolbachia* is successfully being utilized in the field to reduce dengue virus transmission by *Aedes aegypti* (L. in Hasselquist, 1762) (Ritchie 2014). Prelimiary surveys of the

microbiome of Culicodies have identified *Cardinium* and *Wolbachia* (Lewis *et al.* 2014, Mee *et al.* 2015, Morag *et al.* 2012, Nakamura *et al.* 2009) together with a range of other microbiota (Campbell *et al.* 2004) inhabiting *Culicoides*. It is unclear whether *Wolbachia* or other endosymbiont infection(s) in *Culicoides* have any influence on viral blocking, fecundity, parthenogenesis, sex ratios and/or, mate discrimination, traits which have been observed in other *Wolbachia*-infected arthropods, and are the basis of *Wolbachia*'s utility in vector control (Hoffmann *et al.* 2015, Werren *et al.* 2008).

2. 9. 2. 4. Genetic control

These new genetic and genomic resources have not been used yet to either optimise currently available vector control strategies via, for example, screening for genetic markers of insecticide resistance in *Culicoides* or for the development of a genetic modification-based vector control system. Vector control strategies, which are based on genetic modification, can be utilised to either suppress the abundance of the target vector population or promote a refractory phenotype within the target vector population (Alphey 2014). Recent proof-of-principal studies have demonstrated the potential to induce RNA interference (RNAi) in *C. sonorensis* both *in vivo*, using larval cell lines (Schnettler *et al.* 2013), and *in vitro* (Mills *et al.* 2015). The availability of genomic resources or the techniques to implement for example gene-drive systems are, however, not the greatest limitation to the application of genetic control strategies to *Culicoides*. Instead, they are limitations in the availability of basic biological resources for the vectors of interest, principally colonies of vector species of *Culicoides*. *C. sonorensis* is currently the only major vector of arboviruses in colony worldwide (Nayduch *et al.* 2014).

2.9.3. Vaccination

Polyvalent live attenuated vaccine (PLAV) that is used to control the disease in Africa was considered unsafe due to the probability of re-assortment between

AHSV serotypes. In addition, serotypes 5 and 9 are normally excluded from the (LAV) formulations as serotype 5 is difficult to attenuate and partially cross reacts with serotype 8 and serotype 9 partially cross-reacts with serotype 6 (*Wilson et al., 2009*; *Van de Water et al., 2015*; *Fall et al., 2015*; *Bachanek-Bankowska et al., 2014*; *Guthrie et al., 2015* and *Matsuo et al., 2010*). A recent approach applied is the recombinant technology that was used in the development of recombinant modified vaccinia ankara (MVA-VP2) vaccine which is highly protective against (AHSV), but the scale of it is limited to only two serotypes; (AHSV-4) and (AHSV-9) (*Lulla et al., 2016*; *Potgieter et al*., 2015; *Alberca et al., 2014*; *De la Poza et al., 2013*; *E Calvo-Pinilla et al., 2014* and *Crafford et al., 2014*). Presently, live attenuated vaccine for the disease is locally manufactured in the central laboratory in Sudan and contains four strains (types 1, 3, 6 and 9). Although there is no routine vaccination of horses officially, in 2016 about 124880 doses of the vaccine were produced and the total number of vaccinated horses was 4301 heads (OIE, 2016).

CHAPTER THREE

MATERIALS and METHODS

3.1. Study design:

Two studies were conducted. The first study was cross-sectional observation in a multistage sampling technique. Four regions; Northern, River Nile, Khartoum and Southern Darfur were randomly selected from the hole country. Then from each region, four localities were selected. Finally, animals were investigated by visiting markets, farms and villages *(Martin et al., 1987*).

The second study was carried out during the autumn in September 2018 and completed in November 2019. The sampling sites were selected according to the history of the disease outbreaks by monitoring the epidemiological situation of the disease and following up the monthly reports of the government veterinary clinics in the areas where the disease reoccurred. The study involved a cross-sectional observation in simple random sampling technique (*Martin et al., 1987*). Samples were collected from private stables during the autumn of the year 2018 in Khartoum North and Khartoum governorates in Khartoum state. In South Darfur state, the samples were collected at the end of the autumn of the year 2019 from livestock markets in Nyala, Edd al Fursan and Rehed al Birdi. The samples were collected from horses offered for sale from neighboring villages. All horses were in good health condition and didn't show symptoms of the disease. All data of the owner of the animals and the source of these animals were recorded, in addition to animal-related information such as sex, age and vaccination against the disease.

3.2. Study animals:

Sudan's equines are important in pastoralism and agriculture as riding, work and transport animals and in urban areas for transport. In 2010 Sudan had 7.5 million donkeys and 0.8 million horses. All donkeys and most horses are local types. The horse population in Sudan was estimated about

784 thousand heads; most of them are in South Darfur state (MARF, 2009). All the original Sudanese horses are small, lightweight with exception of very few sports horses; they are always working animals. They are used for rides in rural areas, but in urban areas, mainly used for local transportation attached to waggons or carts (Wilson, 2007). Live attenuated vaccine for the disease is locally manufactured in the central laboratory in Sudan and contains four strains (types 1, 3, 6 and 9). Although there is no routine vaccination of horses officially, in 2016 about 124880 doses of the vaccine were produced and the total number of vaccinated horses was 4301 heads (OIE, 2016).

3.2. Collection of blood samples:

A total of 920 blood samples were collected from equines (590 horses and 330 donkeys) in a quiet, calm environment. All samples were collected from the jugular vein, which is large and easily visualized in most horses. The blood was taken aseptically by jugular vein puncture from each animal into sterile vaccutainers without anti coagulants, allowed to colt for 1-2 hours at room temperature, stored horizontally overnight at 4^{o} C and then centrifuged at 4000 rpm for 5 minutes. The sera were taken in sterile bijou bottles and inactivated in water bath at 56^{o} C for 30 minutes, allowed to cool and then stored at -20^{o} C until assayed in the laboratory (Barrelet and Ricketts 2002). Non-coagulated whole blood samples were also collected from 16 suspected horses by jugular vein puncture from each horse into sterile ethylene-diamine-tetra-acetic acid (EDTA) vacutainer tubes, stored at 4^{o}C then stored at -20^{o}C until assayed in the laboratory (Barrelet and Ricketts 2002).

3.1 Cullicoides Collection:

Culicoides (Diptera: Ceratopogonidae) were collected during daylight hours by using a locally made Aerial net handheld (Michael, 2001), in the

morning half hour before sunrise and continuing for an hour after sunrise as well in the evening last hour before sunset and continue collecting after sun set until dark. The horses were inspected for 5 min each and visualized insects that resembled Culicoides midges were manually aspirated. The horses were consistently evaluated from cranial to caudal with emphasis on the dorsal regions of the neck, back and rump, which have been described as preferred landing sites of C. imicola (Braverman, 1988). After collection the net had been kept tiding until morning and the aspirated insects were immediately immobilized by placing the tube in a portable cooler box. In addition, *Culicoides* were collected using a light trap (Homemade as explained by *Venter et al., 2009*) close to the horse stables. The insects were collected into a container with water to which 0.5% Savlon Antiseptic (Chlorohexidine Gluconate Solution 0.3% w/v, Benzyl Benzoate, D-Gluconolactone, Sodium Hydroxide, Cetrimide 3.0%w/v) (Govindpuri, Delhi India) was added (*Venter et al., 2009*). The biting midges were sorted from the collections based on the wings, which have contrasting dark and milky white spots, using the taxonomic key of Glukhova (1989) under an SZX 16 stereoscopic microscope (Olympus) and counted. Collected *Culicoides* were preserved in 70% ethanol until tested in the laboratory.

3.4. Laboratory procedure

3.4.1. ELISA protocol:

Competitive Enzyme-Linked Immuno sorbent Assay (c-ELISA) was used to detect the presence of specific antibodies against the AHS virus in the collected sera samples following manufactures protocol.

Preparation of reagents:

- Washing solution: one part of the concentrate washing solution which provided in the kit was diluted in 24 parts of distilled or deionized water (40 ml of concentrate solution and 960 ml of water) and remained stable at +4°C.
- Controls (+) and (-): were ready to use and didn't need any preparation. 100 µl of each were Dispensed.
- Conjugate and substrate was ready to use and didn't need any preparation.

1. All reagents were brought to room temperature before use.

2. 100 µl of positive control was dispensed into two wells and 100 µl of negative control into two wells. Sera samples were diluted 1/5 in provided diluents. This step was made directly in the wells of the plate by adding 80 µl of the diluents and 20 µl of serum samples and the plate was shacked carefully for homogenization. The plate was covered and incubated for 1 hour at 37 °C.

3. Washing steps:

- The content of the plate was Thrower out by a brusque turnover of the plate to avoid the possible mixture of the content from one well to another.
- A volume of 300 µl of washing solution was dispensed on each well. The plate was shacked delicately, avoiding the contamination between wells. The plate was brusquely turned over to empty the wells. The process was repeated five times as indicated on the kit instructions. After the last step of washing the plate was shacked and turned over on absorbent filter paper.

4. 100 µ / well of conjugate added and incubated for 30 minutes at 37 °C.

5. The plate was washed 5 times as described before in step (3).

6. 100 µl/well of substrate solution was dispensed using a multi-channel pipette. The plate was incubated for 10 minutes at room temperature.

7. 100 µl well of stop solution was dispensed.

8. The result was read at 405 nm using spectrophotometer.

9. Validation criteria:

The ELISA test validation was checked for each plate based on two criteria set by the manufacturer for the mean optical density (OD) of the positive and negative control. The OD of the positive control must be less than 0.2 and the OD of the negative control must be higher than 1.0.

10. Interpretation:

Blocking percentage (BP) of each sample was calculated based on OD value applying the following formula:

$$BP = \frac{OD\ (\text{-control}) - OD\ (\text{sample})}{OD\ (\text{-control}) - OD\ (\text{+control})} \times 100$$

- Samples showing BP value lower than 45% are considered to be negative for antibodies of AHS virus. Samples showing BP value higher than 50% are considered as positive for antibodies of AHS virus. Samples with BP value between 45% and 50% are considered doubtful and they must be retested. If the result is the same, another extraction must be made and tested 2 weeks later.

3.4.2. Viral nucleic acid extraction from whole blood:

The innuPREP Virus RNA extraction kit (analytic jena, Jena, Germany) was used to extract viral nucleic acids. Before starting there was preparing of Washing Solution HS, Washing Solution LS. RNAs were extracted as per manufacturer's instructions. Briefly, 150 µl of blood were added to 450 µl of Lysis Solution RL/Carrier Mix and 20 µl Proteinase K into a 1.5 ml micro-centrifuge tube, mixed vigorously by pulsed vortexing for 10 seconds and incubated at room temperature for 10 minutes. 600 µl of Binding Solution VL were added to the lysed sample and mixed by pulse-vortexing for getting a homogeneous solution. 650 µl of the sample was added to Spin filter which was located in a 2.0 ml Receiver Tube and centrifuged at 10000 $\times g$ for 1 min. The Spin Filter was placed into a new 2.0 ml

Receiver Tube for loading the residual sample onto Spin Filter and centrifuged at 10000 ×*g* for 1 min. 500 µl of Washing Solution HS was added and centrifuged at 10000 ×*g* for 1 min. 650 µl of Washing Solution LS was added in new Receiver Tube and centrifuged at 10000 ×*g* for 1 min. The Receiver Tube with the filtrate were discarded, the Spin Filter was placed into a new 2.0 ml Receiver Tube and centrifuged at maximum speed for 3 minutes to remove all traces of ethanol. The Receiver Tube with the filtrate were discarded, the Spin Filter was placed into a new 1.5 ml Elution Tube, 100 µl of RNase- free water was added and incubated at room temperature for 2 minutes and centrifuged at 8000 ×*g* for 1 min. Two elution steps with equal volumes of RNase-free water (e.g. 30 µl + 30 µl) were conducted to increase the yield of extracted viral RNA. The final concentration of RNA was stored at -80 °C.

3.4.3. Viral nucleic acid extraction from Culicoides:

Before starting there was preparing of Washing Solutions the HS and LS. *Culicoides* were removed from ethanol and pools were homogenized in TRIzol® LS reagent (Invitrogen) using 3 mm stainless steel beads for 2 min in a TissueLyser (Qiagen). 10% (w/v) suspension (PBS) were prepared and added into 1.5 ml reaction tube, and centrifuged at maximum speed for 2 minutes. 150 µl supernatant was transferred to 1.5 ml reaction tube. The innuPREP Virus RNA extraction kit (analytic jena, Jena, Germany) according to manufacturer's instructions, was used to extract viral nucleic acids. RNAs were extracted from *Culicoides* according to the manufacturer's instructions and the final concentration of RNA was stored at -80 °C.

3.4.4. Primer selection and RT-PCR:

The reaction was performed using set of primer which were as follows: (Forward: 5′- GCGCCACCAATTGGAGATGT-3′) and (Reverse: 5′-

TCCCTCTCCTCCTCTGTGT-3′) (*Hussien et al., 2019*). The reaction was performed in a final volume 20 µl using one step RT-PCR kits (Analytik jena, Germany) containing 10 µl 2x RT-PCR buffer, 1 µl Reverse transcriptase (RT) enzyme, 2 µl primers mix in a concentration of a 5 pmol, 5 µl RNA template and 2 µl nuclease – free water. Amplifications were carried out at 45°C for 15 min and 95^{o}C for 10 min for RT enzyme initial activation followed by 40 cycles (94°C for 30 sec, 57°C for 30 sec, and 72°C for 45 sec) and final extension 72°C for 10 min. PCR product was subjected to electrophoresis on a 1.5% agarose gel at 120 V for 30 min and then visualized under UV light after staining with ethidium bromide. The expected size of band is 219 bp.

3.5. Data collection

A pre-tested structured questionnaire with the primary objective of elucidating the multi factorial background of African horse sickness disease was conducted in an interactive manner at every individual owner of horses and donkeys. The format was used to assess the predisposing factors: seasons, presence of equine biting insects and result after vaccinations at study areas and the knowledge base of equine owners about AHS.

3.6. Statistical analysis:

All data collected were entered into Microsoft excel spreadsheet. For analysis of the data, SPSS version 21 software was used. Data were analyzed descriptively in the first step, using the frequency table and cross tabulation. Then the association of the potential risk factors with AHSV at the individual level was analyzed using the Chi-square test. The level of significance was set at $P<0.05$. For the investigation of the association between the prevalence in response to individual animal characteristics, a multivariate analysis was performed in which logistic regression was used. The strength of association between the risk factors and the

prevalence of AHSV was quantified using the odds ratio (OR) and the level of significance was set at $p \leq 0.05$.

CHAPTER FOUR

RESULTS

4.1. Sero-prevalence and associated risk factors:

In a total of 920 equines (590 horses and 330 donkeys) were sampled from four states (Northern, River Nile, Khartoum and Southern Darfur) and examined. The overall sero-prevalence of AHS was 72.2 %., within states the highest sero-prevalence of AHS was reported in Khartoum and Southern Darfur states (81%) and (78.8%), respectively. Table (1) shows the univariate analysis of the association of potential risk factors with the sero-prevalence of AHS. The table shows the risk factors which have been investigated, number of animals tested, number of animals positive with their percent (%), the value of Chi-square (χ^2) and the P value. The sero-prevalence of AHS showed statistically significant association with state ($\chi^2 = 47.434$, $p<0.001$), species ($\chi^2 = 50.163$, $p <0.001$), sex ($\chi^2 = 26.206$, $p<0.001$), housing ($\chi^2 = 26.477$, $p<0.001$), vaccination ($\chi^2 = 44.466$, $p<0.001$), breed ($\chi^2 = 57.256$, $p<0.001$), the presence of water bodies ($\chi^2 = 26.271$, $p<0.001$), presence of cullicoides ($\chi^2 = 42.658$, $p<0.001$), presence of ticks ($\chi^2 = 23.608$, $p<0.001$), activity of animals ($\chi^2 = 41.435$, $p<0.001$), awareness of owners ($\chi^2 = 25.639$, $p<0.001$), age ($\chi^2 = 20.186$, $p<0.001$), health score($\chi^2 = 12.038$, $p<0.001$),pregnancy status($\chi^2 = 3.249$, $p = 0.0355$), and infection with other diseases($\chi^2 = 14.637$, $p<0.001$). However, risk factors of presence of other animals and pervious infection with AHS did not show statistically significant associations ($p>0.05$). In table 2 the final multivariate model revealed that equines in Khartoum state were almost five times more likely to be sero-positive compared with equines in Northern state (OR = 4.909, p = 0.017). Local equines were two times and half more likely to be sero-positive compared with cross equines (OR = 2.532, p = 0.004).

Table 1: Univariate analysis of potential risk factors which having association with Abs of AHS in Equids in some regions in Sudan.

variables	No. Tested	+ve (%)	χ^2	p- value
State:			47.434	0.001
Northern	263	150 (57%)		
River Nile	92	63 (68.5%)		
Khartoum	253	209 (81%)		
Southern Darfur	312	242 (78.8%)		
Species:			50.163	0.001
Horses	590	472 (80%)		
Donkeys	330	198 (58.2%)		
Sex:			26.206	0.001
Male	587	455 (77.9%)		
Female	333	209 (62.2%)		
Vaccination:			44.466	0.001
Yes	197	105 (53.3%)		
No	723	559 (77.3%)		
Health Score:			12.038	0.001
Good	776	523 (69.7%)		
Bad	164	141 (82.9%)		
Housing:			26.477	0.001
Barn	170	99 (58.6%)		
Backyard	622	482 (77.2%)		
Farm	128	83 (65.4%)		
Age:			20.186	0.001
1 – 3 years	319	285 (80.3%)		
4 – 8 years	547	333 (67.8%)		
8 – 12 years	54	34 (60.7%)		
>12 years	18	12 (66.7%)		
Breed:			57.256	0.001
Local	836	633(75.7%)		
Cross	84	31 (36.9%)		
Pregnancy:			3.249	0.035
Yes	28	16 (57.1%)		
No	892	648 (72.6%)		

Table 1: continued

variables	No. Tested	+ve (%)	χ^2	p- value
Presence of water bodies:			26.271	0.001
Yes	293	369 (79.7%)		
No	627	295 (64.6%)		
Presence of cullicoides:			42.658	0.001
Yes	383	451 (79.8%)		
No	537	213 (60%)		
Presence of ticks:			23.608	0.001
Yes	189	167 (86.1%)		
No	731	497 (68.5%)		
Infection with other diseases:			14.637	0.001
Yes	77	74 (90.2%)		
No	843	590 (70.4%)		
Presence of other animals:			0.944	0.165
Yes	8	7 (87.5%)		
No	912	657 (72%)		
Pervious infection with AHS:			0.095	0.379
Yes	16	11 (68.8%)		
No	904	653(72.2%)		
Activity of animals:			41.435	0.001
Racing	131	63 (49.2%)		
Ridding	352	286 (78.6%)		
Working	437	315 (73.6%)		
Awareness of owners:			25.639	0.001
Yes	521	408 (78.8%)		
No	399	256 (63.7%)		

Regarding species, horses were 3.8 times more likely to be sero-positive compared with donkeys (OR = 3.776, p = 0.017). Equines raised in areas with water bodies were two times more likely to be sero-positive compared with those in dry areas

(OR = 2.172, p = 0.033). Non vaccinated equines were 17 times more likely to be sero-positive compared with vaccinated ones (OR =17.298, p<0.001).

Table 2: Multivariate analysis of potential risk factors which having association with Abs of AHS in Equids in some states of Sudan.

variable	No. tested	+ve (%)	OR	Cl 95%	P-value
states:					
Northern	263	150 (57%)	Ref.		
River Nile	92	63 (68.5%)	1.637	0.986 – 2.719	0.057
Khartoum	253	209 (81%)	4.909	1.329 – 18.133	0.017
Darfur	312	242 (78.8%)	1.349	0.359 – 5.070	0.658
Breed:					
Local	836	633(75.7%)	2.532	1.339 – 4.790	0.004
Cross	84	31 (36.9%)	Ref.		
Species:					
Horses	590	472 (80%)	3.776	1.264 – 11.281	0.017
Donkeys	330	198 (58.2%)	Ref.		
Presence of water bodies:					
Yes	463	369 (79.7%)	2.172	1.063 – 4.437	0.033
No	457	295 (64.6%)	Ref.		
Vaccination:					
Yes	197	105 (53.3%)	Ref.		
No	723	559 (77.3%)	17.298	8.673 – 34.501	0.001

4.2. **Results of RT PCR:**

The analytical sensitivity of the RT PCR used in this study was a ten-fold dilutions made from AHSV vaccine strain (3.7 $TACID_{50}$/ml) commonly used in Sudan, and used as templates for the PCR reaction. The detection limit of the PCR was $10^{\wedge 4}$ (figure 1). AHSV RNA was successfully detected in whole blood samples and *Culicoides* pools in the study areas. A total of 184 horses (16 crossbred vaccinated

horses and 168 not vaccinated local breed horses) from two governorates in Khartoum State and three localities in Southern Darfur State were randomly sampled. In addition, 1916 insects in 18 pools, the pool range 70 - 150 non engorged females of different species of *Culicoides* biting midges were tested for the presence of AHSV RNA using one-step reverse transcriptase polymerase chain reaction (RT-PCR) technique. The overall prevalence of AHSV RNA in horses was 38.6% and from the 18 pools of *Cullicoides* the virus was detected in 6 pools (33.3%). The highest prevalence of the AHSV RNA in horses was reported in Edd al Fursan (86.4%), while Khartoum North governorate in Khartoum State showed the lowest prevalence (14.8%) (Figure 2).

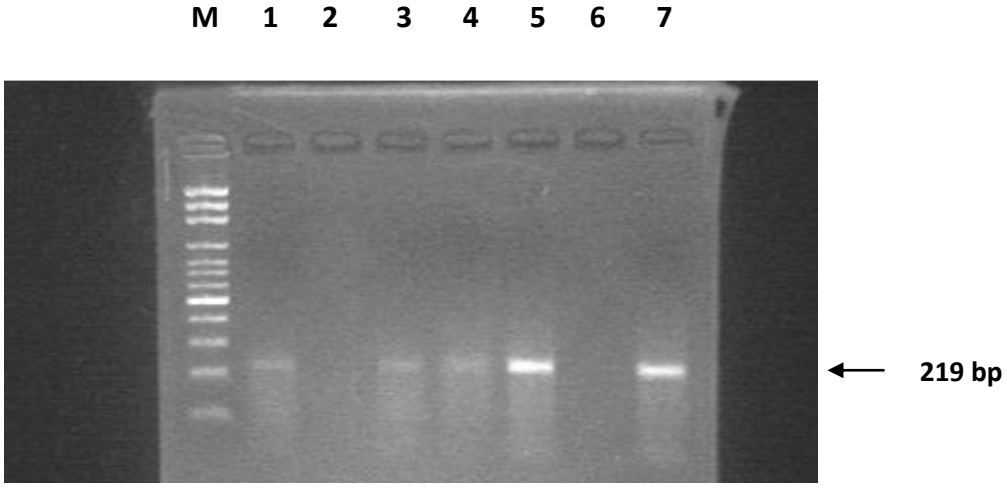

Figure 1: Agarose gel electrophoresis of the products amplified with RT-PCR using specific primers for AHSV. M; 100 bp DNA ladder, Lane 1,3,4,5; positive samples, Lane 2; negative sample, Lane 6; negative control, Lane 7; positive control.

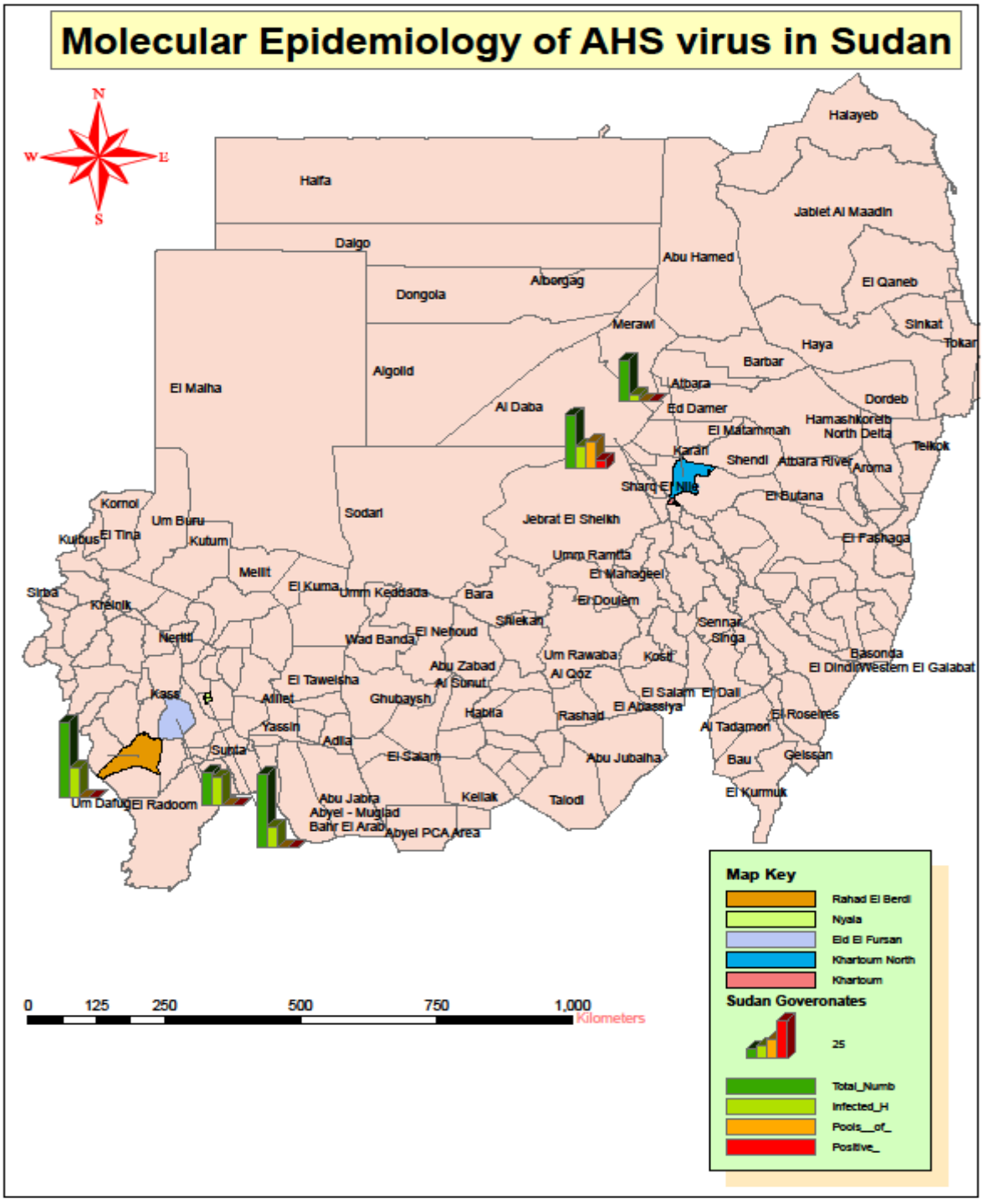

Figure 2: The map of Sudan showing the prevalence of African horse sickness virus in different governorates and localities of Sudan and the detection of the virus in Culicoides biting midges in Khartoum govern.

The prevalence of the virus was high in female horses (51.7%) and within age groups; the prevalence was high in old horses (47.7%). Regarding breed of horses the prevalence was high in crossbred horses (50 %) (Table 3). There was a coincidence between the presence of the virus in insects and the high prevalence of the disease (82.3%) in horses was observed only in Khartoum governorate. Where there was 14/17 (82.3%) of horses were infected in the stable, which close to the location where AHSV RNA was detected in *Culicoides.*

Associated risk factors of AHSV:

Table 3 shows the univariate analysis of the association of potential risk factors with the prevalence of AHSV. The prevalence of AHSV showed statistically significant association with locality ($\chi^2 = 29.86$, $p<0.00$), age ($\chi^2 = 5.62$, $p = 0.01$) and sex ($\chi^2 = 6.42$, $p = 0.01$). However, the breed of horses did not show statistically significant associations ($p>0.05$). As shown in Table 4 the final multivariate model which used to examine the variation in variables simultaneously. The strength of association between the risk factors and the prevalence of AHSV was quantified using the odds ratio (OR) and the level of significance was set at $p \leq 0.05$. Out of the three risk factors that showed significant statistical association ($p \leq 0.05$) with the prevalence of AHSV in the univariate analysis were entered in the multivariate analysis. Only one risk factor (locality) has a significant statistical association with the disease. Horses in Edd al Fursan was almost 28 times more likely to be infected compared with horses in Khartoum North governorate (OR = 28.27, p = 0.00). Also, horses in Khartoum governorate were almost 3 times more likely to be infected compared with horses in Khartoum North governorate (OR = 3.42, p = 0.06).

Table 3: Univariate analysis of the association of potential risk factors for AHSV in horses in some localities in Sudan.

Variables	No. Tested	+ve (%)	χ^2	P- value
Locality:			29.86	0.00
Khartoum North	27	4 (14.8%)		
Khartoum	36	15 (21.7%)		
Nyala	49	14 (28.6%)		
Rehed al Birdi	50	19 (38.0%)		
Edd al Fursan	22	19 (86.4%)		
Total	184	71 (38.6%)		
Breed:			0.96	0.33
Local	168	63 (37.5%)		
Gross bred	16	8 (50.0 %)		
Sex:			6.42	0.01
Male	124	40 (32.3%)		
Female	60	31 (51.7%)		
Age:			5.62	0.01
Young (1-4 years)	98	30 (30.6%)		
Old (above 4 years)	86	41 (47.7%)		

Table 4: Multivariate analysis of potential risk factors associated with the AHSV in horses in some localities in Sudan.

Risk Factors	No. tested	+ve (%)	OR	95% Cl for OR	P-value
Locality:					
Khartoum	27	4 (14.8%)	Ref.		
North Khartoum	36	15 (21.7%)	3.42	0.96 – 12.21	0.06
Nyala	49	14 (28.6%)	1.73	0.48 – 6.23	0.39
Rehed al Birdi	50	19 (38.0%)	2.70	0.77 – 9.40	0.11
Edd al Fursan	22	19 (86.4%)	28.27	5.49 – 145.56	0.00
Sex:					
Male	124	40 (32.3%)	Ref.		
Female	60	31 (51.7%)	1.88	0.92 – 3.85	0.07

CHAPTER FIVE
DISCUSSION

5.1. DISCUSSION

AHS is an arthropod-borne disease primarily affecting equids and is transmitted by *Cullicoides* (Diptera: Ceratopogonidae) (*Meiswinkel et al., 2004* and *Mellor et al., 2000*). This disease has a high mortality rate in susceptible horses and there is a risk for expansion beyond endemic ranges (OIE, 2019). The first study was cross sectional planned to determine the sero-prevalence of the disease by using ELISA test of (920) serum samples and the second study was a rapid, sensitive and specific RT-PCR assay for the detection of AHSV in horses and Culicoides spp.

The cross sectional study indicated an overall sero-prevalence of AHS in equines at the study states of 72.2 %. This result is higher than those reported in Khartoum and South Darfur states (Elghazali and Ali 2013; Ihsan 2004), different regions of Ethiopia (*Tesfaye et al., 2012*; *Ende et al., 2013*; Kassa 2006) and Zimbabwe (*Gordon et al. 2013*). In contrast, the finding of the sero-prevalence of AHS in donkeys was lower than the previous of *Abu Elzein et al. (1989)* in Khartoum state. The difference in sero-prevalence of AHS in the present study and other previous studies could be probably due to differences in season of sampling, geographic location, study methods and diagnostic techniques employed by the investigators.

Out of 15 risk factors that showed significant statistical association ($p \leq 0.05$) with sero-prevalence of AHS in the univariate analysis and entered in the multivariate analysis, only five risk factors (state, species, breed, water bodies and vaccination) showed significant statistical associations ($p \leq 0.05$) with sero-prevalence of AHS. The results indicated higher sero-prevalence in Khartoum state and South Darfur state which characterized by high rainfall, the presence of water bodies and good vegetation with overabundance of midges, while River Nile state and Northern state were located in semi-desert and desert climate, respectively. The variations in sero-prevalence of AHS at different states in our study were significant ($p \leq 0.05$), and this result is in close agreement with the results reported by *Ende et al. (2013)* and *Tesfaye et al. (2012)* in different regions in Ethiopia.

Furthermore, there was a significant variation of sero-prevalence between the two species of equines (horses and donkeys). A higher sero-prevalence was observed in horses as compared with donkeys, and the difference was statistically significant ($p \leq 0.05$). This finding is in agreement with a couple of previous studies (Alemayehu and Benti, 2009; Yeshitila and Bekele, 2017). Also, by the OIE (2019) reports which stated that among equines horses were the most susceptible to AHS with a mortality rate of 50-95% followed by mules with a mortality rate around 50% and donkeys with a mortality rate of 5-10%.

The cross sectional study further revealed that there is a statistically significant variation ($p \leq 0.05$) of sero-prevalence between the local and cross breed. A higher sero-prevalence was observed in the local compared with the cross breed. However, this significant difference could be attributed to the fact that the cross horses receive high care by raising them in safe stables that protect them from the infestation of vectors and vaccinated annually against the disease.

The findings indicated a statistically significant variation between areas with water bodies and dry areas ($p \leq 0.05$). A higher sero-prevalence of AHS was observed in equines raised in areas with water bodies, and this result is in close agreement with the results reported by Coetzer and Guthrie (2004); Demissie (2013) and *Radostitis et al. (2007)* who explained that AHS endemic areas are more likely to be low lying, flooded by rain water, warm and marsh regions that create favorable environment for multiplication of Culicoides and mechanical vectors.

Furthermore, the final multivariate model revealed a statistically significant ($p \leq 0.05$) variation between non-vaccinated and vaccinated equines. A higher sero-prevalence of AHS was observed in non vaccinated equines. Lack of vaccination is the most strong risk factor (OR =17.298, P <0.001) among the risk factors investigated and found statistically significantly associated with AHS. However, the observed association could be confounded with poor management and lack of

knowledge of equines owners about the disease and the importance of vaccination as a protective tool against AHS. Unfortunately, both risk factors were not investigated.

Regarding sex, the findings showed significant variation ($p \leq 0$ .05) in the sero-prevalence between sexes in the univariate analysis, with a higher sero-prevalence in males than in females. This result is in close agreement with the result reported in Ethiopia by Yeshitila and Bekele (2017). However, the risk factor of sex didn't remains statistically significant in the final multivariate model. In this study, the odds ratio was used to quantify the strength of association between potential risk factors and the sero-prevalence of AHS. However, although the discussed risk factors were statistically significantly associated with AHS in the final multivariate model, it is difficult to consider them as necessarily causally related and should be interpreted in light of the causal criteria that have been proposed by Thrusfield (2005).

In the univariate analysis the awareness of owners showed significant statistical associations ($p \leq 0.05$) with sero-prevalence of AHS but, didn't remain significant in the multivariate analysis. The horse owners in South Darfur and Khartoum states were fully aware of the disease compared to those owners in Nile River and Northern states. However, their experience was not sufficient to provide adequate protection for their animals when the disease appeared, through commitment to

stopping movement, using insect repellents and mosquito nets, and vaccinating them annually with the appropriate vaccine.

There have been some molecular studies in Khartoum state in Sudan developed to detect the virus in cell culture (*Aradaib et al. 2006*; Aradaib, 2009). However, the molecular study is conducted to estimate the prevalence of the virus and investigate the contribution of major risk factors for the occurrence of the disease in horses in several governorates in Sudan, where the disease has appeared recently. The molecular study indicated an overall prevalence of AHSV in horses as 38.6% and from the 18 pools of *Cullicoides* the virus was detected in 6 pools (33.3%). Although all sampled horses were apparently healthy, AHSV RNA was detected (38.6%). This may be attributed to that AHSV circulates only for a relative short period before the antibody response begins. In horses viraemia usually lasts for only four to eight days and has not been detected after 21 days (Coetzer and Erasmus, 1994).

Despite the early appearance of the disease in eastern Sudan and its transmission to the Blue Nile Governorate, it has recently become endemic in most areas in the states of Khartoum and South Darfur. The prevalence of the virus was high in the localities of Edd al Fursan, Rehed al Birdi and Nyala in Southern Darfur state 86.4%, 38% and 28.6% respectively. In this state, most horses belonged to nomads and weren’t available for veterinary treatment during the

critical rainy season. The disease in this state undoubtedly continued to be a problem in native horses. Most owners of affected horses and the authorities were surely aware of this as evidenced by the vaccine offered (national production consist 4 serotypes 1, 3, 6 and 9). The higher prevalence of the disease in this state may refer to all local horses, which sampled in this study, weren't vaccinated two years ago. There were statistical significant differences of the prevalence between the various sites in Khartoum and South Darfur states ($p \leq 0.05$) and the same result was reported in the cross sectional study.

The molecular study further revealed in the univariate analysis (Table 3) that there is a statistically significant variation ($p \leq 0.05$) of the prevalence of the virus between the local and crossbred horses, a higher prevalence observed in crossbred compared with the local breed. Although the crossbred horses receive high, care by raising them in vector protected stables and vaccinated annually against the disease the prevalence was high. This is probably due to the existence of a problem in the vaccination process or the inefficiency of the vaccine to provide protection against strains not included in it.

Regarding sex, the findings showed significant variation ($p \leq 0$ .05) in the prevalence of the virus, with a higher prevalence of the virus in females than in males. The high susceptibility of females to the disease was probably due to the availability of several predisposing factors decreasing the immunity during

pregnancy and lactation because of some physiological changes (*Merlot et al., 2013*).

The presence of AHSV in *Culicuides* was found to be higher than those reported in different regions of South Africa (*Scheffer et al., 2012*; *Venter et al., 2014*). This difference in the present study and other previous studies could probably be due to differences in season of sampling, geographic location, study methods and instruments employed by the investigators. One of the observations in this study is the biting midges were caught by the Aerial net handheld trap were visible, clear and easy to examine, compared to the ones that were caught in light trap. This observation was in close agreement with results reported by *Scheffer et al.* (2012).

Culicoides abundance was relative high during September to mid-October, and after that the abundance started to decrease during late October and early November. Trapping was attempted again in January during the winter, but the abundance was very low or not available. These observations were described in previous studies conducted in Sudan and South Africa (Mohammed and Mellor, 1990; *Venter et al., 2014*).

The coincidence between the presence of the virus in *Culicoides* and the higher prevalence of the disease in horses in Khartoum governorate confirmed the virus circulation in this area. Research is needed to find out which was the first to appear in the infected area, the *Culicoides* as predisposing factor or the introduction of an

infected animal (*Martin et al., 1987*). Recently, some studies were designed based on the diagnosis of the virus in *Culicoides*, with the aim of identifying endemic areas. Moreover, this procedure helps towards applying rapid action to limit the transmission of the disease to free areas through the adult *Culicoides* control program and destruction of habitat for insect reproduction (*Grewar et al., 2013*; *Venter et al., 2014*; *De Waal et al., 2016*).

5.2. Conclusion:

The present study indicated that AHS Abs and Ags were highly prevalent in equines and Culicoides spp. in the study states of Sudan. Furthermore, the sero-prevalence of AHS was statistically significantly associated with the vaccination status of equines, geographic location of state, species, breed, and presence of water bodies. Also, the prevalence of AHSV was statistically significantly associated with the sex and age of horses. These identified risk factors should be carefully considered when control strategies for the disease will be implemented. The high abundance of the Culicoides spp. recognized as potential AHSV vectors suggests possible risk of the emergence of this still recognized as endemic disease in the country. Most horse owners and horse breeders were well aware of the disease however, they were less awareness of the importance of vaccination and the good practice of appropriate insect control methods to prevent disease. There is

a need to identify the different species of *Culicoides* to know which species are most closely related to disease transmission in equine in Sudan.

5.3. **Recommendations:**

The results showed that AHS is widely disturbed among equine population. Hence vaccine production to the more prevalent types should be produced. Further more regular surveillance for AHS should be conducted and more studies are recommended to cover all the parts of the Sudan to provide data about the disease. Movement restrictions of animal should be enforced to prevent infected animals initiating new foci of infection. Habitat modification methods involve either the removal or alteration of habitats utilized by *Culicoides*, in order to reduce or eradicate larval populations.

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