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The Epidemiology of Bluetongue Virus in Mnisi, South Africa

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Abstract The role of cattle in the epidemiology of bluetongue (BT) as well as the distribution of different vector species throughout South Africa is not well understood. Mnisi, a rural area located in Mpumalanga, South Africa, was selected for an epidemiological study. The prevalence of *Culicoides* species associated with this area as well as whether bluetongue virus (BTV) is circulating in the area is unclear. Sera were collected from 1 260 cattle and screened with a BTV-specific cELISA. Light traps operated during autumn and winter periods. Midges were identified to species level, pooled (n = 200) and screened for BTV RNA with a real-time RT-qPCR. Blood samples from seronegative cattle were also screened for BTV RNA. Antibodies specific to BTV were detected in 95.7% of the sera with a statistical significant difference ($p < 0.05$) between age groups and villages. Twenty-five different *Culicoides* species were identified of which *C. imicola* was the most abundant. Bluetongue virus RNA was detected in 51.2% (autumn) and 75.9% (winter) of the midges collected. A total of 35.5% seronegative cattle tested positive for the presence of BTV RNA. These results demonstrate that BTV as well as different vectors are circulating in the Mnisi area.

Keywords: *bluetongue virus, Culicoides, cattle, cELISA, epidemiology, real-time RT-qPCR, segment 10*

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1. Introduction

During the last decade bluetongue virus (BTV) has become a major concern worldwide as well as the focus point of many epidemiological studies and surveillance programmes. Since 1998 the global geographical distribution of BTV has changed substantially after its northwards expansion into previously non endemic areas [24]. During the 2006-2008 outbreaks of BT in Europe the role of cattle was re-emphasised after thousands of cattle developed clinical disease [7,27]. This outbreak also demonstrated possible overwintering of BTV when it re-emerged after the 2006 winter as well as novel vectors responsible for spreading the virus [13].

Adult female haematophagous midges belonging to the genus *Culicoides* (Diptera: Ceratopogonidae) are the primary vector responsible for BTV transmission [19]. The spatial and temporal distribution of BTV is determined by the availability of susceptible ruminants, vector-competent midge species, genetic makeup of virus and suitable climatic conditions [20].

In South Africa, bluetongue (BT) is most commonly reported in late summer and autumn (February to May). The occurrence of multiple BTV serotypes circulating at the same time during vector seasons has been demonstrated although the occurrences of serotypes are unpredictable and probably determined by herd immunity amongst others [37]. Outbreaks are more common in areas with high rainfall, even though serosurveys provided evidence of BTV in all vegetational zones, with different rainfall parameters, throughout the country [1]. In cooler high lying areas such as the southern Free State Province, BT usually disappear with the first winter frost, while the disease is likely to occur year round in warmer regions such as KwaZulu-Natal and Limpopo Provinces.

The geographical distribution and relative abundance of *Culicoides* midges in South Africa varies between regions. In South Africa, *C. imicola* is the major vector of BTV followed by *C. bolitinos* [18,21,35]. Under laboratory conditions *Culicoides bolitinos* has been shown to be significantly more susceptible to oral infection with BTV than *C. imicola* [34]. Following feeding on a BTV infected blood meal virus has been isolated under laboratory conditions from *C. imicola, C. bolitinos, C. milnei, C. leucostictus, C. pycnostictus, C. expectator, C. gulbenkiani, C. zuluensis, C. magnus, C. enderleni, C. bedfordi and C. huambensis.* In addition, BTV has been isolated from six field collected *Culicoides* species namely, *C. imicola, C. bolitinos, C. milnei, C. leucostictus C. pycnostictus and C. expectator* [36].

The susceptibility of cattle to BTV was already demonstrated in 1905 [26] when calves were experimentally infected with BTV. Since then, BTV has

been isolated from single cases of cattle suffering from clinical signs similar to BT as well as from subclinically infected cattle throughout South Africa [2]. Studies on BTV in cattle are not well reported and the role of cattle in the epidemiology of the disease in South Africa is not clear. Viraemia in ruminants are highly cell-associated, especially to erythrocytes at late stage of infection. The prolonged viraemia in cattle (up to 60 days) could be due to the adsorption of BTV to blood cells and not because of prolonged replication of the virus in infected tissue [8,14,27]. Prolonged viraemia within the cattle may allow for an extended transmission of the virus to the vector. Subclinically infected cattle could potentially be important amplifying hosts of BTV and aid in the spread and maintenance of the virus.

This present study focused on the epidemiology of BTV in the north-eastern corner of the Bushbuckridge in a rural area, Mnisi in Mpumalanga, South Africa. In this area livestock are mainly cattle with a few goats and donkeys. This area also forms a wildlife-livestock interface surrounded by game reserves.

2. Material and Methods

2.1. Study Area and Sample Collection

Mnisi is a rural area located in the north-eastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province of South Africa (Figure 1). With its subtropical climate and large numbers of livestock, this area makes an ideal environment for the maintenance of arboviruses. The study area (Figure 1) comprises a savannah ecosystem and a semi-arid region with regards to water and grazing availability.

Serum samples and blood in EDTA were collected from cattle from April 2013 to September 2013 at 11 sites/ villages (Figure 1). Sampling months were chosen based on BTV outbreaks in South Africa *i.e.* late summer/ autumn versus winter when BTV usually disappears. Sampling of *Culicoides* midges were conducted once every four nights from 23 April to 3 May 2013 (autumn) and again from 17 June to 15 July 2013 (winter). Two 220 V Onderstepoort light traps equipped with 8 W black light tubes were used to collect midges into 500 ml plastic beakers according to Venter *et al.*, [33]. Traps were placed near 10 to 30 cattle, depending on collection site. Mosquito netting placed around the trap excluded large insects. The beakers were collected every morning after a trapping night. *Culicoides* midges were then separated from other insects. Large collections were sub-sampled [30] and identified to species level using morphological keys and a wing picture atlas of Afrotropical *Culicoides* (R. Meiswinkel 1994, unpublished data). Males and females were separated and females age-graded into freshly blood-fed, parous, nulliparous or gravid, based on abdominal pigmentation and morphological differences [9].

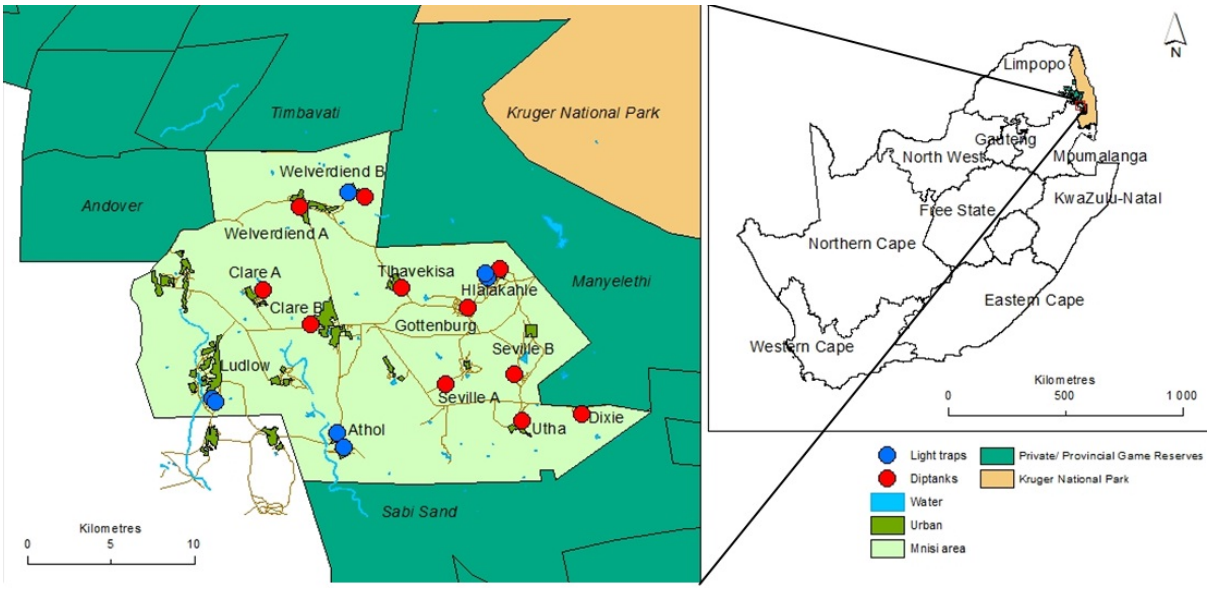

Figure 1. Map of South Africa including an enlarged map of Mnisi. Source: Drawn by J. Steyn on ArcGIS 10.2

2.2. Competitive Enzyme-linked Immunosorbent Assay

A total of 1 260 cattle serum samples were tested for BTV-specific antibodies using a cELISA (Veterinary Medical Research and Diagnostics, Bluetongue Virus Antibody Test Kit, Inc., Pullman, U.S.A). Serum samples included males and females of four different cattle breeds and different age groups.

2.3. Culicoides Processing

Midge catches were divided into pools of 200 individuals resulting in 82 and 79 pools in autumn and winter respectively. Pools consisted of freshly blood-fed, parous, nulliparous and gravid females. Midge pools were washed in 500 µl PBS (+) solution containing 1 ml Gentamycin (50 mg/ml) (Virbac) and 0.125 ml Fungizone (10 mg/ml) (Bristol-Myers Squibb) per 1000 ml stock solution (v/v) by centrifuging the midge suspension for 30 seconds at 1300 g. Midges were subsequently macerated in 1000 µl reconstituted Eagle's minimum essential medium (MEM) (Highveld Biological (Pty) LTD) (without serum) containing a sterile glass bead. Following maceration, insect debris was collected by centrifuging at 1 300 g for 1 minute and the supernatant aliquoted into two 0.5 ml microcentrifuge tubes. One aliquot was used to

conduct real-time RT-qPCR and one stored at -80°C as a backup. Midge suspensions were tested for BTV using real-time RT-qPCR [34]. Meteorological data for the four months (April, May, June and July) were obtained from the Southern African Wildlife College.

2.4. RNA Extraction and Real-time RT-qPCR

Total RNA was extracted from *Culicoides* samples collected in autumn with an Express Magnetic Particle Processor, the MagMAX™ Express (Ambion®) using the MagMAX™ Total Nucleic Acid Isolation Kit (Applied Biosystems part number AM1830) according to manufacturers instruction. Total RNA was extracted from winter midge pools and cattle blood samples using TRIzol® Reagent (Ambion®) according to manufacturer's instructions. Extraction methods differ due to limited access to the MagMAX™ Express (Ambion®).

In total, 82 autumn midge pools and 79 winter midge pools from the different collection sites were randomly selected and screened for BTV with a real-time RT-qPCR. A total of 45 cattle blood samples that demonstrated a serum percentage negativity of less than 50% were also screened. Real-time RT-qPCR was performed on a StepOnePlus™ PCR thermocycler (Applied Biosystems) using a published assay targeting segment 10 (coding protein NS3/A) of the BTV genome [31]. Double-stranded RNA was denatured by heating for 3 minutes at 95°C and snap cooled by placing it at -20°C for 5 minutes. Five µl RNA was added to the MicroAmp® Fast Optical 96-well Reaction Plate (Applied Biosystems) along with 5 µl of a 4x TaqMan® Fast Virus Master Mix reagent and 200 nM of the forward (5'- GTGTCGCTGCCATGCTATC-3') and reverse primer (5'- CGTACGATGCGAATGCA -3') respectively, and 100 nM of hydrolysis probe (5'-FAM-CGAACCTTTGGATCAGCCCGGA-MGB-3'). The samples were centrifuged briefly and the following cycling program was used: 50°C for 5 minutes, 95°C for 20 seconds and 40 cycles of 95°C for 2 seconds and 60°C for 30 seconds.

2.5. Data and Statistical Analyses

Species diversity and evenness were calculated by the Shannon Weiner index (http://lbsite.zxq.net/programs/diversity.html). To compromise for localised abundance, vector rating was calculated by averaging relative abundance and percentage of positive sites for all collections for a specific species [35]. A vector rating > 25% was used to indicate that a species was both abundant and wide spread in the area [35]. The percentages of parous individuals of specific species in the population were also determined by dividing the number of parous females of a specific species by the total number of individuals of that species collected. This was done separately for autumn and winter collections. Field infection prevalence in each sampling period was calculated using the Chiang and Reeves's [6] formula. The Wilcoxon rank sum test was used to compare the mean of midges collected between autumn and winter and a cut-off with a 5% significance level ($p<0.05$) was used. The same test was also used to determine any significant difference in the mean of parous females collected by sites between sampling periods.

The Shapiro- Wilk test and a histogram were used to determine the normality of the data where $p > 0.05$ indicated normal distribution. Non-parametric Kruskal-Wallis one-way analysis of variance test was used to calculate significant difference in antibody percentages between different breeds, sex, age groups and collection site (villages) respectively. Significant differences were confirmed or excluded using Cochran-Mantel-Haenszel (CMH) statistical test to exclude any influence of covariates.

3. Results

Table 1. Significance of different factors that might play a role in the presence of antibodies specific to BTV in samples collected from cattle in Mnisi. Non-parametric Kruskal-Wallis test was used to determine any statistical significant differences within groups where p < 0.05 indicates a significant difference

Factor	Group	No. of cattle tested	Number of positive animals	Number of negative animals	p-value
Breed	Brahman (cross)	694	659	35	0.4
	Brahman (typical)	43	42	1	
	Sanga (typical)	522	504	18	
	Other	1	0	1	
Sex	Male	339	324	15	0.8
	Female	921	882	39	
Age	<1	334	294	40	2.92E-15
	1-2	212	211	1	
	2-3	111	110	1	
	3-4	117	114	3	
	4-5	120	117	3	
	5-10	309	304	5	
	10-15	57	56	1	
Village	Clare A	121	120	1	1.15E-13
	Clare B	173	169	4	
	Dixie	20	20	0	
	Gottenburg	154	141	13	
	Hlalakahle	61	54	7	
	Seville A	126	120	6	
	Seville B	96	93	3	
	Tlhavekisa	85	78	7	
	Utha A	108	101	7	
	Welverdiend A	265	263	2	
	Welverdiend B	51	47	4	

3.1. BTV Seroprevalence in Cattle

A total of 1 260 serum samples were obtained from cattle at 11 villages (Figure 1). Animals were placed within an age group according to year of birth. Seven age groups were identified spanning from less than 1 year to more than 10 years (Table 1). Antibodies specific to BTV were detected in 1 206 (95.7 %) of the serum samples tested. Non-parametric tests were used to establish any statistical significant differences between seroprevalence and breed, sex, age and specific villages (Table 1). There was a significant difference between the seroprevalence of BTV antibodies in the different villages sampled (Table 1), as well as between ages of the animals ($p < 0.05$) (Table 1) where young animals demonstrated less seroconversion. No significant difference for BTV antibody seroprevalence in either breed ($p = 0.4$) (Table 1) or sex ($p = 0.8$) (Table 1) were found. There was a significant difference ($p < 0.05$) with regards to seroprevalence when age groups were separated into sex. The Cochran-Mantel-Haenszel test/ analysis confirmed that the significant difference found between age groups and sex with regards to antibody inhibition is caused by covariates as there are more young bulls than older bulls.

3.2. Weather Conditions

During *Culicoides* collection, the average outside temperatures varied between 17.4°C and 20.8°C with an average rainfall of 0.02 mm during the four months. In April the average outside temperature was 20.5°C with 0.05 mm rain. Throughout the sampling months, the relative humidity was above 50% but lower than 87% with an average wind speed of 2.9 km/h.

3.2. Species Analysis and Screening of *Culicoides* Midges

Species analysis of *Culicoides* midges collected in the Mnisi area revealed 25 different *Culicoides* species (including a number of taxonomically yet undescribed species) (Table 2). A total number of 91 659 *Culicoides* midges were collected in 16 light trap collections made at eight sites (Table 2, Figure 1) between 23 April to 3 May 2013 (autumn) and 17 June to 15 July 2013 (winter). In autumn, 73 321 *Culicoides* midges belonging to 23 different species were collected while in winter 18 338 midges were collected revealing 20 species (Table 2). *Culicoides dekeyseri* and *Culicoides glabripennis* were only collected in winter while *Culicoides engubandei, Culicoides kanagai, Culicoides milnei,* Culicoides. sp # 50 and Culicoides sp. # 54 p/f were absent. Species diversity as represented by Shannon-Weiner index for autumn and winter were H' = 0.5 and H' = 1.2 respectively. Species evenness were calculated to be J' = 0.1 and J' = 0.4 for autumn and winter respectively. The largest number of midges collected in a single trap in one night was 68 928 midges during autumn, whereas the second largest collection consisted of 12 024 individuals collected during winter. No statistical significant difference ($p = 0.96$) was found in the mean number of *Culicoides* midges collected in autumn and winter by site.

Culicoides imicola was the most abundant species present during both autumn (91%) and winter (75%) sampling. The second and third most abundant species collected during autumn was *Culicoides nevilli* (4.0%) and *Culicoides enderleini* (1.3%), respectively. During winter collection, *C. bolitinos* (4.4%) was the second most abundant with *C. nevilli* (3.4%) being the third most abundant. *Culicoides imicola* was also most abundant at all sites during both sampling periods except at one site in winter, were *C. bolitinos* replaced *C. imicola* as the most abundant species.

Of the 25 *Culicoides* species sampled, 13 species in autumn and 16 species in winter, demonstrated a vector rating higher than 25%. These species are considered as being both abundant and wide spread. *Culicoides imicola* demonstrated the highest vector rating i.e. 95.7% and 87.5% in autumn and winter, respectively. Other potentially important species that demonstrated a vector rating of more than 25% and that have demonstrated to be susceptible to BTV infection under either laboratory conditions or in the field are *C. bolitinos, C. bedfordi, C. exspectator, C. leucostictus, C. pycnostictus* and *C. subschultzei* [21,23,34].

Parous females were present throughout both sampling periods. A total of 19 out of the 25 species yielded parous females with 18 of these 19 species collected in autumn and 16 species in winter. The percentage of parous females in the population differed between autumn and winter i.e. 3.4% and 1.8% respectively. Culicoides. sp. # 50 d/f had the highest number of parous females in winter (29%) and the second highest during autumn (49%), while *Culicoides brucei* yielded the most parous females in autumn (50%). The mean number of parous females collected in autumn ($n = 101$) was significantly higher ($p > 0.05$) than that collected in winter ($n = 13$). Parous females are an indication of blood feeding behaviour in the population and could theoretically give an indication of the rate of virus transmission.

3.3. Real-time RT-qPCR Analyses

The real-time RT-qPCR targeting segment 10 was tested before the start of the study with two BTV positive controls composed of extracted RNA from a BTV-infected (BTV-5) cell culture (VERO) and from a *C. imicola* midge that fed on blood infected with BTV-5. PCR grade water was used as negative control and was included in all steps from RNA extraction to real-time RT-qPCR.

A total of 82 and 79 pools (200 midges/ pool) collected during autumn and winter respectively were tested for the presence of BTV RNA (Table 3). Bluetongue virus positive results were obtained in 51.2% of the midge pools collected in autumn (42/82 positive) and 75.9% (60/79 positive) of the midge pools collected during winter (Table 3). Midges caught during autumn demonstrated a field infection prevalence of 0.3% while midges collected during winter showed an infection rate of 0.7%. The quantification cycle (C_q) reflected by the real-time PCR is an indication of viral load. Viral loads differ between autumn and winter catches (Table 3).

A total of 16 out of 45 seronegative cattle blood samples tested positive for BTV RNA (35.5%). Viral RNA loads ranged from the highest C_q - values of 31.4 to the lowest value of 37.0 with an average C_q - value of 34.5. A C_q - value of ≥ 40 indicated the cut-off point for analyses.

Table 2. Summary of different *Culicoides* species associated with 16 collections, at the eight collection sites, per season, in Mnisi. Midges collected in winter and autumn are presented separately. Species absence is indicated by * in June/July (winter) and ** in April/May (autumn)

	Winter sampling (June/July)								
Site no.	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Total
Culicoides spp									
C.bedfordi	1.9	0.0	0.0	1.0	456.6	84.6	2.0	1.0	547.1
C.bolitinos	27.2	17.0	243.4	5.0	456.6	25.4	14.0	11.0	799.5
C.brucei	0.0	0.0	0.0	0.0	76.1	42.3	0.0	0.0	118.4
C.coarctutus	0.0	0.0	0.0	0.0	76.1	42.3	0.0	0.0	118.4
*C.dekeyseri***	0.0	0.0	0.0	0.0	0.0	8.5	0.0	0.0	8.5
C.enderleini	3.9	3.4	9.5	1.0	152.2	0.0	2.0	3.0	175.0
*C.engubendei**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.exspectator	3.9	45.8	88.5	2.0	76.1	25.4	3.0	6.0	250.7
*C.glabripernis***	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0
C.imicola	279.4	101.8	85.3	55.0	12024.1	1116.4	39.0	49.0	13750.0
*C.kanagai**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.leucostictus	21.3	61.1	50.6	43.0	152.2	33.8	7.0	10.0	379.0
*C.milnei**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.neavei	0.0	1.7	0.0	1.0	152.2	84.6	0.0	0.0	239.5
C.nevilli	1.9	0.0	31.6	0.0	532.7	50.7	0.0	0.0	617.0
C.nivosus	3.9	8.5	25.3	13.0	0.0	8.5	3.0	1.0	63.1
C.pycnostictus	17.5	5.1	25.3	5.0	0.0	16.9	4.0	3.0	76.8
C.simillis	7.8	22.1	22.1	3.0	152.2	33.8	2.0	6.0	249.0
*C.sp # 50**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.sp # 54 d/f	1.9	1.7	0.0	3.0	0.0	8.5	10.0	9.0	34.1
*C.sp # 54 p/f**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.subschltzei	7.8	11.9	12.6	6.0	152.2	33.8	0.0	0.0	224.3
C.tropicalis	7.8	42.4	9.5	5.0	228.3	25.4	17.0	17.0	352.3
C.tutttifruitti	1.9	11.9	6.3	33.0	152.2	33.8	4.0	4.0	247.2
C.zuluensis	0.0	1.7	0.0	0.0	76.1	8.5	1.0	0.0	87.3
Total:25	388.0	336.0	610.0	176.0	14916.0	1683.0	109.0	120.0	18338.0
	Autumn sampling (April/May)								
Site no.	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8	Total
Culicoides spp									
C.bedfordi	0.0	0.5	0.0	0.0	142.9	0.0	1.0	0.0	144.4
C.bolitinos	3.0	6.5	0.5	0.0	285.7	0.0	44.5	31.0	371.2
C.brucei	0.0	0.0	0.0	0.0	0.0	4.0	0.0	0.0	4.0
C.coarctutus	0.0	0.0	1.0	0.0	0.0	44.0	0.0	0.0	45.0
*C.dekeyseri***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.enderleini	7.5	12.5	4.0	0.5	857.1	102.0	23.0	3.5	1010.1
*C.engubendei**	0.0	0.0	0.0	0.0	0.0	2.0	0.0	0.0	2.0
C.exspectator	2.0	9.5	0.0	1.5	142.9	8.0	20.0	4.5	188.4
*C.glabripernis***	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
C.imicola	268.5	231.0	31.0	18.5	63428.6	2318.0	307.0	360.0	66962.6
*C.kanagai**	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.0	1.0
C.leucostictus	1.5	7.0	3.0	3.0	714.3	10.0	15.0	14.0	767.8
*C.milnei**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	1.0
C.neavei	0.0	0.5	0.0	0.5	142.9	2.0	0.0	0.0	145.9
C.nevilli	0.5	3.5	0.0	0.0	2785.7	202.0	1.0	1.0	2993.7
C.nivosus	0.0	0.0	1.0	0.0	0.0	0.0	0.0	0.0	1.0
C.pycnostictus	0.0	0.5	0.0	0.5	0.0	0.0	3.5	4.0	8.5
C.simillis	1.0	0.5	1.5	0.0	0.0	0.0	3.0	1.5	7.5
*C.sp # 50**	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.5	0.5
C.sp # 54 d/f	0.0	0.0	0.0	0.0	0.0	0.0	46.5	0.5	47.0
*C.sp # 54 p/f**	0.0	0.0	0.0	0.0	0.0	0.0	32.5	0.0	32.5
C.subschltzei	1.5	5.0	4.0	0.5	357.1	58.0	5.5	1.5	433.1
C.tropicalis	0.5	7.0	0.5	2.5	0.0	6.0	27.0	1.0	44.5
C.tutttifruitti	0.0	1.5	7.0	0.0	0.0	10.0	16.0	1.0	35.5
C.zuluensis	0.5	0.0	0.0	0.0	71.4	2.0	0.0	0.0	73.9
Total:25	286.5	285.5	53.5	27.5	68928.6	2768.0	546.5	425.0	73321.1

'df' (Dark Form) and 'pf' (Pale Form) refers to the colour of the wing of the unidentified species. (Numbering system is that of R. Meiswinkel and refers to yet taxonomically undescribed *Culicoides* species)

Table 3. Percentage of BTV positive midge pools (n = 200), viral load and field infection prevalence between autumn and winter catches

Season	Total # of midge pools tested	C_q – value low	C_q – value high	C_q – value average	BTV positive pools (%)	Field infection prevalence (%)
Autumn	82	27.4	37.8	35.0	51.2	0.3
Winter	79	26.7	38.5	30.5	75.9	0.7

4. Discussion

The role of cattle in the epidemiology of BT in South Africa is not clear. The first natural outbreak of BTV in bovine in South Africa was described in field roaming cattle in 1933 [3]. Bluetongue virus was isolated from cattle although no clinical signs were present at that stage. The BTV outbreak in South Africa during 1995 to 1997 confirmed the presence of this disease in cattle after serological tests verified antibodies in 84% of the cattle tested. During this outbreak BTV was also isolated from 12 cattle as well as from *C. imicola* and *C. bolitinos* [2]. Unusual wet conditions due to an above average rainfall were identified as the main trigger for the BTV outbreak. Bluetongue virus antibodies have also been detected in wild ruminants in different vegetational zones and rainfall areas in South Africa with a lower incidence in semi-desert areas. This demonstrates that the disease occurs countrywide and is likely to affect domestic ruminants throughout South Africa [1].

Antibodies specific to BTV was detected in 95.7% of the 1 260 cattle tested. This revealed a significant difference in antibodies prevalence to BTV among various age groups and different villages sampled ($p < 0.05$) respectively. The significant difference between age groups could be due to the number of young animals (< 1 year) that demonstrated a lower seroprevalence. Young animals are less likely to have become infected with BTV, but as animals mature the chance of becoming infected with BTV increases as they are exposed to more BTV-infected vector periods. The significant difference between sex in age groups with regards to antibody inhibition was explained due to fewer males present in the community. In Mnisi, farmers sell the bulls after weaning resulting in higher numbers of older females than older males. This could also possibly explain previous results indicating a higher seroprevalence in females than males [10]. Alternative possibilities include olfactory response such as cattle derived volatile compounds that might influence host seeking activities of *Culicoides* should also be investigated [22]. The significant difference in seroprevalence between villages is harder to explain, although this could be due to sample size bias. Factors such as vegetation, interface areas e.g. Welverdiend B and Hlalakahle or the presence of water in village e.g. Tlhavekisa and Seville B could also possibly affect the results (Figure 1). No significant variations for prevalence of BTV antibodies were observed between cattle breeds ($p > 0.05$). Similar results were obtained in a study conducted on dairy cattle breeds in Sudan [10]. During the BTV outbreak (2006-2007) in Europe clinical disease associated with BTV-8 in Dutch dairy herds were reported [8,25]. These herds demonstrated a higher seroprevalence than small-scale and suckler herds [8,25]. Housing and grazing management of different cattle breeds might play a role on *Culicoides* exposure/ bites and therefore seroprevalence [25]. Limited studies have been done on the affect that cattle breeds might have on BTV susceptibility unlike in sheep where European breeds have been demonstrated to be more susceptible [26]. Antibody prevalence on its own is not sufficient to determine the role of a species in disease epidemiology as seroprevalence might be under or over represented in a population. The need to include several diagnostic tests such as PCR and virus isolation as well as vector surveillance programs is therefore essential in epidemiological studies [1].

Mnisi is located in the subtropical Mpumalanga Province of South Africa where the summers are hot and rainy and the winters are warm and dry seldom reaching sub-zero temperatures. Temperature fluctuations are therefore small and the area is not known for being windy. Throughout the vector surveillance periods the average outside temperature never exceeded 25°C or fell below 15°C. Temperatures above the former or below the latter have been shown to decrease the biting rate of *Culicoides* midges [19,29]. The average wind speed was also lower than the wind speed at which *Culicoides* are expected to stop flying (3 m/s) [11]. Except for June, the relative humidity was in optimal range for *Culicoides* species i.e. 50-70% [32]. Numerous studies have been conducted on the affect of meteorological factors such as temperature, wind speed and humidity on the presence of *Culicoides* biting midges [4,17]. These factors have been shown to influence the distribution and vectorial capacity of *Culicoides* midges and could influence the occurrence as well as the number of midges collected [5,17].

A total of 25 *Culicoides* species were identified in Mnisi. This is less than 23% of the 112 *Culicoides* species found in South Africa. The dominant *Culicoides* species collected throughout the study was *C. imicola* which accounted for 91% and 75% of midges collected during autumn and winter, respectively. The Shannon-Weiner index indicated that collections made during autumn is concentrated to one dominant species with more rare species while winter has greater species diversity. Species evenness were also determined for both sampling efforts and indicated that there is a variation in midge species collected between the seasons. The great abundance of *C. imicola* throughout the study area confirms that this species is probably still the most prevalent *Culicoides* species throughout South Africa and highlights the importance of this species as a vector for BTV and other related orbiviruses such as AHSV [18].

In winter, *C. bolitinos* was the second most abundant species collected (4.4%). The relatively low abundance, compared to *C. imicola*, could be due to the fact that this species occurs mostly in cooler, highland areas with higher rainfall such as the eastern Free State Province. The large number of cattle together with the bordering wildlife reserves could contribute to the abundance of this species outside its normal distribution range. Previous studies have found that *C. bolitinos* breed in the dung of large herbivores such as cattle breeds (various *Bos* races) as well as African buffalo (*Syncerus caffer*) and blue wildebeest (*Connochaetes taurinus*) [15]. *Culicoides*

nevilli were both the second most abundant species during autumn (4%) and the third most abundant during winter (3.4%). Although the vector competence of this midge species for BTV has not been established, the closely related epizootic hemorrhagic disease virus has been isolated from *C. nevilli* [2]. In contrast, *C. enderleini* the third most abundant species in autumn, has demonstrated to be susceptible to BTV under laboratory conditions and is considered as a livestock associated species [18,21,34]. The presence of C. sp. # 54 d/f could be attributed due to their breeding preferences. This species breed in the dung of African elephants (*Loxodonta Africana*), white rhinoceros (*Ceratotherium simum*), black rhinoceros (*Diceros bicornis*) and Burchell's zebras (*Equus burchelli*) [16]. *Culicoides* species that were both widespread and abundant, resulting in a vector rating of more than 25%, were present in both autumn and winter. *Culicoides imicola* demonstrated the highest vector rating in both seasons and supports previous studies on BTV and related orbiviruses in South Africa [21,35].

Parous females were present in both autumn and winter sampling. Although significantly lower ($p > 0.05$), the presence of parous females in winter indicates that blood feeding and therefore the potential to transmit BTV continues throughout the colder months in the Mnisi, Mpumalanga region. This could prove useful in understanding the overwintering mechanism of BTV and other related orbiviruses. Further, the presence of males during the winter is an indication that mating continues throughout the year [33].

Bluetongue virus RNA was detected in 51.2% of the autumn and 75.9% of the winter *Culicoides* pools. The higher occurrence/ prevalence of BTV within the vectors during winter could be due to the fact the infected midges will survive longer at the lower temperatures [19]. Future studies need to focus on determining the relationship between C_q - values and viral RNA load within *Culicoides.* The results contradict previous findings where it was established that the field infection prevalence of BTV in *Culicoides* is a mere 0.06% [34]. Variation in results could possibly be explained by the different diagnostic tools used. Venter *et al*., [34] used cell culture to detect the presence of the virus. This relies on virus isolation and the detection of cytopathic effect that represents infectious virus. The use of real-time RT-qPCR is therefore more sensitive but allows for the detection of very low levels of viral RNA, even if the virus is non-infectious. Blood samples from seronegative animals were taken on the same day from the same animal as the serum samples. Seronegative animals were expected to remain PCR negative although results could indicate early infection with BTV. Similar results were obtained where BTV was detected with RT-PCR although no antibodies were present at the time [28]. The PCR results do not indicate infectious virus in the animal as real-time RT-qPCR can detect viral nucleic acid long after viraemia. In rams, BTV-8 RNA was detected for up to 116 days post infection with real-time RT-qPCR [12].

The results indicate that BTV is circulating in Mnisi between *Culicoides* vectors and cattle although subclinically infected. The high seroprevalence, BTV RNA positive blood and the lack of clinical disease in cattle support the hypothesis that cattle could potentially be important amplifying hosts of BTV in South Africa.

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