Schmallenberg virus detection in *Culicoides* biting midges in Spain. First laboratory evidence for highly efficient infection of *C. imicola* and *C. obsoletus* s.l.

Short title: SBV infection in *Culicoides*, Spain.

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Summary

Since Schmallenberg disease was discovered in 2011, the disease rapidly spread across Europe silently. *Culicoides* biting midges have been implicated as putative Schmallenberg vectors in Europe. The detection of Schmallenberg virus (SBV) in field collected *Culicoides* was evaluated through retrospective (2011-2012) collections and prospective (2013) captures. The present study represents the first detection of SBV in field collected *Culicoides* in Spain. Infectious midges were detected at the foothills of Pyrenees, Aramunt, in the summer 2012. All the specimens infected with Schmallenberg in nature were of the species *C.obsoletus* s.s. confirming its putative vector status in Spain. Experimental infection on field collected *Culicoides* evidenced atypical high efficiency for SBV vector infection and transmission potential in local populations of *C. imicola* and in *Culicoides* of the Obsoletus group. However captured individuals of *C. imicola* were more sensible to SBV infection than *C. obsoletus* s.l. ($p<0,001$).

Keywords: SBV, *Culicoides*, vector, arbovirus, outbreak, vector competence, infection.

Introduction

In autumn 2011 a novel *Orthobunyavirus* virus, family *Bunyaviridae*, was described in ruminants in Germany (Hoffmann et al, 2013). The virus was named *Schmallenberg* (SBV) and is placed within the Simbu serogroup, known to be arthropod-borne. *Culicoides* biting midges have been implicated as putative SBV vectors in Europe (Balenghien et al, 2012). This statement is in accordance with other SBV-related viruses in the Simbu serogroup which have been isolated from midges or mosquitoes (Saeed et al, 2001). The disease is characterized by a rather mild or subclinical infection with short viremia. Animals could suffer from fever, milk yield reduction, diarrhoea, congenital foetal malformations and still birthds (Hoffmann et al, 2013)

Schmallenberg disease was prevalent in central and northern Europe in 2011. The disease provoked a major and silent epizootic that rapidly spread across Europe. In Spain the first SBV case was officially declared in Córdova (Andalusia) by March 2012 (EFSA, 2013). Several studies detected SBV in collected *Culicoides* in central and northern Europe. Such studies suggested that *Culicoides* species in the Obsoletus group were the putative SBV vectors involved in outbreaks (reviewed in Balenghien et al, 2012).

In order to determine which *Culicoides* species were involved in SBV transmission in Spain, we adopted two strategies. First, a longitudinal retrospective analysis was implemented in two farms of the Bluetongue (BT) Entomological Surveillance Program. Secondly, prospective collections were performed in a farm at the moment where seroconversions were detected. Subsequent experiments were devoted to assess, in laboratory conditions, SBV vector competence in field collected *Culicoides* in order to assess their likelihood for virus infection and transmission.

Materials and Methods

A retrospective screening on *Culicoides* biting midges was performed in order to detect the presence of SBV. *Culicoides* were weekly collected for one night since January 2011 until December 2012 at two livestock farms, Aramunt, Lleida (X: 0.987801, Y: 42.20609; 650 meters above sea level (masl)) and Caldes de Malavella, Barcelona (X: 2.849819, Y: 41.843908; 84 masl). Both farms belong to the Spanish BT Entomological Surveillance network and were settled with sheep. A prospective screening was performed in *Culicoides* in a farm, Vic, Barcelona (X: 2.235692, Y: 41.903272; 484 masl) at that time cattle were seroconverting for SBV (May 2013). *Culicoides* collections (n=10) were implemented at Vic for two months during May (n=4) and June (n=6) 2013. Aramunt, Caldes de Malvella and Vic are located in Catalonia Autonomous Region (Spain). *Culicoides* were collected using CDC black-light traps (John W. Hock Company, Gainesville, USA).

Culicoides were identified under stereomicroscope according to their pattern of wing pigmentation (Delécolle, 1985). Individuals were age graded by physiological status through observation of abdominal pigmentation according to Dyce (1999). *Culicoides* parous females belonging to the subgenera *Avaritia* and *Culicoides* were used for SBV screening. Briefly, for each individual *Culicoides*, the head was excised and removed from the rest of the body midge using sterilized material under stereomicroscope. Both parts, head and body, were stored as paired samples at -20ºC until further molecular processing.

Culicoides heads were pooled according to species, farm and date, not exceeding the amount of 50 heads/pool. Viral RNA was extracted using NucleoSpin RNA Virus (Macherey Nagel, Germany) following the manufacturer's instructions and quantified using the one step real-time reverse transcription quantitative PCR (rRT-PCR) targeting

85 the SBV S3 genomic fragment (Bilk et al, 2012). Samples with cut-off (Ct) values
86 comprised between 38 and 42 were considered doubtful and retested for confirmation.
87 When a pool of heads was confirmed positive to SBV, the corresponding individual
88 bodies were tested for SBV by rRT-PCR. SBV positive bodies from the Obsoletus
89 group were identified to species with a COI (Cytochrome oxidase I) specific PCR test
90 (Nolan et al, 2007).
91 *Culicoides* midges were experimentally infected with SBV using the blood of a
92 viraemic sheep. The blood had a viral load of 10^5 $TCID_{50}$/ml and was used directly to
93 feed *C. imicola* and *Culicoides* of the Obsoletus group that were collected at Caldes de
94 Malavella and Massanes (X: 2.638871 ,Y: 41.765207; 100masl) respectively. One
95 laboratory colony of *C. nubeculosus* was tested as well. Bloodfed females were
96 maintained for an extrinsic incubation period (EIP) of 10 days and supplemented with
97 5% sucrose *at libitum*. A constant regime of temperature (24±2ºC), humidity (80%RH)
98 and photoperiod (14:10; light:dark) was used. After the EIP females were processed for
99 SBV RNA detection as described above but head and body individually tested.
100 Presence of antibodies to SBV on cattle was tested (Vic calves) with an ELISA SBV
101 test (ID Screen® Schmallenberg virus Indirect ELISA kit, Id-Vet, France) according to
102 manufacturer recommendations.

103

104

105 **Results**

106 The retrospective analysis revealed the presence of SBV RNA in two of the 309 pools
107 of *Culicoides* heads tested by rRT-PCR (Table 1). The positive pools, collected during
108 summer 2012 in a (sheep) farm, contained heads of *Culicodies* assigned to the
109 Obsoletus group, which were the most abundant during 2011 and 2012 in Aramunt.

110 None of the 88 pools (1967 parous females) of the *Culicoides* trapped during 2011
111 season was positive for SBV (Table 1). However, in 2012, two out of 66 pools were
112 positive for SBV. One pool contained the heads of 25 specimens trapped at week 27
113 (W27; 05/07/2012) with Ct value 25.27 (Table 2). The individual bodies corresponding
114 to the heads in the positive pool were tested to determine real infection rates leading to
115 seven positives for SBV, with Ct values comprised between 24.4 and 41.37. However,
116 the sample with initial Ct value 41.37 was negative in the second SBV test. The six
117 confirmed SBV positive bodies were genetically diagnosed as *C. obsoletus* s.s. (Table
118 2). The second SBV positive pool had 50 heads from specimens trapped at week 28
119 (W28, 12/07/2012) and a Ct value of 37.27 (Table 2). In that case, a single body
120 (diagnosed as *C. obsoletus* s.s., Table 2) was positive with Ct value 30.14. The same
121 week (W28), another pool of the Obsoletus group with 15 heads tested negative for
122 SBV. Other SBV negative species trapped at Aramunt during 2011-2012 were of the
123 Pulicaris and Newsteadi groups, and *C. flavipulicaris* (Table 1).

124 In Caldes de Malavella, *C. imicola* was the most abundant species in 2011 whereas in
125 2012 it was the Obsoletus group (Table 1). In that farm, 4208 *Culicoides* (heads) were
126 grouped in 155 pools and all tested negative for SBV. Details on the number of pools
127 and individuals per year and species are depicted in Table 1.

All the 136 *Culicoides* pools from the prospective collections at Vic farm tested negative for SBV (Table 1).

Laboratory SBV vector competence assays were performed using the blood of a viraemic sheep to better understand transmission patterns via host to vector. Individuals of a *C. nubeculosus* laboratory colony failed to maintain SBV at detectable levels after the EIP (table 3). Contrarily, field collected *C. imicola* and *Culicoides* species of the Obsoletus group showed highly efficient infection rates, with a mean rate of 0.81 and 0.50 for *C. imicola* and the Obsoletus group respectively on individual excised heads (table 3). The species *C. imicola* was more sensible, or competent, towards SBV infection than Obsoletus group (Fisher exact test between percentages $p<0.001$). After the EIP, Ct values were lower (in bodies and heads) than values obtained for newly engorged females (0 dpi; table 3 and figure 1).

Discussion

The retrospective analysis detected SBV RNA in *Culicoides* collected at the foothills of the Pyrenees in summer 2002 (July). Interestingly, the temporal detection of infected *Culicoides* was coincident with the earliest serological evidence of SBV in domestic and wild ruminants in the neighbouring National Game Reserve of Freser-Setcases in 2012 (Fernandez-Aguilar et al, 2014). There, serology was negative for wild and domestic ruminants in 2011. It is worth to mention that the current putative SBV vectors of the Obsoletus group were described to be in close contact with wild and domestic ruminants in the same mountainous region (Talavera et al, 2015). Our results are in agreement with those obtained in northern and central Europe where SBV was primarily detected in pools of the Obsoletus complex (reviewed in Balenghien et al, 2014). Thus, results supported the implication of *Culicoides* of the Obsoletus group in the SBV large scale dissemination across Europe. Important differences are found between countries considering the particular infected species. In our study all infected specimens were genetically identified as *C. obsoletus* s.s. In Belgium and France a prominent role was attributed to *C. obsoletus* s.s. as well. However, in the Netherlands most positive samples were of *C. scoticus* s.s. and in Denmark, species involved were *C. dewulfi* and *C. chiopterus*. Interestingly, Belgium and the Netherlands performed tests on heads as well.

When SBV seroconvertions were detected in cattle at Vic farm, entomological prospective collections were implemented. *Culicoides* collected tested negative for SBV. This fact was not surprising as *Culicoides* collections were implemented short after animals began to seroconvert. Similarly, *Culicoides* (most *C. imicola*) tested negative at farms reporting SBV abortions in Italy (Balenghien et al, 2014). In Denmark, however, a longer time frame (5 weeks) for SBV positive pools detection was

reported (Rasmussen et al, 2014). Unfortunately, in this study no information on SBV seroprevalence or seroconversion was made available. At Vic farm, two blood serial samples were taken from 8th to 28th April 2013. Although 6 out 36 calves seroconverted to SBV specific antibodies, no SBV detection was done on *Culicoides* captured at the same time.

The presence of SBV RNA in the head of parous females would imply that the infection successfully reached salivary glands (located at the fore-thorax) and SBV was released into saliva. Thus, the field collected specimens examined here, were expected to infect a naïve host through bite. Nevertheless, similar studies as such performed on BT transmission (Pagès et al, 2014) are necessary to confirm SBV transmission dynamics via vector to host. In order to better understand SBV transmission patterns via host to vector, *Culicoides* were orally infected with SBV using the blood of a viraemic sheep. The *C. nubeculosus* colony was refractory to SBV infection. This was in accordance with a previous study that indicated low SBV vector competence for the same colony (Balenghien et al, 2014). However, high susceptibility to infection was evidenced for field collected *Culicoides* of the species *C. imicola* and *Culicoides* of the Obsoletus group. Results suggested that midge secondary tissues supported an efficient replication of SBV. Moreover, infection values obtained for excised heads suggested the virus infecting the salivary glands (at thorax) would be released into the saliva as evidenced by low Ct values of heads.

The transmission cycle of SBV seems to be ephemeral at individual herd level. This would result from interaction of two factors influencing SBV transmission dynamics: short viremia in hosts (Wernike et al, 2012; Poskin et al, 2014) and early appearance of long lasting host immunity (Poskin et al, 2015). However, the disease spread rapidly from North to South Europe suggesting either, rapid movement of host and/or vectors or

a high SBV infection and transmission rates for certain *Culicoides* species (see table 3). Our preliminary results on SBV vector competence would conciliate the above apparent contradictory observations. Thus, *Culicoides* would infect the vast majority of naïve animals at herd in few days. Infected animals rapidly produce neutralizing Ab that block further effective host to vector transmission. Before herd immunity, during the short viraemia, high vector competence will insure geographical dissemination of sufficient infected vectors to propagate SBV in naive animals. Such scenario was described when most ruminants in affected herds seroconverted after SBV incursion (Elbers et al, 2012; Elbers et al, 2015; Meroc et al, 2013; Rodríguez-Prieto et al, 2014). Our data supports such hypothesis. At laboratory, experimental infection assays revealed that field collected *Culicoides* had very high SBV infection rates (ranging from 0.5 to 0.81). At farm, a high infection rate of 0.24 (6/25 *Culicoides*) was detected, suddenly, in Obsoletus group midges collected at W27 in Aramunt. One week after (W28), the infection rate decreased to 0.015 (1/65 *Culicoides*) and no additional infected *Culicoides* were detected in the 14 pools collected between W29 and W47. The approach we used to detect SBV RNA in *Culicoides* heads, as opposed to the entire midge, gives a better approximation not only on the number of infective vectors but also in identifying potential SBV transmitting species.

The present study provides not only the first insights on the detection of SBV naturally infected *Culicoides* in Spain but also on SBV vector competence laboratory assays for Palaearctic *Culicoides* field populations. The assays revealed a very high susceptibility to SBV infection for *Culicoides* of the Obsoletus group and *C. imicola*. The Obsoletus group is virtually present all over Europe whereas *C. imicola* remains restricted to the Mediterranean basin for requiring warmer conditions. We hypothesised that the short SBV viraemia of hosts and fast generation of neutralizing Abs was counterbalanced by

the high vector competence of *Culicoides* vectors. Most likely, combined with the absence of SBV controls to international ruminant movements, the high SBV vector competence and presence of the former competent vectors all over Europe would have lead to the fast spread and vast geographic incidence of SBV epizootic across Europe.

Efforts should be made to determine the spatio-temporal range of Schmallenberg circulation among livestock, wild ruminants and *Culicoides* in order to better understand the dynamics of Schmallenberg disease in Spain and Europe. Better information will enhance prevention strategies once herd immunity overcome.

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292 **Tables and Figures**

293 Table 1. *Culicoides* biting midges tested for SBV in the retrospective and prospective
294 studies.

	Midges tested	Relative abundance	Pools tested	Positive pools
ARAMUNT [1]				
2011	1967		88	0
Obsoletus_group	1622	82,5%	50	0
Pulicaris_group	297	15,1%	24	0
Newsteadi_group	3	0,2%	3	0
C. flavipulicaris	45	2,3%	11	0
2012	850		66	2
Obsoletus_group	705	82,9%	35	2
Pulicaris_group	124	14,6%	23	0
C. flavipulicaris	21	2,5%	8	0
Subtotal Aramunt	2817		154	2
CALDES MALAVELLA [1]				
2011	3581		104	0
C. imicola	2995	83,6%	69	0
Obsoletus_group	544	15,2%	21	0
Pulicaris_group	1	0,0%	1	0
Newsteadi_group	36	1,0%	9	0
C. flavipulicaris	5	0,1%	4	0
2012	627		51	0
C. imicola	156	24,9%	12	0
Obsoletus_group	382	60,9%	24	0
Pulicaris_group	1	0,2%	1	0
Newsteadi_group	85	13,6%	13	0
C. flavipulicaris	3	0,5%	1	0
Subtotal Caldes Malavella	4208		154	0
VIC [2]				
2013	566		136	0
Obsoletus_group	550	97,2%	128	0
Pulicaris_group	9	1,6%	4	0
Newsteadi_group	4	0,7%	3	0
C. flavipulicaris	3	0,5%	1	0
Subtotal Vic	566		136	0
TOTAL	7591	-	445	2

295

296 [1] Farm of retrospective study, [2] Farm of prospective study

297 Table 2. *Culicoides* head pools positive for SBV with details on individual bodies tested for SBV.

Pool nº	Site	Trapping date	Pooled heads				Individual bodies (without heads)			
			Morphological identification	nº heads/ pool	Ct value		Molecular identification	Ct value		Bodies positive (tested)
					1st Test	2nd Test		First test	Second test	
1655	Aramunt	05/07/2012	Obsoletus group	25	25.27	25.51	*C. obsoletus*	24.24	ND	6 (25)
							Undet	41.37	Undet	
							C. obsoletus	34.44	35.11	
							C. obsoletus.	39.76	34.67	
							C. obsoletus.	36.06	36.13	
							C. obsoletus.	34.28	34.44	
							C. obsoletus	24.6	NA	
1765	Aramunt	12/07/2012	Obsoletus group	50	37.27	ND	*C. obsoletus*	30.14	NA	1 (50)

Ct, cycle threshold; ND, not done; Undet, undetermined

298

299

300 Table 3. Vector competence test for SBV on laboratory (*C. nubeculosus*) and field
301 collected *Culicoides* (Obsoletus group and *C. imicola*).

	EIP	Tested	Positive	Mean Ct	Desvest Ct	IR
C. nubeculosus						
Entire	0	3	3	33.5	0.40	-
Body	10	75	0	-	-	-
Head	10	75	0	-	-	-
Obsoletus group						
Entire	0	2	1	31.5	-	-
Body	10	16	10	24.7	4.84	0.63
Head	10	16	8	27.0	3.10	0.50
C. imicola						
Body	10	32	30	22.7	4.77	0.94
Head	10	32	26	23.6	3.16	0.81

302

303 EIP, Extrinsic Incubation Period; IR, Infection Ratio

304 Figure 1. Mean Ct values and standard deviation for SBV RNA detection (rRT-PCR) in

305 experimentally infected *Culicoides* of the Obsoletus group and *C. imicola*.