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Equine *Culicoides* Hypersensitivity: Evaluation of a Skin Test and of Humoral Response

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With 1 figure and 2 tables

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Summary

Intradermal tests were carried out on 18 horses with clinical signs of *Culicoides* hypersensitivity (CHS) and 23 horses without clinical signs of CHS, and sera from these horses were analysed by SDS-PAGE and Western blotting (W-B). Intradermal injections of 0.1 ml of 25 µg/µl sterile *Culicoides* extract, 0.1 ml of 1 : 10 000 histamine (positive control) and 0.1 ml of physiological saline (negative control) were made in the dermis of the middle region of the neck. Analysis of reactions indicated that a 1 cm wheal and a skinfold thickness > 10% at 24 h represented a valid cut-off between horses with and without CHS. In these conditions the test, even in winter when clinical signs were absent, had 100% sensitivity and specificity. The W-B was performed after running *Culicoides* extract on a 12% polyacrylamide gel. The test revealed the presence of several bands with molecular weight ranging from 6 to 200 kDa. In particular, a band of 65 kDa was predominantly found in hypersensitive horses by using an anti-IgE antibody while in normal horses the same band was mainly detected by using an anti-IgG antibody. Our results demonstrated that the skin test is a valid diagnostic test, with high sensitivity and specificity and that the band of about 65 kDa probably corresponds to the allergen involved in the pathogenesis of CHS.

Introduction

Culicoides hypersensitivity (CHS), called 'sweet itch' in the USA and Australia and 'summer eczema' or 'allergic urticaria' in Europe, is a worldwide chronic, recurrent and seasonal dermatitis of horses caused by the allergy to the bite of *Culicoides* (Braveman et al., 1983; Bromstrom et al., 1987; Anderson et al., 1988). The disease is characterized by intense irritation, scratching and rubbing, leading to serous effusion, hair loss and the development of secondary skin lesions. Affected horses may cause severe injury to themselves and their owners in an effort to relieve itch. It has been suggested that genetic factors can play a role in the pathogenesis of CHS, because the prevalence of CHS seems to be higher in some

breeds, such as Icelandic horses born in Iceland (Bromstrom et al., 1987). However, the prevalence of CHS in Icelandic horses born in other countries is not significantly different from the prevalence in other breeds (Reiher and Bjornsdóttir, 2004); therefore, the role of genetic factor needs further studies. The diagnosis of CHS is usually based on clinical signs after elimination of other potential causes while more accurate tools, such as the skin test, are rarely used (Fadok and Greiner, 1990; Anderson et al., 1991). In Italy the occurrence of CHS has long been suspected, but only recently confirmed by means of a skin test characterized by a 100% sensitivity, but only an 88% specificity (Cagnasso et al., 1996). A recent study using Western blotting (W-B) showed that there are specific antibodies to *Culicoides* salivary gland antigens in affected horses (Wilson et al., 2001), but this test has been found unsuitable for the diagnosis of CHS.

Affected horses do not usually show any clinical signs of CHS and in winter could be sold as normal horses. In view of this it would be useful to develop a test for the diagnosis of CHS in the absence of clinical signs.

The aim of the present study was to develop a skin test and to employ W-B to investigate the pattern of *Culicoides* antigen recognized by IgG and IgE in sera from hypersensitive and normal horses exposed to *Culicoides*. We suggest that the improvement of knowledge in the pathogenesis of CHS could also favour the development of better diagnostic methods and new therapy protocols. As successful desensitization has been reported by some authors (Mansfield et al., 1998), the production of a recombinant antigen involved in triggering CHS could allow the development of a desensitizing protocol.

Materials and Methods

Collection of *Culicoides*

Culicoides specimens were captured in summer using CDC traps (John W. Hock Company, Gainesville, FL, USA). The captures were performed in stables with horses during the twilight hours, mainly on warm and windless days, with the traps placed on the walls of the stable and emptied out every

morning. Insects were collected and classified as *Culicoides obsoletus* according to the identification key of wings patterns (Meiswinkel et al., 1994), eyes distance, palpus segments differences, antennal and genital morphology. *Culicoides* with evidence of a blood meal were eliminated and the others were frozen at -18°C .

Extract preparation

The insect extract was obtained from the whole insects and from the insect heads separated from the body by using a thin needle.

The whole bodies and the insect heads were crushed in physiological saline, centrifuged at 2000 g for 10 min and the protein concentration of the supernatant was estimated by the DC Protein Assay (Bio-Rad, Hercules, CA, USA). After several trials to evaluate the optimal extract concentration, the extracts used in the intradermal test were diluted to 25 $\mu\text{g}/\mu\text{l}$, sterilised by filtering through decreasing sterile Millipore filters (Millipore, Billerica, MA, USA) with a pore diameter of 22 μm and then measured into aliquots and kept frozen in single-dose vials at -18°C until use.

Collection of serum samples

Eighteen horses with reported signs of CHS and 23 horses that had never exhibited clinical signs of CHS but were kept in the same area as the diseased ones were selected. The positive ones were horses with a reported seasonal summer and recurrent itch, causing scratching, hair loss, crusts, ulcerations and sometimes cutaneous sores. The blood was collected into plain vacutainers in early spring before *Culicoides* season. After collection it was spun at 1000 g for 10 min, the serum was harvested and stored at -18°C .

Intradermal reaction

The intradermal skin test was performed in winter in the middle region of the neck of both healthy and hypersensitive horses. The skin of the horses was clipped and skinfold thickness of the injection site was measured immediately before the injection with a calliper (Hauptner, Solingen, Germany). Intradermal injections of 0.1 ml of the sterile *Culicoides* whole body extract, physiological saline as negative control and 1 : 10 000 histamine (w/v in physiological solution) as positive control were made 10 cm apart using 25-gauge needles. Site of injection has been randomized and skinfold

thickness and maximum wheal width increases were measured at 1, 5 and 24 h after injections by a person different from the one who did the injections. Skinfold thickness and maximum wheal width increases were measured at 1, 5 and 24 h after injections. The evaluation of the test sensitivity and specificity was performed using WinEpiscope ver.2.0 (Veterinary Faculty of University of Zaragoza, Spain and Wageningen Agriculture University, the Netherlands). The GraphPad InStat (Graph-Pad Software, San Diego, CA, USA; <http://www.graph-pad.com>) statistical package was used to compare mean skinfold thickness and mean maximum wheal width with an unpaired Chi-squared test. Differences were considered as significant when $P \leq 0.05$.

Western blotting

Western blotting procedure was effected by using 200 μg of both head and whole body *Culicoides* extracts that were boiled for 5 min in a reducing sample buffer and run using a BioRad Mini-PROTEAN II apparatus (Bio-Rad, Hercules, CA, USA) on a 12% polyacrylamide gel under denaturizing condition (SDS-PAGE). Molecular weight markers (Biotinylated SDS-PAGE standards; Bio-Rad) were used in a separate track.

Fractionated proteins were electroblotted overnight (90 mA) onto nitrocellulose sheets that were saturated (3 h) with 5% TBS-BSA. Horses' sera were diluted, after various calibration tests, 1 : 4 in TBS and incubated for 3 h in a Multiscreen Apparatus (Bio-Rad). After washing three times with 0.05% Tween 20-TBS, the secondary antibody (goat anti-Horse IgE 1 : 1500; Immunology Consultants Lab., Sherwood, OR, USA or goat anti-Horse IgG HRP-conjugated 1 : 6000; Bethyl, Montgomery, TX, USA) was incubated for 1 h. The track incubated with the Goat anti-Horse IgE, after washing three times with 0.05% Tween 20-TBS, was incubated for another hour with horseradish peroxidase labelled Rabbit anti-goat antibody diluted 1 : 5000 (Sigma-Aldrich, St Louis, MO, USA).

Nitrocellulose membrane was washed twice with 0.05% Tween 20-TBS and the presence of anti-*Culicoides* antibodies was revealed on a Hyperfilm ECL (ECL System, Amersham, Uppsala, Sweden) using a chemiluminescent W-B detection kit (ECL System).

Results

The results of skinfold thickness increase and wheal width are reported in Tables 1 and 2 respectively.

Table 1. Percentage of mean skinfold thickness increase at 1, 5 and 24 h after injection of 0.1 ml of *Culicoides* extract (25 $\mu\text{g}/\mu\text{l}$), 0.1 ml of physiological saline and 0.1 ml of histamine (1 : 10 000 w/v) in 18 hypersensitive [*Culicoides* hypersensitive (CHS positive)] horses and 23 normal (CHS negative) horses

	Clinical status	Physiological saline	Histamine	<i>Culicoides</i> antigen
1 h	CHS positive	6.84% $\pm$ 8.29 (0–25)	47.89% $\pm$ 36.83 (0–113.3)	36.06% $\pm$ 20.14*** (8.3–94.4)
	CHS negative	6.08% $\pm$ 8.86 (0–26.7)	31.19% $\pm$ 29.04 (4.2–111.8)	7.98% $\pm$ 7.48 (0–32.5)
5 h	CHS positive	6.09% $\pm$ 8.78 (0–28.1)	30.72% $\pm$ 22.54* (0–77.3)	42.54% $\pm$ 19.63*** (15.4–86.1)
	CHS negative	4.58% $\pm$ 6.2 (0–20)	18.35% $\pm$ 16.92 (0–70.6)	9.94% $\pm$ 11.52 (0–47.5)
24 h	CHS positive	5.28% $\pm$ 8.91 (0–29)	19.12% $\pm$ 21.39** (0–83.3)	39.6% $\pm$ 29.08*** (11.5–127.7)
	CHS negative	2.59% $\pm$ 5.33 (0–17.6)	3.76% $\pm$ 5.37 (0–17.6)	1.28% $\pm$ 1.89 (0–6.9)

Results are expressed as mean $\pm$ SD (range). Comparison between CHS-positive and CHS-negative horses was carried out with an unpaired *t*-test (with Welch correction when needed).

* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$.

Table 2. Wheal width (mm) at 1, 5 and 24 h after injection of 0.1 ml of *Culicoides* extract (25 µg/µl), 0.1 ml of physiological saline and 0.1 ml of histamine (1 : 10 000 w/v) in 18 *Culicoides* hypersensitive (CHS positive) horses and 23 normal (CHS negative) horses

	Clinical status	Physiological saline	Histamine	<i>Culicoides</i> antigen
1 h	CHS positive	7.55 ± 5.8** (0–18)	23.61 ± 10.6** (11–47)	22.39 ± 4.09*** (12–28)
	CHS negative	2.91 ± 3.3 (0–11)	12.83 ± 6.91 (7–33)	4.22 ± 5.25 (0–15)
5 h	CHS positive	1.83 ± 3.71 (0–11)	24.06 ± 12.31*** (9–56)	29.61 ± 6.6*** (19–43)
	CHS negative	1.13 ± 2.6 (0–10)	7.87 ± 8.44 (0–32)	5.04 ± 6.61 (0–19)
24 h	CHS positive	0.72 ± 2.61 (0–11)	4.78 ± 9.39* (0–27)	28.61 ± 12.9*** (9–68)
	CHS negative	0 (0)	0 (0)	0 (0)

Results are expressed as mean ± SD (range). Comparison between CHS-positive and CHS-negative horses at a given time was carried out with an unpaired *t*-test (with Welch correction when needed).

P* ≤ 0.05; *P* ≤ 0.01; ****P* ≤ 0.0001.

As regards intradermal reaction, at 1 and 5 h after injections, the skinfold thickness increased and the wheal width was greater in hypersensitive than in normal horses, but it was not possible to find out a discriminating cut off. At 24 h a welt of at least 1 cm wide was present in all hypersensitive horses but absent in the normal ones using *Culicoides* extracts. At the same time the cut off value of 10% skinfold thickness increase allowed discrimination between hypersensitive (minimum increase 11.5%) and normal (maximum increase 6.9%) horses. Therefore, at 24 h, at the above mentioned cut-off values – 1 cm wheal and 10% thickness increase – sensitivity, specificity and the positive- and negative-predictive values of the skin test were 100% (with IC = 95%). Using *Culicoides* extracts differences between CHS-positive and CHS-negative horses were highly significant (*P* ≤ 0.0001) at every time both for wheal width and skinfold increase. While both *Culicoides* extract and histamine have significant differences when compared with physiological saline at every time, differences between *Culicoides* extract and histamine were significant for wheal width (*P* ≤ 0.0001) and skinfold increase (*P* ≤ 0.05) only at 24 h.

Western blotting assays indicated that, by using anti-Horse IgG, normal horses had different bands, generally of 6, 50, 65, 70 and 200 kDa. However, a band of 65 kDa was present in all of them (Fig. 1). This band was absent or poorly appreciable in hypersensitive horses when an anti-Horse IgG antibody was used. In contrast, the band of 65 kDa was appreciable in all hypersensitive horses when an anti-Horse IgE antibody was used, while it was absent or poorly appreciable in normal

horses. No differences were observed comparing whole body and head extracts in W-B.

Discussion

The skin test demonstrated that all affected horses react more severely to the *Culicoides* extract than normal ones. Previous reports did not get a standardization of the test because authors did not use a precise protein concentration of the antigen (Halldorsdottir and Larsen, 1989). Conversely, our results with a 25 µg/µl of *Culicoides* extract showed that, even in winter when clinical signs were absent, all affected horses are characterized by the presence of, at least, a 1 cm welt and a skinfold thickness increase > 10% as cut-off at 24 h. In these conditions the test has 100% sensitivity and specificity, and differences between CHS-positive and CHS-negative horses were highly significant (*P* ≤ 0.0001) at every time both for wheal width and skinfold increase.

The increase of skinfold thickness at 5 and 24 h and the fact that differences between *Culicoides* extract and histamine were significant for wheal width (*P* ≤ 0.0001) and skinfold increase (*P* ≤ 0.05) only at 24 h, confirmed the presence of both immediate (type I) and delayed (type IV) hypersensitivity reactions in affected horses, as reported by other authors (Fadok and Greiner, 1990) and suggests the validity of this skin test for diagnosis.

The W-B showed that all tested horses present a band of 65 kDa. A similar band was found by other authors (Wilson et al., 2001), who did not find differences between normal and hypersensitive horses. Our results demonstrate that anti-*Culicoides* IgG antibodies are more evident in normal horses, while only hypersensitive horses have anti-*Culicoides* IgE antibodies, particularly against the 65 kDa antigen. The finding of anti-IgE antibodies in CHS horses confirms the allergic nature of the disease, and the antigen of 65 kDa seems to be the allergen involved in the pathogenesis of CHS. No differences were observed by using head or whole body antigen in W-B, therefore, it could mean that the concentration of antigen is sufficient in both cases and we can affirm that our test has a positive-serological diagnostic value. Some authors (Anderson et al., 1991) tried to hyposensitize horses by injecting increasing doses of *Culicoides* extract, but the quantity of necessary insects was very high (about 15 000 insects/horse). Therefore, the possibility of producing the allergen by molecular biology could be very useful in diagnosis and in therapy, so more studies have to be carried out in order to isolate and characterise the 65 kDa protein and to evaluate its use in diagnostic tests and hyposensitizing protocols.

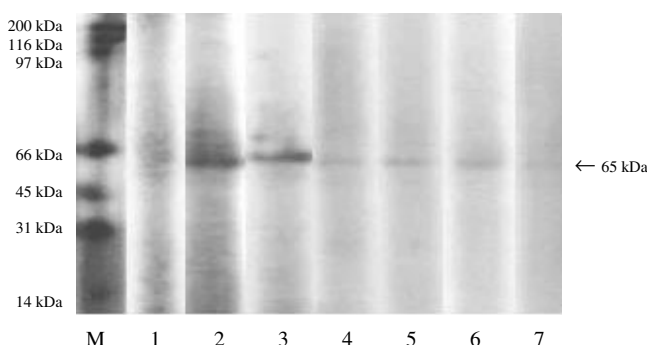


Fig. 1. Western blotting on the serum of *Culicoides* hypersensitive horses (lanes 4–6, anti-IgE antibody; lane 7, anti-IgG antibody) and normal horses (lane 1, anti-IgE antibodies lanes 2, 3, anti-IgG antibody). Right arrow indicates the band of about 65 kDa cited in the text. M, biotinylated SDS-PAGE molecular weight marker.

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