Orbivirus *detection from* Culicoides *collected on African horse sickness outbreaks in Namibia*

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Veterinaria Italiana 2015, **51** (1), 17-23. doi: 10.12834/VetIt.275.1016.2
Accepted: 14.09.2014 | Available on line: 31.03.2015

Keywords
African horse sickness, Bluetongue, *Culicoides imicola*, Namibia.

Summary
African horse sickness (AHS), a non-contagious infectious disease caused by a RNA virus in the *Orbivirus* genus within the *Reoviridae* family affecting all equids, is endemic in sub-Saharan Africa. The virus is transmitted by some species of biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae). In April 2011, 8 *Culicoides* collections were performed in 6 districts of 4 regions of the Republic of Namibia (Africa), all within a 400 km radious from the capital Windhoek. Six farms - Khomas (Windhoek and Steinhausen), Erongo (Karibib and Omaruru), Otjozondjupa (Okahandja), and Omaheke (Gobabis) involved in the AHS outbreaks, were sampled. Overall 194,211 *Culicoides* were collected and identified. *Culicoides imicola* was largely the most abundant species at all farms (99.4%). A total of 18,687 parous and gravid *Culicoides* females were assayed for AHS virus (AHSV) by real time RT-PCR. Of the 248 assayed pools, 227 consisted of *C. imicola*, 13 of *Culicoides pycnostictus* and 5 of Schultzei complex. Only 1 pool each of *Culicoides nivosus*, *Culicoides leucostictus*, and *Culicoides tropicalis* was assayed. Of the 248 pools examined by real time RT-PCR, 81 tested positive for AHSV, all consisting of *C. imicola* collected at Omaruru, resulting in a field vector infection rate of 0.91%. No viable AHSV could be isolated from 88 of the tested pools (n = 1,463). However, bluetongue virus (BTV) serotype-1 and 10 were isolated from 3 of these pools, each consisting of 100 *C. imicola* collected at Windhoek. The present study confirms the relative low infection prevalence in field collected *Culicoides* and the strict relationship between the high abundance of *C. imicola* and outbreaks of AHSV.

Indagini virologiche in Culicoides *catturati in focolai di Peste equina in Namibia*

Parole chiave
Bluetongue, *Culicoides imicola*, Namibia, Peste equina (PE).

Riassunto
La Peste equina (PE) è una malattia infettiva non contagiosa degli Equidi. È causata da *Orbivirus* e trasmessa da vettori del genere *Culicoides*. Nel 2011 è stata svolta un'indagine entomologica in sei allevamenti sede di focolaio di PE, situati in quattro regioni della Namibia, nel raggio di circa 400 km dalla capitale Windhoek: Khomas (Windhoek e Steinhausen), Erongo (Karibib e Omaruru), Otjozondjupa (Okahandja) e Omaheke (Gobabis). Sono stati catturati e identificati 194.211 *Culicoides*. In tutti gli allevamenti, la specie *Culicoides imicola* è risultata quella più abbondante (99,4%). In totale 18.687 *Culicoides*, divisi in 248 pool, sono stati analizzati per PE tramite real time RT-PCR: 227 pool composti da *C. imicola*, 13 da *C. pycnostictus*, 5 da Schultzei complex, 1 da *C. nivosus*, 1 da *C. leucostictus* e 1 da *C. tropicalis*. Tra questi, 81 pool sono risultati positivi (tasso d'infezione 0,91%), tutti composti da *C. imicola* e tutti provenienti dal sito di Omaruru. L'isolamento virale è stato tentato su 88 pool (n=1.463) con esito negativo. Tuttavia sono stati isolati due sierotipi del virus della Bluetongue (BTV 1 e BTV 10) da tre pool, composti ognuno da 100 esemplari di *C. imicola* provenienti dal sito di Windhoek.

Introduction

African horse sickness (AHS) is a non-contagious infectious vector borne viral disease affecting all equids. It is caused by AHS virus (AHSV), a RNA virus in the *Orbivirus* genus within the *Reoviridae* family, transmitted by certain species of blood feeding *Culicoides* midges (Diptera: Ceratopogonidae). In addition to AHSV, *Culicoides* midges are able to transmit veterinary important viral diseases such as Bluetongue, Epizootic haemorrhagic disease, Equine encephalosis, Bovine ephemeral fever, Schmallenberg (Meiswinkel *et al.* 2004, EFSA 2014).

African horse sickness has already been isolated in Africa in several *Culicoides* species, among which *Culicoides bolitinos* in South Africa, *Culicoides imicola* in South Africa and Zimbabwe, *Culicoides* spp. (not identified at species level) in Kenya and South Africa, and mixed pools of *Culicoides nivosus*/*Culicoides leucostictus*/*C. bolitinos* in South Africa (Blackburn *et al.* 1985, Davies *et al.* 1979, Meiswinkel and Paweska 2003, Nevill *et al.* 1992a, Scheffer *et al.* 2012, Venter *et al.* 2006).

In Spain AHSV has been isolated from *C. imicola* and from mixed pools of *Culicoides obsoletus*/*Culicoides pulicaris* (Mellor *et al.* 1990).

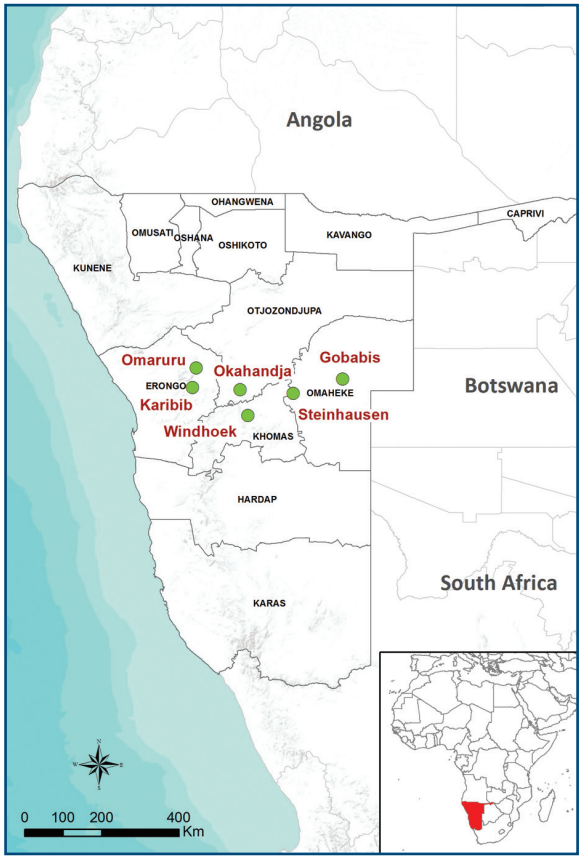

Figure 1. *Location of* Culicoides *collection sites in the Windhoek, Steinhausen, Karibib, Omaruru, Okahandja and Gobabis districts (Namibia 2011).*

African horse sickness is endemic in sub-Saharan Africa (Howell 1963, Coetzer *et al.* 2004), however epizootic events have occurred outside this area, such as in Asia, North Africa, Spain, and Portugal (MacLachlan and Guthrie 2010). A limited serological study in the Windhoek district (Namibia, Africa), revealed the presence of antibodies against AHSV in 50% of the tested donkeys (Venter *et al.* 1999). In addition, the 1987 AHS outbreak in, Spain, was ascribed to zebras imported from Namibia (Lubroth 1988, Hamblin *et al.* 1991).

In the AHS outbreaks investigated between 2006 and 2011, 7 (1, 2, 4, 6, 7, 8 and 9) of the 9 AHSV serotypes were detected in the affected horses (Scacchia *et al.* 2009, Scacchia personal communication, 2011).

Even though the first 2 described species of sub-Saharan *Culicoides* (*Culicoides shultzei* and *Culicoides herero*) originated from Namibia (Enderlein 1908), there is a relatively scanty literature surveying the presence of *Culicoides* in this region (Becker *et al.* 2012, Becker *et al.* 2013), thus leaving the *Culicoides* species composition at farm level still greatly unknown. In April 2011, an entomological survey was performed on 6 farms in the AHSV infected area surrounding Windhoek (Namibia), with the goal of defining the species composition of the *Culicoides* population and to detect AHSV in the insects. It is noteworthy that outbreaks of AHS were occurring in the sampled farms during the survey.

Materials and methods

The study included 6 farms located in the regions of Khomas (Windhoek and Steinhausen), Erongo (Karibib and Omaruru), Otjozondjupa (Okahandja) and Omaheke (Gobabis) (Namibia, Africa). All the farms are located within a radius of about 400 km around Windhoek (Figure 1). In 4 of the surveyed farms, along with horses, other domestic and/or wild animal species were also present (Table I). Apart from Gobabis, the horses of the other farms were regularly vaccinated for AHS with a live attenuated polyvalent vaccine. Despite this, AHS cases had been detected in 6 farms, during the sample period (Molini personal communication, 2014).

Onderstepoort-type blacklight traps, particularly attractive for *Culicoides*, were used (Wieser-Schimpf *et al.* 1990, Goffredo and Meiswinkel 2004, Venter and Hermanides 2006). In each farm, the trap was positioned close to the horses. The field activities and the analysis of the collected samples were performed as described by Goffredo and Meiswinkel (Goffredo and Meiswinkel 2004). Eight light trap collections were conducted in April 2011 (Table I). The collected insects were stored refrigerated in phosphate-buffered saline (PBS) as medium, before being pooled for virus isolation. The remaining

Table I. *Location and description of the* Culicoides *collection sites in the Windhoek, Steinhausen, Karibib, Omaruru, Okahandja and Gobabis districts (Namibia 2011).*

Region	District	Geographical coordinates	Mammal species (number)	Type of farming	Collection
Khomas	Windhoek	S 22,575816° E 17,126126°	Horses (40)	Manège and riding school	11 April 2011 20 April 2011
Khomas	Steinhausen	S 22.06° E 18.19°	Horses (154)	Breeding horse farm	29 April 2011
Erongo	Karibib	S 21,94479722° E 15,84054444°	Horses (39)	Commercial animal farm and manège	27 April 2011
			Cattle (130)		
			Sheep (120)		
			Goats (80)		
			Antelopes		
			Warthogs		
Erongo	Omaruru	S 21,46821111° E 15,92056111°	Horses (16)	Commercial animal farm	27 April 2011
			Zebras		
			Antelopes		
			Warthogs		
			Baboons		
Otjozondjupa	Okahandja	S 21,967883° E 16,94561°	Horses (32)	Hunting farm	13 April 2011
			Donkeys(1)		
			Cattle (90)		
			Zebras		
			Antelopes		
			Warthogs		
Omaheke	Gobabis	S 21,72331667° E 19,34855°	Horses (25)	Commercial animal farm and wild animal reserve	7 April 2011 29 April 2011
			Cattle (250)		
			Sheep (124)		
			Goats (32)		
			Zebras (2)		
			Antelopes		
			Warthogs		
			Hyenas		
			Lions		
			Leopards		

insects were then put in 70% ethanol and stored at room temperature.

The species were identified using the wing morphology according to the atlas of African species (Meiswinkel 1996) (Afrotropical *Culicoides*, unpublished data). Based on abdominal pigmentation (Dyce 1969), *Culicoides* classified as parous or gravid females were sorted out and divided in pools (max 100 individuals).

From midges collected and stored in PBS, virus isolation was attempted on African green monkey kidney (VERO) cell lines, according to the method described in the Manual of diagnostic tests and vaccines for terrestrial animals (OIE 2012). Virus neutralization test using type specific antisera was used to identify and determine the serotype (OIE 2012).

The pools in ethanol were tested for AHSV by real time RT-PCR, samples with Ct-values less than 40 were considered positive (Aguero *et al.* 2008). The infection rate in vector population was calculated using a maximum likelihood estimation method, which takes into account the size of each tested pool (Biggerstaff 2009).

Results

During the 8 light trap collections conducted on the 6 Namibian farms, 194,211 *Culicoides* were collected. *Culicoides imicola* was largely the most abundant species, representing 99.39% of total midge population, followed by *Culicoides pycnostictus* (0.35%), Schultzei complex (*C. schultzei* and *Culicoides subschultzei*) (0.09%), *Culicoides tropicalis* (0.06%), *C. leucostictus* (0.06%), *C. nivosus* (0.02%) and other species, such as *Culicoides ravus* and *Culicoides macintoshi* (0.03%). The largest collection was the one conducted at the Omaruru

Table II. *Abundance of* Culicoides *species collected at 6 farms in the Windhoek, Steinhausen, Karibib, Omaruru, Okahandja and Gobabis districts (Namibia 2011).*

Species	Collection site						
	Windhoek (%)	Steinhausen (%)	Karabib (%)	Omaruru (%)	Okahandja (%)	Gobabis (%)	Total (%)
C. imicola	13,439(98.54)	7,788 (99.99)	604 (98.53)	167,793 (99.87)	1,571 (96.9)	1839 (72.40)	193,034 (99.39)
C. pycnostictus	19 (0.16)	1 (0.01)	1 (0.16)	0	35 (2.2)	621 (24.45)	677 (0.35)
Schultzei complex	11(0.08)	0	8 (1.31)	145 (0.09)	5 (0.3)	2 (0.08)	171 (0.09)
C. tropicalis	48(0.37)	0	0	72 (0.04)	0	0	120 (0.06)
C. leucostictus	108(0.75)	0	0	0	0	1 (0.04)	109 (0.06)
C. nivosus	1(0.01)	0	0	0	1 (0.1)	44 (1.731)	46 (0.02)
Other species	12(0.09)	0	0	0	9 (0.6)	33 (1.30)	54 (0.03)
Total *Culicoides*	13,638	7,789	613	168,010	1,621	2,540	194,211

Table III. Culicoides *tested for AHSV by RT-PCR in Namibia in 2011.*

Collection site	Species	Number of midges	Number of positive/tested pools
Windhoek	*C. imicola*	3,537	0/36
	Schultzei complex	5	0/1
	C. pycnostictus	4	0/1
	C. leucostictus	7	0/1
Omaruru	*C. imicola*	12,449	81*/152
	Schultzei complex	3	0/2
	C. tropicalis	1	0/1
Okahandja	*C. imicola*	873	0/9
	Schultzei complex	5	0/1
	C. pycnostictus	5	0/1
Gobabis	*C. imicola*	1,178	0/30
	C. pycnostictus	580	0/11
	C. nivosus	39	0/1
	Schultzei complex	1	0/1
Total		18,687	81/248

* 46 pools of 100 and 35 pools of 50 midges.

Table IV. Culicoides *tested for AHSV by virus isolation in Namibia in 2011.*

Collection site	Species	Number of midges	Number of pools	Virus isolation
Windhoek	*C. imicola*	511	22	BTV1 (1 pool) BTV10 (2 pools)
Gobabis	*C. imicola*	209	5	-
	C. pycnostictus	13	1	-
Karabib	*C. imicola*	291	8	-
	Schultzei complex	8	1	-
Stheinhausen	*C. imicola*	247	9	-
Omaruru	*C. imicola*	184	42	-
Total		1,463	88	-

site, where in the span of 1 night 168,010 *Culicoides* midges (99.87% *C. imicola*) were collected (Table II).

Culicoides imicola was found on all the 6 collection sites, with a relative abundance ranging from 72.40% (Gobabis) to 99.99% (Steinhausen). The second most common species, *C. pycnostictus*, was collected at 5 sites. Its relative abundance ranged from 24.5%, at Gobabis, to 2.2%, at Okahandjia, to <0.3% at further 3 sites, Windhoek, Steinhausen and Karibib (Table II).

A total of 18,687 parous/gravid females were divided in 248 pools and tested by RT-PCR. Two hundred and twenty-seven of the considered specimens consisted of *C. imicola* (n = 18,037), 13 of *C. pycnostictus* (n = 589), 5 of Schultzei complex (n = 14) and only 1 each of *C. nivosus* (n = 39), *C. leucostictus* (n = 7) and *C. tropicalis* (n = 1). Eighty-one of the 248 pools tested by RT-PCR were positive for AHSV. The positive samples consisted of *C. imicola* pools collected at Omaruru. Of these pools, 46 were composed of 100 *C. imicola* and 35 pools by 50 *C. imicola* (Table III). The Ct-values ranged from 29 to 39 (mean 35.6).

No AHSV could be isolated from the 88 assayed pools (1,463 midges). However, in 3 pools, each consisting of 100 *C. imicola* collected at the Windhoek farm, bluetongue virus (BTV) serotype-1 and -10 were isolated (Table IV). Virus neutralization test using type specific antisera was used to identify and determine the serotype.

Within *C. imicola* population, the AHSV infection rate at Omaruru was 0.91%. At Windhoek farm, where BTV was also isolated, the infection rate was 0.15%. In particular, the infection rate was 0.05% for BTV-1 and 0.1% for BTV-10.

Discussion

Despite the sampling being run only once or twice on the farms, this survey allowed to collect a huge

number of *Culicoides* and provided a clear picture of the *Culicoides* species composition in the livestock populating the study area.

This study clearly showed that *C. imicola* is the most widespread and abundant species in Namibia or at least in the selected and investigated areas. It accounted for more than 99% of the collected *Culicoides* midges. The trap sites, in close proximity to animal nocturnal shelters, and the collection period, the Summer, when *C. imicola* becomes abundant and the peak AHS season occurs, might have affected these results.

However, a study performed from July to September 2009 (Winter season) in the Khomas area, reported a relative abundance of *C. imicola* of 93.9% in the Windhoek region (Becker *et al.* 2012), which is in agreement with the abundance of *C. imicola* collected in the same region in the present study. These authors attributed this high value to a combined effect of breeding sites availability and host density. *Culicoides imicola* is known to be closely associated with livestock (large mammals) and to breed at farm level, in man-made larval sites, partially muddy areas near watering or irrigation points, whereas the species does not breed in sandy soil where water drains quickly (Braverman *et al.* 1974, Meiswinkel *et al.* 2004). In the South-Western Khomas Region, anthropogenic impacted/ homestead sites have been shown more favorable for *C. imicola* than veld sites (Becker *et al.* 2013). In this study, the most abundant collection, with nearly 170 thousands *Culicoides*, mostly *C. imicola*, was carried out at Omaruru, where only 16 horses were hosted. It is, however, worth noticing that at this site, wild animals, such as zebras and various species of antelopes, used to approach the farm at dusk in search for water. Apart from enhancing the availability of vertebrate for blood meals, the presence of zebras might be epidemiologically relevant as these animals act as reservoirs for AHSV (Binepal *et al.* 1992).

The AHSV infection rate in *C. imicola* (0.91%) was comparable with that recently reported in South Africa (0.98%) (Scheffer *et al.* 2012). The low infection rate in vector populations is typical of arbovirus infections (Venter *et al.* 2006), this rate could be even lower if determined by virus isolation whose sensitivity is much lower than RT-PCR. The field AHSV prevalence in vectors, obtained in other studies using virus isolation during outbreaks of AHSV, does not exceed 0.005% (Venter *et al.* 2006).

The second most abundant species, *C. pycnostictus*, was found at 5 of the 6 sites. Compared to *C. imicola* abundance, the presence of *C. pycnostictus* was relatively low, with the exception of the Gobabis district, where it represented 24.45% of the collected midges. *Culicoides pycnostictus* feeds mainly on birds and, occasionally, on mammals and BTV was isolated from field-collected specimens in South Africa (Nevill *et al.* 1992a, Nevill *et al.* 1992b). None of the 589 *C. pycnostictus* was positive for AHSV.

Similarly, AHSV was not detected in pools of *C. schultzei* and *C. subschultzei*, which belong to the Schultzei complex. In Africa, this complex includes also *Culicoides nevilli*, *Culicoides enderleini* and *Culicoides kingi*. Species of this complex are considered potential vectors of arboviruses. In Kenya, species of the Schultzei group are considered potential vectors of BTV and Ephemeral Fever virus (BEF) (Davies and Walker 1974, Walker and Boreham 1976, Davies *et al.* 1979). In Sudan, Epizootic Haemorrhagic Disease virus (EHDV) was isolated from *C. kingi* (Mellor *et al.* 1984). More recently, BTV (in India), and other arboviruses (in Japan) have been isolated from *Culicoides oxystoma*, an Asian species belonging to the Schultzei complex (Dadawala *et al.* 2012, Yanase *et al.* 2005).

Other species not abundant in the study area, and from which AHSV was not detected, include *C. nivosus, C. leucostictus*, and *C. tropicalis*. These species are mainly birdfeeders, although they also feed on livestock (Nevill *et al.* 1992b). In South Africa, a mixed pool of *Culicoides* species, including *C. bolitinos*, *C. nivosus*, and *C. leucostictus* was found positive for AHSV (Scheffer *et al.* 2012). This finding can probably be ascribed to the presence of *C. bolitinos* in the pool, however the possibility that *C. nivosus* and *C. leucostictus* might be involved in the transmission of AHSV cannot be discarded.

In previous surveys in the Khomas region, *C. bolitinos*, a species morphological closely related to *C. imicola* belonging to the Imicola complex, was reported in Namibia (Becker *et al.* 2013). It breeds in cattle manure and plays a potential role as AHS vector in South Africa (Meiswinkel and Paweska 1998, Meiswinkel and Paweska 2003, Meiswinkel *et al.* 2004). In the present survey, even though cattle were present at some of the sites, *C. bolitinos* was not collected in any of the AHSV affected farms and it probably does not play a significant role as a vector of AHSV in the surveyed areas.

Clinical cases of AHS occurred in all selected farms despite vaccination, involving in 2 regions (Otjozondjupa and Khomas) the majority (90%) of horses. Live attenuated polyvalent vaccine was the product used and animals were vaccinated between August and November. Therefore, it seems unlikely that the AHSV RNA fragments found in *C. imicola* pools collected at Omaruru in April derived from the vaccine administration.

Interestingly, BTV was isolated at Windhoek during an outbreak of AHSV. This might suggest that the same vector population is able to sustain

contemporaneously the circulation of at least 2 arboviruses. Clinical cases of BT were not reported in the area, where all the sheep of commercial farms are annually vaccinated with a polyvalent vaccine containing live attenuated bluetongue virus strains (Molini, personal communication 2014). Forty horses were kept at the Windhoek farm, however numerous domestic and wild ruminants, considered as reservoir hosts for BTV, were also present in the surrounding area and this might have accounted for the presence of BTV in the *Culicoides* collected on this farm.

In conclusion, this study confirms the relative low infection prevalence in field collected *Culicoides* and the strict relationship between the high abundance of *C. imicola* and outbreaks of AHSV (Venter *et al.* 2006) in Namibia. According to these results, *C. imicola* is likely to play a crucial role in spreading AHSV and BTV in Namibia or at least in the investigated areas. In Khomas region this role could be played all year round, as indicated by this and previous studies (Becker *et al.* 2012).

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