Schmallenberg virus detected by RT-PCR in *Culicoides* biting midges captured during the 2011 epidemic in The Netherlands

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Infections with Schmallenberg virus (SBV) are associated with congenital malformations in ruminants. To identify potential vectors for SBV, *Culicoides* collected in the autumn of 2011 in the Netherlands were tested retrospectively by RT-PCR. A total of 610 pools of heads (10 *Culicoides*/pool) from 6,100 female *Culicoides* were analyzed. Twelve pools of the Obsoletus Complex and two pools of *C. chiopterus* tested positive, the majority with C_t-values of between 20 and 30. Molecular sequencing of positive Obsoletus Complex midges revealed eleven to be *C. scoticus* and one *C. obsoletus sensu stricto*. Prevalence of SBV in midges of the Obsoletus Complex was 56 per 10,000, ten times higher when compared to bluetongue virus detection in the same *Culicoides* species in Europe during 2002-2008. Vector biology might have been positively influenced by climatological circumstances in 2011 with a prolonged vector season (several weeks) and a higher survival rate and increased vector abundance (rain in summer and higher temperatures in autumn).

*Keywords*: Schmallenberg virus, *Culicoides*, RT-PCR

Commencing in the late summer of 2011, a previously unknown pathogenic agent, provisionally named Schmallenberg virus (SBV) or Shamonda-like virus, spread silently across much of northern Europe, infecting ruminant livestock in Germany, Denmark, the Netherlands, Belgium, the United Kingdom, Luxembourg, France, Italy and Spain. In November 2011, the Friedrich Loeffler
Institute (FLI) in Germany reported the discovery of SBV and that this Orthobunyavirus - new to science - was linked to clinical problems in cattle (1). Studies since have confirmed SBV to be associated with congenital malformations in newly born lambs, goat kids and calves (2).

More than 300 viruses comprise the family Bunyaviridae and are assigned amongst five genera. Bunyavirus is the largest of these genera and embraces more than 170 viruses divided into 18 serogroups (3) and amongst which is found the Simbu serogroup of 25 viruses. Along with Schmallenburg virus, this serogroup includes also Shamonda virus (SHAV), Akabane virus (AKAV), Sathuperi virus (SATV) and Aino virus (AINOV), all known for their teratologic effects in ruminants. As far as is known all Simbu serogroup viruses are arthropod-borne, with the majority isolated from mosquitoes and Culicoides biting midges (3). SBV is an RNA virus, and based on the three gene segments (S, M and L), shares a 97% identity with SHAV (S segment), 71% with AINOV (M segment), and 69% with AKAV (L segment) (4). A recent phylogenetic study indicates SBV to be ancestral to SHAV (5).

Three recent preliminary reports have indicated one or more species of Culicoides biting midges to be possible field vectors for SBV in northern Europe (6,7,8). During the autumn of 2011 Culicoides were collected at three separate livestock holdings situated in the eastern and the north-eastern parts of the Netherlands.

MATERIAL AND METHODS

Trapping locations

Within the framework of an ongoing bluetongue research project managed by the Central Veterinary Institute (CVI), Lelystad, and conducted at a dairy in the municipality of Ermelo (province of Gelderland) in the east of the Netherlands, Culicoides were trapped almost daily throughout September and early October, 2011. The dairy herd consisted of approximately 100 dairy cows and young stock. In addition, and as part of ongoing research projects (9,10) of the Faculty of Veterinary Medicine, University of Utrecht, Culicoides were trapped also in the vicinity of sheep in the municipality of Bilthoven (province of Utrecht) in the central part of the Netherlands, and at a second sheep flock in the municipality of Midden-Drenthe (province of Drenthe), in the north-eastern part of the Netherlands.

Climatological characteristics of 2011

The year 2011 ranked among the three warmest years since 1901 in the Netherlands (source: Royal Netherlands Meteorological Institute, de Bilt, Netherlands; http://www.knmi.nl/klimatologie/maand_en_seizoensoverzicht-en/jaar/jaar11.html).

Spring was very mild and dry, summer was very wet, followed by an extremely mild autumn. November was a record dry month and December ranked fourth among the mildest winter months in the last 100 years.
**Culicoides trapping**

In the province of Gelderland, *Culicoides* biting midges were collected in September and early October of 2011 using various methods that included the ‘golden standard’ Onderstepoort-type blacklight trap (11). The trapping of midges in the vicinity of sheep in the provinces of Utrecht and Drenthe was done using the Onderstepoort-type trap and a drop-tent cage in which an ewe and her lamb had been placed; collections using both methods were made over several days in August and September of 2011. Captured *Culicoides* were stored in 70% ethanol. Prior to assay for SBV, female midges were age-graded as either nulliparous, parous, gravid or freshly blood fed (engorged) (11).

**Culicoides identification**

*Culicoides* were identified morphologically using primarily the published keys of Campbell and Pelham-Clinton (12), Glukhova (13), and the unpublished PhD thesis of Jean-Claude Delécolle (14). The accurate morphological separation of the females of *C. obsoletus sensu stricto* from *C. scoticus* is unreliable, and for this reason their data are combined and referred to as ‘the Obsoletus Complex’. The four remaining species assayed were *C. dewulfi*, *C. chiopterus*, *C. punctatus* and *C. pulicaris*. After assay the identity of each SBV-positive midge pool was confirmed molecularly (described below).

**Preparation of Culicoides pools**

As noted earlier, only parous and gravid female midges not engorged with fresh blood, were selected for assay. After age-grading, the 6,100 midges were divided into 610 species-specific pools, i.e. 10 midges/pool. The number of pools assayed for each species was as follows: Obsoletus Complex (230), *C. chiopterus* (144), *C. dewulfi* (130), *C. punctatus* (105), and *C. pulicaris* (1). In total 556 of the pools originated from the dairy in the municipality of Ermelo, 44 (39 pools of *C. chiopterus* and five of the Obsoletus Complex) from the sheep flock in the municipality of Midden-Drente, and 10 pools (Obsoletus Complex) from a sheep flock in the municipality of Bilthoven.

Before being assayed, and working under a dissecting microscope using a scalpel, the head of each midge was separated away from the body in 70% ethanol; 10 heads were then pooled and assayed for SBV, while the corresponding abdomens (also pooled) were stored in 70% ethanol. Once a pool of 10 heads was found SBV-positive the corresponding pool of dissected abdomens was retrieved and assayed also; in this instance the 10 abdomens were assayed individually, so that the individual abdomen that proved to be SBV-positive could be identified also molecularly, to establish exactly which one of the two species of the Obsoletus Complex was involved, and to confirm or refute the morphological identifications of the remaining *Culicoides* species made while the midge pools were selected some weeks earlier.
RNA extraction and RT-PCR

RNA extraction was performed using a protocol developed by the Veterinary and Agrochemical Research Centre (CODA-CERVA), Brussels, Belgium, which in turn is an adaptation of protocols described by Mehlhorn et al. (15) and Vanbinst et al. (16) for detection of Bluetongue virus in Culicoides. Total nucleic acid was isolated using the MagnaPureLC isolation robot (Roche-Diagnostics©) according to the manufacturer instructions. RT-PCR detection of SBV was done according to the protocol for SBV-S segment detection provided by the Friedrich Loeffler Institute (FLI), Germany.

Cut-off value

The cut-off value of the RT-PCR was set at a cycle threshold (Ct) value of 35 for the pools of the heads. Pools with C_t values >35 were retested (duplo samples in Table 1) and considered positive when confirmed in the retest. Reported C_t values, using the same RT-PCR, from blood samples from infected cattle in Germany were 24-35 (4), confirming our choice for the cut-off. No C_t values above 40 were observed. If a specific pool of Culicoides heads tested positive, the corresponding pool of stored bodies was retracted and each separate body was than tested by RT-PCR. Briefly, separate bodies were thoroughly rinsed with 70% ethanol and subsequently total nucleic acid was isolated as described above.

Culicoides species determination by sequencing

The 18S-ITS1-5.8S region was amplified using the PanCulF and PanCulR primer set adapted from Cêtre-Sossah et al. (17). Briefly reactions were done in 50μl total volume consisting of 5x PCR buffer, 2.5 mM of MgCl2, 2 μM of each primer, 100μM dNTP’s and 0.5 μl of GoTaq® Hot Start Polymerase (5 U/μl; Promega, Madison USA) and 5 μl of DNA template. PCR was carried out with the following cycling conditions: an initial denaturation step at 94°C for 3 min followed by

<table>
<thead>
<tr>
<th>Municipality (Province)</th>
<th>C. obsoletus complex</th>
<th>C. dewulfi</th>
<th>C. chiopterus</th>
<th>C. punctatus</th>
<th>C. pulicaris</th>
<th>Total pools</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bilthoven (Utrecht)</td>
<td>0/10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0/10</td>
</tr>
<tr>
<td>Midden-Drenthe (Drenthe)</td>
<td>0/5</td>
<td>1/39</td>
<td>-</td>
<td>-</td>
<td>1/44</td>
<td></td>
</tr>
<tr>
<td>Ermelo (Gelderland)</td>
<td>12/215</td>
<td>0/130</td>
<td>1/105</td>
<td>0/105</td>
<td>0/1</td>
<td>13/556</td>
</tr>
<tr>
<td>Total</td>
<td>12/230</td>
<td>0/130</td>
<td>2/144</td>
<td>0/105</td>
<td>0/1</td>
<td>14/610</td>
</tr>
</tbody>
</table>
40 cycles at 94°C, 1 min; 45°C, 1 min; 72°C, 1 min and a final extension phase at 72°C for 4 min. PCR products were examined by electrophoresis in a 1.0% agarose gel containing ethidium bromide. Bands of the appropriate size (ca. 400 bp) were excised and isolated using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA). Amplicons were sequenced with forward and reverse primers by using the BigDye Terminator v1.1 Cycle Sequencing Kit in a ABI PRISM® 3130 Genetic Analyzer (both supplied by Applied Biosystems, Foster City, CA, USA). Sequences were analysed and aligned using the Sequencer 4.10.1 software package (Ann Arbor, MI, USA). Consensus sequences from each individual Culicoides body were then aligned using Seaview 4.2.7 (CNRS, Lyon, France) (18,19,20,21,22). Trees were generated in Seaview using the PHYLIP package.

RESULTS

Livestock at Culicoides trapping locations

The entire dairy herd at Ermelo was blood sampled on April 19, 2012 and tested for antibodies to SBV using the virus neutralisation test (VNT) described by Loeffen et al. (23). Of 107 cattle tested, 103 were seropositive (VNT titer ≥ 8); the four seronegative animals (VNT titer < 4) were six-month-old calves. None of the animals in this herd displayed clinical signs indicative of an SBV infection, neither in 2011, nor in early 2012, when some calves were born, but without congenital malformations. At a dairy herd, situated <100 m from the sheep where Culicoides were collected in the municipality of Midden-Drenthe, a malformed calf was born that displayed arthrogryposis hydranencephaly syndrome (AHS), typical of an infection with SBV. Though brain material from this calf tested negative in the PCR, it does not exclude SBV as only a fraction of calves infected with the virus test PCR-positive (24). The owner of the flock did not allow his sheep to be tested for antibodies against SBV in May 2012.

RT-PCR detection of SBV in Culicoides

Fourteen (2.3%) of the 610 Culicoides pools (consisting of heads) tested SBV-positive by RT-PCR (Table 1). Twelve of these represented two species of the Obsoletus Complex, eleven C. scoticus and one C. obsoletus sensu stricto; all emanated from the dairy at the municipality of Ermelo and were collected in September. The two remaining pools were C. chiopterus, one from the municipality of Midden-Drente collected around sheep in August, and the second from the municipality of Ermelo collected around cattle in September. Twelve of these 14 pools had C_t values ranging between 19.6 and 30, while the two remaining pools were weak positive, with C_t values of 34.98 and 36 (Table 2). Subsequent testing of the individual bodies linked to each SBV-positive heads pool, revealed 13 pools to have C_t values lower (meaning a higher viral load) than
Table 2. PCR Ct-values of Schmallenberg virus positive *Culicoides* pools (heads) and individual *Culicoides* bodies, and species identification by DNA sequencing.

<table>
<thead>
<tr>
<th>Municipality (livestock species)</th>
<th>Pool (heads) identification</th>
<th>Morphological identification of <em>Culicoides</em> spp.</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;-value pools (heads) single</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;-value pools (heads) duplo</th>
<th><em>Culicoides</em> body identification</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;-values individual <em>Culicoides</em> bodies Body 1 single</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;-values individual <em>Culicoides</em> bodies Body 2 single</th>
<th>C&lt;sub&gt;T&lt;/sub&gt;-values individual <em>Culicoides</em> bodies Body 2 duplo</th>
<th>Species identification by DNA sequencing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mid-Drente (sheep) 95-D</td>
<td>C. chiopterus</td>
<td>27.88</td>
<td>95-D</td>
<td>24.59</td>
<td>C. chiopterus</td>
<td>C. chiopterus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 501</td>
<td>C. chiopterus</td>
<td>35.36</td>
<td>34.98</td>
<td>501</td>
<td>36.45</td>
<td>35.07</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 9-A</td>
<td>C. obsoletus complex</td>
<td>30.44</td>
<td>9-A</td>
<td>24.75</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 18-C</td>
<td>C. obsoletus complex</td>
<td>28.24</td>
<td>18-C</td>
<td>24.95</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 32-B</td>
<td>C. obsoletus complex</td>
<td>21.84</td>
<td>32-B</td>
<td>18.32</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 259</td>
<td>C. obsoletus complex</td>
<td>19.60</td>
<td>259</td>
<td>18.16</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 275</td>
<td>C. obsoletus complex</td>
<td>20.72</td>
<td>275</td>
<td>20.39</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 276</td>
<td>C. obsoletus complex</td>
<td>36.02</td>
<td>276</td>
<td>36.68</td>
<td>C. scoticus s.s.</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 293</td>
<td>C. obsoletus complex</td>
<td>20.43</td>
<td>293</td>
<td>19.95</td>
<td>No reliable sequence</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 294</td>
<td>C. obsoletus complex</td>
<td>24.60</td>
<td>294</td>
<td>20.06</td>
<td>C. obsoletus s.s.</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 368</td>
<td>C. obsoletus complex</td>
<td>25.21</td>
<td>368</td>
<td>21.80</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 385</td>
<td>C. obsoletus complex</td>
<td>20.67</td>
<td>385</td>
<td>20.25</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 405</td>
<td>C. obsoletus complex</td>
<td>23.38</td>
<td>405</td>
<td>21.64</td>
<td>C. scoticus</td>
<td>C. scoticus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ermelo (cattle) 434</td>
<td>C. obsoletus complex</td>
<td>23.68</td>
<td>434-1 and 434-2</td>
<td>35.75</td>
<td>35.37</td>
<td>C. scoticus</td>
<td></td>
<td></td>
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</tbody>
</table>
those obtained for their corresponding heads. In one pool of ten bodies of the Obsoletus Complex, two of the bodies tested SBV-positive, one strongly and the other weakly. All 130 pools of C. dewulfi tested RT-PCR-negative for SBV, as well as the 105 pools of C. punctatus and the single pool of C. pulicaris.

**Culicoides species determination by sequencing**

All but one individual Culicoides body could be identified molecularly to species using the ITS1 region as discriminant (Table 2) and confirmed the morphological identifications that had been made originally during the age-grading and pooling process. The molecular were superior in being able to clarify which species of the Obsoletus Complex were involved and indicates that C. scoticus played a more prominent role than C. obsoletus ss in the transmission of SBV. The ITS1 sequences obtained from samples 95-D and 501 were almost identical to the published sequences of C. chiopterus; the same applied to sample 294 and representing C. obsoletus sensu stricto (25). While sequence polymorphism in C. scoticus was diverse, we were able to assign unambiguously each of the eleven SBV-positive bodies to this species.

**DISCUSSION**

Our study demonstrates that SBV was harboured in three species of field-collected Culicoides, namely C. scoticus, C. chiopterus and C. obsoletus sensu stricto. These species were amongst the more abundant of the 15 species found on the livestock holdings sampled during August and September of 2011 in the eastern and north-eastern parts of the Netherlands. The holdings were situated in the heart of the epidemic and of the approximately 100 animals at the dairy in Ermelo more than 96% had seroconverted to SBV. On this same farm large numbers of Culicoides - up to 800/minute at certain hours of the day - were caught regularly while the cattle were at pasture by day, followed by similarly large numbers in light traps operated at the buildings where they were housed each night. Culicoides were particularly abundant in September and October of 2011 and though midge activity declined thereafter, it continued until the beginning of December. The heightened midge abundances were preceded by climatic circumstances that for the Netherlands seemed ideal for Culicoides to propagate in: a wet summer followed by a very mild autumn.

The low Cₜ values obtained by RT-PCR indicate high concentrations of SBV were present in the Culicoides; the fact that Cₜ values in the heads of midges closely matched those obtained from their associated abdomens renders it certain that SBV had replicated to transmissible levels in the midges, and supports the contention that two species of the Obsoletus Complex and C. chiopterus act as natural vectors for SBV. In particular, our findings consolidate the vectorial involvement of the Obsoletus Complex as they mirror preliminary findings made earlier by Rasmussen et al. (7) in Denmark and Goffredo et al. (8) in Italy.
In spite of the relatively large numbers of pools tested and found negative, our findings should not be interpreted to exclude the involvement of other species, such as C. dewulfi or C. punctatus, in the field transmission of SBV.

Besides SBV at least eight other Simbu serogroup viruses have been isolated from Culicoides in various parts of the Old World, predominantly in Japan and Australia and in Africa (26). These include AKAV, AINOV, Douglas virus, Peaton virus and Tinaroo virus, all isolated in Australia from C. brevitarsis (27,28). AKAV has been isolated also from C. wadai (29) in Australia, from C. oxystoma in Japan (30,31), from C. imicola in Oman (32), from C. milnei and C. imicola in Zimbabwe (33) and from a mixed pool comprising mainly C. imicola in South Africa (34). AINOV has been isolated from C. oxystoma and C. punctatus in Japan (31), while SHAV has been isolated from C. imicola in Nigeria (35) and from a mixed species pool of unidentified Culicoides in Japan (36). Finally, Sango virus and Shuni virus have been isolated from unidentified Culicoides in Nigeria (37). Except for C. punctatus the remaining five species of Culicoides listed above, and implicated in the transmission of viruses belonging to the Simbu serogroup, do not occur in north-western Europe.

Experimental infection studies first provided evidence of the involvement of Culicoides in the transmission of a Simbu serogroup virus, namely AKAV (38). It replicated in a laboratory strain of C. variipennis infected orally; virus loads increased 1000-fold and could be transmitted 7-10 days after incubation at 25 °C. In Australia, AKAV has been shown to replicate also in C. brevitarsis, with virus reaching midge salivary glands following 10 days incubation (39).

An issue bedeviling vector research is that nearly all biting midge species involved in the transmission of veterinary pathogens belong to groups of closely related species and so are difficult to identify accurately. To resolve the uncertainty that surrounds their taxonomy, vector midges are being identified increasingly using molecular markers derived from gene regions such as CO1, ITS1, ITS2 and so forth. In this study, the molecular differentiation of C. scoticus and C. obsoletus ss was hampered by the presence of heterozygous alleles within ITS1 and singled out recently as problematic (25). However, significant sequence differences occurred outside of these short polymorphic regions and helped differentiate C. obsoletus ss from C. scoticus. Nevertheless, additional studies are needed to elucidate the exact nature of the polymorphisms within ITS1, especially with regard to the six or more species of the Obsoletus Complex that occur throughout the Palaearctic and Nearctic regions and of which the females are notoriously difficult to identify.

The prevalence of bluetongue virus (BTV) in midges of the Obsoletus Complex collected in Italy in 2002 was approximately 4 to 7 per 10,000, assuming one positive midge per positive pool (40), while during the BT epidemic in the Netherlands in 2006-2007, this was approximately 3 to 16 per 10,000 C. chiopterus and C. dewulfi midges tested respectively, assuming one positive midge
per positive pool (41, 42). In Australia three separate studies showed the prevalence of AKAV in parous, non-engorged Culicoides brevitarsis to be 6 per 10,000 (27), 13 per 10,000 (29) and 19 to 56 per 10,000 (43, 44).

The prevalence of SBV detected in Culicoides midges overall from our study was 25 per 10,000 (15 per 6,100) Culicoides tested. More specifically, the prevalence of SBV in midges from the Obsoletus Complex was 56 per 10,000 (13 per 2,300). This is on the upper side of aforementioned data for AKAV and about ten times higher than those reported for BTV (40). For C. chiopterus our results were 14 per 10,000 (2 per 1,440) midges tested, which is about 5 times higher than those reported for BTV (41).

The high proportion of SBV-infected Culicoides midges is a result of the interplay between host, vector and environment. Lack of transport restrictions in 2011 might have spread infection by infected hosts over long distances and may have started several foci of local transmission. SBV might replicate more efficiently in Culicoides midges or specific Culicoides species compared to other viruses. This has to be determined by experimental vector competence studies, which is currently under investigation at Pirbright (UK) and Wageningen University. Vector biology might have been positively influenced by climatological circumstances in 2011 with a prolonged vector season (several weeks) and a higher survival rate and increased vector abundance (rain in summer and higher temperatures in autumn).

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