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

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 (Diptera: Ceratopogonidae) 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 haemocoeol 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 TCID₅₀ 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 °C 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 TCID₅₀/mL through a chicken skin membrane (Venter & Paweska 2007). AHSV, African horse sickness virus; CT, cycle threshold for AHSV VP7.

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 (D₀), whilst the other group was dissected after 10 days’ incubation (D₁₀). 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) and rapidly chilled at –20 °C for if stored for a longer period.

RESULTS

There was a significant (p < 0.001) decrease in the number of midges in which AHSV was detected after 10 days’ incubation (51.5%) than after 10 days’ incubation (51.5%). Based on the CT values no significant difference was identified in the AHSV concentration between heads/thoraxes and abdomens of D₀ C. imicola (p > 0.05). Only one of the four positive D₀ heads/thoraces (25%) had a Ct value below the D₀ mean, whereas 18 of the 24 positive abdomens (75%) had Ct values below the mean of D₀.

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

<table>
<thead>
<tr>
<th>Category</th>
<th>Test group</th>
<th>D₀ (n = 47)</th>
<th>D₁₀ (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Head/thorax</td>
<td>Abdomen</td>
<td>Head/thorax or abdomen</td>
</tr>
<tr>
<td>Positive samples</td>
<td>16 (34.0%)</td>
<td>42 (89.4%)</td>
<td>45 (95.7%)</td>
</tr>
<tr>
<td>Number of midges below mean Cₜ of D₀</td>
<td>7</td>
<td>23</td>
<td>–</td>
</tr>
</tbody>
</table>

AHSV, African horse sickness virus; Cₜ, cycle threshold for AHSV VP7.

1. One group of midges was dissected on the day of feeding (D₀), whilst the other group was dissected after 10 days’ incubation (D₁₀).

Analysis of variance (ANOVA) was used to differentiate between mean cycle threshold (Cₜ) values. Statistical differences between experimental groups were analysed using Fisher’s exact test and/or χ² 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 D₀ and 49 D₁₀ C. imicola, respectively, are provided in Table 1. AHSV was detected in 45 (95.7%) D₀ 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 D₁₀ midges (51%), with a significantly higher number being PCR positive for AHSV in the abdomen (49%) than in the head/thorax (8.2%).

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 1991). After an acclimatising period of 2–3 days at 23.5 °C 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 TCID₅₀/mL through a chicken skin membrane (Venter & Paweska 2007).
Taking up the blood meal. The AHSV loads detected in the thorax were probably harvested and immobilised whilst still three head/thorax, virus was also detected in the abdomen. The thoraxes. However, this study does not indicate whether taken place in the salivary glands. The salivary glands were lower in D10 midges (32.52) than in D0 midges (34.67). A drop of 3.32 in C 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 D10 midges compared to D0 midges. The results are even more prominent if one looks at the lowest C value of the abdomens (31.49 in D0 and 26.55 in D10 midges, respectively). This difference of almost five C 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 before 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 D0 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 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 D0 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 CT value 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 D0 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 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 value of the heads/thoraxes of the D0 midges was not significantly different from that of the D10 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.

Acknowledgments

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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. and E.G.S. wrote the manuscript with contributions from all authors.

References


