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RAPID COMMUNICATION

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Evidence of extensive renewed Schmallenberg virus circulation in Belgium during summer of 2016 – increase in arthrogryposis-hydranencephaly cases expected

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Summary

A seroprevalence study carried out between June and September 2016 in the Belgian sheep population showed a significant increase in overall (from 25% to 62%) and between-herd (from 60% to 96%) seroprevalence against Schmallenberg virus (SBV) during this period, indicating the most extensive recirculation of SBV since its original emergence in 2011. SBV recirculation was confirmed by the detection of SBV RNA-positive *Culicoides obsoletus* complex midges collected in the region of Antwerp in August 2016, reaching a minimum infection rate of 3%. The recirculation of SBV in the largely unprotected ruminant population during summer 2016 will likely cause an increase in the number of arthrogryposis-hydranencephaly cases in newborn ruminants during the coming months.

KEYWORDS

Belgium, *Culicoides*, entomological monitoring, Schmallenberg virus, seroprevalence, sheep

1 | INTRODUCTION

Schmallenberg virus (SBV) belongs to the family *Bunyaviridae*, genus *Orthobunyavirus*, Simbu serogroup (Hoffmann et al., 2012) and emerged for the first time at the end of 2011 in North-western Europe. SBV induces only mild symptoms in adult ruminants but leads to congenital malformations in lambs, goat kids, and new born calves (Bayrou, Garigliany, Cassart, Jolly, & Desmecht, 2013). Significant production losses related to SBV infection have been reported in sheep (Barrett, O'Neill, Sammin, Clegg, & More, 2015; Poskin et al., 2017). Its pathogenesis and epidemiology closely resembles that of Akabane virus (AKAV), the most studied member of the Simbu serogroup seen its veterinary importance. *Culicoides* midges are assumed to be the most important biological vectors of SBV. *Culicoides obsoletus* complex (including *C. obsoletus* s.s. and *Culicoides scoticus*), *Culicoides chiopterus*, and *Culicoides dewulfi* midges have been proposed to be putative SBV vectors (De Regge et al., 2012, 2015; Elbers, Meiswinkel, van Weezep, Kooi, & van der Poel, 2013; Elbers, Meiswinkel, van Weezep, van Oldruitenborgh-Oosterbaan, & Kooi, 2013; Goffredo et al., 2013; Larska, Lechowski, Grochowska, & Zmudziński, 2013; Larska, Polak et al., 2013; Rasmussen et al., 2012).

After the first SBV identification in Germany in 2011, SBV spread rapidly and widely over a large part of Europe (EFSA, 2014). Belgium was one of the first and most SBV affected countries, whereby virtually all Belgian sheep and cattle herds had been in contact with SBV at the end of the first vector season of 2011 (Méroc, Poskin, Van Loo, Quinet et al., 2013; Méroc et al., 2014). Evidence for renewed SBV circulation was found in 2012 and SBV seropositive animals were still detected at each cattle farm at that time but the overall seroprevalence in cattle had dropped from 86% to 65% (Méroc, Poskin, Van Loo, Van Dressche et al., 2013). Since 2013, no more confirmed SBV cases were reported and efforts to follow the SBV situation in Belgium were strongly reduced (Poskin et al., 2016). The lack of significant SBV circulation and associated losses since that time makes that governments, veterinarians, and farmers tend to lose their awareness for this disease (De Regge 2016; De Regge et al., 2015).

Detection of SBV RNA in three aborted calves in April 2016, however, provided evidence of limited renewed SBV circulation in Belgium in 2015 (Delooz et al., 2016). It was therefore decided to perform a cross-sectional seroprevalence study in sheep between June and September 2016 to study the evolution of seroprevalence

against SBV. The potential circulation of SBV was also studied by entomological monitoring of *Culicoides* midges caught during that period.

2 | MATERIALS AND METHODS

2.1 | Seroprevalence study design and ELISA testing of collected serum samples

This study represents an opportunistic sampling of sheep serum collected between June and September 2016 as part of the Maedi-Visna programme for trade certification (Royal Decree 24-03-1993). Within this voluntary program, all sheep older than 1 year are sampled if flocks contain less than 50 animals. According to the data in the Sanitel database of 2011, the Belgian sheep population accounted 216.018 animals distributed over 27.253 herds, indicating a mean herd size of eight animals. The median herd size however is four animals, being in line with the fact that Belgium has mostly small-scale sheep holders having only a few animals. All participating farms to the program that had four or more animals were included in this study, and we tested a maximum of seven samples per farm. The choice to omit farms with less than four animals from the study was made for budgetary reasons, and it should be considered that this might have introduced some bias to the results. The median number of sheep in the tested herds was 12, while the medium number of tested samples per herd was 7. A total of 501 samples originating from 81 farms distributed all over Belgium (Figure 1) were tested for the presence of SBV-specific antibodies. A commercially available ELISA kit (ID Screen®, Schmallenberg virus, competition multi-species, ID-vet, France) was used for the detection of SBV-specific antibodies following manufacturer's instructions. Doubtful ELISA results were considered as negative during data analysis. The results were only valid if the control samples included in the kit fulfilled the prescribed conditions. For the purpose of this study, a herd was considered positive if at least one of the sampled animals was positive.

2.2 | *Culicoides* trapping and morphological identification

The *Culicoides* analyzed in this study were caught at five different locations in the region of Antwerp (Varendonk, Berlaar, Nijlen, Olen, and Viersel) in Belgium (Figure 1). All are situated in the neighborhood of cattle farms. *Culicoides* were caught using an "Onderstepoort Veterinary Institute" (OVI) trap (Venter et al., 2009). Collection was carried out once a month (30/6-01/07/16, 08/08-10/08/16, 5/09-07/09/16) during the study period, and traps were deployed for two consecutive nights. Attracted insects were trapped in a container

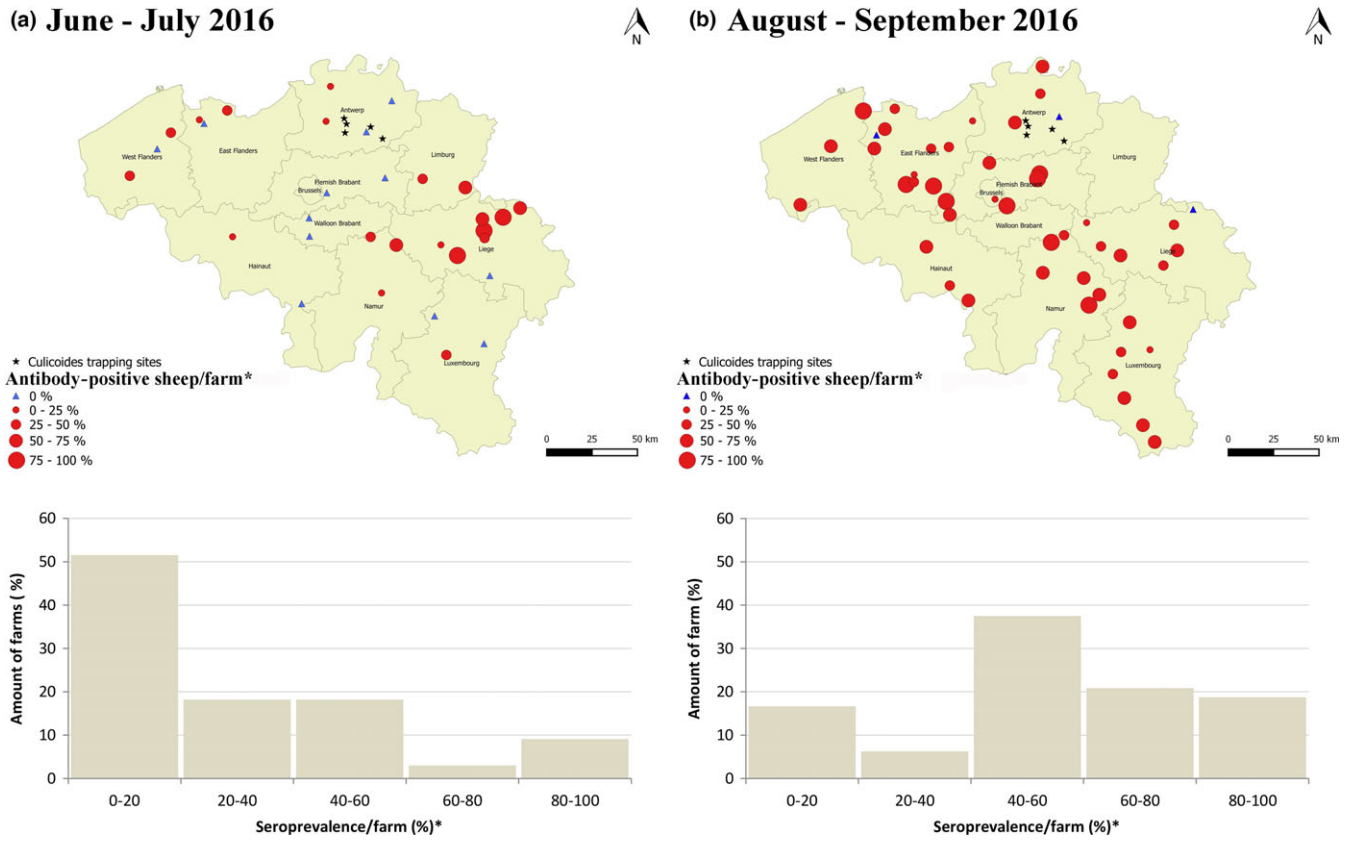

FIGURE 1 Geographical location of studied farms and antibody-positive sheep/farm in the period June-July 2016 (a) and August-September (b) 2016 in Belgium. *Determined on $4 \leq x \leq 7$ animals sampled per farm

TABLE 1 Estimated overall and estimated between-herd seroprevalence

Month	Farms	Animals	Estimated overall seroprevalence (%)	Estimated between-herd seroprevalence (%)
June	20	121	25 (95% CI: 15–39)	60 (95% CI: 38–79)
July	13	79	30 (95% CI: 17–48)	62 (95% CI: 34–85)
August	23	142	46 (95% CI: 36–56)	96 (95% CI: 75–99)
September	25	159	61 (95% CI: 51–70)	96 (95% CI: 77–99)

with 60% ethanol. The biting midges were morphologically identified at species level with a stereomicroscope using a dichotomous key (Delécolle, 1985) and stored in 80% ethanol. Only individuals belonging to the *C. obsoletus* complex, comprising *C. obsoletus* s.s. and *C. scoticus* that are difficult to distinguish morphologically, were selected and pooled. Per collection place and time point, a maximum of 10 pools of 20 parous females without signs of a recent bloodmeal of the *C. obsoletus* complex were prepared.

2.3 | rRT-PCR analysis of pools of *Culicoides*

Pools of *C. obsoletus* complex midges were analyzed by real-time reverse transcription PCR (rRT-PCR) for the presence of SBV RNA as described before (De Regge et al., 2012). Briefly, each pool was homogenized in Trizol (Life Technologies, Paisley, UK) and 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 extracts were analyzed for the presence of SBV RNA by a duplex rRT-PCR combining detection of the SBV S segment (Bilk et al., 2012) and 18S rRNA of *Culicoides* (Vanbinst et al., 2009), using the AgPath-ID One Step RT-PCR kit (Life Technologies).

2.4 | Statistical analysis and maps

The generalized estimating equations (GEE) model (Liang & Zeger, 1986) was used to estimate the overall seroprevalence and 95% confidence intervals for each month as this model allows taking into account the correlation between animals that belong to the same herd. The between-herd seroprevalence and 95% confidence intervals for each month were estimated using a generalized linear model. For the purpose of this study, a herd was considered positive if at least one of the sampled animals was positive. All statistical analyses were performed using SPSS statistics software version 23 (IBM). Maps showing positive and negative sheep farms, *Culicoides* trapping sites and the within-herd seroprevalence per farm were produced using QGIS, version 2.12.3.

3 | RESULTS AND DISCUSSION

A total of 501 sheep sera originating from 81 farms distributed all over Belgium (Figure 1) collected between June and September 2016 were tested. Five percent of the samples were classified as doubtful using manufacturer's instructions and were considered negative in our analysis.

During June and July, the overall and between-herd seroprevalence was constant at about 25% and 60%, respectively (Table 1). This is clearly lower than the previously reported overall seroprevalence and between-herd seroprevalence of, respectively, 65% and 100% reported at the end of the vector season of 2012 (Méroc, Poskin, Van Loo, Van Driessche et al., 2013). This seems to be in line with the general assumption that the SBV circulation in Belgium and other European countries has ceased since 2012, leading to a decline in the proportion of sheep that have been into contact with the virus and harbor SBV-specific antibodies (Collins, Barrett, Doherty, Larska, & Mee, 2016; Gache et al., 2017; Poskin et al., 2015; Stokes, Baylis, & Duncan, 2016; Wernike, Holsteg, Sasserath, & Beer, 2015).

Interestingly, an increase in both overall and between-herd seroprevalence occurred in August 2016. The overall seroprevalence rose to 46% and SBV seropositive animals were found in almost every herd (96%) under examination, indicating a fast transmission of the virus all over the country within a time period of 1 month (Figure 1). In September, the overall seroprevalence increased further to 62% (Table 1), being significantly higher than the overall seroprevalence in June and July and reaching similar levels as observed after the initial emergence in 2011 (Méroc et al., 2014). Figure 1a shows that most sampled animals were SBV negative in June and July or that only few SBV antibody-positive animals were detected. These latter probably reflect remaining animals that got infected during the initial SBV emergence in 2011 or in the years thereafter. In some farms from Liège and Namur, however, between 50% and 100% of the sampled animals were found SBV antibody-positive at that time. The high within-herd seroprevalence in these provinces seems to be in line with the detection of some aborted SBV RNA-positive calves in those regions in spring 2016 by the Belgian reference laboratory (CODA-CERVA (Veterinary and Agrochemical Research Centre), personal communication; Delooz et al., 2016), further indicating that an SBV recirculation took already place in this region in 2015.

It is important to mention that although we used all available samples that were submitted within the Maedi/Visna programme, the limited number of tested herds leads to less precise estimations

TABLE 2 Overview of pools of *Culicoides obsoletus* complex midges tested for Schmallenberg virus in rRT-PCR, () SBV-positive samples

	July 2016	August 2016	September 2016
Varendonk	9	2	7
Berlaar	10	1 (1)	1
Nijlen	10	2 (2)	3
Olen	10	10 (6)	10
Viersel	10	10 (6)	10
Total	49	25 (15)	31

Number of positive pools is indicated between brackets—each pool consists out of 20 parous females.

of overall and between-herd seroprevalence and that this has to be taken into account during interpretation of the results. The number of animals that were tested per flock is however appropriate, given that the median size of Belgian sheep flocks is 4 and that a median number of seven animals per flock were tested here. Furthermore, as the seroprevalence study had a cross-sectional design and animals over 1 year old were included, it cannot be completely ruled out that the significantly higher percentage of SBV seropositive animals detected from August 2016 onwards is not due to sampling of animals exposed to SBV before 2016. Therefore, we further investigated the possibility of active SBV transmission during August 2016 via entomological surveillance.

A total of 105 pools representing 2,100 midges of the *C. obsoletus* complex collected in the region of Antwerp were tested between July and September 2016 (Table 2). Among the 105 pools, 15 pools were found positive in the rRT-PCR detecting the S segment with C_t values varying from 26 to 36 (Table 3), thereby irrefutably showing that active SBV circulation has taken place in 2016. Interestingly, SBV-positive pools were only detected in midges caught in August. Positive pools were found at four of the five trapping locations and 60% (15 of 25) of the pools were positive at that time point (Table 2). If one considers that each positive pool contained only one positive midge, a minimum infection rate of 3% (15/500) was present in *C. obsoletus* complex midges in August in the region of Antwerp. Similar minimum infection rates of 3.6% in *C. obsoletus* s.s. midges in September 2011 in Antwerp and 3.1% in *C. obsoletus* complex midges in October 2011 in Liège have been reported in Belgium during the initial SBV emergence (De Regge et al., 2012). Despite the examination of a higher number of midges in July (49 pools–980 midges) and September (31 pools–620 midges), no SBV-positive pools were found at those time points. Our results therefore confirm that entomological SBV surveillance is an important non-invasive surveillance method but that it must be done on a continuous basis in order not to miss the mostly short-lived virus circulation in a specific region (De Regge et al., 2014; Kameke, Werner, Hoffmann, Lutz, & Kampen, 2016).

Taken together, our results strongly suggest that 5 years after the initial emergence of SBV in Belgium and the rest of Europe, a renewed large-scale circulation took place in the summer of 2016. This again establishes a clear homology between the epidemiology of SBV and the related Akabane virus as it is described that epizootics of Akabane virus also tend to occur at 4- to 6-years intervals, coinciding with a decrease in population immunity against the virus (CFSPH, 2016; Kono et al., 2008).

A follow-up of the situation in the field will be necessary to measure the consequences of this renewed virus circulation in the summer of 2016, but based on the outcome of the initial SBV outbreak in 2011, it is to be expected that we will be confronted with an increase in the number of arthrogryposis-hydranencephaly cases in newborn ruminants in the coming months. Compared to the initial outbreak of 2011 where the highest SBV circulation in Belgium was detected in September and October (De Regge et al., 2015), the most important virus circulation in 2016 seems to have taken place in August. This could imply that many sheep might have already been protected against infection before the mating season started in autumn 2016, thereby potentially leading to a lower impact on the ovine sector than observed during the 2011 epidemic.

TABLE 3 Detailed overview of C_t values obtained for Schmallenberg virus RNA-positive pools of *Culicoides obsoletus* complex midges collected between 8 August 2016 and 10 August 2016

Sampling site	Internal control	S segment
Viersel	14.1	26.07
Viersel	14.48	33.76
Viersel	14.23	27.69
Viersel	12.6	31.84
Viersel	13.88	32.85
Viersel	15.82	31.93
Nijlen	13	32.96
Nijlen	13.76	36.5
Berlaar	13.12	33.93
Olen	13.47	30.24
Olen	13.58	33.67
Olen	15.1	33.57
Olen	13.73	33.91
Olen	13.44	32.62
Olen	14.38	34.18

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