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Short Communication

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Hyaluronidase Activity in Saliva of European *Culicoides* (Diptera: Ceratopogonidae)

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Abstract

Biting midges of the genus *Culicoides* transmit pathogens of veterinary importance such as bluetongue virus (Reoviridae: Orbivirus). The saliva of *Culicoides* is known to contain bioactive molecules including peptides and proteins with vasodilatory and immunomodulative properties. In this study, we detected activity of enzyme hyaluronidase in six *Culicoides* species that commonly occur in Europe and that are putative vectors of arboviruses. Hyaluronidase was present in all species studied, although its molecular size, sensitivity to SDS, and substrate specificity differed between species. Further studies on the potential effect of hyaluronidase activity on the vector competence of *Culicoides* species for arboviruses would be beneficial.

Key words: *Culicoides*, hyaluronidase, saliva

Biting midges of the genus *Culicoides* (Diptera: Ceratopogonidae) transmit arboviruses of global medical and veterinary importance, including bluetongue virus (BTV), Schmallenberg virus (SBV), and Oropouche virus (Purse et al. 2015). Their biting activity is also the primary causative agent of a seasonally recurrent chronic dermatological condition, commonly termed "sweet itch". The study of bioactive molecules and antigens in *Culicoides* saliva is an increasingly important area of research, both in understanding their impact on arbovirus transmission between vector and host and in examining the immunological response of the host to the biting activity.

Culicoides saliva has been hypothesized to trigger BTV viremia from the noninfectious status in cattle (Akey et al. 1985); however, the supposed "latent" period in infection has been criticized. More recently, following the development of techniques to bulk harvest saliva from colony lines of *Culicoides*, treatment of BTV particles with saliva collected from the BTV vector *Culicoides sonorensis* Wirth & Jones was shown to lead to the formation of highly infectious subviral particles (Darpel et al. 2011). In addition, it was also demonstrated that the feeding activity of *Culicoides* can increase the titer of BTV-infected host viremia and the severity of clinical signs in sheep (Pages et al. 2014).

In parallel, *Culicoides* saliva has been found to contain powerful allergens including those ascribed to the immunoglobulin E (IgE)-mediated type 1 hypersensitivity response occurring in livestock after *Culicoides* bites (Yeruham et al. 1993, Wilson et al. 2001). Salivary proteins including maltase, D7-related protein, trypsin, and hyaluronidase have been described as the primary allergens (Schaffartzik et al. 2011, van der Meide et al. 2013).

Hyaluronidases are ubiquitous group of hydrolytic enzymes found in both vertebrates and invertebrates. In phlebotomine sand flies and other bloodsucking insects, they have been detected in saliva and are hypothesized to promote the distribution of other pharmacologically active salivary compounds (Charlab et al. 1999, Volfova et al. 2008). In *Culicoides*, hyaluronidase transcripts or enzyme activities have been detected in *Culicoides sonorensis*, *Culicoides nubeculosus* Meigen, and *Culicoides obsoletus* Meigen (Campbell et al. 2005, Volfova et al. 2008, Wilson et al. 2008, Russell et al. 2009). Here, we examine and directly compare the hyaluronidase properties in two confirmed (*Culicoides imicola* Kieffer and *C. obsoletus*) and four potential (*Culicoides pulicaris* L., *Culicoides punctatus* Meigen, *Culicoides newsteadi* Austen, and *C. nubeculosus*) vectors of BTV and SBV in Europe.

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Materials and Methods

Processing of *Culicoides*

C. nubeculosus originated from the colony maintained at CIRAD (Agricultural Research Centre for International Development) Montpellier, France, originally established from the line maintained at the Pirbright Institute, and maintained under standard conditions (Boorman 1974, Nayduch et al. 2014). Other *Culicoides* species were collected using light-suction trapping in the field; *C. obsoletus*, *C. pulicaris*, and *C. punctatus* were captured in Libkova Voda and Mezihori, Czech Republic; *C. newsteadi* in Mas du Pont and Saint Georges đOrques, France; and *C. imicola* on Réunion, France. Insects were determined using the keys of Campbell and Pelham-Clinton (1960) and Delécolle (1985). *C. obsoletus* complex was distinguished by a multiplex PCR analysis as described in Nolan et al. (2004). Additional control insects were also used: *Culex quinquefasciatus* Say and *Phlebotomus duboscqi* Neveu-Lemaire originated from laboratory colonies at Charles University in Prague, Czech Republic.

As for logistical reasons it was impossible to obtain alive specimens of *C. imicola* for salivary gland dissection, a body extraction (BE) was made from heads and thoraxes of 20 females homogenized using pestles in 20 µl of Tris buffer saline (20 mM Tris, 150 mM NaCl, pH 7.8), three freeze–thaw cycles in liquid nitrogen, and centrifugation (12,000 × *g* for 5 min). For other species tested, salivary glands were dissected from insects knocked-down on ice, pooled in Tris buffer saline (10 glands in 10 µl), and stored at –80°C until required. Immediately prior to experiments, glands were processed as for BE, creating a salivary gland extract (SGE). Pure saliva (SAL) of *C. nubeculosus* was also obtained in bulk from the Pirbright Institute laboratory colony line as described in Langner et al. (2007). Protein concentrations in SGE, BE, and SAL were determined using Quibit equipment (Invitrogen, Carlsbad, CA).

Detection of Hyaluronidase Activity

Hyaluronidase activity was studied on substrate gels using a dot method and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) zymography as described in Volfova et al. (2008). The dot method was performed on gels with copolymerized 0.002% hyaluronic acid (HA; ICN Pharmaceutical, Costa Mesa, CA) or 0.002% chondroitin sulfate (CHS; Sigma, Oakville, ON, Canada) at pH 5.5, as optimized by Ribeiro et al. (2000) and Volfova et al. (2008). In order to compare hyaluronidase activity across species, SGE equivalent to a pair of glands of six *Culicoides* species and 2 µl of *C. imicola* BE were dotted on substrate gels in 2-µl volumes. SGEs of *P. duboscqi* and *Cx. quinquefasciatus* were used as positive controls, with Tris buffer saline as a negative control.

SDS-PAGE zymography was performed on female individuals of five European species (*C. obsoletus*, *C. nubeculosus*, *C. pulicaris*, *C. punctatus*, and *C. newsteadi*) using a method described in Volfova et al. (2008). In the case of *C. obsoletus*, females were separated into individuals with unpigmented (nulliparous) or pigmented abdomens (parous females; Dyce 1969). The quantity of SGE was optimized by preliminary experiments. Variable equivalents of salivary glands were loaded per lane for each *Culicoides* species SGE as follows: *C. nubeculosus* and *C. pulicaris*: equivalent to two salivary glands; *C. punctatus* and *C. newsteadi* five glands; *C. obsoletus*: 10 glands. For *C. nubeculosus*, also 1 µg of SAL was loaded per lane. In positive controls, the equivalents of two salivary glands of *P. duboscqi* and *Cx. quinquefasciatus* were loaded per lane. Both experiments, dot method and SDS-PAGE zymography, were repeated at least three times for each species.

Affinity Blotting

N-glycoproteins were studied in *C. nubeculosus* and *C. pulicaris* SGEs. The equivalent of 5 and 28 salivary glands were used in each lane for *C. nubeculosus* and *C. pulicaris*, respectively. Samples were separated by SDS-PAGE on 10% gel under nonreducing conditions. One part of the gel was stained by silver and the second transferred to nitrocellulose membrane and cut into strips. The strips were then incubated with biotinylated lectin from *Canavalia ensiformis* (ConA, Sigma, Oakville, ON, Canada) and processed as described in Vlkova et al. (2014). Inhibitory sugar (0.5 M methyl-α-D-mannopyranoside) was added in control strips to ensure the specificity of reaction.

Results and Discussion

Protein concentrations detected varied according to extraction method and species. The greatest quantity was found in the BE of *C. imicola* (2.115 µg/µl) while, as expected, SGE preparations for *C. obsoletus* nulliparous (<0.1 µg per one salivary gland), *C. obsoletus* parous (0.103 µg per gland), *C. pulicaris* (0.170 µg per gland), *C. punctatus* (0.294 µg per gland), *C. newsteadi* (0.167 µg per gland), and *C. nubeculosus* (0.513 µg per gland) yielded lower protein concentrations. The SGE preparations gave comparable protein quantities to those from *P. duboscqi* (0.562 µg per gland) and *Cx. quinquefasciatus* (0.394 µg per gland).

Protein content in SAL of *C. nubeculosus* was 0.320 µg/µl.

Enzymatic activity reflected these quantities on a gel with incorporated HA, the greatest activity being observed with SGE of *C. nubeculosus*; moderate activity in *C. pulicaris*, *C. punctatus*, and *C. newsteadi*; and the least in *C. obsoletus* (both parous and nulliparous; Fig. 1A). The BE of *C. imicola* showed a moderate response that was also correlated with protein yield.

Interestingly, on a gel with copolymerized CHS, the strongest reaction was achieved with SGE of *C. newsteadi*, a medium response was recorded in *C. nubeculosus* and *C. pulicaris*, and a low response was found in *C. punctatus*. No hydrolysis of CHS was observed in *C. obsoletus*, regardless of examining unpigmented and pigmented females (Fig. 1B). Moderate hyaluronidase activity was also detected in *C. imicola* BE (Fig. 1A and B). The experiment was repeated three times with the same result. Experiments suggest that hyaluronidases of most species (in our experiments *C. nubeculosus*, *C. pulicaris*, and *C. punctatus*) hydrolyze both substrates in a comparable way. Similar hyaluronidase activity to HA and CHS was found also in a previous study using BE of *Culicoides kibunensis* (Volfova et al. 2008). All repeats showed the same results.

SGE of five *Culicoides* species and SAL of *C. nubeculosus* were analyzed by SDS-PAGE zymography on a gel with incorporated HA (Fig. 2). Hyaluronidases of *C. pulicaris* and *C. newsteadi* appeared as a single band with a molecular weight of 42 kDa and 45 kDa, respectively (Fig. 2). Three bands with an approximate molecular size of 38, 40, and 45 kDa were detected in *C. nubeculosus* SGE under nonreducing conditions. The 45 kDa band is in accordance with previous data (Russell et al. 2009). In SAL of *C. nubeculosus*, one broad band with a molecular weight of 38 kDa was demonstrated. The intensity of activity bands slightly differed between repeated experiments but the molecular weight was highly reproducible.

No activity was detected in SGEs of *C. punctatus* and *C. obsoletus* (Fig. 2). To elucidate the discrepancy between the results of the

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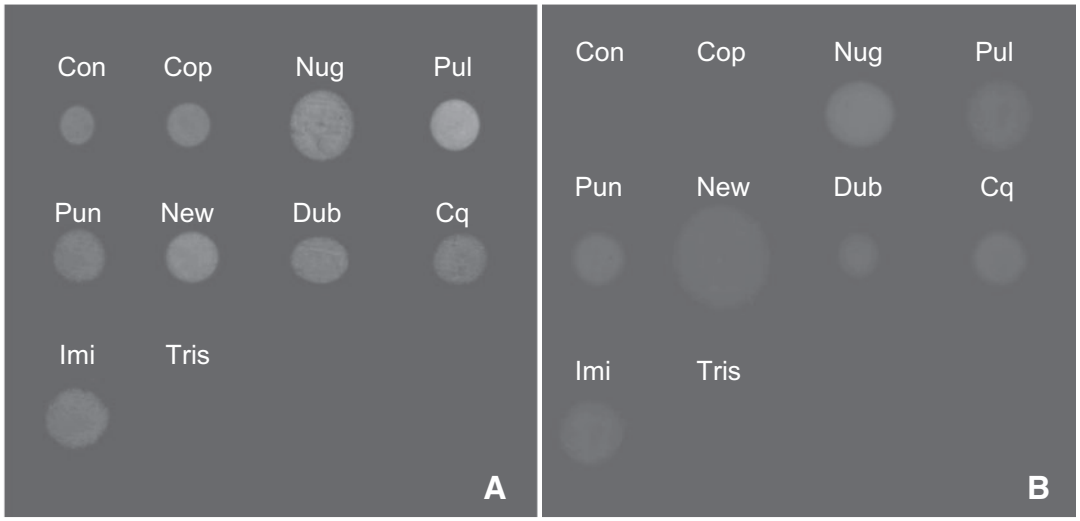


Fig. 1. Hyaluronidase activity in SGs of *Culicoides* spp. and other insects tested by the dot method on polyacrylamide gel with copolymerized hyaluronan (A) and chondroitin sulfate (B). Con—*C. obsoletus* nulliparous; Cop—*C. obsoletus* parous (SGE); Nug—*C. nubeculosus* (SGE); Pul—*C. pulicaris* (SGE); Pun—*C. punctatus* (SGE); New—*C. newsteadi* (SGE); Imi—*C. imicola* (BE); Dub—*P. duboscqi* (SGE); Cq—*Cx. quinquefasciatus* (SGE); Tris—Tris buffer saline.

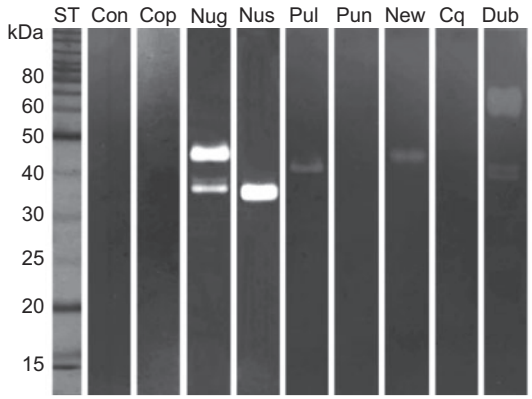


Fig. 2. SDS-PAGE zymography conducted under nonreducing conditions on a polyacrylamide gel with copolymerized hyaluronan. ST—marker; Con—*C. obsoletus* nulliparous (SGE); Cop—*C. obsoletus* parous (SGE); Nug—*C. nubeculosus* (SGE); Nus—*C. nubeculosus* (SAL); Pul—*C. pulicaris* (SGE); Pun—*C. punctatus* (SGE); New—*C. newsteadi* (SGE); Dub—*P. duboscqi* (SGE); Cq—*Cx. quinquefasciatus* (SGE).

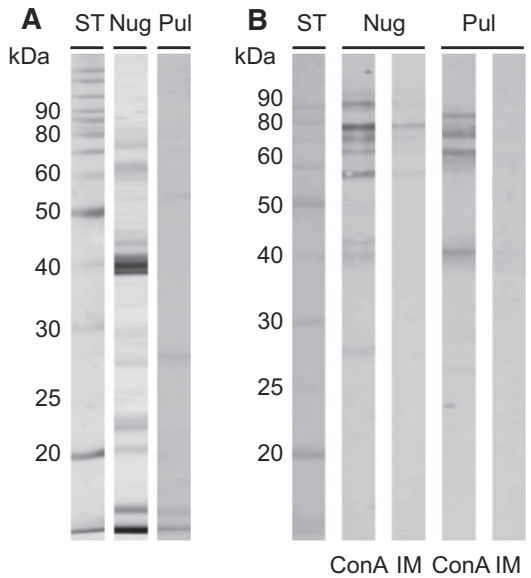


Fig. 3. Gel with 10% polyacrylamide (SDS-PAGE) silver-stained (A) and affinity blotting with lectin ConA and inhibition by saccharide inhibitor (B). ST—marker; Nug—*C. nubeculosus* (SGE); Pul—*C. pulicaris* (SGE); ConA—biotinylated lectin concanavalin A; IM, inhibitory mannose.

dot method and SDS-PAGE zymography, SGE of *C. obsoletus* was dotted on a polyacrylamide gel with copolymerized HA in the presence or absence of SDS. Hyaluronidase activity was repeatedly observed in the sample without SDS, while no activity was repeatedly found in the sample mixed with SDS (data not shown). Such sensitivity of salivary hyaluronidase to SDS was previously demonstrated by Volfova et al. (2008) in *Culex* mosquitoes. It is, however, interesting to find striking differences in sensitivity to SDS between salivary hyaluronidases of various *Culicoides* species. Both, SDS-PAGE zymography and SDS-sensitivity tests gave reproducible results.

Protein profiles of *C. nubeculosus* and *C. pulicaris* SGEs were repeatedly studied by silver-stained SDS-PAGE (Fig. 3A). Major salivary protein bands ranged in weight from 16 to 83 kDa, and 18 and 19 major polypeptides were found in *C. nubeculosus* and *C. pulicaris*, respectively. In *C. nubeculosus*, the strongest protein bands had approximate molecular size of 20, 22, 38–40, and 65 kDa, whereas in *C. pulicaris*, the strongest staining was observed in 17, 28, 59, 62, and 64 kDa protein bands (Fig. 3A). Salivary hyaluronidases are known to be highly glycosylated proteins (Vlkova et al. 2014). Therefore, glycosylation in *C. nubeculosus* and *C. pulicaris* SGEs was studied by affinity blotting with lectin ConA, which recognizes mannose in *N*-glycosylated proteins. In all repeats, the most intense response in *C. nubeculosus* was observed with protein bands of 55, 76, and 83 kDa, whereas in *C. pulicaris*, ConA bound mainly to the protein bands of 42, 64, 71, and 83 kDa. Specificity of the reaction was confirmed by full inhibition of ConA binding in control strips where 0.5 M mannose was added (Fig. 3B). The poor *N*-glycosylation of the *C. nubeculosus* band, coincident with the hyaluronidase

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molecular mass, is in agreement with the NetNGlyc prediction server, which determined a single putative *N*-glycosylation site for the enzyme. In *C. pulicaris*, such a prediction is impossible, as, contrary to *C. nubeculosus*, the cDNA library or salivary proteome of this species has not been produced.

In some studies, a proinflammatory activity was induced by hyaluronidase and low molecular weight (LMW) HA fragments under stress conditions (Termeer et al. 2003, Chiarella et al. 2013). On the other hand, Huang and colleagues (2014) found that neither PH20 nor LMW HA fragments in situ stimulate cytokine and chemokine production; highly purified recombinant human hyaluronidase PH20 inhibited some aspects of inflammation, such as neutrophil accumulation, therefore possessing potential role as an anti-inflammatory agent (Huang et al. 2014) which may facilitate pathogen transmission.

Our previous studies on sand flies revealed that hyaluronidase concentration does not correlate with enzyme activity or ability to transmit *Leishmania* parasites (Černá et al. 2002, Hostomská et al. 2009, Rohoušová et al. 2012). In *Culicoides*, results by Volfova et al. (2008) allow to hypothesize about a possible effect of hyaluronidase activity on arbovirus transmission, but such functional studies require significantly more saliva to purify the enzyme and thus were beyond the scope of this work.

In conclusion, we characterized hyaluronidase activity in six *Culicoides* species of significant veterinary importance. In contrast to mosquitoes, in which hyaluronidase activity or the genes coding for hyaluronidase are missing in some species (Calvo et al. 2004, 2007; Ribeiro et al. 2007; Volfova et al. 2008), we demonstrated that this enzyme is a common component of *Culicoides* saliva. In this aspect, biting midges are close to sand flies, belonging to pool feeders, in contrast to mosquitoes known as vessel feeders. We detected substantial differences in the properties of salivary hyaluronidase among various *Culicoides* species and we suggest that further studies would be beneficial to elucidate a possible effect of hyaluronidase activity on pathogen transmission by biting midges.

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