

Short communication

Comparison of recombinant *Culicoides* allergens produced in different expression systems for IgE serology of insect bite hypersensitivity in horses of different origins

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ABSTRACT

Insect bite hypersensitivity (IBH) is an allergic dermatitis of horses, an IgE-mediated reaction to *Culicoides* midges. Causative *Culicoides* spp. are not indigenous in Iceland resulting in high prevalence of IBH in horses born in Iceland and exported as compared to Icelandic horses born in a *Culicoides* rich environment. The aims were (i) to compare IgE levels in sera of IBH-affected horses born in Iceland (n = 47) with horses of the Icelandic breed (n = 23) and of other breeds (n = 27) born in *Culicoides* infested area; (ii) to investigate if barley could be a useful production system of allergens for IBH immunoassays.

IgE binding in sera was tested by ELISA on two recombinant *Culicoides* allergens, rCul n 3 and rCul n 4, each produced in *E. coli*, insect cells and barley. Significantly more IgE was detected against all allergens in sera from IBH-affected compared to healthy horses. Icelandic-born Icelandic horses stand out with higher IgE levels against the allergens and higher area under the curve (AUC) on rCul n 4 as compared to the European-born horses. The barley and *E. coli* produced allergens had very similar performance in distinguishing between IBH-affected and healthy horses.

1. Introduction

Insect bite hypersensitivity (IBH) is a recurrent allergic dermatitis of horses characterized by severe itching leading to a loss of hair and formation of lesions, especially on the mane, neck and tail area (Brostrom et al., 1987). IBH is caused by bites of blood feeding midges (*Culicoides* spp.). All breeds of horses can get affected but Icelandic horses are especially prone to develop this allergy when exported from Iceland to *Culicoides* infested area. This is probably due to the fact that most of these horses are only exposed to *Culicoides* after export at adult age, as the causative *Culicoides* species are not indigenous in Iceland (Sommer-Locher et al., 2012). Interestingly, previous studies indicate that Icelandic-born IBH-affected horses respond to more *Culicoides* salivary gland allergens than horses born in Europe (Hellberg et al., 2006; Novotny et al., 2021) and have stronger Th2 response (Hamza et al.,

2007).

The causative allergens of IBH are salivary gland proteins from *Culicoides* (reviewed in Schaffartzik et al., 2012). *Culicoides* whole body extracts are commonly used as allergen source for immunological studies of IBH-affected horses (Baselgia et al., 2006; Langner et al., 2008; van der Meide et al., 2012, 2014; Wagner et al., 2008). However, such crude allergen extracts are difficult to standardize and confirming the presence of the most important allergens is laborious (reviewed in Curin et al., 2017). Furthermore, *Culicoides* whole body extracts are still used in commercial IgE serological tests, often resulting in a low sensitivity and specificity of these assays for diagnosis of IBH (Frey et al., 2008). A large number of *Culicoides* allergens have been produced as recombinant proteins (reviewed in Jonsdóttir et al., 2019) and the most relevant allergens for IBH have recently been identified using a protein chip array (Novotny et al., 2021). Availability of the major *Culicoides* allergens as

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Table 1

Total number of horses and their groups with gender and age.

	IBH			Healthy		
	N	Gender	Median of age (Range)	N	Gender	Median of age (Range)
Icelandic-born Icelandic horses	47	F = 20, M = 27	17 (7–24)	23	F = 7, M = 16	11 (6–22)
European-born Icelandic horses	23	F = 12, M = 11	9 (3–25)	24	F = 11, M = 13	7.5 (4–26)
European-born various breeds*	27	F = 15, M = 12	6 (2–19)	24	F = 7, M = 17	10 (3–19)
Total horses (N)	97			71		

* Warmbloods [n = 18], Franches-Montagnes [n = 23], Ponies [n = 6], Tinker [n = 1], Arabian [n = 1], Andalusian [n = 1], Haflinger [n = 1].

pure recombinant proteins offers the opportunity to analyze further the immune response of IBH-affected horses, to develop a better allergen specific immunotherapy and to improve diagnostic tests (Novotny et al., 2021; van der Meide et al., 2014). *E. coli* is the most straightforward expression system as it is usually easy and cost-effective. However, the disadvantage of *E. coli* expressed allergens is that they are often not suitable for cellular assay due to unspecific stimulation (Jonsdottir et al., 2018). Using insect cells for expression of *Culicoides* allergens has overcome the problem of *E. coli* expressed allergens in cellular test (Jonsdottir et al., 2018) but is not suitable for large scale production due to cost. Expression of recombinant proteins in barley grains has been developed by ORF Genetics in Iceland. It is a cost-effective system, has low risk of contamination and the grains are natural protein storage, thus it could be an ideal allergen source with capacity for mass production and long-term storage (Magnusdottir et al., 2013). We have shown previously that allergens produced in barley show equally good or better performance as the corresponding proteins produced in *E. coli* and insect cells for analysis of the antibody response following vaccination of horses in Iceland with *Culicoides* allergens (Jonsdottir et al., 2018).

The aims for our study were to investigate if the increased IgE response observed in Icelandic horses is breed-specific or linked to lack of exposure to *Culicoides* at young age, and to evaluate the performance of barley-expressed *Culicoides* allergens. For that purpose serum IgE levels against two recombinant *Culicoides* allergens expressed in *E. coli*, insect cells and barley were determined in sera of three groups of horses; Icelandic breed born in Iceland an exported to Europe, Icelandic horses born in Europe and horses from various other breeds born in Europe.

2. Materials and methods

2.1. Horse sera for evaluation of IgE

Sera from 168 adult horses (97 IBH-affected and 71 non-affected healthy control horses) located in Switzerland or Sweden, sampled during *Culicoides* season, i.e. between April and November, were used for the IgE ELISA. Blood samples had been taken between 2001 and 2015 and sera had been kept frozen at -80°C until used. These 168 horses were split up into three groups based on breed and origin. Group 1; Icelandic-born Icelandic horses that had been living in Switzerland or Sweden in an environment where *Culicoides* were present for at least 4 summers. Group 2; Icelandic-horses born in Europe (living in Switzerland or Sweden). Group 3; horses belonging to various breeds (living in Switzerland) (Table 1). Diagnosis of IBH was made based on history and the typical clinical signs of IBH, i.e. strong pruritus, broken hair, excoriations and thickening of the skin along the dorsal midline. In

some cases the ventral midline and/or the head were also affected. Clinical signs recurred during the *Culicoides* season, between April and November with a period of remission in winter. The horses assigned to the healthy group had no history of skin problems. To ensure that the healthy control horses were living in presence of *Culicoides* spp., at least one IBH-affected horse had to be living at the same farm. Furthermore, no preventive measures, such as use of eczema blankets or repellents were used for the healthy control horses.

2.2. Recombinant rCul n 3 and rCul n 4 allergens

The *Culicoides* allergens Cul n 3 and Cul n 4 were expressed in *E. coli*, insect cells and barley grains as described by Jonsdottir et al., 2018. In short; *E. coli*-rCul n 3 and *E. coli*-rCul n 4 were purified under denatured conditions and dialyzed against 5% trehalose. Insect cell expressed; Bac-rCul n 3 and Bac-rCul n 4, and barley expressed; Barley-rCul n 3 and Barley-rCul n 4 were purified under native conditions and dialyzed against 2xPBS (Jonsdottir et al., 2018).

2.3. IgE ELISA

The IgE ELISA was performed as described in Jonsdottir et al. (Jonsdottir et al., 2015). Briefly, ELISA plate was coated with $2\text{ }\mu\text{g/mL}$ of the recombinant proteins and unspecific binding sites blocked with blocking buffer (5% dried milk powder and 5% Tween 20 in PBS) for 2 h at 37°C . The sera were diluted 1:5 in blocking buffer, added to the plate in duplicate and incubated overnight at 4°C . After washing monoclonal mouse-anti equine-IgE ($1\text{ }\mu\text{g/mL}$ clone 3H10 (Wilson et al., 2006)) was applied. Plates were washed again, an AP-conjugated goat-anti mouse IgG (Jackson ImmunoResearch, Ely, Cambridgeshire, UK) diluted 1:2000 added and incubated for $1\frac{1}{2}\text{ h}$ at RT. After washing, plates were developed with 1.5 mg/mL phosphatase substrate (Sigma-Aldrich, St. Louis, MO, USA) in 10 % diethanolamine (Fluka) and absorption measured at 405 nm after 2 h. After subtraction of the blank, OD values were corrected for differences between the plates coated with the same allergen. The correction factor for each plate was calculated based on the average of the OD values of the positive controls included on all plates as in Jonsdottir et al. (Jonsdottir et al., 2016). The results are shown as corrected OD values.

2.4. Statistical analysis

2.4.1. Comparison of disease status and origin of the horses

A linear mixed effect model was used to analyze the data. The data was log-transformed before statistical analysis and analyzed separately for rCul n 3 and rCul n 4. Two sample *t*-test was used to test for difference between healthy and IBH horses for each expression system. Statistical analysis was performed in R and we fitted linear random effect models using the nlme-package (Pinheiro et al., 2017).

We fitted 4 models for each allergen using maximum likelihood and compared them with likelihood ratio tests and the Akaike Information Criterion (AIC). The full model (FM) contained disease status (Healthy or IBH-affected), expression system and the breed and origin group as fixed factors and all two-way and three-way interactions between them. We included the individual horse as a random factor and had different variance for the IBH-affected and healthy horses. The first reduced model (R1) had the same structure for the random effect but we excluded the three-way interaction between the fixed factors. The second reduced model (R2) had the same structure for fixed factors as FM but the structure of the random effect was simpler with the same variance for IBH and healthy horses. The final reduced model (R3) had the same fixed effect structure as the FM but no random effect. The full model had the lowest AIC and outperformed all the simpler models in the likelihood ratio test and was used for downstream analysis (data not shown). The multcomp-package (Hothorn et al., 2008) was used for postHOC *t*-tests to analyze which parameters were different. Differences

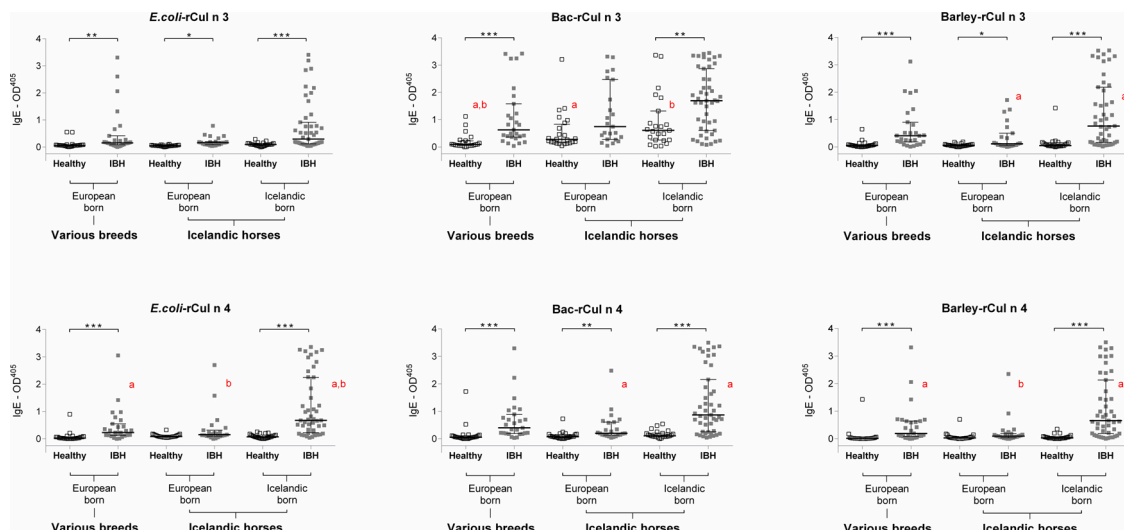


Fig. 1. Serum IgE level of healthy and IBH-affected horses, grouped according to their origin and breed.

Serum IgE levels against rCul n 3 and 4 produced in the three expression systems, *E.coli*, insect cells (Bac) and barley, tested by ELISA. Corrected IgE OD values for healthy (□) and IBH-affected (■) horses. Values of each horse plotted and shown with the median and interquartile ranges for the group. Linear mixed effect models were used to compare the different groups of origin and the difference between healthy and IBH-affected horses within each group. Asterisk indicates statistically significant difference between healthy and IBH within group of origin (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$). Groups (within IBH or H) that are statistically different ($p \leq 0.05$) within the same allergen are highlighted with the same letter (a or b in red). See Supplementary Table 1 and 2 for more details on statistical results.

between healthy and IBH horses was tested within each expression system for each group and for differences between groups within each diseases status for each expression system. The multcomp-package adjusted P-values for multiple testing.

2.4.2. Comparison of expression system of allergens

As the data was not normally distributed Spearman's rank correlation coefficient was used to calculate the correlation between IgE levels against Cul n 3 and rCul n 4 in the different expression systems. For evaluation of the performance of the IgE ELISA with rCul n 3 or rCul n 4 produced in the three expression systems, non-parametric receiver operating characteristic (ROC) analysis was done and calculations of the area under the empirical curve (AUC) were carried out using the statistical software program NCSS 11 (NCAA Statistical Software). The AUC for r-allergens expressed in different systems were compared using the empirical (nonparametric) methods (DeLong et al., 1988), in NCSS). The ROC curves were used to select the optimal cut-off value for each allergen for Icelandic- and European-born horses (European born Icelandic and non-Icelandic horses were pooled as there was no significant difference between those groups). The value with the best Youden Index was chosen to determine the ELISA cut-off values.

Agreement between binary IgE ELISA results (positive/negative) using the r-allergens expressed in the three different expression systems was assessed by Kappa statistic (κ), which evaluate the proportion of agreement that occurred beyond that expected due to chance alone. Values of Kappa between 0.4 and 0.5 indicate a moderate level of agreement, those between 0.5 and 0.6 can be regarded as acceptable, those 0.6 and 0.8 are good. If Kappa = 1, there is perfect agreement (Noordhuizen et al., 1997). Statistical significance was defined as $p < 0.05$.

3. Results and discussion

3.1. IgE response of Icelandic-born Icelandic horses differ from European-born Icelandic horses

A first overall analysis of Cul n 3- and Cul n 4 specific IgE showed that IBH-affected horses had significantly higher IgE level in serum against both allergens expressed in the three systems, *E.coli*, insect cells and

barley as compared to healthy controls (Supplementary Fig. 1). The horses were then split into three groups based on their origin and breed; Icelandic-born Icelandic horses, European-born Icelandic horses and European-born various breeds (Table 1), because previous studies showed that IBH-affected Icelandic horses imported from Iceland had IgE to a higher number of *Culicoides* allergens than horses born in Europe (Hellberg et al., 2006).

A significant difference in IgE levels was observed between IBH-affected horses and healthy controls against both proteins in all expression systems for all groups except for European-born Icelandic horses against Bac-rCul n 3, *E. coli*- and Barley-rCul n 4 (Fig. 1 and supplementary Table 1). This indicates a difference in the response between European- and Icelandic born Icelandic horses, where Icelandic-born Icelandic horses respond more strongly. Furthermore, when the response of the IBH-affected horses from the three groups was compared, the IBH-affected Icelandic-born horses stand out as having significantly higher IgE levels against rCul n 4, produced in any of the three systems, as compared to those born in Europe. This difference between the European- and Icelandic-born Icelandic horses was not as obvious against rCul n 3 as it was only seen for Barley-rCul n 3 (Fig. 1 and supplementary Table 2). In line with our finding, it has been demonstrated previously that IBH-affected horses imported from Iceland as adults have strong Th2-biased immune response (Hamza et al., 2007). The horses in the group IBH-Icelandic-born Icelandic have the highest median age (supplementary Fig. 2 A). This can be explained by the fact that Icelandic-born Icelandic horses are mostly exported as adults (mean and median age 7, supplementary Fig. 2B) and following export it takes them 2–3 years to develop IBH. Horses born in Europe usually develop IBH at the age of 2–5 years. Importantly for our study, there is no significant age difference between the main two groups being compared, Icelandic-born and European-born IBH-affected Icelandic horses. However, the group of IBH-affected Icelandic-born Icelandic horses was significantly older than European-born horses of various breeds (IBH and healthy) and European-born Icelandic horses (Supplementary Fig. 2A). It is important to note that within each IBH group, no significant correlation between age and IgE level was observed for any of the tested allergens nor any association between serum IgE levels and gender (data not shown). Since Icelandic horses are exported as adults, when their immune system has “fully” developed they are probably

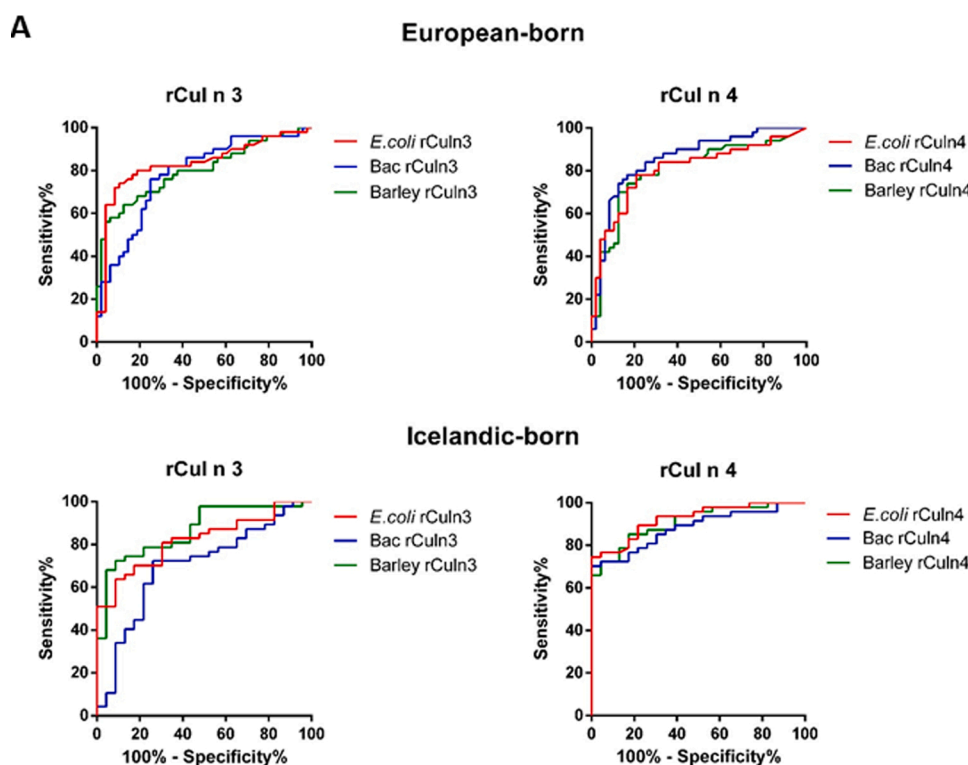


Fig. 2. A) Receiver operator characteristic (ROC) curve of serum IgE levels against the *Culicoides* allergens rCul n 3 and rCul n 4 expressed in *E. coli* (—), insect cells (—) and barley (—) for European-born and Icelandic-born horses B) Comparison of the performance of IgE ELISAs with allergens expressed in three different expression systems. Data from European born and Iceland-born horses were analyzed separately based on the results from Fig. 1. Results from ROC analyses are shown: area under the curve (AUC) with lower and upper confidence limits. The cut off values were selected to obtain the best Youden index and the resulting accuracy, sensitivity and specificity are displayed. ^a indicates a significantly difference from Bac-rCul n 3.

B

European born

	AUC	95% CI	Cut off (OD ₄₀₅)	Accuracy	Sensitivity	Specificity
<i>E.coli</i> -rCul n 3	0.83	0.72-0.90	0.09	82%	72%	92%
Bac-rCul n 3	0.78	0.67-0.86	0.37	76%	76%	75%
Barley-rCul n 3	0.80	0.69-0.87	0.19	76%	56%	96%
<i>E.coli</i> -rCul n 4	0.81	0.70-0.88	0.11	79%	78%	79%
Bac-rCul n 4	0.86	0.76-0.92	0.15	80%	80%	79%
Barley-rCul n 4	0.81	0.69-0.88	0.07	79%	70%	88%

Icelandic born

	AUC	95% CI	Cut off (OD ₄₀₅)	Accuracy	Sensitivity	Specificity
<i>E.coli</i> -rCul n 3	0.82 ^a	0.70-0.90	0.14	74%	70%	83%
Bac-rCul n 3	0.70	0.54-0.81	0.9	73%	72%	74%
Barley-rCul n 3	0.87 ^a	0.75-0.93	0.22	77%	68%	96%
<i>E.coli</i> -rCul n 4	0.93	0.84-0.97	0.32	83%	75%	100%
Bac-rCul n 4	0.88	0.78-0.94	0.55	80%	70%	100%
Barley-rCul n 4	0.91	0.81-0.96	0.22	80%	72%	96%

more prone to develop IBH due to lack of exposure to the midges early in life. Only 7% of foals born in Iceland and exported to *Culicoides* infested areas at the age of 6–9 months develop IBH later in life (Sommer-Locher et al., 2012). The overall frequency of IBH in exported Icelandic horses have been found to be around 25–35 % (Björnsdóttir et al., 2006; Halldórsdóttir and Larsen, 1991). However, 80 % of the horses born in Iceland and exported at the age of 12 or more years develop this allergy (Sommer-Locher et al., 2012). This is in accordance with studies in human allergy showing that early life exposure to allergens can decrease the likelihood of allergy later in life (reviewed in Reynolds and Finlay, 2017). Early life exposure thus seems to be important for development of tolerance to allergens.

3.2. Comparison of the performance of the IgE ELISA using *Culicoides* allergens produced in different expression systems

Reliable diagnostic tests for IBH are lacking as commercially available tests are all based on *Culicoides* whole body extracts, which is very difficult to standardize, and contains large quantities of irrelevant proteins. Reliable sources of recombinant allergens are paramount for developing standardized diagnostic tests. As collection of saliva or production of salivary gland extracts from *Culicoides* is extremely laborious, their production as recombinant proteins is the method of choice for obtaining pure *Culicoides* allergens (Peeters et al., 2013; van der Meide et al., 2013). Various expression systems can be used for that

Table 2

Comparison of the IgE ELISA with rCul n 3 and rCul n 4 produced in different expression systems.

Expression systems	Spearman's-Rank correlation coefficient		Kappa	
	rCul n 3	rCul n 4	rCul n 3	rCul n 4
Barley/ <i>E.coli</i>	0.73	0.88	0.86	0.86
Barley/Bac	0.65	0.86	0.75	0.86
Bac/ <i>E.coli</i>	0.60	0.85	0.71	0.82

purpose and each of them has its advantages and limitations (Wells and Robinson, 2017). We have here compared the performance of IgE ELISAs for *in vitro* diagnostic of IBH with *Culicoides* allergens produced in three different expression systems. For that purpose we used the two allergens rCul n 3 and rCul n 4, each expressed in *E.coli*, insect cells and barley. As we saw previously that IgE response are similar between European-born horses of the Icelandic breed and other breeds (Fig. 2) but those imported from Iceland often have higher IgE levels to these allergens, ROC analysis was carried out for the Icelandic-born horses and for European-born horses separately. The six r-proteins showed in general good performance in distinguishing between IBH and healthy horses with AUC ranging from 0.7–0.93, with Bac-rCul n 3 showing the lowest performance in Icelandic-born horses (AUC = 0.70). In this group, the AUC of Bac-rCuln3 was significantly lower compared to *E.coli*-rCul n 3 and Barley-rCul n 3 (0.82 and 0.87, respectively). The low performance of Bac-rCul n 3 can be explained by the high number of positive horses in the healthy group as was seen in supplementary Fig. 1 and Fig. 2.

For rCul n 4 the AUC were generally higher (0.81–0.93) than for rCul n 3 and there were no significant differences between the three expression systems for this allergen. Accordingly, correlation (Table 2) between IgE levels against *E.coli*-, Bac- and Barley expressed rCul n 4 was very good (> 0.85) and was slightly lower between Barley- and *E.coli*-rCul n 3 (0.73), while correlation with Bac-rCul n 3 was clearly lower (0.65 with Barley and 0.60 with *E.coli*). Within each allergen, *E.coli*- and Barley expressed allergens correlated best. The agreement between the expression systems was further evaluated with Kappa values confirming Spearman correlation (Table 2).

We have shown previously that barley expressed allergens are an excellent source of recombinant proteins for evaluation of IgG antibodies in serum of vaccinated horses (Jonsdottir et al., 2018). Here we show that they can also be used for detection of allergen-specific IgE in serum from IBH-affected horses. IgE serology with Barley expressed allergens has good performance and is similar to IgE serology with *E.coli* expressed proteins.

4. Conclusion

Our results indicate that IBH-affected Icelandic-born horses have stronger allergen-specific IgE response than Icelandic horses born in *Culicoides* infested areas, indicating that lack of exposure early in life is an important factor in allergy development. Additionally, barley expressed allergens perform similarly well as allergens expressed in other systems in IgE serology for IBH. Production of the recently identified major causative allergens for IBH in barley should be considered, as barley is an attractive source of reliable, cost effective allergens performing well in both serological and cellular immune assays.

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Declaration of Competing Interest

The authors report no declarations of interest.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.vetimm.2021.110289>.

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Supplementary table 1: Difference between IBH and healthy horses within each group of origin.

PostHoc test to analyze the difference in IgE level against rCul n 3 and rCul n 4 between IBH-affected and healthy horses within each group of origin. The difference was analyzed for each expression system. The est. diff are on log scale.

Expression system	Group	rCul n 3			rCul n 4		
		Esti. diff	p-value	Significant	Esti. diff	p-value	Significant
<i>E.coli</i>	European born Icelandic	0,881	0,017	*	0,501	0,229	ns
	Icelandic born Icelandic	1,260	≤ 0,001	***	1,746	0	***
	European born various breeds	0,924	0,01	**	1,257	≤ 0,001	***
Insect cells	European born Icelandic	0,718	0,092	ns	0,869	0,003	**
	Icelandic born Icelandic	0,779	0,007	**	1,520	≤ 0,001	***
	European born various breeds	1,587	≤ 0,001	***	1,297	≤ 0,001	***
Barley seeds	European born Icelandic	0,896	0,014	*	0,550	0,149	ns
	Icelandic born Icelandic	1,810	≤ 0,001	***	1,868	0	***
	European born various breeds	1,554	≤ 0,001	***	1,284	≤ 0,001	***

Supplementary table 2: Difference between groups of origin.

PostHoc test to analyze the difference in IgE level against rCul n 3 and rCul n 4 between the groups of origin. The difference was analyzed for each expression system. The est. diff are on log scale.

		rCul n 3			rCul n 4		
Contrast		Esti. diff	p-value	Significant	Esti. diff	p-value	Significant
<i>E.coli</i>	H	Euro. born Icelandic - Ice. born Icelandic	-0,404	0,655	0,136	0,994	
		Euro. born Icelandic -Euro. born various breeds	-0,244	0,980	0,504	0,053	.
		Ice. born Icelandic - Euro. born various breeds	0,160	1,000	0,369	0,338	
	IBH	Euro. born Icelandic - Ice. born Icelandic	-0,784	0,088	-1,110	≤ 0,001	***
		Euro. born Icelandic -Euro. born various breeds	-0,287	0,995	-0,252	0,984	
		Ice. born Icelandic - Euro. born various breeds	0,496	0,590	0,857	0,007	**
Bac	H	Euro. born Icelandic - Ice. born Icelandic	-0,422	0,596	-0,313	0,549	
		Euro. born Icelandic -Euro. born various breeds	0,944	0,001	0,052	1,000	
		Ice. born Icelandic - Euro. born various breeds	1,366	≤ 0,001	0,365	0,349	
	IBH	Euro. born Icelandic - Ice. born Icelandic	-0,483	0,696	-0,963	0,003	**
		Euro. born Icelandic -Euro. born various breeds	0,076	1	-0,376	0,850	
		Ice. born Icelandic - Euro. born various breeds	0,559	0,415	0,588	0,175	
Barley	H	Euro. born Icelandic - Ice. born Icelandic	-0,282	0,948	0,007	1	
		Euro. born Icelandic -Euro. born various breeds	-0,059	1,000	0,179	0,959	
		Ice. born Icelandic - Euro. born various breeds	0,223	0,991	0,171	0,970	
	IBH	Euro. born Icelandic - Ice. born Icelandic	-1,196	≤ 0,001	-1,311	≤ 0,001	***
		Euro. born Icelandic -Euro. born various breeds	-0,718	0,289	-0,555	0,432	
		Ice. born Icelandic - Euro. born various breeds	0,478	0,641	0,756	0,028	*

Supplementary figure 1: Performance of rCul n 3 and rCul n 4 in IgE ELISA. rCul n 3 and rCul n 4 expressed in *E. coli* (○/●), insect cells (□/■) and barley (Δ/▲) tested by ELISA. Corrected IgE OD values for healthy (H) and IBH-affected horses, each horse plotted and shown with the mean and standard deviations for the group. The asterisks (*) indicate statistical significance in t-test. *** $p \leq 0.001$.

Supplementary figure 2:

A) Age of the horses at the time of bleeding within each group. The age is plotted from Min to Max. Kruskal-Wallis and Dunn's multiple comparison test was used to compare the groups, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$. **B)** Age of Icelandic horses exported from Iceland to Switzerland, 2015-2021 compared to the IBH-affected Icelandic-born Icelandic horses used in this study.

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