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Use of real-time quantitative reverse transcription polymerase chain reaction for the detection of African horse sickness virus replication in *Culicoides imicola*

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Despite its important role as vector for African horse sickness virus (AHSV), very little information is available on the dissemination of this virus in *Culicoides* (*Avaritia*) *imicola* Kieffer (Diptera: Ceratopogonidae). This study reports on the applicability of a real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) to detect AHSV in dissected midges. A total of 96 midges were fed on AHSV-infected blood, after which one test group was dissected into head/thorax and abdomen segments immediately after feeding and the other only after 10 days of incubation. The majority of the midges (96%) ingested the virus successfully and there was no significant difference between the virus concentration in the heads/thoraxes and the abdomens immediately after feeding. After incubation, virus was detected in 51% of the midges and it was confined to the abdomen in the majority of these. The fact that virus was detected only in the heads/thoraxes of four *Culicoides* midges after incubation suggests the presence of a mesenteronal escape barrier. Replication in the salivary glands was not shown. An increase of the mean virus concentration in the abdomen after incubation indicates localised viral replication. The real-time RT-qPCR is recommended for further studies investigating the replication and dissemination of AHSV in *Culicoides* midges.

Introduction

Small biting midges in the genus *Culicoides* (Diptera: Ceratopogonidae) are involved in the epidemiology and transmission of a number of orbiviruses of veterinary importance, including African horse sickness virus (AHSV) with nine known serotypes (Howell 1962). This virus causes an infectious, non-contagious disease, African horse sickness (AHS), which is endemic in sub-Saharan Africa and can have a mortality rate of up to 95% in susceptible horses.

Based on its confirmed vector status, wide geographical distribution, abundance and host preference for larger mammals, the Afro-Asiatic *Culicoides* (*Avaritia*) *imicola* Kieffer is considered the principle vector of AHSV in South Africa (Meiswinkel, Venter & Nevill 2004; Nevill, Venter & Edwardes 1992). This species is also the most important vector of orbiviruses across vast geographic regions in Africa, the Mediterranean and southern Europe (Mellor, Boorman & Baylis 2000). Following ingestion by a susceptible midge, AHSV infects and replicates in cells of the mesenteron before entering the haemocoel and infecting secondary target organs such as the fat body and salivary glands (Mellor 2000; Wittmann & Baylis 2000). A number of barriers to arbovirus infection appear to exist in *Culicoides* midges, including the mesenteronal infection and escape barriers and the dissemination barrier. A salivary gland barrier has not been shown to be present in *Culicoides* species (Fu *et al*. 1999; Mellor 1990). Studies involving the North American vector *Culicoides* (*Monoculicoides*) *sonorensis* Wirth and Jones and bluetongue virus (BTV) indicate infection of the salivary glands to be an essential prerequisite for the transmission of virus (Jennings & Mellor 1987). No comparable studies have been performed for *C. imicola* and/or AHSV.

A number of real-time reverse transcription polymerase chain reaction (RT-PCR) assays have been described for AHSV (Fernández-Pinero *et al*. 2009; Quan *et al*. 2010; Rodríguez-Sanchez *et al*. 2008), all with high sensitivity and a detection limit of 0.001–0.15 $TCID_{50}$ per reaction. A real-time quantitative RT-PCR (RT-qPCR) with a unique approach of using circulating field isolates of AHSV (Quan *et al*. 2010) has recently been used to determine the infection prevalence of AHSV in *Culicoides* midges. The use of PCR to investigate the replication and distribution of AHSV in *Culicoides* midges has not been described.

The objective of this study was to investigate the replication and dissemination of AHSV in field-collected *C. imicola* by feeding, incubating and dissecting individuals and performing real-time RT-qPCR on the abdomens and the heads/thoraxes.

Research method and design

Materials and method

Culicoides biting midges were collected alive using 220 V Onderstepoort downdraught suction light traps (Venter *et al*. 1998) at various sites near cattle at the ARC-Onderstepoort Veterinary Institute, South Africa (25°39′S, 28°11′E; 1 219 m above sea level). After an acclimatising period of 2–3 days at 23.5 ºC and a relative humidity of 50% – 70%, field-collected midges were fed on defibrinated sheep blood containing AHSV serotype 6 at a concentration of $10^{6.1}$ $TCID_{50}$/mL through a chicken skin membrane (Venter *et al*. 1991). After a feeding period of 30–40 min the blood-engorged females were separated into two groups: one group was dissected within hours after blood feeding (D_0), whilst the other group was dissected after 10 days' incubation (D_{10}). The blood-engorged females were maintained on a 5% (w/v) sucrose solution containing antibiotics (500 IU penicillin, 500 μg streptomycin and 1.25 μg fungizone per 1 mL sucrose solution) at 23.5 °C (Venter & Paweska 2007). Midges were identified as *C. imicola* by examination of wing pattern. Straight Vanna's microscissors (Agar Scientific, Essex, UK) were used to separate the abdomen (containing the midgut) from the head/thorax (containing the salivary glands). Midges that could not be dissected immediately after feeding or incubation were stored overnight in a refrigerator at 4 °C or in a freezer at –70 °C if stored for a longer period.

The dissected midges were subjected to real-time RT-qPCR following an adaption of the protocol described by Quan *et al*. (2010). *Culicoides* parts were placed separately in MagNA Lyser green beads (Roche Products, South Africa), containing 300 μL lysis/binding solution (AM8500) from the Ambion total nucleic acid extraction kit (AM1836), to which 2.1 μL β-mercaptoethanol was added, or in 300 μL phosphate-buffered saline. After homogenisation in a MagNA Lyser (Roche Products, South Africa), 100 μL of each sample was mixed with 1 μL carrier RNA, 60 μL isopropanol and 20 μL bead mix, the latter consisting of lysis/binding enhancer and magnetic beads. RNA extraction was performed using either the MagMAX Express Magnetic Particle Processor (Life Technologies, USA) or the Kingfisher Flex Automated Purification System (ThermoFisher Scientific, Finland). Purified water and blood from a clinical case of AHS were used as negative and positive controls, respectively. An aliquot of 5 μL of each extract was mixed with 5 μL primers and probe for part of segment 8, which codes for the structural protein VP7 (Quan *et al*. 2010), to obtain final concentrations of 400 nM for each primer and 180 nM for the probe in the 25 μL reaction. The samples were centrifuged, denatured at 95 °C for 1 min using a PCR machine (GeneAmp 9700, Life Technologies, USA) and rapidly chilled at –20 °C for 5 min. A total volume of 15 μL master mix (12.5 μL 2x RT-PCR buffer, 1 μL 25x RT-PCR enzyme and 1.5 μL purified water) was added before the samples were centrifuged again. RT-qPCR was performed using the StepOne Plus Real Time PCR system (Life Technologies, USA) according to the manufacturer's instructions.

Analysis of variance (ANOVA) was used to differentiate between mean cycle threshold (C_T) values. Statistical differences between experimental groups were analysed using Fisher's exact test and/or χ^2 analysis. *P*-values < 0.05 were considered statistically significant.

Results

The results of the RT-qPCR assays on the abdomens and heads/thoraxes of 47 D_0 and 49 D_{10} *C. imicola*, respectively, are provided in Table 1. AHSV was detected in 45 (95.7%) D_0 midges, three of which (6%) contained virus only in the head/thorax. There was a significant difference between the number of *Culicoides* that tested PCR positive for AHSV in the abdomen (89.4%) and in the head/thorax (34%). AHSV was detected in 25 D_{10} midges (51%), with a significantly higher number being PCR positive for AHSV in the abdomen (49%) than in the head/thorax (8.2%).

There was a significant ($p < 0.001$) decrease in the number of midges in which AHSV was detected in either the head/thorax or the abdomen immediately after blood feeding (95.7%) than after 10 days' incubation (51%). Based on the C_T values no significant difference was identified in the AHSV concentration between heads/thoraxes and abdomens of D_0 *C. imicola* ($p > 0.05$). Only one of the four positive D_{10} heads/thoraxes (25%) had a C_T value below the D_0 mean, whereas 18 of the 24 positive abdomens (75%) had C_T values below the mean of D_0.

Discussion

With use of RT-qPCR, AHSV RNA was detected in 95.7% of the *Culicoides* midges assayed immediately after feeding on an AHSV-infected blood meal. In previous studies, where similar infection techniques were used, AHSV was isolated only in 44% – 64% of the midges tested immediately after feeding when using cell culture systems (Venter & Paweska

TABLE 1: Summary of real-time RT-qPCR results for body segments of *Culicoides imicola* after feeding on AHSV-6 infected blood.

Category	Test group[a]					
	D_0 (n = 47)			D_{10} (n = 49)		
	Head/thorax	Abdomen	Head/thorax or abdomen	Head/thorax	Abdomen	Head/thorax or abdomen
Positive samples	16 (34.0%)	42 (89.4%)	45 (95.7%)	4 (8.2%)	24 (49.0%)	25 (51.0%)
Mean C_T (range)	35.83 (31.76–39.39)	34.67 (31.49–39.27)	–	36.74 (30.94–39.95)	32.52 (26.55–39.60)	–
Number of midges below mean C_T of D_0	7	23	–	1	18	–

AHSV, African horse sickness virus; C_T, cycle threshold for AHSV VP7.
[a], One group of midges was dissected on the day of feeding (D_0), whilst the other group was dissected after 10 days' incubation (D_{10}).

2007; Venter, Graham & Hamblin 2000). In the present study, AHSV RNA was detected in 51% of the midges assayed after incubation. Previous oral susceptibility studies using identical incubation conditions reported markedly lower virus recovery. Depending on the virus isolate used, results for *C. imicola* ranged from 4.3% to 26.8% (Paweska & Venter 2003; Venter & Paweska 2007; Venter *et al.* 2000). In these studies AHS virions were detected using virus isolation on cell culture systems. RT-qPCR, however, detects viral RNA. This technique has been shown to be substantially more sensitive than virus isolation (Quan *et al*. 2010), which may explain the higher values reported in the present study.

In most of the D_0 midges in which AHSV was found in the head/thorax, virus was also detected in the abdomen. The three *C. imicola* that tested PCR positive only in the head/thorax were probably harvested and immobilised whilst still taking up the blood meal. The AHSV loads detected in the heads/thoraxes and abdomens of D_0 *C. imicola* were similar ($p > 0.05$), implying that no virus replication had taken place yet. However, the mean C_T value for the abdomens was lower in D_{10} midges (32.52) than in D_0 midges (34.67). A drop of 3.32 in C_T values implies a 10-fold increase of double-stranded RNA (Quan *et al*. 2010); the observed decrease of 2.15 therefore reveals approximately five times more viral RNA in the abdomens of D_{10} midges compared to D_0 midges. The results are even more prominent if one looks at the lowest C_T value of the abdomens (31.49 in D_0 and 26.55 in D_{10} midges, respectively). This difference of almost five C_T values indicates more than a 50-fold increase of virus load in the abdomens, which was probably due to virus replication in the midgut cells.

It has been shown that *Culicoides* midges express various barriers that limit virus replication and transmission. The present results clearly illustrate that not all midges in a population are susceptible to infection with AHSV and that some individuals are able to clear the virus to below detectable levels within 10 days after feeding on a virus-infected blood meal. The mesenteronal infection barrier may have played a role in the proportion of D_{10} midges (49%) that were able to eliminate AHSV within 10 days without becoming infected. *Culicoides* midges that were PCR positive in the abdomen but exhibited a C_T value below detectable limits in the head/thorax probably expressed a mesenteronal escape barrier, not allowing the virus to escape from the midgut cells. This result relates to a previous study where 43.6% of *C. sonorensis* exhibited such a barrier to BTV (Jennings & Mellor 1987). In the present study, only four (8.2%) of the D_{10} midges were PCR positive in the head/thorax, indicating that they expressed neither a mesenteronal escape barrier nor a dissemination barrier. Virus that is present in the head/thorax is presumably located in the salivary glands. All four these midges had a higher C_T value in the head/thorax than in the abdomen (i.e. less viral RNA in the head/thorax), implying that no additional viral replication had taken place in the salivary glands. The salivary glands were not specifically dissected but remained part of the heads/thoraxes. However, this study does not indicate whether this could have influenced the results and secondary viral replication in the salivary glands remains unlikely. The mean C_T value of the heads/thoraxes of the D_{10} midges was not significantly different from that of the D_0 midges (p = 0.3847). However, the value was 4.22 units higher than for the abdomens in the former test group, which indicates a substantially lower viral load in their heads/thoraxes. The finding also supports the hypothesis that viral replication did not occur in the salivary glands.

Conclusion

The real-time RT-qPCR used in the present study was an adapted version of the protocol optimised for detection of AHSV in blood and organ samples (Quan *et al*. 2010). This adapted assay has recently been used to quantify viral loads in *Culicoides* midge pools and now it has been shown to be a very sensitive method for investigating AHSV viral load differences in different body parts of *Culicoides* midges as well. Future studies investigating AHSV replication in *Culicoides* midges should include investigations of AHSV viral load in salivary glands and/or saliva.

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Authors' contributions

A.J.G. and N.O. were the project leaders, whilst E.G.S., G.J.V. and A.J.G. were responsible for the experimental and project design. The experiments were performed by E.G.S. and C.J. and statistics were performed by G.J.V., E.G.S. wrote the manuscript with contributions from all authors.

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