

Recovery rates of bluetongue virus serotypes 1, 2, 4 and 8 Spanish strains from orally infected *Culicoides imicola* in South Africa

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Abstract: Bluetongue (BT) is an infectious disease of ruminants that has spread northwards in Europe during the last decade. The aetiological agent of the disease is an arbovirus (Bluetongue) which belongs to the genus *Orbivirus* (family *Reoviridae*) and it is transmitted by certain species of biting midges within the genus *Culicoides* (Diptera: Ceratopogonidae). Information regarding the vector status of the *Culicoides* species in a specific area will be essential to predict the risk of a BTV incursion. Field collected *Culicoides (Avaritia) imicola* Kieffer from South Africa were fed on blood containing several Spanish isolates of BTV. Despite the high virus concentrations in the blood meal (5.1-6.4 log₁₀ TCID₅₀/ml of blood), virus was recovered from <1% of midges assayed after incubation. Virus concentrations >2.5 log₁₀ TCID₅₀/midge in individual infected *C. imicola* suggest virus replication with possible risk of transmission to susceptible vertebrate hosts in the field for at least two of the serotypes assayed (BTV1 and BTV2). A third serotype (BTV4) was very close to the estimated threshold of transmission. The relatively low to near refractory status of *C. imicola* compared to other vector species like *C. bolitinos* supports previous results, indicating that *Culicoides* species other than *C. imicola* may play a more important role in the epidemiology of BTV.

Keywords: *Culicoides imicola*, bluetongue virus, infection rate, oral susceptibility, vector capability

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INTRODUCTION

Bluetongue (BT) is an infectious viral disease of wild and domestic ruminants that can severely affects domestic sheep and sometimes cattle. The causative agent, bluetongue virus

(BTV), genus *Orbivirus*, family Reoviridae (Borden *et al.*, 1971), is transmitted by several *Culicoides* species (Diptera: Ceratopogonidae) (Mellor *et al.*, 2000). Before 2000 BTV was believed to be restricted to an area between 40°N and 35°S (Walton, 2004), however, during the last decade, it has expanded northwards in Europe up to 50°N (OIE, 2006; Carpenter *et al.*, 2009). This expansion of the infection and associated vectors has been postulated as partly ascribed to climate change (Purse *et al.*, 2005).

24 BTV serotypes of the virus are recognised worldwide (OIE, 2009), however, recent studies seem to indicate that two more serotypes could be implicated in the epidemiology of the disease (Hofmann *et al.*, 2008; Maan 2011). BTV-10 was the first to be recorded in mainland Spain between 1956 and 1960. The outbreaks occurred during those years were attributed to infected *Culicoides* transported by wind from North Africa resulting in the death of more than 100000 sheep (Manso-Ribeiro & Noreña, 1958; Campano-Lopez & Sanchez-Botija, 1958, Sellers *et al.*, 1978). Forty years later, in October 2000, bluetongue disease struck Spain again. The serotype was identified as BTV-2 and caused 305 outbreaks in the Balearic Islands (Miranda *et al.*, 2003). Three years later, BTV-4 was reported in the eastern part of Menorca, and in October 2004 in mainland Spain affecting the province of Cádiz and spreading rapidly north and south (Gómez-Tejedor, 2004). Despite vaccination with live-attenuated BTV-4 vaccine (Gómez-Tejedor, 2004), this serotype continued to spread northwards during the summer of 2005 into provinces in Andalucía, Castilla la Mancha, Castilla y León, Extremadura and Madrid. In addition, BTV-2 was also isolated from sentinel cattle in Spain in 2005 (Mellor *et al.*, 2008). In 2007 another serotype, BTV-1, appeared for the first time in Andalucía (Allepuz *et al.*, 2010) and in 2009 it spread to the northwest causing 240 outbreaks in cattle and sheep (Sabirovic *et al.*, 2009). In January 2008, BTV-8 occurred in northern Spain and spread to the southern part of the Iberian Peninsula in October 2008 (Lorca *et al.*, 2011).

Based on its confirmed vector status and host preference for large livestock, the Afro-Asiatic *Culicoides (Avaritia) imicola* Kieffer is considered to be the principal vector of BTV in South Africa (Nevill *et al.*, 1992ab; Meiswinkel *et al.*, 2004) and also across vast geographic regions of Africa and southern Europe (Mellor *et al.*, 1985; Boorman, 1986; Mellor, 1992;

Mellor *et al.*, 2000, 2009). The oral susceptibility of *C. imicola* to various strains of BTV has been demonstrated on several occasions in the laboratory (Venter *et al.*, 2010). Field isolations of BTV from this species also support laboratory results (Meiswinkel *et al.*, 2004; Venter *et al.*, 2006).

Distribution and modelling studies of *C. imicola* in Spain showed that outbreaks of BTV in 2007 were almost exclusively restricted to areas with high numbers of this species (Acevedo *et al.*, 2010). Nevertheless, about 5% of those localities, where *C. imicola* was predicted to be absent, had experienced at least one outbreak of BTV in the past (Acevedo *et al.*, 2010). In addition, recent studies conducted in Europe associate transmission of several serotypes of the virus with *Culicoides* species belonging to the Pulicaris and Obsoletus groups (Mellor & Pitzolis 1979; Savini *et al.*, 2005; Ferrari *et al.*, 2005).

In order to elucidate the role of the proven vector *C. imicola* in the epidemiology of BTV, the oral susceptibility of this species for those BTV serotypes circulating in Spain was determined.

Material and methods

Viruses and insects

In the absence of either large populations or laboratory colonies of *C. imicola* in Spain which are necessary for conducting this type of trial, adults of this species were collected in South Africa using the Onderstepoort black light trap as described by Venter *et al.* (1998). Traps were placed close to cattle stabled at the Agricultural Research Council-Onderstepoort Veterinary Institute (25°29' S, 28°11' E; 1219 m a.s.l.) in South Africa.

Field collected *Culicoides* midges were fed on blood-BTV mixtures between April and May 2009 in nine separate feeding attempts.

The four BTV serotypes responsible for recent outbreaks in Spain (BTV-1, -2, -4 and -8) were obtained from the National Reference Laboratory in Algete, Madrid (Spain). Stocks of virus for infection studies were grown in baby hamster kidney cells (BHK-21). Viruses were titrated and stored as described by Venter *et al.*, 1998. The details of each are shown in Table 1.

Feeding technique

After acclimatization in the insectary for 2-3 days at 23.5°C and 50-70% RH, field-collected midges were held without nutrients nor water for 24 hours before blood feeding. 5ml of stock virus was mixed, immediately before feeding, with 5ml of blood obtained from a BTV antibody negative sheep. Sheep used for oral susceptibility studies were kept under insect free conditions and were monitored daily for disease symptoms in order to prevent any field virus infections of the animals. Flies were fed through one-day-old chicken skin membrane in batches of 250–300 for 30–45 minutes on defibrinated blood containing one of the four serotypes (Venter *et al.*, 1998). During feeding, lighting in the room was dimmed to $\approx 1\%$ daylight (≈ 65 lux) while the blood-virus mixture was maintained at 35.5°C and stirred continuously to prevent settling of blood cells (Venter *et al.*, 1998).

After feeding, midges were immobilized at -20°C for about a minute. Blood-engorged females were separated out on a chill-table and kept in 250 ml unwaxed paper cups, closed with fine mesh, at 23.5°C and 50–70% relative humidity at $\approx 1\%$ daylight for the 10-day extrinsic incubation period. *Culicoides* females were maintained on 5% (w/v) sucrose solution and, in order to improve the survival rate, 500 IU penicillin, 500 μg streptomycin and 1.25 μg fungizone per ml of solution was added. Females surviving the incubation period were sorted into species on a chill-table and stored individually in 1.5 ml microfuge tubes at -70°C until assayed.

Processing of Culicoides and virological assays

Midges were assayed for virus immediately after feeding (10 blood engorged midges per feeding attempt) and also after 10 days extrinsic incubation. Processing of individual midges for microtitration assays was carried out as described by Paweska *et al.* (2002).

The identity of all virus isolates was determined by a microtitre virus-neutralisation procedure (House *et al.*, 1990), using type-specific antisera produced in guinea pigs. Virus concentrations were calculated by the method of Kärber (1931). Statistical differences between experimental groups were analyzed using Chi-square analysis. *P*-values of < 0.05 were used as the cut-off for statistical significance.

RESULTS

Culicoides feeding and survival rates

Feeding rates varied from 10 to 70% and the virus concentrations in blood meals ranged from 5.1 (BTV-2) to 6.4 (BTV-1) \log_{10} TCID₅₀/ml (Table 2). 3573 out of 6063 field collected midges (58.4%), fed on the four serotypes of BTV, survived the 10-day extrinsic incubation period.

Virus recovery in midges

Due to bacterial and fungal contamination, results could not be obtained neither from midges tested immediately after feeding nor from all the surviving midges assayed after incubation. The number of midges from which results could be obtained is shown in Table 2.

Only three (BTV-1, -2, -4), of the four serotypes used, were recovered from *C. imicola* after 10 days at 23.5°C. The highest virus recovery rate (0.5%) was obtained for BTV-2, while recovery rates for BTV-1 and BTV-4 was 0.2%. No significant difference ($\chi^2=2.543$, d.f=3, $P=0.467$) in the infection prevalence for the four different serotypes was obtained. All isolates from infected midges were serotyped as the same used to spike the blood.

Virus titres in the infected midges ranged from 1.4 to 3.9 \log_{10} TCID₅₀/midge (mean 2.2 \log_{10} TCID₅₀/midge). Titres of at least 2.5 \log_{10} TCID₅₀/midge were demonstrated for BTV-1 and BTV-2 (Table 2).

Few specimens of *Culicoides (Avaritia) bolitinos* Meiswinkel, *Culicoides (Remia) enderleini* Cornet and Brunhes, and *Culicoides (Remia) nevillei* Cornet and Brunhes, survived the 10-day incubation period. No virus was isolated from any of those specimens (Table 2).

DISCUSSION

In the present study, field collected *Culicoides* midges were not screened for the presence of field viruses, but virus recovery from field collected vector populations is generally very low (Chiang & Reeves, 1962; Walter *et al.*, 1980; Gerry *et al.*, 2001; Venter *et al.*, 2006a). In a field survey conducted in South Africa (endemic for BT) from 1979 to 1985, the virus was isolated from only 526 out of 4506 *Culicoides* pools (pool size 100-100 000 individuals) (Nevill *et al.*,

1992a). Using an average of 1000 midges per pool, the field infection prevalence would be 0.06%. The whole South Africa is endemic for BT and during the time of the study no cases of BT were reported. For these reasons it is therefore likely that the infection rate obtained reflects the laboratory conditions and that any influence by field infections should be minimal. This is supported by the fact that the identities of all serotypes recovered from the midges were the same as those used to spike the blood meal.

The infection prevalence obtained (i.e. percentage of midges from which virus could be recovered after the incubation period) was very low compared to other susceptibility studies with different strains of the virus, and ranged from 0.0% (BTV-8) to 0.5% (BTV-2). The highest recovery rate (0.5%) was obtained for BTV-2 being also the serotype with the lowest virus titre in the blood meal. Despite the relatively high virus concentration of BTV-8 in the blood meal ($6.1 \log_{10} \text{TCID}_{50}/\text{ml}$), this serotype was not recovered from the midges after the extrinsic incubation period (Table 2). The fact that BTV-8 was not recovered from *C. imicola* does not necessarily mean that this species is refractive to infection with that serotype. Based on the number of midges assayed it can be concluded that the infection prevalence for this strain was <0.2%. Statistically, the virus recovery rate of BTV-8 was not significantly different from that of the other three serotypes.

Low virus recovery rates, as an indication of vector competence, does not necessary translate to a low vector capacity for *C. imicola* with the serotypes/strains used. Based on light trap results, *C. imicola* is a widespread species in South Africa and found in very high numbers near livestock (Meiswinkel *et al.*, 2004). This super abundance and host preference of *C. imicola* can compensate for the low infection rates observed. Indeed, this species is considered to have the highest vector capacity for orbiviruses in South Africa (Nevill *et al.*, 1992b; Meiswinkel *et al.*, 2004). Although *C. imicola* in the Mediterranean basin is not as abundant as in Africa, its role as a competent vector for BTV in this area is enhanced by the presence of relatively high numbers of naïve or unvaccinated hosts due to non persistent vaccinations campaigns. Sheep in these regions, therefore, do not develop effective immunocompetent responses to the infection. The higher susceptibility of European breeds of sheep (Erasmus &

Potgieter, 2009), compared to South African sheep breeds, may also play a role in the epidemiology of this disease in Europe.

The minimum level of viraemia in a host, necessary to infect *Culicoides* vectors, is not known. Based on the blood-meal size for *C. imicola* (0.01-0.06 μ l) (Venter *et al.*, 2005) theoretically a minimum of 5.0 \log_{10} TCID₅₀/ml would be needed to ensure that each individual midge will be exposed to approximately one TCID₅₀/blood-meal. This value does not take into account the elimination of the excess of liquid taken while feeding. It has been said that virus concentrations as low as 2.0 \log_{10} TCID₅₀/ml are sufficient to produce infection rates of 0.2% in *Culicoides (Avaritia) brevitarsis* Kieffer (Muller *et al.*, 1982), a species similar in size to *C. imicola*. In the USA Bonneau *et al* (2002) succeeded in infecting *Culicoides (Monoculicoides) sonorensis* Wirth & Jones by feeding them on a BTV infected sheep with no detectable viraemia (<0.5 \log_{10} TCID₅₀/ml of blood). It is therefore clear that the virus titres used in the present study (>5.1 \log_{10} TCID₅₀/ml of blood) were more than enough to infect all susceptible *Culicoides* and that the infection prevalence obtained is an indication of the susceptibility rate of this *C. imicola* population for the viral strains used.

Some studies show that concentrations of live virus > 2.5 \log_{10} TCID₅₀/midge in its head or body is an indicator of a fully disseminated infection (Jennings & Mellor 1987; Fu *et al.*, 1999; Riegler, 2002). This threshold value (>2.5 \log_{10} TCID₅₀/midge) was derived from *C. sonorensis* studies and may not be applicable for interpretation of vector competence results in other *Culicoides* species like *C. imicola*. The virus titres recorded in individual infected *C. imicola* in the present study indicate that the virus had replicated in the insect to high levels in two of the serotypes assayed (BTV1 and BTV2) and, consequently, onwards transmission to susceptible vertebrate hosts might be possible. A titre of 2.4 TCID₅₀/midge was obtained for BTV4. This value was very close to the estimated threshold of transmission in *C. sonorensis*. Due to the smaller size of *C.imicola* compared to that of *C. sonorensis* and observing that this serotype circulated in several outbreaks in Spain in areas where *C. imicola* was present (Gómez-Tejedor, 2004), it can be postulated that the Spanish strain of BTV4 could also be transmitted by the South African population of *C. imicola*.

Although the current study indicates a very low vector competence of *C. imicola* for the serotypes used, it also shows that this species is susceptible to infection with those isolates and that onwards transmission is possible. In Europe, *C. imicola* has been recorded in the Iberian Peninsula, the Balearic Islands, Greece, Italy, and the South of France and was thought to be the main vector of BTV in those areas (Miranda *et al.*, 2003; Goffredo *et al.*, 2001; Giovannini *et al.*, 2004; Sarto i Monteys *et al.*, 2005; Purse *et al.*, 2005). Climate change has created milder winter conditions in the northern regions, and recent studies describe an apparent on-going range expansion of this species northwards (Mellor & Wittmann, 2002; Calvete *et al.*, 2006). In addition to the range expansion, milder conditions can also influence the vector competence of *Culicoides* species leading to an exponential increase in vector capacity (Mullens *et al.*, 2004).

The relatively low susceptibility results obtained in the present study support the notion that BT might be a “multi-vector” disease and this will add to the complexity of the epidemiology of BTV.

It should also be noted that the susceptibility of a single *Culicoides* population to infection with different strains of orbiviruses may differ significantly over time (e.g. BTV serotypes: Jones & Foster, 1978; African horse sickness virus serotype 7 strains: Venter & Paweska, 2007). Field populations of *Culicoides* species can vary broadly in their genetic susceptibility to BTV infection. Indeed, it has been demonstrated that some populations of the same species might be completely refractory to infection by some serotypes (Ward, 1994; Mellor, 2004, Carpenter *et al.*, 2006). True assessment of vector competence might be difficult to calculate as it would require some level of real-time monitoring, e.g. testing local *Culicoides* populations using variants of orbiviruses in current local circulation. Thus, the current study should be repeated with the European populations of *C. imicola*, and extended to other potential vector species such as those included in the Obsoletus group, in order to assay their implication in the epidemiology of BT in places where several species of either proven or suspected and potential vectors are present. The lack of established laboratory colonies of *C. imicola* in Europe, as well as the lower population abundance of this species compared to that in Africa, may represent a

serious obstacle to develop such type of oral susceptibility trials in the southern European countries.

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Table 1. Virus serotypes, year of isolation and passage history of the Spanish BTV isolates used for the oral infection of field collected *Culicoides* midges

Virus Serotype	Strain identification	Year of isolation	Passage history*
BTV-1	SPA/07	2007	1P Vero
BTV-2	R23/00	2000	2P BHK-21
BTV-4	SPA/04	2004	3P BHK-21
BTV-8	Segovia		2P BHK-21

* number of passages in baby hamster kidney cells (BHK-21) or Vero cells

Table 2. Virus recovery rates and titres in field collected *Culicoides imicola*, *C. bolitinos*, *C. enderleini* and *C. nevillei* maintained for 10 days at 23.5°C after feeding on blood containing different serotypes of BTV

BTV serotype	BTV-1	BTV-2	BTV-4	BTV-8
Virus titre of blood meal	6.4	5.1	5.8	6.1
Log ₁₀ TCID ₅₀ /ml				
<i>C. imicola</i> (%)	1/490 ¹ (0.2%)	4/889 (0.5%)	1/404 (0.2%)	0/492 (0.0%)
Avg Virus titre/midge ²	3.9	2.5	2.4	-
Range in virus titre		1.4-3.2		
<i>C. bolitinos</i>	0/2	0/2	0/11	0/5
<i>C. enderleini</i>	0/1		0/1	0/8
<i>C. nevillei</i>				0/2

¹ No positive/No tested, ² log₁₀TCID₅₀ /midge