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 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 Ondersteapoort downdraught suction light traps (Venter et al. 1998) at various sites near cattle at the ARC-Ondersteapoort 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 106.1 TCID50/mL through a chicken skin membrane (Venter & Pawska 2007). AHSV, African horse sickness virus; CT, cycle threshold for AHSV VP7.

**TABLE 1:** 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 = 49)</th>
<th>D10 (n = 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Head/thorax</td>
<td>Abdomen</td>
<td>Head/thorax</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>Mean C&lt;sub&gt;τ&lt;/sub&gt; (range)</td>
<td>35.83</td>
<td>34.67</td>
<td>31.76–39.39</td>
</tr>
<tr>
<td>Number of midges below mean C&lt;sub&gt;τ&lt;/sub&gt; of D&lt;sub&gt;0&lt;/sub&gt;</td>
<td>7</td>
<td>23</td>
<td>–</td>
</tr>
</tbody>
</table>

AHSV, African horse sickness virus; C<sub>τ</sub>, cycle threshold for AHSV VP7.

*R* One group of midges was dissected on the day of feeding (D<sub>0</sub>), whilst the other group was dissected after 10 days’ incubation (D<sub>10</sub>).

Results

The results of the RT-qPCR assays on the abdomens and heads/thoraxes of 47 D<sub>0</sub> and 49 D<sub>10</sub> *C. imicola*, respectively, are provided in Table 1. AHSV was detected in 45 (95.7%) D<sub>0</sub> 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<sub>10</sub> 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<sub>τ</sub> values no significant difference was identified in the AHSV concentration between heads/thoraxes and abdomens of D<sub>0</sub> *C. imicola* (*p* > 0.05). Only one of the four positive D<sub>0</sub> heads/thoraxes (25%) had a C<sub>τ</sub> value below the D<sub>0</sub> mean, whereas 18 of the 24 positive abdomens (75%) had C<sub>τ</sub> values below the mean of D<sub>0</sub>.

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 & Pawska...
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 D₀ midges in which AHSV was found in the head/thorax, virus was also detected in the abdomen. The AHSV loads detected in the 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 thoraxes. than in the abdomen (i.e. less viral RNA in the head/thorax than in the abdomen (i.e. less viral RNA in the head/thorax vs. abdomen). 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., E.G.S. wrote the manuscript with contributions from all authors.

References


