Transboundary and Emerging Diseases

RAPID COMMUNICATION

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

N. De Regge[1,*], I. Deblauwe[2], R. De Deken[2], P. Vantieghem[2], M. Madder[2,4], D. Geysen[2], F. Smeets[3], B. Losson[3], T. van den Berg[1] and A. B. Cay[1]

[1] Veterinary and Agrochemical Research Centre, Brussel, Belgium
[2] Institute of Tropical Medicine, Antwerp, Belgium
[3] University of Liège, Liège, Belgium
[4] Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, Pretoria, South Africa

Keywords:
Schmallenberg virus; *Culicoides* spp.; vector; transmission

Correspondence:
N. De Regge, Groeselenberg 99, 1180 Brussel, Belgium.
Tel.: 0032 (0)2 379 05 80;
Fax: 0032 (0)2 379 06 70;
E-mail: nick.deregge@coda-cerva.be

Received for publication June 15, 2012

doi:10.1111/tbed.12000

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.

Introduction

In November 2011, a new virus was identified by researchers from the Friedrich Loeffler Institute (FLI, Germany) that had caused milk drop, diarrhoea 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 (St George et al., 1978; Al-Busaidy and Mellor, 1991; Bryant et al., 2005; Yanase et al., 2005), led to the hypothesis that also SBV might be spread by these vectors. We analysed pools of midges caught in Belgium to identify putative local vectors for SBV.

Materials and Methods

Culicoides trapping and morphological identification

The analysed *Culicoides* were caught at nine different locations in Belgium. Seven are located in the region of Antwerp (Berlaar, Eindhout, Kessel, Nijlen, Olen, Varendonk, Betekom) and two in the region of Luik (Boncelles, Bettincourt) (Fig. 1). All are situated in the neighbourhood 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,

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

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 analysed by real-time reverse transcription PCR (rRT-PCR). Therefore, each pool was homogenized in 500 μl Trizol (Life Technologies, Paisley, UK) with a 5mm steel bead (Qiagen) by high-speed shaking (3 min, 25 Hz) in a TissueLyser (Qiagen, Hilden, Germany). 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 analysed 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 were kindly provided by FLI, Germany). Briefly, a master mix consisting of 2.5 μl RNase-free water, 12.5 μl 2× RT-PCR buffer, 1.0 μl 25× RT-PCR enzyme mix, 2 μl SBV-specific primer-probe-mix (10 μM SBV-specific primers +2 μM SBV-specific probes) and 2 μl IC-specific primer-probe-mix (10 μM 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 15 s at 95°C (denaturation) and 45 s at 60°C (annealing and elongation).

Results and Discussion

Among the 134 and 44 pools of heads of parous females caught respectively in the region of Antwerp and Luik

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

a. Overview of number of *Culicoides* and pools() analysed

	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 C_t values in rRT-PCR

		C_t value		
	Collection date	Internal control	L segment	S segment
C. obsoletus s.s.(1)	07 September 2011	31.7	37.0	34.9
C. obsoletus s.s.(2)	20 September 2011	16.3	Neg	36.45
C. obsoletus s.s.(3)	06 September 2011/20 September 2011	17.6	Neg	36.31
C. pulicaris(1)	07 September 2011/04 October 2011	14.3	Neg	37.9
C. dewulfi(1)	04 October 2011	16.7	Neg	38.1

()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).

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

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

a. Overview of number of *Culicoides* and pools() analysed

	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 C_t values in rRT-PCR

		C_t value		
	Collection date	Internal control	L segment	S segment
C. obsoletus complex(1)	10 August 2011	15.09	Neg	35.41
C. obsoletus complex(2)	23 August 2011	18.09	Neg	35.96
C. dewulfi(2)	23 August 2011	17.11	34.12	32.21
C. obsoletus complex(1)	25 September 2011	17.96	32.81	30.7
C. obsoletus complex(1)	05 October 2011	26.78	33.66	34.78
C. obsoletus complex(1)	05 October 2011	26.67	33.67	32.51
C. chiopterus(2)	28 October 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).

(Fig. 1; Tables 1a and 2a) that were tested for the presence of SBV, 12 pools (five from the region of Antwerp and seven from the region of Luik) were found positive in the rRT-PCR detecting the S segment with C_t values varying from 28 to 38 (Tables 1b and 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 (Tables 1b and 2b). The pools with a C_t 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 because 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 with the rRT-PCR detecting the S segment probably explains why these pools were not confirmed for the L segment and therefore 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 because for the moment, only one pool of this species showed a positive signal in the rRT-PCR detecting the S segment with a high C_t 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 because the virus has reached their salivary glands and was 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; Savini et al., 2005; Mehlhorn et al., 2007; Meiswinkel et al., 2007; Dijkstra et al., 2008; 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 diarrhoea 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 (ProMED-Mail, 2012).

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

Acknowledgements

We gratefully thank Martin Beer and Bernd Hoffmann from the FLI for the provision of primer/probe sequences for SBV detection by rRT-PCR. We thank Flavien Riocreux for the preparation of the map with *Culicoides* trapping locations. Monitoring of *Culicoides* spp. in Belgium is financed by the Belgian Federal Agency for the Safety of the Food Chain (FAVV-AFSCA).

References

Al-Busaidy, S. M., and P. S. Mellor, 1991: Isolation and identification of arboviruses from the Sultanate of Oman. *Epidemiol. Infect.* 106, 403–413.

Bilk, S., C. Schulze, M. Fischer, M. Beer, A. Hlinak, and B. Hoffmann, 2012: Organ distribution of Schmallenberg virus RNA in malformed newborns. *Vet. Microbiol.* 159, 236–238.

Bryant, J. E., M. B. Crabtree, V. S. Nam, N. T. Yen, H. M. Duc, and B. R. Miller, 2005: Isolation of arboviruses from mosquitoes collected in northern Vietnam. *Am. J. Trop. Med. Hyg.* 73, 470–473.

De Liberato, C., G. Scavia, R. Lorenzetti, P. Scaramozzino, D. Amaddeo, G. Cardeti, M. Scicluna, G. Ferrari, and G. L. Autorino, 2005: Identification of *Culicoidesobsoletus* (Diptera: ceratopogonidae) as a vector of bluetongue virus in central Italy. *Vet. Rec.* 156, 301–304.

Delécolle, J. C. 1985: Nouvelle contribution à l'étude systématique et iconographique des espèces du genre *Culicoides* (Diptera: Ceratopogonidae) du nord-est de la France. PhD thesis, University of Strasbourg, Strasbourg.

Dijkstra, E., I. J. van der Ven, R. Meiswinkel, D. R. Hölzel, P. A. Van Rijn, and R. Meiswinkel, 2008: *Culicoideschiopterus* as a potential vector of bluetongue virus in Europe. *Vet. Rec.* 162, 422.

Herder, V., P. Wohlsein, M. Peters, F. Hansmann, and W. Baumgärtner, 2012: Salient lesions in domestic ruminants infected with the emerging so-called Schmallenberg virus in Germany. *Vet. Pathol.* 49, 588–591.

Hoffmann, B., M. Scheuch, D. Höper, R. Jungblut, M. Holsteg, H. Schirrmeier, M. Eschbaumer, K. V. Goller, K. Wernike, M. Fischer, A. Breithaupt, T. C. Mettenleiter, and M. Beer, 2012: Novel orthobunyavirus in cattle, Europe, 2011. *Emerg. Infect. Dis.* 18, 469–472.

Mehlhorn, H., V. Walldorf, S. Klimpel, B. Jahn, F. Jaeger, J. Eschweiler, B. Hoffmann, and M. Beer, 2007: First occurence of *Culicoidesobsoletus*-transmitted Bluetongue virus epidemic in Central Europe. *Parasitol. Res.* 101, 219–228.

Meiswinkel, R., P. Van Rijn, P. Leijs, and M. Goffredo, 2007: Potential new *Culicoides* vector of bluetongue virus in northern Europe. *Vet. Rec.* 161, 564–565.

ProMED-Mail. 2012. Schmallenberg virus – Europe (06): update, Belgium [cited 2012 Jan 17]. Available at http://www.promedmail.org, archive no. 20120117.1012402 (accessed 21 September 2012).

Rasmussen, L. D., B. Kristensen, C. Kirkeby, T. B. Rasmussen, G. J. Belsham, R. Bødker, and A. Bøtner, 2012. Culicoids as vectors of Schmallenberg virus [letter]. *Emerg. Infect. Dis*. 18, 1204–1206.

Savini, G., M. Goffredo, F. Monaco, A. Di Gennaro, M. A. Cafiero, L. Baldi, P. de Santis, R. Meiswinkel, and V. Caporale, 2005: Bluetongue virus isolations from midges belonging to the *Obsoletus complex* (Culicoides, Diptera: Ceratopogonidae) in Italy. *Vet. Rec.* 157, 133–139.

St George, T. G., H. A. Standfast, and D. H. Cybinski, 1978: Isolations of akabane virus from sentinel cattle and *Culicoidesbrevitarsis*. *Aust. Vet. J.* 54, 558–561.

Van den Brom, R., S. J. Luttikholt, K. Lievaart-Peterson, N. H. Peperkamp, M. H. Mars, W. H. van der Poel, and P. Vellema, 2012: Epizootic of ovine congenital malformations associated with Schmallenbergvirus infection. *Tijdschr. Diergeneeskd.* 137, 106–111.

Vanbinst, T., F. Vandenbussche, E. Vandemeulebroucke, I. De Leeuw, I. Deblauwe, G. De Deken, M. Madder, E. Haubruge, B. Losson, and K. De Clercq, 2009: Bluetongue virus detection by real-time RT-PCR in *Culcoides* captured during the 2006 epizootic in Belgium and development of an internal control. *Transbound Emerg. Dis.* 56, 170–177.

Venter, G. J., K. Labuschagne, K. G. Hermanides, S. N. B. Boikanyo, D. M. Majatladi, and L. Morey, 2009: Comparison of the efficiency of five suction light traps under field conditions in South Africa for the collection of Culicoides species. *Vet. Parasitol.* 166, 299–307.

Yanase, T., T. Kato, T. Kubo, K. Yoshida, S. Ohashi, M. Yamakawa, Y. Miura, and T. Tsuda, 2005: Isolation of bovine arboviruses from *Culicoides* biting midges (Diptera: Ceratopogonidae) in southern Japan: 1985–2002. *J. Med. Entomol.* 42, 63–67.