Blood meal analysis in Central European *Culicoides* biting midge species (Diptera: Ceratopogonidae) by reverse line blot hybridization

Helge Kampen [1] & Doreen Werner [2]

[1] Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Greifswald – Insel Riems, Germany

[2] Leibniz-Center for Agricultural Landscape Research, Muencheberg, Germany

Zusammenfassung: Die Identifizierung von Blutwirten hämatophager Arthropoden kann nicht nur Informationen über die Biologie und Ökologie der blutsaugenden Spezies vermitteln, sondern möglicherweise auch Rückschlüsse über Transmissionszyklen von Pathogenen, wenn die Arthropoden als Vektoren von Krankheitserregern dienen. Blutgesogene Gnitzenweibchen der Gattung *Culicoides*, die von 2007 bis 2010 mit Hilfe von UV-Lichtfallen in ganz Deutschland gefangen worden waren, wurden daher per Reverse Line Blot-Hybridisierung auf die Herkunft ihrer Blutmahlzeiten untersucht. Dazu wurden PCR-Produkte der 12S Vertebraten-rDNA, die aus 207 Gnitzenexemplaren gewonnen wurde, gegen 18 DNA-Sonden getestet. Drei der Sonden waren Gruppen-spezifisch für Säuger, Vögel und Nager, während 15 Sonden die häufigsten Wirbeltierarten, die in Deutschland als Blutwirte für Gnitzen denkbar sind, erfassten. Der Großteil der Gnitzen saugte an Rindern, Schafen und Menschen, was angesichts der Lokalisation der Fallen auf oder in der Nähe von landwirtschaftlichen Betrieben mit entsprechender Tierhaltung nicht verwunderlich ist. Weitere Untersuchungen zur Blutmahlanalyse in natürlichen Habitaten der Gnitzen sollten folgen, um Hinweise auf mögliche Reservoirwirte von Blauzungen- und Schmallenberg-Virus zu bekommen, die von diesen Gnitzenarten übertragen werden.

Keywords: Blood meal, bluetongue, Central Europe, *Culicoides*, DNA probes, reverse line blot hybridization, Schmallenberg

[1] Helge Kampen, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Suedufer 10, 17493 Greifswald – Insel Riems, Germany; E-Mail: helge.kampen@fli.de

[2] Doreen Werner, Leibniz-Center for Agricultural Landscape Research, Muencheberg, Germany, Eberswalder Straße 84, 15374 Muencheberg, Germany; E-Mail: doreen.werner@zalf.de

Introduction

Culicoides biting midges (Diptera, Ceratopogonidae) are the vectors of numerous viruses some of which may cause severe disease in vertebrates, such as Oropouche virus in humans, bluetongue and Schmallenberg viruses (BTV, SBV) in ruminants, and African horse sickness virus in equids (Sick & al. 2019). With regard to ceratopogonid-borne diseases, Europe has historically been affected only by bluetongue disease which broke out irregularly during the past few decades in the Mediterranean (Mellor & al. 2008). The transmission pressure increased after the establishment on the Iberian peninsula and the subsequent spread of the Asioafrican biting midge species *Culicoides imicola*, the Old World's most important culicoid vector, in the early 1980s (Mellor & al. 1983, Purse & al. 2005). However, so far *C. imicola* has obviously remained restricted to Europe south of the Alps and has only once (one specimen) been found as far north as southern Switzerland (Cagienard & al. 2006). In more northerly parts of Europe, biting midges were recognized as potential vectors of disease agents only in 2006 when bluetongue disease broke out for the first time north of the distribution area of *C. imicola* (Wilson & al. 2009). Until that time, the pathogen transmission potential of indigenous biting midge species was largely ignored, although several studies had previously suggested widely-distributed European species to be vector-competent for BTV (Caracappa & al. 2003, Savini & al. 2004). Only due to their aggressive biting behaviour and being a heavy nuisance, ceratopogonids had been reported to be of severe public

health relevance in some northern regions seasonally (Bröström & al. 1987, Hendry & Godwin 1988). Following the BTV serotype 8 (BTV-8) crisis that eventually ebbed away in 2009 after large-scale vaccination campaigns (Baetza 2014), a completely knew biting midge-borne disease agent, SBV, appeared in Central Europe in 2011 (Beer & al. 2013). During the management of this epidemic, it became obvious once more that European biting midge species had generally been neglected as research objects and knowledge on the biology and ecology of this group of insects in Central and northern Europe was scarce. While SBV, or antibodies against it, could be identified throughout the years in Central Europe suggesting that the virus persisted (Wernike & Beer 2017), BTV-8 disappeared in 2010 but re-emerged in 2018 (FLI 2019).

According to Havelka (1978), 162 taxonomically valid *Culicoides* species existed in Europe in the late 1970s. Meanwhile, numerous additional species have probably been identified genetically. For some of them, notably those of the Obsoletus and Pulicaris Complexes, consent exists that they are the vectors of BTV and SBV. Field infections with both viruses have been demonstrated multiple times (e.g., Caracappa & al. 2003, Mehlhorn & al. 2007, Meiswinkel & al. 2007, Dijkstra & al. 2008, Carpenter & al. 2009a, Hoffmann & al. 2009, De Regge & al. 2012, Rasmussen & al. 2012, Elbers & al. 2013), but final evidence for vector competence from the laboratory is still missing, in the first place due to failure in rearing the species.

While the identification of possible European virus vectors is progressing, various biological and ecological features of the indigenous biting midge fauna that may be relevant to disease epidemiology and control, however, remain obscure. For example, little is known about the blood host preferences of the midges. Identification of major blood host species of the putative culicoid vector species could point to natural virus reservoirs and help solving the miracle of virus overwintering. Hibernation in a vertebrate host reservoir would be one possible strategy of overwintering when natural transmission ceases at the end of the season due to vector inactivity (Takamatsu & al. 2003, Wilson & al. 2008). Serological studies on antibody prevalences in wild ruminants that might be virus reservoir candidates are rare and do not allow conclusions yet (EFSA 2007, Ruiz-Fons & al. 2008, Linden & al. 2010).

Blood host identification is also important when it comes to vector control which has to take into account the ecological characteristics of the target species such as breeding requirements. *Culicoides* species are probably adapted to their preferred hosts in that they breed close to their feeding or resting places or take advantage of any peculiarities of their habitat.

Various methodologies have been established to identify the origin of a haematophagous arthropod's ingested blood. While immunological approaches (e.g. Beier & al. 1988, Hunter & Bayly 1991) have lost importance, molecular methods (particularly PCR techniques and DNA sequencing) have become standard (Kent 2009). Diagnostic DNA markers targeted at the identification of blood host species are commonly mitochondrial genes (e.g. cytochrome b, cytochrome c oxidase subunit 1), ribosomal genes (e.g. internal transcribed spacers 1 and 2, mitochondrial 12S) and nuclear genes (e.g. prepronociceptin) (Kent 2009). Using these markers, blood meal identification has been successfully conducted in mosquitoes (e.g. Molaei & Andreadis 2006), black flies (e.g. Imura & al. 2010), sand flies (e.g. Haouas & al. 2007), tsetse flies (Steuber & al. 2005) and ticks (Humair & al. 2007).

In our study, we targeted the vertebrate 12S rDNA within a blood meal ingested by *Culicoides* biting midges. We applied a reverse line blot hybridization assay that, using a multiple-lane blotter, is able to process numerous biting midge/blood samples against the same number of DNA targets (potential blood meal sources) at the same time.

Materials and methods

Biting midge collection and identification

Biting midges were collected in 80 % ethanol between 2007 and 2010 by UV-light traps (BG Sentinel, Biogents, Germany) located on rural animal holdings all over Germany. After morphological identification, the midges were sorted visually according to 'blood-fed' and 'non blood-fed', using a dissecting microscope. *Culicoides* specimens belonging to the Obsoletus Complex were processed by PCR for species differentiation (Lehmann & al. 2012).

DNA extraction and amplification

Prior to DNA extraction, the biting midges were removed from their ethanol fixative and air-dried. Tissue homogenization was achieved by shaking single insect specimens 3 min at 30 Hz in 200 µl AL buffer (Qiagen) together with three steal beads (3 mm diameter) in a TissueLyser (Qiagen). The supernatant was used for DNA extraction by means of the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol. In a nested PCR, a≈145 bp fragment of vertebrate 12S rDNA was amplified using primers and protocols as described by Humair & al. (2007). A small volume of the PCR mixtures were checked on agarose gels, and positive samples were stored at 4 °C until hybridization.

Reverse line blot hybridization

Group- and species-specific DNA probes were either adopted from Humair & al. (2007) or newly designed (Table 1), based on GenBank DNA sequence entries, for the most prevalent domesticated and wild indigenous vertebrate species which might serve as blood hosts for biting midges. A *Lama*-specific probe was included as one insect trap was operated on an alpaca farm.

Using a 45-lane miniblotter (Immunetics, MA, USA), the probes were covalently linked to an activated Biodyne C membrane (Pall, Germany) as described by Rijpkema & al. (1995). 50 pmol of probe in 150 µl 500 mM $NaHCO_3$ were loaded per lane. The hybridization procedure was also copied from Rijpkema & al. (1995), using 5-10 µl of PCR product, depending on the intensity of the amplicon band on the agarose gel. Detection was done using the ECL detection system (GE Healthcare) according to the manufacturer's instructions, and visualization by exposing the membrane to an X-ray film (Hyperfilm, GE Healthcare) for 1-20 min.

Table 1: Sequences of oligonucleotides used as DNA probes for RLB hybridization

Target group or species	Nucleotide sequence (5'-3')
Mammal	5'-Amino-AAACTCAAAGGACTTGGC
Rodent (Muroidea)	5'-Amino-GGCGGTACTTTATATCCAT
Bird (Aves)	5'-Amino-TACGAGCACAAACGCTTAA
Cattle (*Bos taurus*)	5'-Amino-ACACAGATAATTACATAAACAA
Sheep (*Ovis aries*)	5'-Amino-CCTAAACACAAATAATTATAAA
Goat (*Capra aegagrus*)	5'-Amino-CTAAACACAAATAATTACAG
Roe deer (*Capreolus capreolus*)	5'-Amino-CCTAAACACAAGTAATTAATATAACAA
Red deer (*Cervus elaphus*)	5'-Amino-CACAAATAGTTATGCAAACAAAAC
Fallow deer (*Dama dama*)	5'-Amino-CACAAATAGTTGTGTAAACAAAAC
Alpaca (*Lama pacos*)	5'-Amino-TTTAAGTGATTACAATAACAAAATC
Man (*Homo sapiens*)	5'-Amino-ACCTCAACAGTTAAATCAACA
Horse (*Equus ferus*)	5'-Amino-CACAAATAGTTGTGTAAACAAAAC
Pig (*Sus scrofa*)	5'-Amino-ACCCAAATAGTTACATAACAAAA
Fox (*Vulpes vulpes*)	5'-Amino-CTATAACAAAACAATTCGCCA
Dog (*Canis lupus*)	5'-Amino-CCTAAACATAGATAATTTTAC
Cat (*Felis silvestris*)	5'-Amino-TAGATAGTTACCCTAAACAAAACT
Hare (*Lepus europaeus*)	5'-Amino-TTAAACCTAAATAATTTCCTAACAAA
Rabbit (*Oryctolagus cuniculus*)	5'-Amino-TAAACTTTGATAATTTCATAACAAAATT

Controls

Positive controls were constructed using amplicons generated by PCRs on DNA extracted from blood samples of the various vertebrate species. Products of PCRs run with water instead of DNA served as negative controls.

Results

Preliminary experiments were successful in demonstrating the specificity of the RLB. Only the dog-specific probe showed some cross-reactivity with cat DNA in the controls (Fig. 1). Vertebrate DNA recovered from blood ingested by culicoids could also be detected (Fig. 1). Rarely, faint secondary hybridization signals appeared with biting midge samples and human probe, probably as a consequence of handling (sorting, identification, DNA extraction, hybridization), but never in the controls. Such faint and obviously incorrect signals were considered negative. By contrast, human signals were considered positive when strong and unique. Using this approach, no biting midge presented two blood meal origins. The biting midges analysed belonged to 16 species (Table 2), with *C. obsoletus* and *C. scoticus* by far being the most frequent ones, followed by *C. punctatus* and *C. pulicaris* (Table 2). The majority of the biting midges fed on sheep (n = 96), cattle (n = 54) and humans (n = 56), which was not surprising given the localization of the traps on or close to sheep and cattle holdings. Some midges collected on an alpaca farm contained alpaca blood (n = 8), and few also fed on horses (n = 5). Only one midge was found to have imbibed rodent blood.

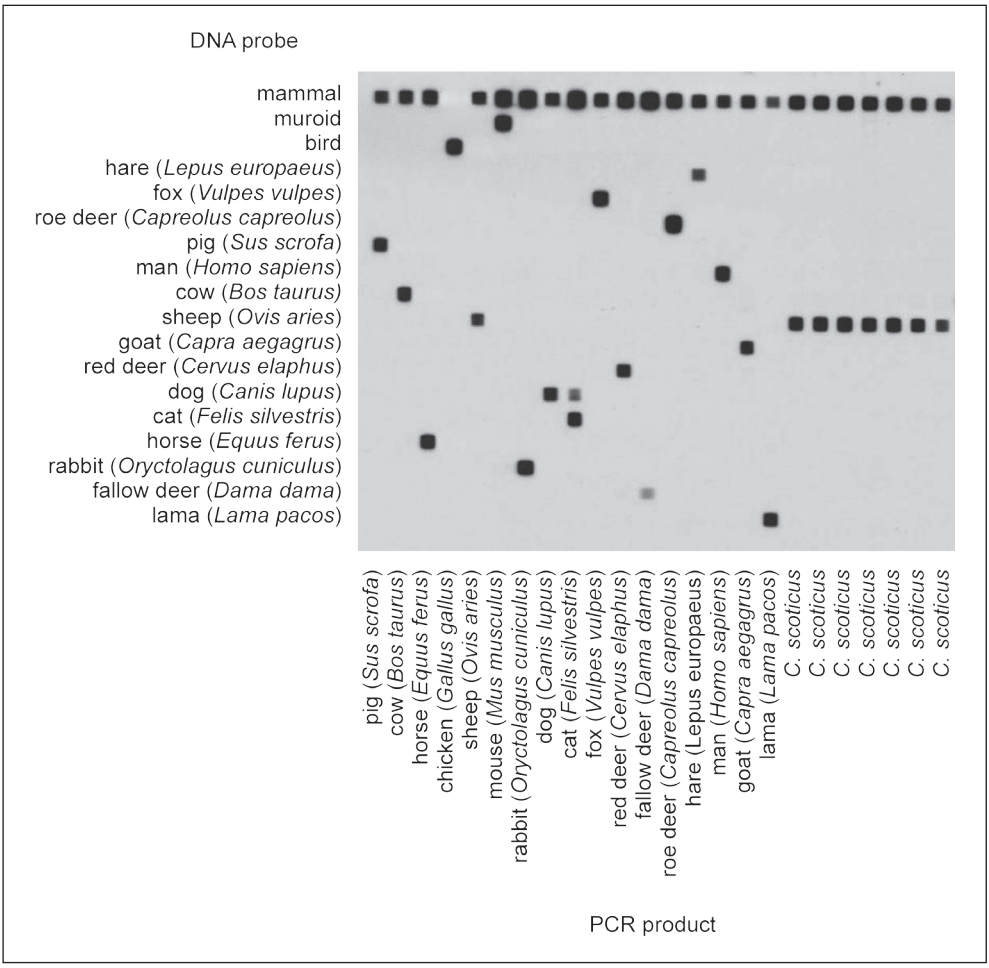

Fig. 1: Chemiluminograph of RLB hybridization of species-specific DNA probes with PCR products generated from control blood samples and from blood-fed *Culicoides* specimens

Table 2: *Culicoides* species (number of specimens) found feeding on vertebrate blood tested

Biting midge species	Blood meal source																
	Bird	Muroid	Cattle	Sheep	Goat	Roe deer	Red deer	Fallow deer	Alpaca	Man	Horse	Pig	Fox	Dog	Cat	Hare	Rabbit	Total
C. albicans			1	1						3								5
C. achrayi			1	2						2								5
C. brunnicans			1							1								2
C. circumscriptus										3								3
C. festivipennis										2								2
C. griseidorsum										1								1
C. nubeculosus			2							3								5
C. parroti			1															1
C. vexans			2							1								3
Obsoletus Complex																		
C. obsoletus		1	24	3	1				1	14	2							46
C. scoticus			11	86	2					17								116
C. chiopterus			2															2
C. dewulfi			1	1						1								3
Pulicaris Complex																		
C. pulicaris			1	1					7	2	1							12
C. punctatus			5	2	1					6	2							16
C. newsteadi			2															2
Total			1	54	96	4				8	56	5						224

Discussion

Little information exists about blood host preferences of Central European culicoid biting midges. The most comprehensive compilation is provided by Santiago-Alarcon & al. (2012), which is in accordance with the findings presented here that humans and cattle belong to the major blood hosts of *C. obsoletus, C. scoticus, C. punctatus* and *C. pulicaris*. Not included in that contribution are the studies by Lassen & al. (2011), which is in agreement regarding the preference of the four biting midge species for cattle but did not consider human hosts, and by Ninio & al. (2011), who found horse and rabbit to be major host species, in addition to cattle. Our results suggest that the host specificity of these four most frequent biting midge species, which are among the most important putative BTV and SBV vectors (Carpenter & al. 2009b, Balenghien & al. 2014), is not very high and host choice probably rather depends on host species availability and density within a certain distance from breeding habitats. Further tests on the host preferences of biting midges should include blood-fed specimens collected in more natural habitats.

RLB hybridization seems to be suitable to determine the blood meal origin of engorged biting midge females, provided species-specific DNA probes are available. The latter, however, is in fact a methodological limitation of this approach, since the pre-selection of probes excludes the detection of possible hosts: vertebrate species or groups of species can only be detected if probes specific for them are used in the assay. RLB hybridization must therefore be balanced against PCR amplification of vertebrate DNA, followed by DNA sequencing and databases alignment, considering information content of expected results as well as efforts, practicability, duration and costs of the study.

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