

Research paper

Culicoides obsoletus extract relevant for diagnostics of insect bite hypersensitivity in horses

Nathalie M.A. van der Meide^a, Chantal Meulenbroeks^b, Christine van Altena^a, Anouk Schurink^c, Bart J. Ducro^c, Bettina Wagner^d, Wolfgang Leibold^e, Jens Rohwer^e, Frans Jacobs^f, Marianne M. Sloet van Oldruitenborgh-Oosterbaan^g, Huub F.J. Savelkoul^a, Edwin Tijhaar^{a,*}

^a Cell Biology and Immunology Group, Wageningen University, Wageningen, The Netherlands

^b Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

^c Animal Breeding and Genomics Centre, Wageningen University, Wageningen, The Netherlands

^d Population Medicine and Diagnostic Sciences, Cornell University, Ithaca, NY, United States

^e Immunology Unit, University of Veterinary Medicine Hannover, Hannover, Germany

^f Laboratory of Entomology, Wageningen University, Wageningen, The Netherlands

^g Department of Equine Sciences, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands

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ABSTRACT

Insect bite hypersensitivity (IBH) is an allergic dermatitis in horses caused by the bites of *Culicoides* species.

The aim of the present study was to evaluate the applicability of whole body extracts of *C. obsoletus* (the main species found feeding on horses in the Netherlands), *C. nubeculosus* (rarely found in The Netherlands) and *C. sonorensis* (typical for North America) for diagnosis of IBH in horses in The Netherlands.

Blood and serum samples of 10 clinically confirmed IBH affected and 10 healthy control horses were used to evaluate the IgE titers (ELISA) against the *Culicoides* whole body extracts of the three *Culicoides* species. Basophil degranulation was assessed by histamine release test (HRT) after stimulation with these extracts at 5, 0.5 and 0.05 µg/ml.

IBH affected horses had significantly higher IgE titers against *C. obsoletus* than against *C. nubeculosus* and *C. sonorensis*. Furthermore, *C. obsoletus* induced significantly higher histamine release in whole blood of IBH affected horses compared to the other extracts at 0.5 µg/ml. Western blot data revealed IgE binding to many proteins in *C. obsoletus* extract. This interaction was absent or weak in *C. nubeculosus* and *C. sonorensis* extracts for IBH affected horses.

Results on individual level indicate that the HRT is more sensitive than ELISA in diagnosing IBH. However, ELISA is more practical as a routine test, therefore the ELISA was further evaluated using *C. obsoletus* extract on 103 IBH affected and 100 healthy horses, which resulted in a test sensitivity and specificity of 93.2% and 90.0%, respectively. The IgE ELISA readings enabled the analysis of the predicted probability of being IBH affected. From an optical density 450 nm value of 0.33 onwards, the probability of IBH affected was more than 0.9. The results presented in this paper show that the use of native *Culicoides* spp. that

Abbreviations: ASR, allergen specific release; AUC, area under the curve; *C. nubeculosus*, *Culicoides nubeculosus*; *C. obsoletus*, *Culicoides obsoletus*; *C. sonorensis*, *Culicoides sonorensis*; HRT, histamine release test; IBH, insect bite hypersensitivity; WBE, whole body extract.

* Corresponding author at: Cell Biology and Immunology Group, P.O. Box 338, 6700AH Wageningen, The Netherlands. Tel.: +31 317 483967; fax: +31 317 483962.

E-mail address: Edwin.tijhaar@wur.nl (E. Tijhaar).

feed on horse, is important for improved diagnosis and that the described ELISA based on *C. obsoletus* can be used routinely to diagnose IBH in countries where this species is the main *Culicoides* feeding on horses.

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1. Introduction

Insect bite hypersensitivity (IBH), also called ‘sweet itch’ or ‘summer eczema’, is a seasonal recurrent allergic dermatitis in horses caused by an allergy against the bites of midges (*Culicoides* spp.), or sometimes black flies (*Simulium* spp.) and to an even lesser extend other insects (Anderson et al., 1988; Braveman et al., 1983; Broström et al., 1987; Mullens et al., 2005; Wilson et al., 2008). IBH is found in many countries of the world with a prevalence ranging from 3 to 11.6% in areas in the UK (McCaig, 1973; Littlewood, 1998), 10–60% in areas of Queensland, Australia (Riek, 1954) and 0–71.4% in regions of The Netherlands (Van Grevenhof et al., 2007).

Insect bite hypersensitivity is clinically characterized by strong pruritus and irritation, leading to alopecia and even secondary lesions due to scratching and rubbing. These symptoms are particularly found along the preferred feeding sites of the insect, which is the ventral midline, mane and tail region of the horse (Anderson et al., 1988; Braverman, 1988). Several studies indicate that the allergic reaction is predominantly IgE-mediated (Hellberg et al., 2006; Wilson et al., 2001). However, IgG(T) also seems to be involved (Wagner et al., 2006).

Intradermal injections with *Culicoides* extracts often induce immediate and delayed type skin reactions in allergic horses (Ferroglio et al., 2006; Sloet van Oldruitenborgh-Oosterbaan et al., 2009). In Iceland, where *Culicoides* spp. do not occur, IBH has never been reported (Bjornsdottir et al., 2006; Wilson et al., 2006). Several *Culicoides* species have been associated with IBH, including *C. sonorensis*, *C. nubeculosus*, *C. imicola*, *C. obsoletus* and *C. pulicaris* (Halldorsdottir et al., 1989; Mellor and McCraig, 1974; Mullens et al., 2005; Sloet van Oldruitenborgh-Oosterbaan et al., 2009; Townley et al., 1984). Intradermal tests on allergic and healthy control horses in Northern Germany and British Columbia with extracts and saliva of native and exotic *Culicoides* species, showed no difference between the native and exotic *Culicoides* species, indicating the presence of species-shared allergens (Anderson et al., 1993; Langner et al., 2008). However, intradermal skin tests in The Netherlands, with a commercial extract of *C. nubeculosus* and wild-caught *C. obsoletus*, showed the lack of cross reactivity between these *Culicoides* species (Sloet van Oldruitenborgh-Oosterbaan, 2006; Sloet van Oldruitenborgh-Oosterbaan et al., 2009).

The aim of the present study was to evaluate three *Culicoides* species for their applicability in diagnostic tests for IBH of horses in The Netherlands: *C. obsoletus* which is most frequently found on horses in The Netherlands (De Raat et al., 2008; Van der Rijt et al., 2008), *C. nubeculosus*, widely distributed in Europe, but only occasionally detected in The Netherlands (Takken et al., 2008) and not found to be attracted to horses (Van der Rijt et al., 2008)

and *C. sonorensis* which is only present in North America. Currently, *C. sonorensis* and *C. nubeculosus* are often used in studies about IBH, because they can be successfully maintained in laboratory bred colonies (Boorman, 1974). *C. obsoletus*, however, are not available from laboratory bred colonies and have to be collected from the wild. An attempt to breed *C. obsoletus* has been made, but was not very efficient (Boorman, 1985). Results presented in this report show that the use of native *Culicoides* spp. that feed on horse, is important for improved diagnosis and possibly, for future immunotherapy development. A diagnostic ELISA for IBH based on *C. obsoletus* is described that can be used routinely and has a high specificity and sensitivity.

2. Materials and methods

2.1. Animals

A total of 223 horses located in different regions of The Netherlands were included in this study. Pairs of clinically confirmed IBH affected and healthy control horses kept at the same location were formed (Schurink et al., 2009). Ten clinically confirmed IBH affected and 10 healthy control Shetland ponies were used to compare the different *Culicoides* whole body extracts in different *in vitro* diagnostic tests. The remaining 203 horses (76 Icelandic horses and 127 Shetland ponies) were used to evaluate the predictive value and test sensitivity and specificity of an ELISA using *C. obsoletus* whole body extract.

Blood samples were taken from all horses and serum was frozen in aliquots not later than 24 h after blood sampling and stored at -20°C until use. Blood sampling was approved by the Board on Animal Ethics and Experiments from Wageningen University and Utrecht University.

2.2. Collection of *Culicoides* insects

C. obsoletus insects were captured during spring and summer months using a pooter (aspirator to collect insects) (Supplementary Fig. 1) or an “Onderstepoort” suction light trap kindly provided by Laboratory of Entomology, Wageningen University.

Horses wearing an anti-insect blanket were put outside around dawn hours on warm ($>20^{\circ}\text{C}$), dry and low wind days and *Culicoides* insects were collected directly from the horses using the pooter. The insects were collected and completely frozen alive at -80°C and stored at that temperature until preparation of the extracts. A small fraction (5%) of the insects collected with the pooter was checked under a stereo microscope to confirm the species. Identification of *C. obsoletus* was based on size and wing patterns (Campbell and Pelham-Clinton, 1960) (Supplementary Fig. 2).

The “Onderstepoort” suction light trap was operated from before dusk to far after dawn near a horse stable for 19 days at different locations in The Netherlands in the summer months of 2009. Insects were captured in 100% alcohol and frozen the next day in alcohol at -80°C until determination. *Culicoides obsoletus* insects were selected and separated from the other captured insects using a stereo microscope as described above. Separated *C. obsoletus* insects were used for the preparation of extracts.

Three-day-old laboratory bred *C. sonorensis* were a kind gift from Arthropod Borne Animal Diseases Research Unit Center for Grain and Animal Health, Manhattan, USA. *C. nubeculosus* insects were kindly donated by the Institute for Animal Health, Pirbright, UK. All insects were kept frozen (without alcohol) at -80°C until preparation of the extracts.

2.3. Preparation of *Culicoides* protein extracts

Whole body extracts (WBE) were prepared from about 300 insects that were transferred to a 2 ml Eppendorf tube with 1 ml of PBS containing a protease inhibitor cocktail (Sigma–Aldrich, P8849) and crushed with a micro pestle. The insoluble material was removed by centrifugation at $13,000 \times g$ for 10 min at 4°C . Supernatant was collected and filtered through sterile Millex-GV filters (Millipore) with a pore diameter of $0.22 \mu\text{m}$ and protein content of the filtrate was determined by $\text{OD}_{280\text{nm}}$ measurement on a Nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific). Samples were aliquoted, directly frozen in liquid nitrogen and stored at -80°C until use. Quality of the protein WBE was checked by protein staining with Gelcode Coomassie blue staining (Thermo Scientific) after proteins were separated by 15% SDS–PAGE.

2.4. SDS–PAGE and Western blotting

Whole body protein extract samples, $20 \mu\text{g/lane}$ (Western blotting) or $60 \mu\text{g/lane}$ (Coomassie staining) were heated at 96°C for 5 min with sample buffer containing dithiothreitol (DTT) and separated by SDS–PAGE (15% gel). These separated proteins were transferred to a nitrocellulose membrane (Protrans, Schleicher & Schuell, Bioscience GmbH) by means of electrophoresis. Membranes were blocked with 5% non-fat cow's milk in Tris buffered saline (TBS)–Tween (10 mM Tris, 150 mM NaCl, pH 7.5, 0.05% (v/v) Tween 20) for 1 h at room temperature (RT) and then incubated overnight with horse sera from allergic or control horses diluted 1:10 in 5% non-fat cow's milk in TBS–Tween. Membranes were then incubated for 1.5 h with a mAb against horse IgE ($\alpha\text{IgE-176}$) (Wagner et al., 2003) followed by goat anti-mouse IgG horseradish peroxidase (Dako, 1:1000 in milk powder/TBS–Tween). Between each incubation step, membranes were washed three times with TBS–Tween.

Signal was detected by development with an enhanced chemiluminescence (ECL) western blotting detection reagent (Amersham, GE Healthcare) according to the manufacturer's protocol and visualized by the use of Lumni-til

chemiluminescent Detection Film (Roche, Woerden, The Netherlands).

2.5. Histamine release test (HRT)

The histamine release by basophils was determined by a modified method of Kaul (1998). Blood samples were collected in anticoagulant tubes (EDTA) and kept at RT in the dark until further use within 24 h. The total blood cells were washed twice ($500 \times g$ for 10 min) with PBS to remove non-cell bound antibodies. Supernatant was discarded and the cell pellet was resuspended in PBS to its original blood sample volume. Endogenous histamine from whole body *Culicoides* extracts was removed by PD-10 Desalting columns (GE Healthcare) according to manufacturer's recommendations. The antigen induced histamine release was obtained by incubating $250 \mu\text{l}$ of washed blood cells with $250 \mu\text{l}$ of PIPES buffer (110 mM NaCl, 5 mM KCl, 40 mM NaOH, 2 mM CaCl_2 , 25 mM PIPES and 2 mM MgCl_2) containing the histamine depleted *Culicoides* whole body extracts at final concentrations of $5 \mu\text{g/ml}$, $0.5 \mu\text{g/ml}$ and $0.05 \mu\text{g/ml}$ at 37°C for 60 min. Spontaneous release was obtained by incubating $250 \mu\text{l}$ of PIPES B buffer with $250 \mu\text{l}$ of washed blood cells at 37°C for 60 min. Physical maximum release was obtained by boiling $200 \mu\text{l}$ of washed blood cells with $800 \mu\text{l}$ of PIPES buffer for 10 min. After incubation, all samples were chilled on ice for 5 min and pelleted by spinning down at $700 \times g$ for 10 min. The cell-free supernatants were collected and stored at -20°C . Subsequently, competitive RIA was carried out as per the manufacturer's instructions (LDN Nordhorn, Germany) to determine the histamine content of the supernatants.

The maximum amount of histamine obtained by boiling was set to 100%. The histamine content of each test sample was calculated from this maximum histamine release. The allergen specific release (ASR) is calculated as: $\text{ASR} = (\text{sample induced release} - \text{spontaneous release}) / (\text{maximum release} - \text{spontaneous release}) \times 100\%$. Net-histamine releases that were equal or greater than 10% of the maximum release were considered as positive.

2.6. *Culicoides*-specific IgE ELISA

Specific IgE levels in sera of 10 IBH affected and 10 healthy control Shetland ponies, binding the different *Culicoides* WBE, were measured by ELISA. Optimal coating concentration, serum dilution and antibody concentrations were determined prior to the experiment by titration of the different components. Costar 96-well microtiter plates were coated with $100 \mu\text{l/well}$ of $10 \mu\text{g/ml}$ *C. obsoletus*, *C. nubeculosus* or *C. sonorensis* extract, diluted in PBS, and incubated overnight at 4°C and afterwards blocked with $150 \mu\text{l}$ of a 1.5% casein buffer (SDT, Germany) for 1.5 h at RT. Plates were washed 5 times with PBS containing 0.05% Tween 20, followed by incubation for 1.5 h at RT with $100 \mu\text{l}$ of horse serum samples diluted 1:5 in a 1.5% casein buffer. After washing, wells were incubated for 1 h at RT with $100 \mu\text{l}$ of $2.5 \mu\text{g/ml}$ mouse monoclonal anti-equine IgE-176 (Wagner et al., 2003) diluted in casein buffer. After washing the plates 5 times with PBS containing 0.05% Tween 20, goat

anti-mouse peroxidase conjugate (multispecies adsorbed, Serotec) diluted 1000 times in casein buffer, was applied to the wells and incubated for 1 h at RT. After 5 washes with PBS/0.05% Tween 20, 100 µl tetramethylbenzidine (high sensitivity, SDT, Germany) was added to the wells and incubated for 10 min at room temperature. The reaction was stopped with 100 µl/well of 1% HCl. Absorbance was measured with a multi-mode microplate reader (SpectraMax M5, Molecular Devices) at a wave length of 450 nm corrected for 650 nm. Based on the preliminary experiments a standard serum dilution of 1:5 was selected as suitable for comparison of OD_{450nm} values in the IgE ELISA. The cut off level was assigned as the mean + 3 times the standard deviation (SD) of the IgE levels of the healthy control horses.

An additional 203 horse serum samples, 103 IBH affected and 100 healthy control horses, were evaluated for *C. obsoletus* specific IgE (OD_{450nm}) values in a 384 wells plate to determine the sensitivity and specificity of this *Culicoides*-specific ELISA. The same conditions as described for the 96-wells plate were used, with 20 µl volumes per well. Distribution plots of the healthy and IBH affected horses were obtained by categorizing horses according to their OD_{450nm} values. The first category ranged from 0 to 0.01, the second from 0.01 to 0.02 and the following categories each increased with 0.02 up to 0.32. Then the categories ranged from 0.32 to 0.35, 0.35 to 0.4 and subsequent categories each increased with 0.2 until the maximum OD_{450nm} of 2.2 was reached.

Accuracy of diagnostic tests is often determined from so-called receiver-operating characteristic (ROC) curves. ROC-curves represent the trade-off between sensitivity (i.e. true positive rate) of a test and (1-specificity) (i.e. false positive rate) at all possible positivity cut-off points. The area under the curve summarizes the overall diagnostic accuracy. It takes values from 0 to 1, where a value of 0 indicates a perfectly inaccurate test and a value of 1 reflects a perfectly accurate test. A good first choice for a test cut-off value that results in a balanced optimal sensitivity and specificity is that value which corresponds to a point on the ROC-curve nearest to the upper left corner of the ROC graph.

2.7. Statistical analysis

Analysis of variance was performed on log transformed data obtained by either HRT or ELISA to determine influence of *Culicoides* species on the outcome. Factors included in the model were, *Culicoides*-species (*C. nubeculosus*, *C. sonorensis* and *C. obsoletus*), IBH-status of the horse (yes/no) and interaction between these two factors. Additionally, WBE concentration (0.05, 0.5 and 5 µg/ml) was included in the analysis of HRT results. IBH-status within individual horses was included as random factor since the same set of horses were used in testing the three *Culicoides* types. Analysis was performed using PROC MIXED of SAS (SAS Inc, V9.2).

The relation of OD_{450nm} value in the IgE-ELISA to the IBH-status (negative or positive) was analyzed with a logistic regression. The analysis was performed with the PROC LOGISTIC of SAS (SAS Inc, V9.2).

3. Results

3.1. Collection of *C. obsoletus*

Two different collection methods ("Onderstepoort" light trap and pooter) were used to determine the most selective and efficient way of collecting *C. obsoletus* from the wild.

Determination of insects collected with the "Onderstepoort" light trap revealed that many different insect species were collected in this manner, from which only a small fraction (<1%) belonged to *Culicoides* species. A total of 766 *Culicoides* midges were collected during these 19 days. The large majority of these *Culicoides* were identified as *C. obsoletus* (82%), followed by *C. dewulfii* (6.5%) and *C. punctatus* (5.0%).

Using the pooter hundreds of *Culicoides* insects were easily collected within an hour from a horse wearing an anti-insect blanket. A small part (±5%) of the collected insects was used for identification and these were all identified as *C. obsoletus* based on size and wing patterns.

3.2. Quality of extracts of *Culicoides* captures by different methods

WBE were prepared from *C. obsoletus* insects collected alive with the pooter system, as well as insects collected in alcohol with the Onderstepoort light trap. Insects that were stored in alcohol were excluded in the following experiments due to substantial degradation of the proteins revealed on SDS-PAGE (Supplementary Fig. 3).

The extracts from laboratory bred *C. sonorensis* and *C. nubeculosus* and wild-caught *C. obsoletus* prepared in the same manner from freshly frozen insects, showed some differences in the lower molecular weight regions (indicated by arrows in Fig. 1) on SDS-PAGE (15% gel), but overall pattern and intensity of the protein bands were similar, with no obvious degradation, indicating similar quality of the different extracts.

3.3. IgE-specific antibodies in horse sera specific for *Culicoides* proteins as determined by Western blotting

Western blotting was performed to evaluate sera of clinically confirmed IBH affected and healthy control Shetland ponies for the presence of IgE specific for proteins from *C. obsoletus*, *C. sonorensis* and *C. nubeculosus*. Typical examples of 5 allergic (upper panel) and 5 healthy horses (lower panel) are shown (Fig. 2).

The IgE in sera of all allergic horses reacted strongly to a number of proteins from *C. obsoletus* extract, but much weaker with proteins from *C. sonorensis* and *C. nubeculosus* extract despite the similar quality of the extracts. The antigen recognition pattern for each individual horse was different, but most IBH affected horses reacted with a protein(s) around 20 kDa. IgE from the healthy horses, except for one, hardly recognized any proteins from any of the 3 *Culicoides* species. Proteins that did bind to the IgE in serum of healthy horses all had a Mw of 25 kDa or higher (Fig. 2).

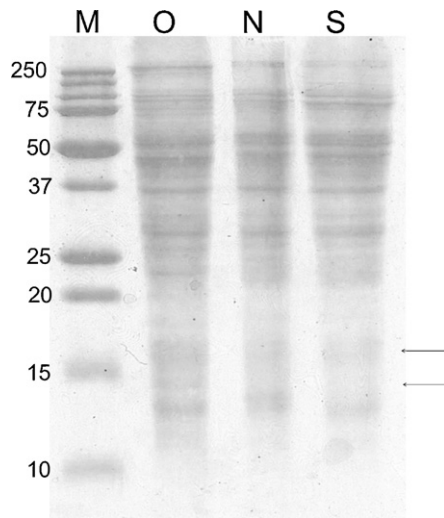


Fig. 1. Coomassie staining of proteins from *C. obsoletus* (O), *C. nubeculosus* (N) and *C. sonorensis* (S) whole body extracts demonstrating similar quality of the extracts. M represents the molecular weight marker.

3.4. Basophil degranulation induced by *Culicoides* whole body extracts determined by histamine release test

For each of the 3 different *Culicoides* species the allergen specific release (ASR) of histamine from basophils was tested at 3 WBE concentrations on whole blood samples of 10 IBH affected and 10 healthy control Shetland ponies (Fig. 3), including the horses that were used for the Western blot analysis.

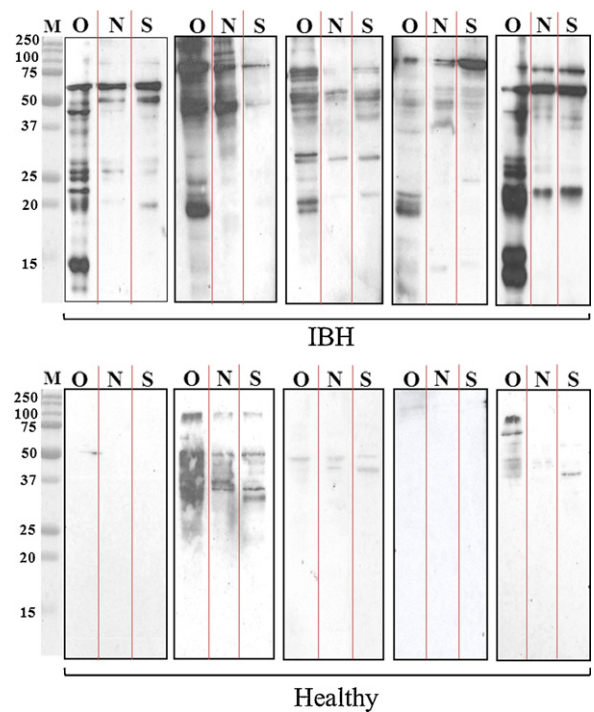


Fig. 2. Immunoblot analysis of 5 IBH affected and 5 healthy horses using whole body extracts of *C. obsoletus* (O), *C. nubeculosus* (N) and *C. sonorensis* (S). Proteins were separated on 15% SDS–PAGE gels and transferred to nitrocellulose membranes. Binding of IgE from horse sera was detected with an anti-equine IgE mouse mAb and HRP goat anti-mouse IgG. The molecular weight marker (M) is indicated on the left in kDa.

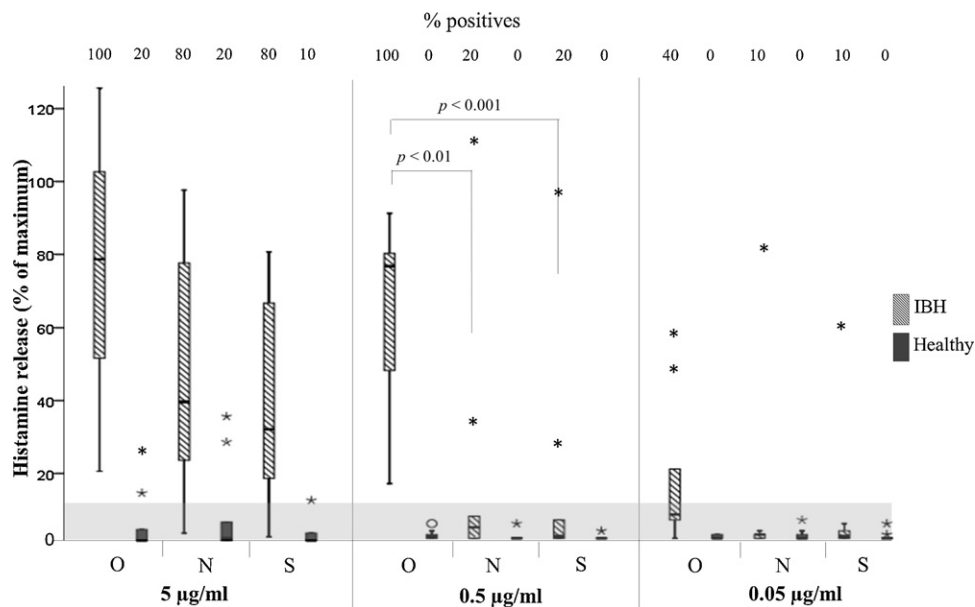


Fig. 3. *Culicoides* induced histamine release as percentage of maximum release of 10 IBH affected horses and 10 healthy control horses. Whole blood samples were analyzed after stimulation with three different *Culicoides* whole body extracts (O = *C. obsoletus*, N = *C. nubeculosus*, S = *C. sonorensis*) tested at three different concentrations ($\mu\text{g/ml}$, x-axis) by a histamine release test (HRT). Results are presented in box plots. The horizontal line near the middle of the box is the median of the measurements. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represents the minimum and maximum value. The stars represent the outliers. Horses with histamine release above the cut off value of 10% (highlighted in gray) were considered positive (Kaul, 1998). Statistical analysis was performed on log transformed data. The p values account for unequal variance.

At the highest WBE concentration of 5 µg/ml 10 out of 10 (100%) of the IBH affected horses scored positive on the *C. obsoletus* WBE, while this was 8 out of 10 (80%) for *C. nubeculosus* and *C. sonorensis*. However, at this highest WBE concentration some of the healthy horses also scored positive on all *Culicoides* species. At a concentration of 0.5 µg/ml none of the healthy control horses scored positive on any of the extracts, but at this concentration only 20% of the IBH affected horses scored positive with *C. nubeculosus* and *C. sonorensis* extract. In contrast, 100% of the IBH affected horses scored positive when the *C. obsoletus* extract was used. At this concentration the reactivity toward *C. obsoletus* was significantly higher than to *C. nubeculosus* ($p < 0.01$) and *C. sonorensis* ($p < 0.001$) (Fig. 3). At the lowest WBE concentration of 0.05 µg/ml 40% of the IBH affected horses were still found to be positive with *C. obsoletus*, whereas for *C. nubeculosus* and *C. sonorensis* this was only 10% of the IBH affected horses. Also at this concentration all healthy control horses had a negative test results for all *Culicoides* extracts.

3.5. IgE measurements in horse sera against *Culicoides* whole body extracts by ELISA

Specific IgE serum levels against WBE of the three different *Culicoides* species were determined in an indirect ELISA (Fig. 4).

IgE levels expressed as OD_{450nm} values of the clinically confirmed IBH affected Shetland ponies against *C. obsoletus* extract were significantly higher compared to OD_{450nm} values of the same IBH affected horses against the other extracts (both $p < 0.0001$). With *C. obsoletus* extract

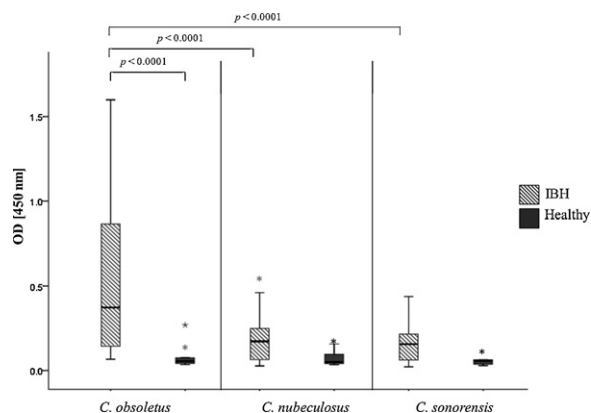


Fig. 4. IgE levels presented as OD_{450nm} values against three different *Culicoides* extracts sera diluted 1:5 of 10 IBH affected horses and 10 healthy control horses. Results are presented in box plots, for a definition see legend of Fig. 3. Statistical analysis was performed on log transformed data. The p values account for unequal variance.

only, specific IgE serum levels of IBH affected horses were significantly higher than specific IgE levels of healthy control horses ($p < 0.0001$).

3.6. Comparison of ELISA and HRT data for individual horses

Individual HRT (Fig. 5 upper panel) and ELISA (Fig. 5 lower panel) responses to the WBE of the different *Culicoides* species were compared for the same Shetland ponies

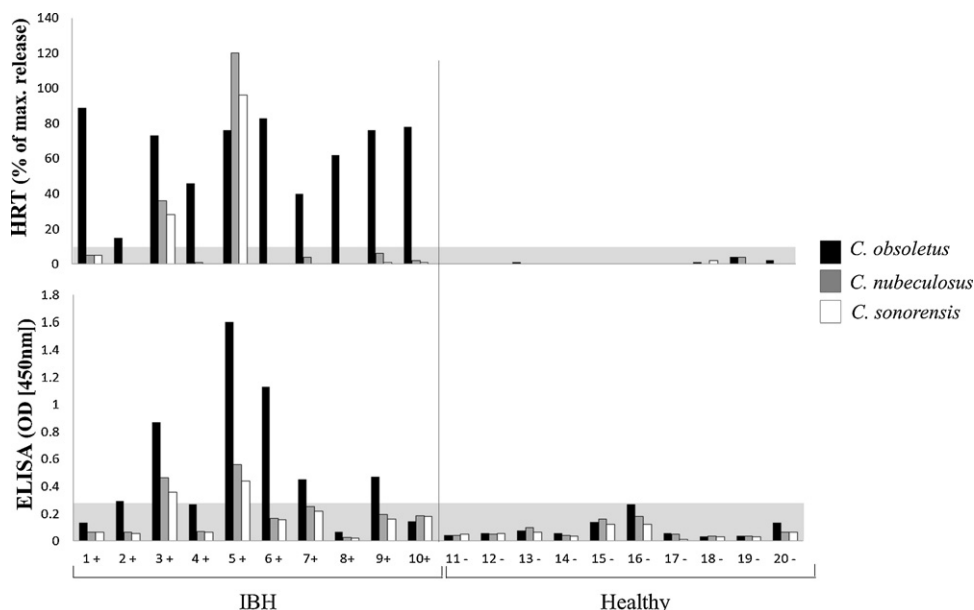


Fig. 5. Comparison between histamine release test (HRT) and ELISA of individual horses. Numbers 1–10 represent IBH affected horses, horses 11–20 represent healthy control horses. IgE levels (ELISA) are presented as OD_{450nm} values; Histamine release after stimulation with whole body extract at a concentration of 0.5 µg/ml, is presented as percentage of maximum release. The cut-off of each test is highlighted in light gray and corresponds to 10% for the HRT and mean + 3 times standard deviation of the *C. obsoletus* values of the healthy control horses for the ELISA.

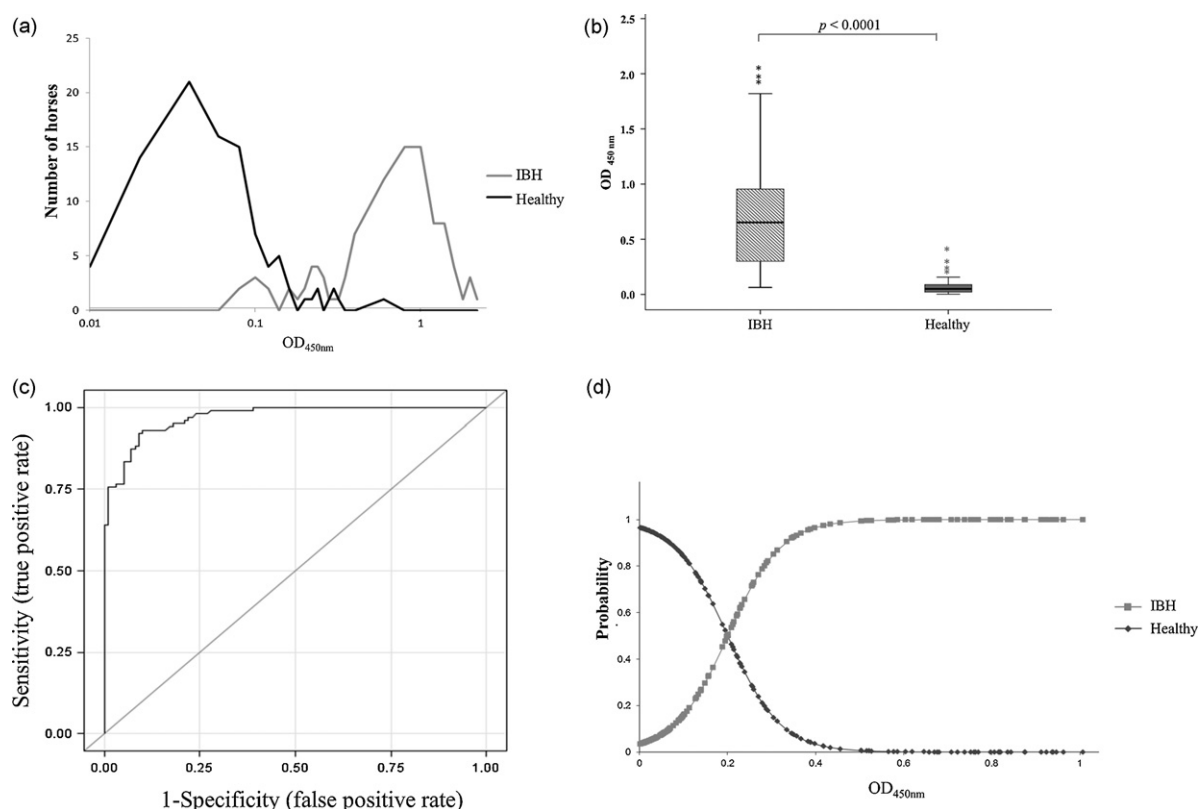


Fig. 6. Validation of the *C. obsoletus*-specific IgE ELISA on serum samples of 100 healthy and 103 IBH-affected horses. (a) Distribution plots of the healthy and IBH-affected horses categorized for their OD_{450nm} ELISA reading. (b) Box plot of IgE levels of the healthy and IBH-affected horses presented as OD_{450nm} values. The horizontal line near the middle of the box is the median of the measurements. The bottom and top of the box are the 25th and 75th percentile, respectively. The end of the whiskers represents the minimum and maximum value. The stars represent the outliers. The p value accounts for unequal variance. (c) ROC curve of *C. obsoletus*-specific IgE ELISA on sera samples of 100 healthy and 103 IBH-affected horses. The area under the curve, indicating diagnostic accuracy of the test was 0.97. (d) Estimated probability to be IBH-affected in relation to the OD_{450nm} value. Determined by logistic regression analysis of the obtained ELISA data.

used in Figs. 3 and 4. The values at a concentration of 0.5 µg/ml per extract were chosen to analyze the horses on individual level for the HRT, because at this concentration the best distinction could be made between IBH affected and healthy control horses (Section 3.5). On individual level, for the *C. obsoletus* extract the histamine release of all IBH affected horses was higher than 10% and therefore positive (Kaul, 1998), whereas the maximum histamine release of all healthy control horses was below 10% of the maximum histamine release. For the ELISA 6 out of 10 IBH affected horses had OD_{450nm} values against *C. obsoletus* extract above the set cut-off level (mean + 3 times the standard deviation SD of the OD_{450nm} values of the healthy control horses). With this cut-off level, all healthy horses were negative. In 6 out of 10 IBH cases (horses 2, 3, 4, 5, 6, and 7) the HRT and ELISA values against *C. obsoletus* WBE correlated with each other. Interestingly, the IBH affected horses with OD_{450nm} values below the cut-off level in the ELISA (horses 1, 4, 8 and 10) did have a high positive histamine release with the HRT using *C. obsoletus* WBE. One horse had a higher histamine release after stimulation with *C. nubeculosus* and *C. sonorensis* WBE compared to *C. obsoletus*, but did have a higher IgE level against *C. obsoletus* when measured by ELISA.

3.7. ELISA test sensitivity and specificity

The results described in this section indicate that the HRT outperforms the ELISA as a diagnostic test for IBH, but the ELISA is more practical as a routine test. It is much less laborious and can be performed on serum samples, while the HRT requires fresh full blood. Therefore the ELISA using *C. obsoletus* extract was further evaluated with sera of 103 clinically confirmed IBH affected horses and 100 healthy control horses (76 Icelandic horses and 127 Shetland ponies).

When categorizing the healthy and IBH affected horses according to their OD_{450nm} values from the IgE ELISA, two distributions with equal variance were observed which are nearly baseline-separated (Fig. 6a). Although the distribution curve of healthy and IBH affected horses overlapped somewhat, the IBH-affected horses had higher serum IgE levels against *C. obsoletus* WBE compared to healthy control horses ($p < 0.0001$) (Fig. 6b).

The pattern of the ROC-curve (Fig. 6c) indicates that the sensitivity sharply increases already at low false positive rates. The sensitivity of the test is therefore high over a large range of cut-off points. The accuracy of the test as evaluated by the area under the curve (AUC) is high and amounted

to 0.97, indicating that high sensitivity is achieved with a high specificity. The point on the ROC-curve nearest to the upper left corner of the curve corresponds with a sensitivity of 93.2% and a specificity of 90.0% and is obtained at an OD_{450nm} cut-off value of 0.2.

Logistic regression was performed to analyze the relation of IgE OD_{450nm} values to the IBH-status. The response IBH-affected or healthy was regressed on OD_{450nm} values.

Fig. 6d shows the predicted probabilities of both IBH-outcomes (i.e. affected or healthy) related to OD_{450nm} values. With IgE-values close to zero the probability of being healthy is much higher than the probability of having IBH; up to an IgE value (OD_{450nm} value) of 0.07 (true for 70% of the healthy horses) the probability of being IBH negative is approximately 10 times higher than being IBH positive and 5 times higher for a value of 0.12 (true for 85% of the healthy horses). At the inflection point at an OD_{450nm} value of approximately 0.2 the probability being IBH-positive or negative is equal. From an OD_{450nm} value of 0.33 onwards (true for 75% of the IBH affected horses) the probability of IBH-positive is more than 0.9.

4. Discussion

In this study we evaluated three different *Culicoides* whole body extracts for their applicability for *in vitro* diagnosis of IBH horses in The Netherlands. We show that *C. obsoletus* (a species found feeding on horses in The Netherlands) whole body extract is much better for *in vitro* diagnosis of IBH by ELISA and HRT, than *C. nubeculosus* and *C. sonorensis* (not found feeding on horses in the Netherlands) whole body extracts. An IgE-ELISA with *C. obsoletus* whole body extract performed with 103 IBH affected horses and 100 healthy horses located in the Netherlands demonstrated a high sensitivity and specificity (93.2% and 90.0%, respectively) and can thus be used as a valuable test to diagnose horses for IBH in countries where *C. obsoletus* is the main species found feeding on horses.

This study describes an easy and selective method of catching *Culicoides* spp. attracted to horses by using a pooter. Although this method is initially more labor intensive than an “Onderstepoort” suction trap, it is a selective way of collecting preferentially those *Culicoides* species attracted to horses. This obviates the need for the labor intensive selection of the desired *Culicoides* species out of a large majority of unwanted insects that is required when using a light trap. The biggest advantage of the pooter method is that the insects are caught in a gentle way that keeps them alive, preventing substantial protein degradation as is observed for insects captured in alcohol by the “Onderstepoort” light trap.

The majority of *Culicoides* spp. that were caught by both capturing methods was found to be *C. obsoletus*: over 80% when using the light trap and nearly 100% when directly collected from the horse by the pooter. This is in agreement with earlier studies performed in The Netherlands that also found *C. obsoletus* to be the main *Culicoides* species attracted to horses (De Raat et al., 2008; Van der Rijt et al., 2008). In other countries, such as England (Wilson et al., 2008) Ireland (Townley et al., 1984), Japan (Yamashita et al.,

1957) and United States (Mullens et al., 2005), *C. obsoletus* was also found to be the most important *Culicoides* species attracted to horses.

Comparison of three *Culicoides* extracts demonstrates that in The Netherlands horses with IBH have more specific IgE directed against whole body extracts of *C. obsoletus* than against *Culicoides* species that do not feed on these horses. Although cross-reactivity between different *Culicoides* species has been reported (Anderson et al., 1993; Langner et al., 2008) our study shows weaker IgE binding in Western blot and ELISA to proteins of non-indigenous *Culicoides* species compared to native *C. obsoletus*, which was also observed before (Wilson et al., 2008). This indicates the importance of using extracts from native *Culicoides* species feeding on horses for reliable diagnostics of IBH.

The binding of IgE from allergic horses with *C. sonorensis* and *C. nubeculosus* proteins might be due to crossreactivity between these proteins and the *C. obsoletus* antigens and maybe also proteins of other insect species that the horses were exposed to. The horses in this study might have been exposed to the native *C. nubeculosus*, but our as well as another study performed in The Netherlands (Van der Rijt et al., 2008) did not find any *C. nubeculosus* insects to be attracted to horses. Although *C. sonorensis* and *C. nubeculosus* antigens have previously been successfully used in different diagnostic tests (Langner et al., 2009; Schaffartzik et al., 2010, 2011) our study clearly shows the importance of using an extract of a *Culicoides* species to which horses have been actually exposed to for diagnosis. Western blot data revealed many IgE binding proteins in *C. obsoletus* extract that were absent in *C. nubeculosus* and *C. sonorensis* extracts. Interestingly, a protein of around 20 kDa from *C. obsoletus* extract was found to be bound by IgE from almost all clinically confirmed IBH affected horses, whereas this was not observed for *C. nubeculosus* and *C. sonorensis* extracts and also not for IgE from healthy horses. This makes this protein an interesting candidate allergen for further characterization.

The *C. obsoletus* insects collected from the wild using the pooter were all female species, since females need blood to reproduce and were trying to feed on the horse when captured. Although WBE of laboratory-bred insects were of both sexes, it is unlikely that this explains the lower allergen-reactivity by *C. nubeculosus* and *C. sonorensis* in this study. Other studies found *Culicoides* extracts made from males only, to be just as effective in stimulating horse basophils (Marti et al., 1999) and non-salivary antigens from the thorax of *Culicoides* spp. have shown IgE-reactivity with IBH-affected horses (Wilson et al., 2001). One of the IBH affected horses reacted even slightly stronger in the HRT with *C. nubeculosus* and *C. sonorensis* than with *C. obsoletus* WBE, and therefore rules out a lack of antigens of lower protein quality in the *C. nubeculosus* and *C. sonorensis* WBE. This was also demonstrated by the similar pattern and intensity of the protein bands of all three WBE on SDS-PAGE.

Surprisingly, some horses had low IgE binding in the ELISA but a high histamine release in response to *Culicoides* extract stimulation in the HRT. IgG(T) has also been observed to bind to skin mast cells and therefore might play an important role in the histamine release reaction in IBH

(Wagner et al., 2006). Therefore, the observation that some horses have low IgE binding in ELISA but a high histamine release in response to *Culicoides* extract stimulation, might be due to cross-linking of allergen specific IgG(T) instead of IgE.

The results in this study indicate that the histamine release test (HRT) might be more sensitive and reliable for diagnosis of IBH than the ELISA. However, this comparison was made on a relatively small number of horses (10 IBH affected and 10 healthy control horses). When tested with a large number of horses, the IgE ELISA, resulted in a high specificity (90.0) and sensitivity (93.2) and proved to be the method of choice for routine screening, because it is more robust, easier to perform and more economical than the HRT. The test clearly discriminates between IBH-affected and healthy controls such that there is little overlap of distributions. The accuracy of the test as evaluated by the area under the curve (AUC) of the ROC-graph is high (0.97), indicating that high sensitivity is achieved with a high specificity. At an OD_{450nm} cut-off value for positivity of 0.2 the test has a sensitivity of 93.2% and a specificity of 90.0%. However, this cut-off value does not take the actual OD_{450nm} value of an individual horse into account, apart from being lower or higher than the determined cut-off value for positivity. Therefore logistic regression analysis was performed to determine the relation of the IgE OD_{450nm} values to the IBH-status. For most horses the ELISA can determine with 90–100% probability the correct IBH-status of the individual tested horse. For those OD_{450nm} values where reliability is less, e.g. around the inflection point at OD_{450nm} value of 0.2, the horse owner can choose for an additional HRT to obtain a more conclusive diagnosis. Western blot analysis on *C. obsoletus* whole body extracts using serum of healthy horses showed some IgE reactivity of these horses against proteins with Mw above 25 kD. Therefore, the use of selected recombinant proteins from *C. obsoletus* might further improve the sensitivity of the ELISA described in this study.

At present, treatment of IBH is based on insect avoidance by stabling, use of anti-midge blankets (pajamas) or insect repellants and suppression of symptoms by the use of corticosteroids. Specific immunotherapy might be possible and some immunotherapy trials have been carried out with *Culicoides* whole body extracts, but with varying results (Anderson et al., 1996; Barbet et al., 1990). The use of purified allergens could further improve diagnostics, but could also be a benefit for immunotherapy. Collecting insects is very time consuming and for immunotherapy over 10,000 insects were necessary per horse (Anderson et al., 1996), therefore recombinant allergens from an infinite source would be a sensible alternative for whole body extracts.

Currently, allergens have been identified and produced from species that are not present or common in The Netherlands, e.g. *C. sonorensis* and *C. nubeculosus* (Langner et al., 2009; Schaffartzik et al., 2011). Implementation of future immunotherapy in horses will depend on the availability of correct allergens and therefore the use of allergens from *Culicoides* spp. to which horses have been exposed, which for The Netherlands is mostly *C. obsoletus*, might be crucial.

In conclusion, our results show that horses with IBH in the Netherlands have much more IgE antibodies against *C. obsoletus* proteins compared to *C. sonorensis* and *C. nubeculosus* proteins which can be routinely detected in different diagnostic tests. The developed ELISA to identify sensitization against *C. obsoletus* allergens provides a valuable diagnostic test to discriminate IBH affected from healthy control horses in The Netherlands, but will also be valuable in other countries where *C. obsoletus* is mostly found feeding on horses.

Conflict of interest statement

None.

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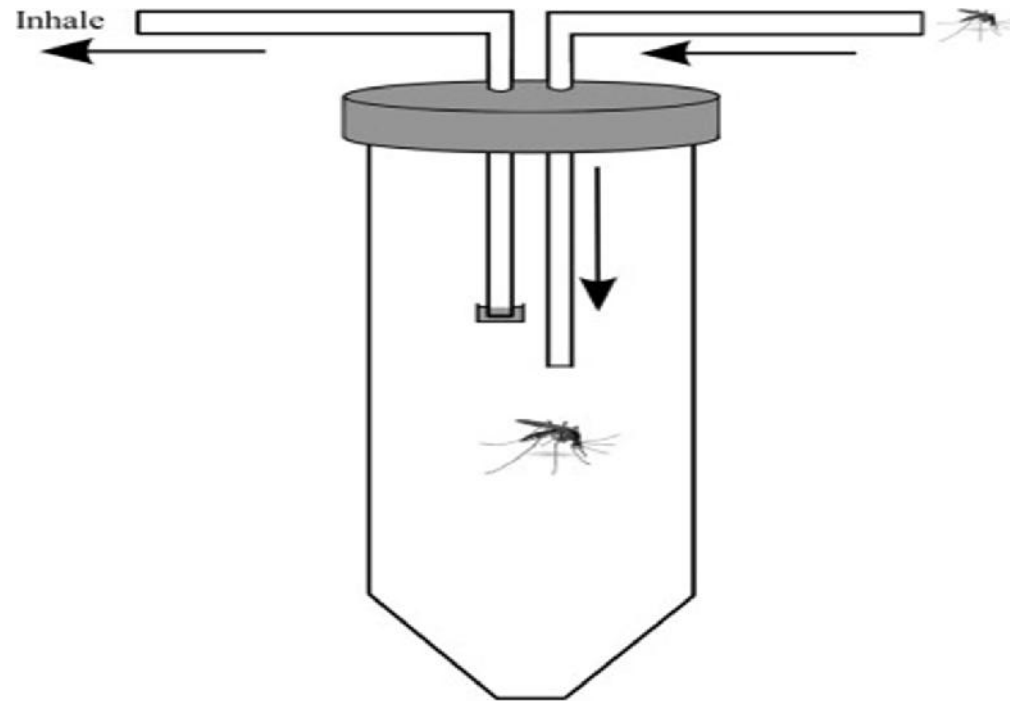
Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2012.07.007>.

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Supplementary Fig. 1 Pooter used to collect *Culicoides* from horses. Two separate straws are inserted in two holes into a 50 ml tube. One straw has a fine mesh covering the end inside of the tube. This straw is us...

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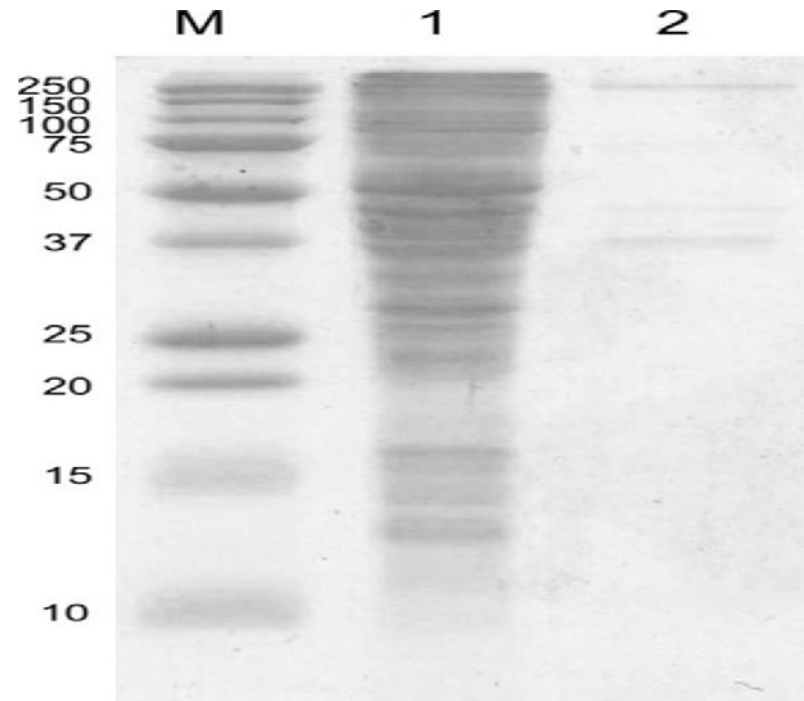
Supplementary Fig. 2 Individual example of a female *C. obsoletus* used in this study and identified on size and wing pattern according to [Campbell and Pelham-Clinton \(1960\)](#) .

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Supplementary Fig. 3 Coomassie staining of proteins from *C. obsoletus* extract (50 µg), from insects collected alive with the pooter (1) and from insects collected in alcohol with the Onderstepoort light trap (2...

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