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Detection of Schmallenberg virus in different Culicoides spp. by real-time RT-PCR.

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Detection of Schmallenberg virus in different *Culicoides spp.* by real time RT-PCR.

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Running title: SBV detection in *Culicoides* spp.

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Summary:

To identify possible vectors of Schmallenberg virus (SBV), we tested pools containing heads of biting midges (*Culicoides*) that were caught during the summer and early autumn of 2011 at several places in Belgium by real time RT-PCR. Pools of heads originating from following species: *C. obsoletus complex*, *C. dewulfi* and *C. chiopterus* were found positive, strongly indicating that these species are relevant vectors for SBV.

Keywords :

Schmallenberg virus, *Culicoides* spp., vector, transmission

Introduction:

In November 2011, a new virus was identified by researchers from the Friedrich Loeffler Institute (FLI, Germany) that had caused milk drop, diarrhea and fever in adult cattle during the summer of 2011 in Germany and the Netherlands (Hoffmann et al, 2012) and later was shown to be involved in congenital malformations in lambs, calves and goat kids (Herder et al, 2012; Van den Brom et al, 2012). The virus was named Schmallenberg virus (SBV) and belongs to the Simbu serogroup of Orthobunyaviruses. The presence of SBV has in the meantime also been confirmed in the Netherlands, Belgium, United Kingdom, France, Luxembourg, Italy and Spain. The rapid and wide expansion of SBV, together with the knowledge that related viruses like Akabane and Aino virus are spread by midges and mosquitoes (Al-Busaidy and Mellor, 1991; Bryant et al, 2005; St George et al, 1978; Yanase et al, 2005), led to the hypothesis that also SBV might be spread by these vectors. We analyzed pools of midges caught in Belgium to identify putative local vectors for SBV.

Material and Methods:

Culicoides trapping and morphological identification:

The analyzed *Culicoides* were caught at 9 different locations in Belgium. Seven are located in the region of Antwerp (Berlaar, Eindhout, Kessel, Nijlen, Olen, Varendonk, Betekom) and 2 in the region of Luik (Boncelles, Bettincourt) (Figure 1). All are situated in the neighborhood

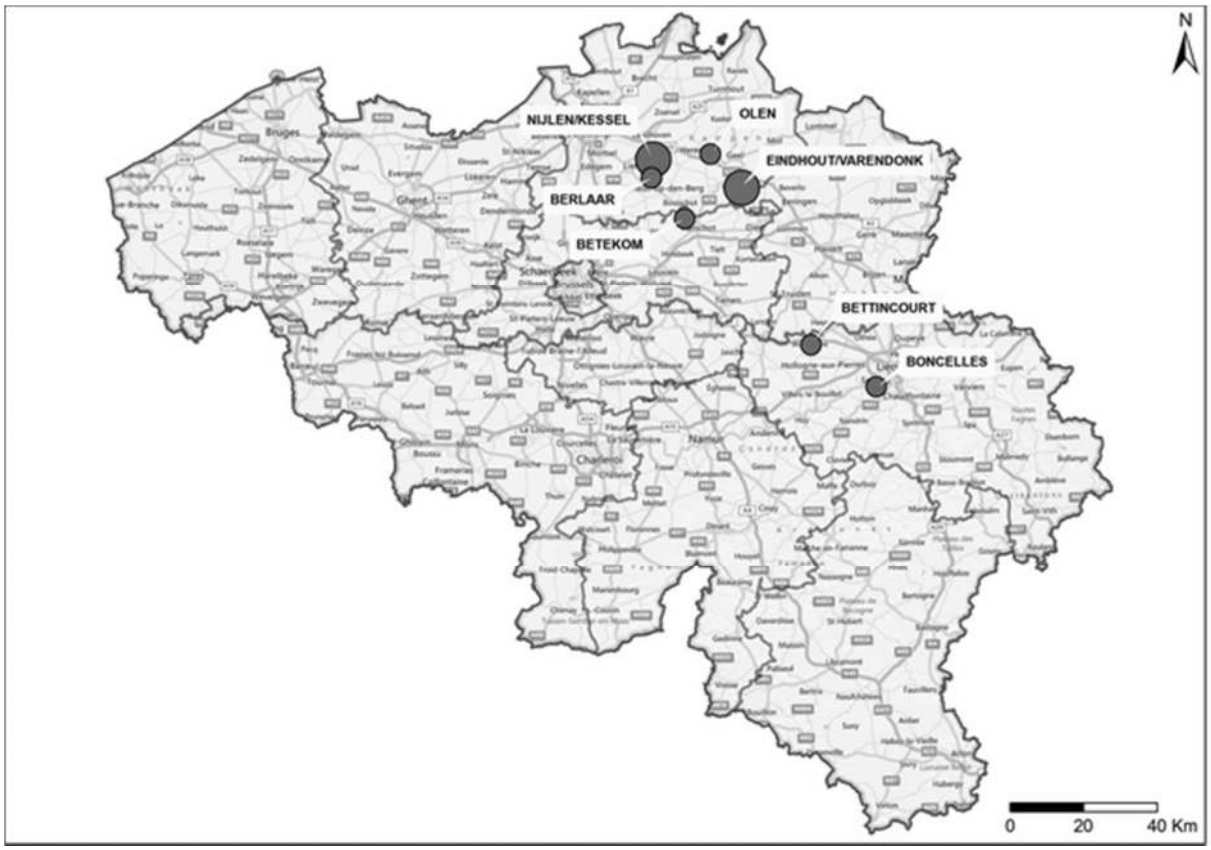

Figure 1: *Culicoides* trapping locations in the regions of Antwerp and Luik.

of sheep and cattle farms where the presence of SBV was confirmed. *Culicoides* were caught using an 'Onderstepoort Veterinary Institute' (OVI) trap (Venter et al, 2009). Traps were deployed for one night each 2 weeks in July, August and September and one night each week in October. Attracted insects were trapped in a container containing 60% ethanol. The biting midges were morphologically identified at species level under the microscope using the key

of Delécolle (Delécolle, 1985) and further stored in 80% ethanol. *C. obsoletus sensu strictu* (*C.obsoletus s.s.*) and *C. scoticus* caught in the region of Antwerp were identified at species level while for the *Culicoides* caught in the region of Luik both species were grouped together in the *C. obsoletus complex*. After the midges were identified, pools of maximum 25 heads of parous females without signs of a recent blood meal were prepared.

rRT-PCR analysis of pools of *Culicoides*:

Pools containing heads of *Culicoides* were analyzed by real-time reverse transcription PCR (rRT-PCR). Therefore each pool was homogenized in 500 µl Trizol (Life Technologies) with a 5mm steel bead (Qiagen) by high speed shaking (3 min, 25 Hz) in a TissueLyser (Qiagen). After phase separation following manufacturer instructions, total RNA in the aqueous phase was extracted using the MagMAX Total Nucleic Acid Isolation kit and the MagMAX Express-24 purification system (Life Technologies). RNA was eluted in 90 µl elution buffer. In each series of 12 homogenizations and RNA extractions, a negative control was included to monitor possible SBV contamination. RNA extracts were analyzed by using the AgPath-ID One Step RT-PCR kit (Life Technologies) in a duplex rRT-PCR combining a SBV rRT-PCR detecting the S segment (Bilk et al, 2012) with a pan-*Culicoides* assay detecting the 18S rRNA (Vanbinst et al, 2009) as an internal control for RNA extraction and amplification. To confirm positive SBV rRT-PCR results, the RNA was subjected to another SBV rRT-PCR detecting the L segment (forward primer: 5'-TTGCCGTTTGATTTTGAAGTTGTG-3'; reverse primer: 5'-TCAGGGATCGCAAATTAAAGAACC-3'; probe: FAM-5'-TCATCCGTGCTGACCCTCTGCGAG-3'-BHQ1; primers and probe sequences werekindly provided by FLI, Germany). Briefly, a master mix consisting of 2,5 µl RNase-free water, 12,5µl 2x RT-PCR buffer, 1,0µl 25x RT-PCR enzyme mix, 2µl SBV-specific primer-probe-mix (10µM SBV-specific primers + 2µM SBV specific probes) and 2 µl IC-specific primer-

probe-mix (10µM 18S rRNA specific primers + 2 µM 18S rRNA specific probe) for one reaction was prepared and 5 µl RNA template was added. For amplification the following temperature profile was used: 10 min at 45°C (reverse transcription), 10 min 95°C (inactivation reverse transcriptase/activation Taq polymerase), followed by 40 cycles of 15s at 95°C (denaturation) and 45s at 60°C (annealing and elongation).

Table 1. (a) Number of *Culicoides* and pools () analyzed by rRT-PCR caught in the region of Antwerp during the period from July to October 2011 and (b) details of pools showing Ct values in rRT-PCR.

a. overview of number of *Culicoides* and pools() analyzed

	July	August	September	October
C. obsoletus s.s.	6 (1)	44 (5)	83 (10)	156 (17)
C. scoticus	-	45 (5)	49 (6)	146 (16)
C. dewulfi	-	20 (2)	29 (5)	83 (10)
C. chiopterus	5 (1)	4 (1)	41 (6)	98 (11)
C. nubeculosus	40 (4)	60 (6)	20 (2)	20 (2)
C. pulicaris	-	3 (1)	12 (2) + 5 (1)*	35 (4) + 4 (1)*
C. punctatus	2 (1)	3 (1)	28 (4)	54 (7)
C. festivipennis	15 (2)	5 (1)	-	-
C. circumscriptus	-	-	10 (1)	-
total	68 (9)	184 (22)	277 (36+1*)	596 (66+1*)

b. overview of pools showing Ct values in rRT-PCR

		Ct value		
	Collection date	Internal control	L segment	S segment
C. obsoletus s.s.(1)	07 Sept 2011	31,7	37,0	34,9
C. obsoletus s.s.(2)	20 Sept 2011	16,3	neg	36,45
C. obsoletus s.s.(3)	06 Sept 2011/ 20 Sept 2011	17,6	neg	36,31
C. pulicaris(1)	07 Sept 2011/ 04 Oct 2011	14,3	neg	37,9
C. dewulfi(1)	04 Oct 2011	16,7	neg	38,1

*This pool contained a mix of heads of *C. pulicaris* caught on September 7 and October 4.

( )This pool contained Culicoides caught at (1)Betekom (51,00200°N; 4,79206°E), (2)Eindhout (51,08618°N; 4,97253°E), (3)Berlaar (51,11861°N; 4,66571°E).

Results and Discussion:

Among the 134 and 44 pools of heads of parous females caught respectively in the region of Antwerp and Luik (Figure 1; Table 1a and Table 2a) that were tested for the presence of SBV,

Table 2. (a) Number of *Culicoides* and pools () analyzed by rRT-PCR caught in the region of Luik during the period from August to October 2011 and (b) details of pools showing Ct values in rRT-PCR.

a. overview of number of *Culicoides* and pools() analyzed

	July	August	September	October
C. obsoletus complex	-	478 (24)	120 (6)	90 (4)
C. dewulfi	-	39 (2)	-	10 (1)
C. chiopterus	-	74 (4)	-	10 (1)
C. pulicaris	-	20 (1)	10 (1)	-
total	-	611 (31)	130 (7)	110 (6)

b. overview of pools showing Ct values in rRT-PCR

		Ct value		
	Collection date	Internal control	L segment	S segment
C. obsoletus complex[(1)]	10 Aug 2011	15,09	neg	35,41
C. obsoletus complex[(2)]	23 Aug 2011	18,09	neg	35,96
C. dewulfi[(2)]	23 Aug 2011	17,11	34,12	32,21
C. obsoletus complex[(1)]	25 Sept 2011	17,96	32,81	30,7
C. obsoletus complex[(1)]	05 Oct 2011	26,78	33,66	34,78
C. obsoletus complex[(1)]	05 Oct 2011	26,67	33,67	32,51
C. chiopterus[(2)]	28 Oct 2011	25,76	29,49	28,69

[( )]This pool contained Culicoides caught at [(1)]Boncelles (50,567739°N; 5,554803°E), [(2)]Bettincourt (50,712872°N; 5,236917°E).

12 pools (5 from the region of Antwerp and 7 from the region of Luik) were found positive in the rRT-PCR detecting the S segment with Ct values varying from 28 to 38 (Table 1b and Table 2b). These positive pools consisted of *C. obsoletus complex*, *C. obsoletus s.s.*, *C. dewulfi*, *C. chiopterus* and *C. pulicaris*. For all species except *C. pulicaris*, at least one pool was also positive in the rRT-PCR detecting the L segment that was performed to confirm the positive status of the pools (Table 1b and Table 2b). The pools with a Ct value of above 35 in the rRT-PCR detecting the S segment that were not confirmed with the rRT-PCR detecting the L segment likely represent low SBV positive pools since all negative controls were continuously negative for SBV and the internal control sequences. The lower sensitivity of the rRT-PCR detecting the L segment in comparison to the rRT-PCR detecting the S segment probably explains why these pools were not confirmed for the L segment and therefor suggest that the rRT-PCR detecting the L segment is not suitable to confirm weak positive pools of

Culicoides. It seems however appropriate that further evidence needs to be obtained before *C. pulicaris* can be considered as a putative vector for SBV since for the moment only one pool of this species showed a positive signal in the rRT-PCR detecting the S segment with a high Ct value of 37,9 which could not be confirmed by the rRT-PCR detecting the L segment (Table 1b).

The finding that pools of *C.obsoletus complex, C. obsoletus s.s, C. dewulfi and C. chiopterus* species were positive for SBV is a strong indication that these species can play an active role in the transmission and spread of SBV. This is further strengthened by the fact that the examined pools consisted exclusively of heads, suggesting that these midges act as real amplification vectors since the virus has reached their salivary glands and were not simply SBV positive after a blood meal on viraemic animals. Our results are in agreement with recent results obtained by Danish colleagues that found 2 pools containing midges belonging to the *C. obsoletus* group caught in Denmark, close to the German border, positive for SBV (Rasmussen et al, 2012). The three species that are identified here as local vectors for SBV have also already been identified previously as vectors for BTV (De Liberato et al, 2005; Dijkstra et al, 2008; Mehlhorn et al, 2007; Meiswinkel et al, 2007; Savini et al, 2005; Vanbinst et al, 2009).

Our results show that SBV already circulated in *Culicoides* in Belgium during August and September 2011. This coincides with the period that the regional Animal Health Care centers (ARSIA and DGZ) in Belgium started receiving notifications of problems such as milk drop and diarrhea on cattle farms where other endemic viruses (e.g. BVD, BTV, IBR) had been excluded. Further on, SBV was also retrospectively confirmed in serum samples collected from 2 Belgian cattle farms in September (ProMedMail, 2012).

Although the number of *Culicoides* tested is limited, our results indicate that during September and October 2011 a considerably high percentage of *Culicoides* were SBV

positive. If one considers that each positive pool of *C. obsoletus s.s.* caught in the region of Antwerp only contained one positive midge, this would result in an infection rate of 3.61% in this species in the month of September in this region. Furthermore, minimum 3 out of 110 *Culicoides* caught in the region of Luik during October 2011 were positive. This relative high number of positive midges could explain the fast and wide spread of SBV during the summer and autumn of 2011.

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