

ORIGINAL ARTICLE

Allergens

Levels of horse allergen Equ c 4 in dander and saliva from ten horse breeds

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Summary

Background: Horses are an important source of allergens, but the distribution of horse allergens is poorly understood. Five horse allergens have been identified, Equ c 1-4 and 6. Equ c 4 seems to be an important allergen, with an IgE-binding frequency of 77% in horse-sensitized individuals.

Objectives: The aim of this study was to investigate levels of horse allergen Equ c 4 in dander, saliva and urine from ten horse breeds.

Method: The study population included 170 horses (87 mares, 27 stallions, 56 geldings) from ten breeds. Horse dander, saliva and urine samples were collected. Levels of horse allergen Equ c 4 were quantified using a two-site sandwich ELISA (mAb 103 and 14G4) and were expressed as Equ c 4 U/ μ g protein.

Results: The horse allergen Equ c 4 was present in all dander and saliva samples from ten horse breeds, with high within-breed and inter-breed variations; GM values were 639 Equ c 4 U/ μ g protein (range 5-15 264) for dander and 39.5 (4-263) for saliva. Equ c 4 was found in 19/21 urine samples. Adjusted for age, sex and changes over time, no differences between breeds could be seen in dander, while in saliva the North Swedish horse showed lower levels of Equ c 4 than any other breed. The levels of Equ c 4 protein in dander and saliva were significantly higher in samples from stallions compared to mares and geldings, independent of breed.

Conclusions and Clinical Relevance: The results show a high variability in allergen levels of Equ c 4 in dander and saliva both within and between breeds. Significantly higher levels were found in stallions compared to mares and geldings, independent of breed. Results suggest that none of the horse breeds studied can be recommended for individuals allergic to Equ c 4.

KEYWORDS

dander, Equ c 4, horse allergen, horse breeds, saliva

1 | INTRODUCTION

Allergies such as those leading to asthma, rhinitis, eczema and even anaphylactic reactions are some of the most common diseases world-wide.¹ In Sweden, the most common allergies are to pollen allergens followed by an increasing incidence of allergies to allergens from furry animals.^{2,3}

The prevalence of horse allergy in the population is not well studied. In one study, where the aim was to investigate the increase in sensitization to common airborne allergens in northern Sweden, the prevalence of positive skin prick tests to horse was approximately 7% and 10% in 1994 and 2009, respectively.⁴ Allergic sensitization to horse allergen, without direct or occupational exposure, has been shown and is more frequent than expected in urban-living subjects.⁵ The spread of horse allergens to the surroundings has been investigated since there is a potential health risk for horse-allergic individuals in the general population.^{6,7}

The existence of animal breeds—cat, dog and horse—with so-called hypoallergenic properties has been suggested and extensively debated. Differences in dog (Can f 1) allergen production have been shown between different dog breeds, but the variability between individuals was very large and it was concluded that a hypoallergenic dog breed does not exist.^{8,9} A study performed in Germany on the most common cattle breeds showed a high variability in allergen levels between individual animals. The results also indicated that allergen production was not related to breed or to gender.¹⁰ Similar studies have been performed for horses and, for example, the Bashkir horse and the American Curly horse have been suggested to be hypoallergenic.¹¹ However, no breed-specific allergens have previously been identified although variations could be seen between and within breeds.¹² In a preliminary study, we compared three breeds: the Bashkir horse, the Icelandic horse and the Swedish warmblood horse (Elfman et al, EAACI 2010). The median levels of airborne horse allergen (Equ c x) were highest for the Iceland horse and the Swedish warm-blooded horse and lowest for the Bashkir horse.

The allergens of *Equus caballus* listed in the World Health Organization and International Union of Immunological Society (WHO/IUIS) Allergen Nomenclature Database (<http://www.allergen.org>) are

- Equ c 1, a 25 kDa lipocalin, which is believed to be the major horse allergen.^{13,14} Up to 76% of horse-allergic patients react to Equ c 1.¹⁵
- Equ c 2, a 17 kDa lipocalin that showed IgE binding, by immunoblotting, in horse-sensitized patients.^{16,17}
- Equ c 3, a 67 kDa horse serum albumin^{17,18} that showed IgE binding in 50% of patients tested.¹⁹
- Equ c 4, a 17 (20.5) kDa protein with latherin function.²⁰⁻²² Approximately 77% (17 of 22) of horse-allergic patients showed IgE binding in ELISA, enzyme-linked immunosorbent assay.²⁰
- Equ c 5 was removed from the databases in January 2015, since the protein was later identified to be Equ c 4.
- Equ c 6, a 15 kDa lysozyme which seems to be both a food and dermal allergen.²³

The horse allergen Equ c 4 belongs to a family of proteins known as latherins, which are present in horse sweat and saliva. The intrinsic surfactant activity of these proteins suggests that they act as wetting agents and play a role in the thermoregulation of equines. Equids are flight animals that can produce large amount of sweat during heavy activity and the detergent-like activity of Equ c 4 seems to facilitate cooling. Latherin in equine saliva might help wet the fibrous feed that equines are adapted to.²⁴ The amino acid sequences of latherins are similar to that of the PLUNCs (palate, lung, nasal epithelium clones) protein family, which are found in mammals.²⁵

ELISA is the gold standard method for quantifying allergens.²⁶ In previous studies, a sandwich ELISA has been used to measure horse allergen, based on the monoclonal antibodies (mAb) 103 and 14G4 (MabTech AB, Stockholm, Sweden).²⁷ To date, it has not been known which horse protein these mAb's recognize, only that the molecule is approximately 16 kDa.⁶ It has been suggested that the target protein is Equ c 4, but this has not been confirmed,²⁸ and until now this allergen has been referred to as Equ c x.

The aim of this study was to investigate levels of horse allergen Equ c 4 in dander, saliva and urine from ten different horse breeds. First, we investigated if native Equ c 4 was detected by the mAb 103 and 14G4, indicating that Equ c x is indeed Equ c 4.

2 | MATERIAL AND METHODS

2.1 | Study population

This study included 170 horses from ten different horse breeds, American Curly (AC), American Quarter horse (AQ), Gotland pony (G), Icelandic horse (I), North Swedish horse (N), Russian Bashkir horse (B), Shetland pony (SP), Standardbred (S), Swedish warmblood (SWB) and Thoroughbred (T). All horses were registered in their respective breed association. A variety of ages (<1-31 years with a mean of 10 years) and sexes (87 mares, 27 stallions and 56 geldings) were selected for allergen sampling. The horses were stabled in the middle part of Sweden, from the east to the west. Samples from the horses were collected at the farms where they lived. The sampling was performed during the summer of 2013 (144 individuals), with a follow-up during the summer of 2014 (108 individuals). With all breeds represented, 82 horses were sampled both in 2013 and in 2014 (44 mares, 11 stallions and 27 geldings). Totally, 252 dander and 248 saliva samples were collected. The distribution of the dander/saliva samples is presented in Table 1.

Discrepancies between dander and saliva samples can be explained as follows: in 2013, twelve dander samples and, in 2014, 2 dander samples had positive Equ c 4 levels, but protein levels were below detection level. Therefore, these samples have not been used in further calculations of Equ c 4 levels and statistical analyses. Furthermore, in 2014 it was not possible to sample saliva from four horses.

Ethical approval was not required, according to Swedish Board of Agriculture (SJVFS 2015:38, chapter 2 §15). Informed consent was received from the horse owners.

Horse dander (HD) was obtained by grooming the horses with a brush, and the dander was kept in Petri dishes at 4-8°C until

TABLE 1 Number of dander/saliva samples from the horse breeds, sampled in 2013 and 2014

Breed	2013				2014			
	Gelding	Mare	Stallion	Total	Gelding	Mare	Stallion	Total
AC	5/7	6/6	1/1	12/14	7/7	5/5	6/6	18/18
AQ	5/5	8/8	0/0	13/13	6/7	9/8	2/2	17/17
B	7/7	12/12	1/1	20/20	7/7	11/11	1/1	19/19
G	9/9	7/7	4/4	20/20	0/0	5/4	5/5	10/9
I	4/6	7/8	2/2	13/16	2/2	3/3	2/2	7/7
N	1/1	3/4	3/5	7/10	1/1	3/2	6/6	10/9
S	4/4	6/7	0/1	10/12	0/0	2/2	0/0	2/2
SP	1/2	10/11	0/0	11/13	1/1	10/10	0/0	11/11
SWB	6/6	8/8	2/2	16/16	4/4	3/3	0/0	7/7
T	4/4	6/6	0/0	10/10	3/3	2/2	0/0	5/5

extracted (maximum 3 days). Extracts of horse dander were performed as in earlier studies.^{6,27} Extracts were kept at -70°C until analysed.

Horse saliva (HS) was obtained using Salivette[®] (Sarstedt, Numbrecht, Germany). The cotton-swab was held by using metal tweezers and placed on the tongue for one minute. The swab was then placed in the Salivette[®] tube and kept cool until centrifugation. Saliva samples were centrifuged within 8 hours, at 1000 g for 10 minutes, and kept at -70°C until analysed.

Horse urine (HU): Urine was collected and kept at -70°C until analysed. Urine samples were collected in 2013 and could only be collected from 21 horses (AC = 1, AQ = 4, B = 4, I = 1, S = 3, SWB = 7, T = 1) included in the study.

2.2 | Purification of natural Equ c 4

In brief, 5 g of horse dander (Allergon, Angelholm, Sweden) was dissolved in 90 mL of Tris-HCl pH 7.4. The protein concentration of the extract was determined to be 4.25 mg/mL (Pierce[™] BCA Protein Assay Kit, Thermo Fisher Scientific, Waltham, MA). The extract was purified using anion exchange chromatography (AIEX) on a HiTrap[®] Q HP 5 mL column (GE Healthcare, Uppsala, Sweden), and the peak fractions were further purified using size exclusion chromatography (SEC) using a HiLoad[®] 16/600 Superdex[®] 75 pg column. The resulting minor peak was further purified by AIEX using a Mono Q[®] 10/100 column, and the purity was determined to be ~98%, analysed by SEC using a Superdex 75 10/300 GL column (GE Healthcare). The Equ c 4 sequence was verified by liquid chromatography-tandem mass spectrometry (LC-MS) at Uppsala Biomedical Centre, Sweden. The yield was calculated to 0.8 mg per gram starting material and 1% of the protein content of the extract.²⁹

2.3 | Investigation of the binding of mAb 103 and 14G4 to native Equ c4

Analysis of the mAb 103 and 14G4 used in the assay included biotinylation of native Equ c 4 (nEqu c 4)²⁹ according to the manual (EZ-Link[™]

Sulfo-NHS-Biotin lot.DK209210. Thermo Fisher Scientific). Desalting was performed on a NAP[™] 5 column using phosphate-buffered saline (PBS) as buffer, according to the manual (GE Healthcare). Ninety-six-well plates (Nunc, Sigma-Aldrich, Saint Louis, MO, USA) were coated overnight at 4°C with mAb 103 and mAb 14G4 with 100 μL /well at a concentration of 2.5 $\mu\text{g}/\text{mL}$ in PBS. The plate was washed with washing buffer (3 mol/L NaCl, 7.5 mmol/L NaH_2PO_4 , 50 mmol/L Na_2HPO_4 , 0.02% Tween 20), blocked with 200 μL /well of blocking buffer (PBS containing 1% BSA, 0.2% Tween) and incubated for 1 hour in room temperature (r.t). Biotinylated native Equ c 4 was added in a threefold dilution, in triplicates, in blocking buffer from 1 $\mu\text{g}/\text{mL}$ down to 0.004 $\mu\text{g}/\text{mL}$, at 100 μL /well, and incubated for 2 hours at r.t. Streptavidin-horseradish peroxidase conjugate, 100 μL /well, was added and incubated for 1 hour at r.t. followed by 100 μL /well of TMB Super Slow substrate (Sigma-Aldrich, Stockholm, Sweden) for 10 minutes. The reaction was stopped by adding 50 μL /well of 0.5 mol/L H_2SO_4 . The absorbance at 450 nm was determined by spectrophotometry using a SpectraMax[®] Plus microplate reader (Molecular Devices, Sunnyvale, CA). To verify the specificity of the monoclonals 14G4 and 103 recombinant Equ c 1, recombinant Equ c 2 and native Equ c 3 was also tested in concentration ranging from 1 $\mu\text{g}/\text{mL}$ down to 0.017 ng/mL. All three allergens were produced and purified according to previously described protocols.²⁹

2.4 | Quantification of the horse allergen Equ c 4 levels by ELISA

Horse allergen Equ c 4 levels were determined using a two-site sandwich ELISA (mAb 103 and mAb 14G4 from Mabtech AB, Stockholm, Sweden).^{6,27} The horse allergen standard (Allergon, Sweden, art. No.2043 batch No. 204610901) was prepared according to the protocol,⁶ except that Thimerosal was no longer used, and therefore, the standard extract was kept at -70°C . Protein content in horse dander, saliva and urine samples was determined using a Pierce[™] BCA Protein Assay Kit. The level of horse allergen in samples was expressed as Equ c 4 U/ μg protein. Arbitrary units (U) were used, where 1 U is equal to 1 ng protein of the horse standard, since no international standard was available. The detection limit

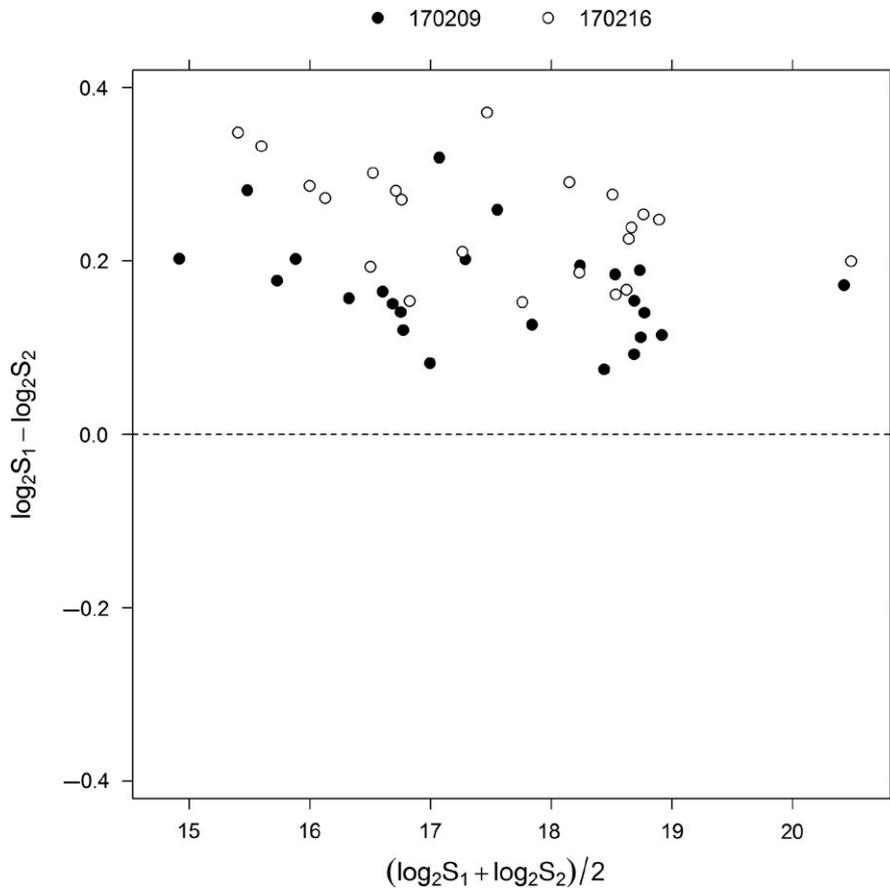


FIGURE 1 Bland and Altman plot showing difference (vertical axis) vs mean value (horizontal axis) of log-transformed sample concentrations between the two standards, S_1 and S_2 analysed on two separate dates represented by filled and open circles

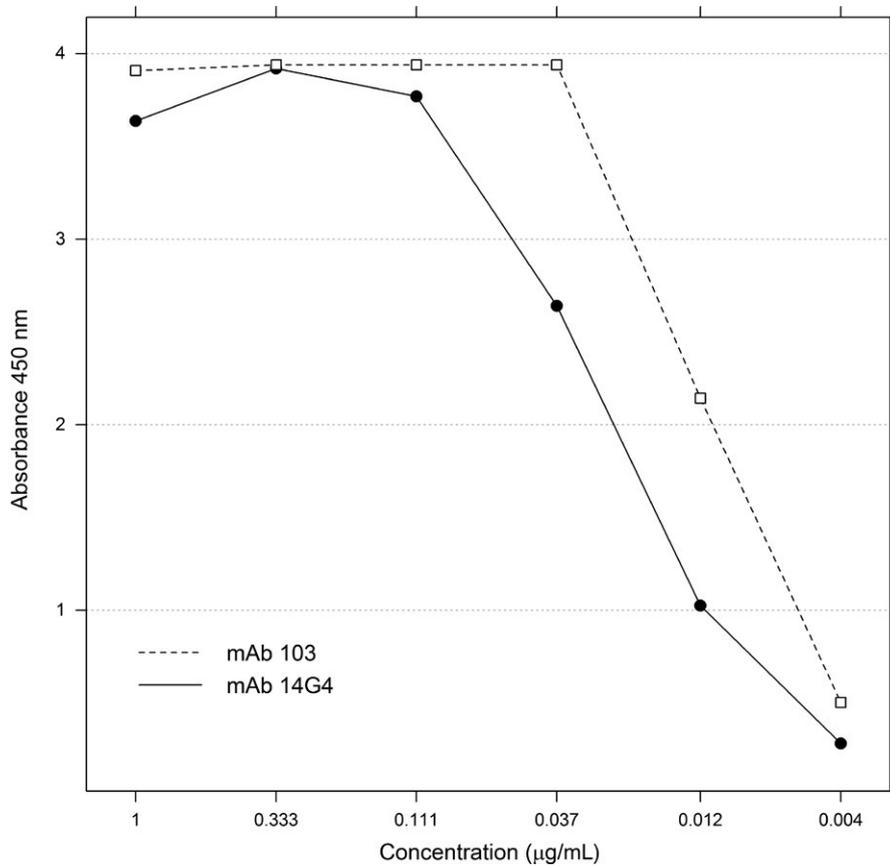


FIGURE 2 Binding of mAb's 103 and 14G4 to nEqu c 4. Biotinylated native Equ c 4 was added in a threefold dilution, in triplicates, from $1 \mu\text{g/mL}$ down to $0.004 \mu\text{g/mL}$

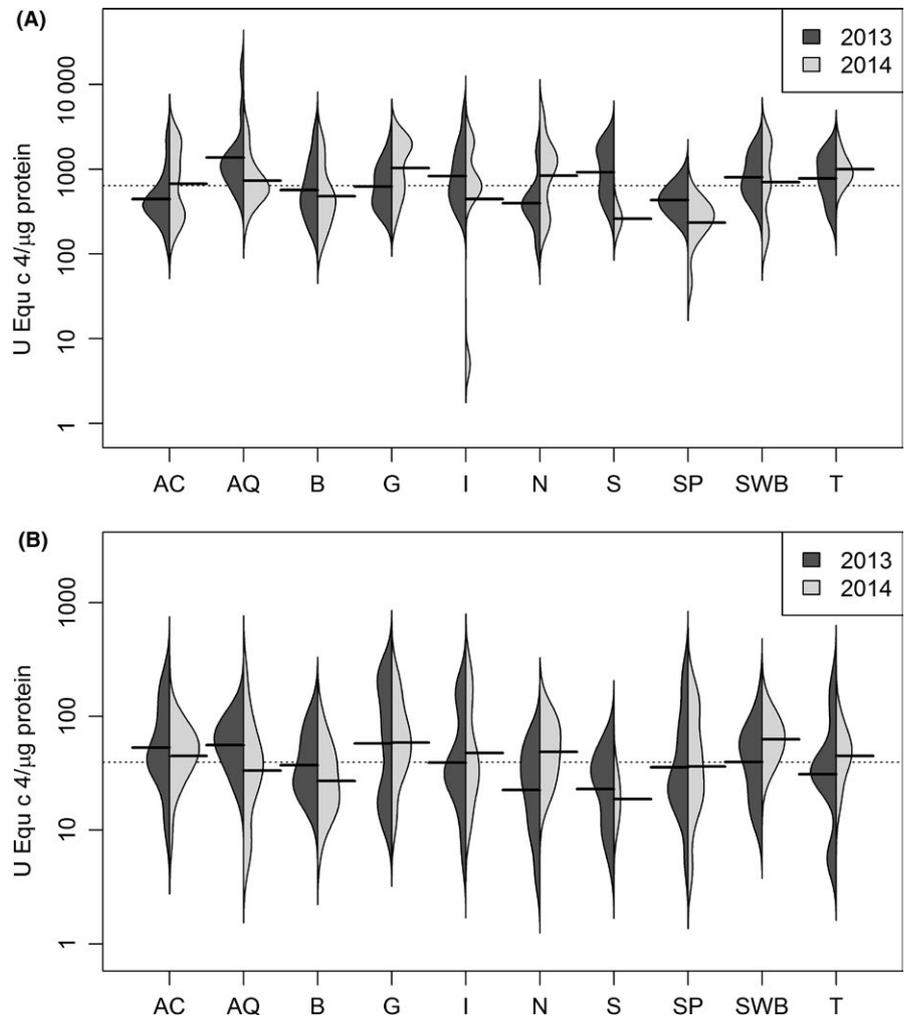


FIGURE 3 Descriptive figures of the levels of Equ c 4 (U/ μ g protein) in A: dander and B: saliva for each year in the breeds studied. The dotted line shows the overall geometric mean value of all the horses, and the black lines show the geometric mean values for each breed and year

was 1.5 U/mL and the limits of quantification were set to 2–100 U/mL. Samples were run in duplicates and at three different dilutions to fall within the linear range of the standard curve. A coefficient of variation (CV) <20% was accepted. Control samples, high and low, with known concentration were included on every plate. Blank samples composed of buffer were also included so background absorbance could be subtracted from the data points.

2.5 | Standard

The standard curve for Equ c 4 was in the range 1.5–198 U/mL for dander and between 1.6 and 206 U/mL for saliva and urine. Dander samples had to be analysed using two standard preparations due to technical problems. Since the standard prepared was not enough to run all samples, therefore, a new standard was prepared from the same batch as the first standard. Agreement of data generated using the two standards was assessed by analysing 25 dander samples using both standards on two days, 2 February and 16 February 2017. The mean value and difference on the log scale for each sample pair were calculated and plotted vs each other as suggested by Bland and Altman.³⁰ Points should be spread around zero and show no systematic trend if the two standards are in good agreement, see Figure 1.

2.6 | Statistical analysis

As seen in Figure 1, the average log difference between standard 1 and standard 2 was about 0.2. This was added to the log-transformed dander values obtained using standard 2 to adjust for this systematic bias. Differences between the breeds were estimated using a linear mixed effects model. Age, gender and breed were included as fixed effects, while horse-specific intercepts and by-breed time trends were included as random effects. Visual inspection of residual plots did not reveal any deviations from the assumptions of constant variance and normality when log-transforming the dander and saliva values. All analyses were done using R³¹ version 3.3.1 using the lme4 package.³² Multiplicity adjusted 95% confidence intervals for the estimated differences were obtained using the multcomp package.³³

3 | RESULTS

3.1 | Monoclonal antibodies 103 and 14G4 bind to native Equ c 4

The mAb's 103 and 14G4 were shown to bind to nEqu c 4 when used in concentrations ranging from 1 μ g/mL down to 0.004 μ g/mL, see

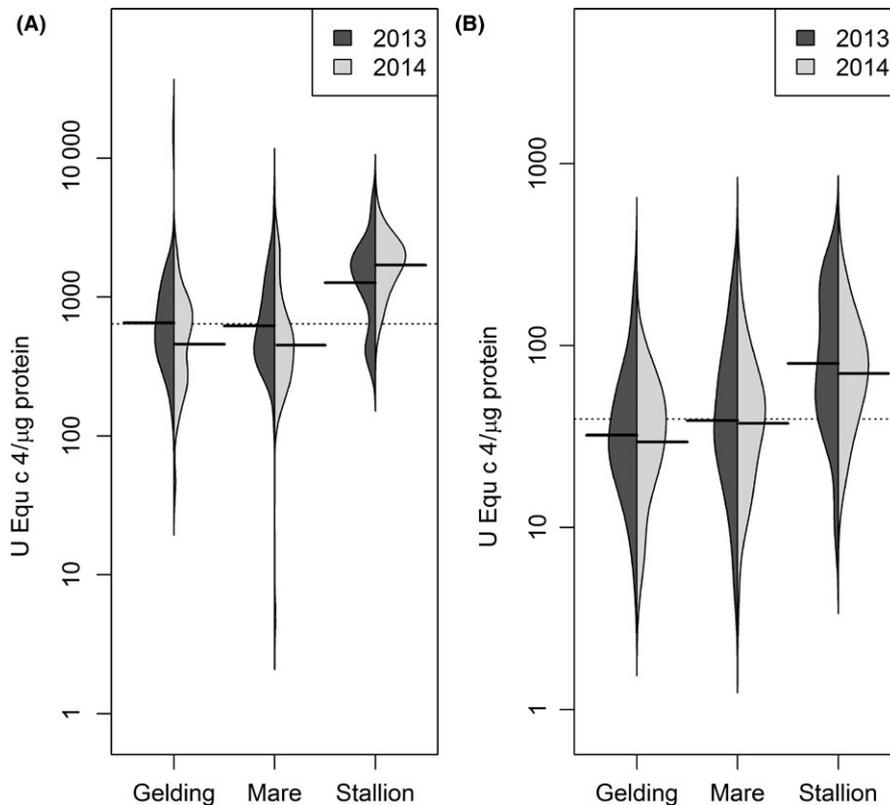


FIGURE 4 Descriptive figure of the levels of Equ c 4 (U/μg protein) in A: dander and B: saliva by sex and year. The dotted line shows the mean value of all the horses in this study, and the black lines show the geometric mean values for each sex and year

Figure 2. No binding was shown by ELISA assay for recombinant Equ c 1, recombinant Equ c 2, and native Equ c 3 to mAbs 103 and 14G4 in concentrations from 1 μg/mL down to 0.017 ng/mL.

3.2 | Horse dander and saliva

The horses included in the study in 2013 varied from foals under one year of age to 31 years with a mean of 10 years. A total of 144 horses were investigated: 77 mares, 16 stallions and 51 geldings. Similarly, a total of 108 horses included in 2014 varied in age from under 1 to 24 years, with a mean of 10 years and included 54 mares, 22 stallions and 32 geldings.

The geometric mean levels of Equ c 4 (U/μg protein) in dander and saliva in the sampled horses were 639 (range 5-15 264) and 39.5 (4-263), respectively. The breed-specific dander geometric mean values ranged between 317 and 1029 Equ c 4 U/μg protein, and saliva values varied between 22 and 59 Equ c 4 U/μg protein. The Gotland pony had the highest average values for both dander and saliva, while the Shetland pony had the lowest average dander value and the Standardbred had the lowest average saliva value.

Descriptive Figure 3A,B illustrates the levels of Equ c 4 (U/μg protein) in dander (Figure 3A) and saliva (Figure 3B) from the ten breeds included in this study.

The dark grey area represents levels from samples taken in 2013 and light grey from 2014. Figure 4A,B shows the levels of Equ c 4 (U/μg protein) in dander (A) and saliva (B) in 2013 and 2014, based on sex. The mean levels from both years are higher in samples from the

stallions compared to mares and geldings without any adjustments. The mean levels of Equ c 4 are about 10-fold higher in dander than in saliva. These plots show descriptive data for all individuals, irrespective of sex and age. The mean levels of Equ c 4 for different breeds cannot be directly compared since the study population is unevenly distributed see Table 1.

Adjusted comparisons between breeds, presented as ratios, are shown in Figure 5 with dander (Figure 5A) and saliva (Figure 5B). Data are arranged from the lowest ratio at the top to the highest at the bottom. For example, at the top of Figure 5A, the mean value for the North Swedish horse (N) was 0.22 (95% CI 0.08-0.58) times lower than the mean value for the American Quarter horse (AQ). Likewise, at the bottom of the figure, the mean value for the Standardbred (S) was 3.15 (1.10-9.04) times higher than the mean value for the North Swedish horse (N). Regarding dander, we could not find any differences between breeds, while for saliva samples the mean value for the North Swedish horse was lower compared to all the other breeds in this study, which is highlighted in Figure 5B.

The estimated differences in levels of Equ c 4 between stallions, mares and geldings adjusted for age, breed and time trend, are shown in Figure 6 for dander (Figure 6A) and saliva (Figure 6B). Stallions showed significantly higher levels of Equ c 4 in both dander and saliva than mares and geldings in this study. A ratio of 1 indicates that the mean values were equal. The geometric mean value in dander for stallions was almost three times higher than that of geldings and mares. Mares had slightly higher levels of Equ c 4 than geldings in saliva.

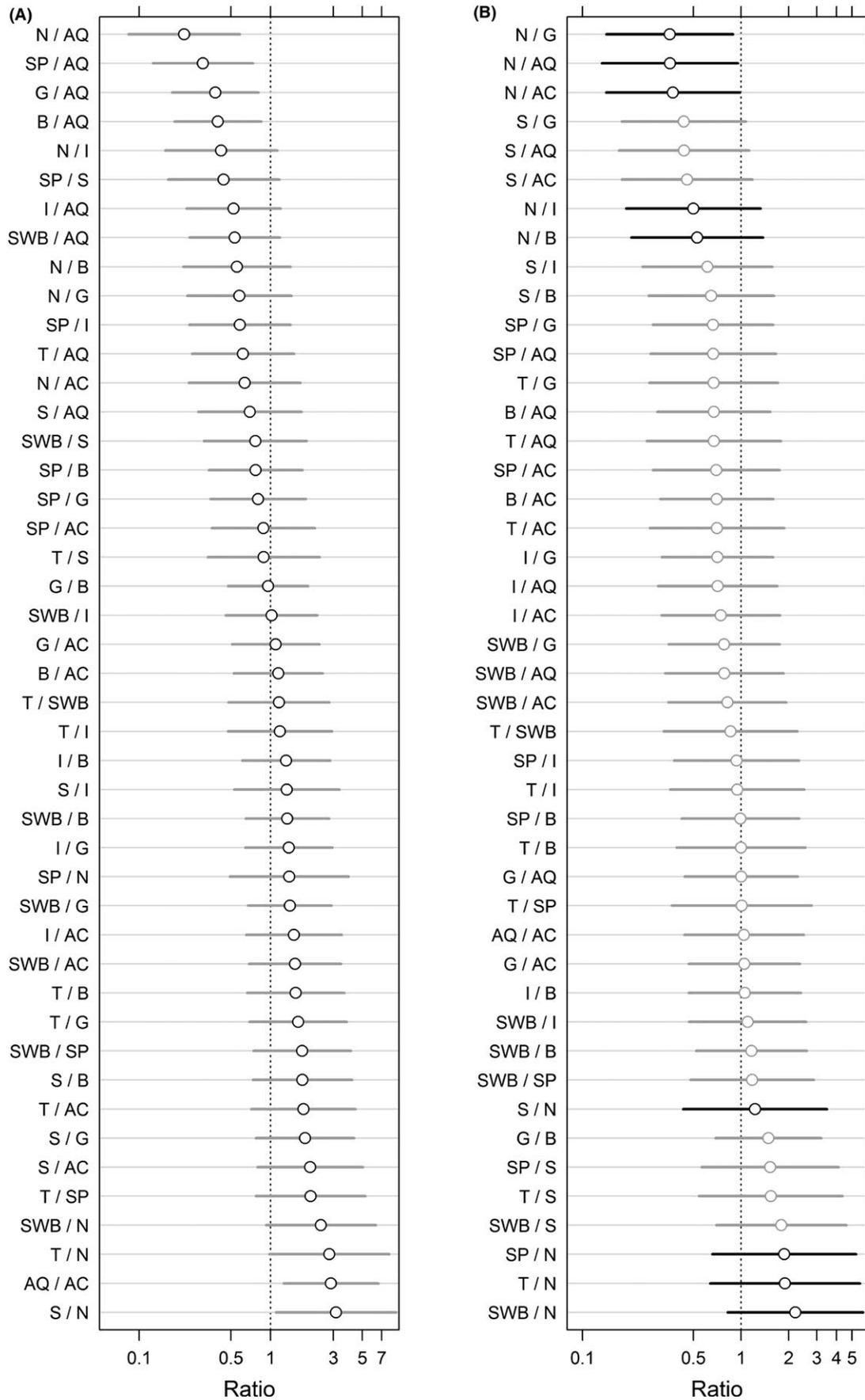


FIGURE 5 Estimated differences, presented as ratios with 95% confidence intervals, of the breed-specific geometric mean levels of Equ c 4 (U/ μ g protein) in A: dander, and B: saliva, adjusted for age, gender and time trend

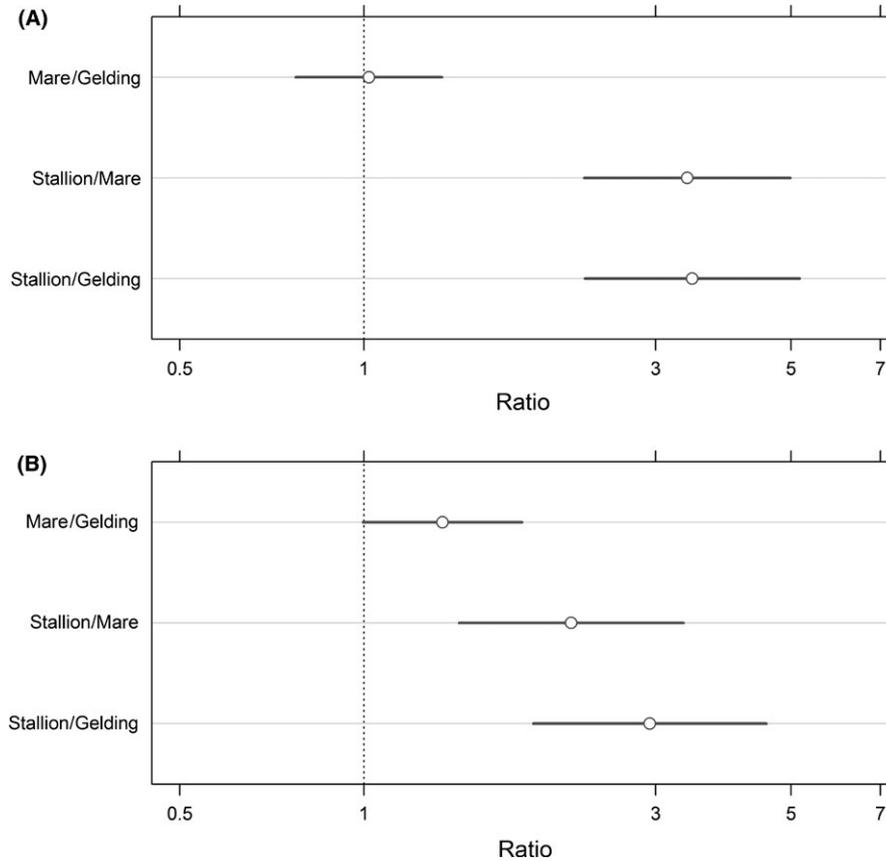


FIGURE 6 Estimated differences, presented as ratios with 95% confidence intervals, of the sex-specific mean levels of Equ c 4 (U/ μ g protein) in A: dander, and B: saliva, adjusted for age, breed and time trend

Eighty-two horses were sampled both in 2013 and in 2014 (44 mares, 11 stallions and 27 geldings), and the correlation between the levels of Equ c 4 (U/ μ g protein) between year 2013 and year 2014 for both dander and saliva samples is shown in Figure 7. Correlation coefficients for dander were $\rho = 0.72$, for saliva $\rho = 0.38$ and totally $\rho = 0.85$.

3.3 | Horse urine

The horse allergen Equ c 4 was detected in urine samples from 19 of 21 horses. The geometric mean value was 0.004 Equ c 4 (U/ μ g protein) with a geometric SD of 5.81. Levels of Equ c 4 in urine were 10 000-fold lower than in saliva samples. It was not possible to compare breeds or sexes because of the low number of urine samples.

4 | DISCUSSION

To our knowledge, this is the largest study of horse (170 horses) allergen profiles performed. We show that the horse allergen Equ c 4 is present in all dander and saliva samples from ten different horse breeds. The presence of the latherin protein Equ c 4 in all horse breeds is understandable since its function in the thermoregulation of equines. Likewise, the presence of the latherin protein Equ c 4 in saliva helps wet the fibrous feed that horses eat. In urine, Equ c 4 was detected in 19 of 21 samples.

The levels of Equ c 4 in both dander and saliva were significantly higher in the samples from stallions compared to mares and geldings in samples taken both in 2013 and in 2014, independent of breed. Estimated differences, presented as ratios, of the sex-specific mean level of Equ c 4 in dander and saliva adjusted for age, breed and time trend, showed that stallions had higher levels than mares and geldings.

In dander samples, our data demonstrate that there were no differences in Equ c 4 levels between breeds. In saliva samples, the North Swedish horse was a breed that stood out in this study, based on the calculation of estimated differences, presented as ratios, of the breed-specific mean levels of Equ c 4 in saliva adjusted for age, gender and time trend. This result was detected despite the relatively high number of stallions in this breed (5 of 10 in 2013, and 6 of 9 in 2014) compared to the other breeds, which most likely could have affected the mean values. However, the higher number of stallions should have increased the mean value, but still this breed showed lower ratios compared to the other breeds. An additional analysis showed that stallions had higher Equ c 4 levels than mares in this group. There was only one gelding in the group making comparisons difficult (See Figure 8). The individuals that were sampled both 2013 and 2014 are marked with a dotted line between the data points.

In Figure 9, the relationship between Equ c 4 (U/mL) and protein content (μ g/mL) in dander and saliva for both year 2013 and year 2014 is shown. Protein content and Equ c 4 content in the samples

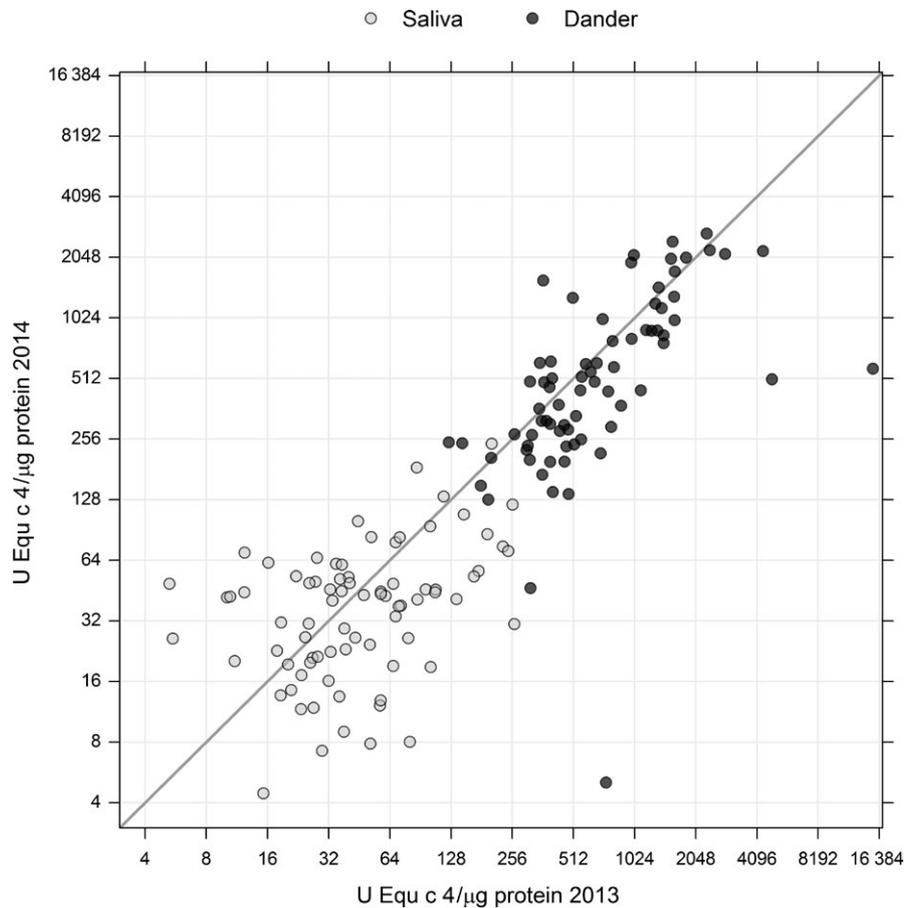


FIGURE 7 Correlation between the levels of Equ c 4 (U/μg protein) between year 2013 and year 2014 for both dander (black dots) and saliva (grey dots) samples. Correlation coefficients for dander were $\rho = 0.72$, for saliva $\rho = 0.38$ and totally $\rho = 0.85$

correlated with a certain degree in dander (2013: $\rho = 0.73$, 2014: $\rho = 0.78$) but less so in saliva (2013: $\rho = 0.25$, 2014: $\rho = 0.26$).

Previous studies have reported quantification of horse allergen with mAb's 103 and 14G4 as units of Equ cx.^{6,27,28} In this study, we have shown that these mAb's bind to native Equ c 4, and not to Equ c 1, Equ c 2 or Equ c 3, which could therefore be used as a reagent to specifically target this allergen.

Generally, dander, fur and skin extracts have been used to identify various allergenic proteins from furry animals, such as cat, dog, cattle and horse. However, animal saliva has also been shown to be an important source of allergens. The major cat allergen, Fel d 1, is present in tears, skin and saliva.³⁴ The dog allergens Can f 1-4 and Can f 6, which belong to the lipocalin and albumin protein family, have been found in dander and saliva.³⁵ In one study, 20% of dog allergic patients were shown to be IgE-negative to dog dander, while they were IgE-positive to saliva.³⁶

Horse dander, saliva and urine are all important allergen sources for people working in the stable environment. However, for people allergic to horses, dander is more likely to be the most relevant source of the horse allergen Equ c 4, since dander is easily airborne.

A pattern between males and females and the effect of castration of male horses, similar to the one observed in this study, has been reported regarding the main cat allergen (Fel d 1) in fur. Male cats seem to produce higher levels of Fel d 1 than female cats.³⁷ The production of Fel d 1 allergen seems to be influenced by the

production of hormones. A decrease could be seen after castration of the cats and the levels increased significantly after injecting testosterone.³⁸ We are not aware of any study showing that testosterone levels in horses affect the production of Equ c 4. Another study showed that working with male rodents may increase the risk of allergy to laboratory animals because male rodents excrete up to 100-fold higher amounts of allergens in urine than females.³⁹

In conclusion, our results show a high variability in levels of the allergen Equ c 4 in dander and saliva between individuals both within breeds and between breeds. Significantly higher levels were found in stallions compared to mares and geldings, independent of breed. Our results suggest that none of the horse breeds studied here can be recommended for individuals allergic to Equ c 4.

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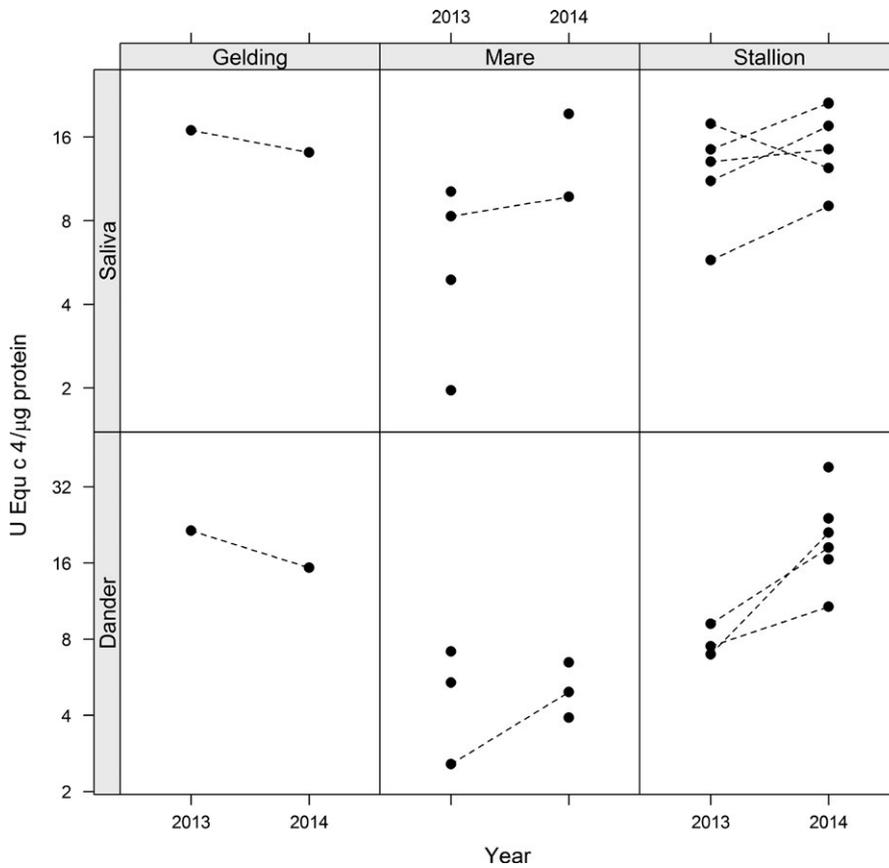


FIGURE 8 Levels of Equ c 4 (U/μg protein) in saliva and dander samples, from the subgroup North Swedish horses. The individuals sampled both 2013 and 2014 are marked with a dotted line between the data points

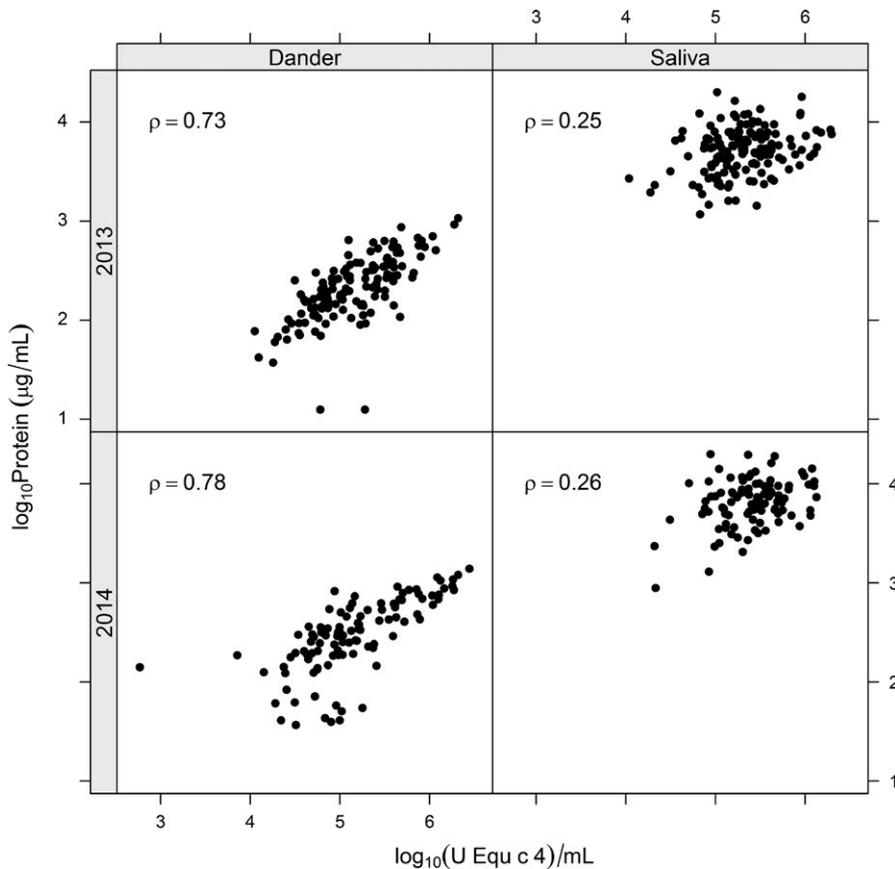


FIGURE 9 Relationship between Equ c 4 (U/mL) and protein content (μg/mL) in dander (left panels) and saliva (right panels) samples from both year 2013 (top panels) and year 2014 (bottom panels). Protein content and Equ c 4 content correlated with a certain degree in dander (2013: $\rho = 0.73$, 2014: $\rho = 0.78$) but less so in saliva (2013: $\rho = 0.25$, 2014: $\rho = 0.26$)

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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REFERENCES

- Roberts G, Lack G. Horse allergy in children. *BMJ*. 2000;321:286-287.
- Rönmark E, Bjerg A, Perzanowski M, Platts-Mills T, Lundbäck B. Major increase in allergic sensitization in schoolchildren from 1996 to 2006 in northern Sweden. *J Allergy Clin Immunol*. 2009;124:357-363.e15.
- Miljöhälsorapport 2017 – Folkhälsomyndigheten. <http://www.folkhalsomyndigheten.se/publicerat-material/publikationsarkiv/m/miljohalsorapport-2017/>. Accessed November 29, 2017.
- Warm K, Lindberg A, Lundbäck B, Rönmark E. Increase in sensitization to common airborne allergens among adults – two population-based studies 15 years apart. *Allergy Asthma Clin Immunol*. 2013;9:20.
- Liccardi G, Salzillo A, Dente B, et al. Horse allergens: an underestimated risk for allergic sensitization in an urban atopic population without occupational exposure. *Respir Med*. 2009;103:414-420.
- Elfman L, Brannstrom J, Smedje G. Detection of horse allergen around a stable. *Int Arch Allergy Immunol*. 2008;145:269-276.
- Haeger-Eugensson M, Ferm M, Elfman L. Use of a 3-D dispersion model for calculation of distribution of horse allergen and odor around horse facilities. *Int J Environ Res Public Health*. 2014;11:3599-3617.
- Ramadour M, Guetat M, Guetat J, El Biaze M, Magnan A, Vervloet D. Dog factor differences in Can f 1 allergen production. *Allergy*. 2005;60:1060-1064.
- Vredegoor DW, Willemse T, Chapman MD, Heederik DJJ, Krop EJM. Can f 1 levels in hair and homes of different dog breeds: lack of evidence to describe any dog breed as hypoallergenic. *J Allergy Clin Immunol*. 2012;130:904-909.e7.
- Zahradnik E, Sander I, Brüning T, Raulf M. Allergen levels in the hair of different cattle breeds. *Int Arch Allergy Immunol*. 2015;167:9-15.
- Mitlehner W, Mitlehner HC, Niggemann B. Horse allergy: curly horses allow horse allergic riders to ride again. *Pneumologie*. 2015;69:711-718.
- Felix K, Ferrándiz R, Einarsson R, Dreborg S. Allergens of horse dander: comparison among breeds and individual animals by immunoblotting. *J Allergy Clin Immunol*. 1996;98:169-171.
- Dandeu JP, Rabillon J, Divanovic A, Carmi-Leroy A, David B. Hydrophobic interaction chromatography for isolation and purification of Equ.c1, the horse major allergen. *J Chromatogr*. 1993;621:23-31.
- Gregoire C, Rosinski-Chupin I, Rabillon J, Alzari PM, David B, Dandeu JP. cDNA cloning and sequencing reveal the major horse allergen Equ c1 to be a glycoprotein member of the lipocalin superfamily. *J Biol Chem*. 1996;271:32951-32959.
- Saarelainen S, Rytönen-Nissinen M, Rouvinen J, et al. Animal-derived lipocalin allergens exhibit immunoglobulin E cross-reactivity. *Clin Exp Allergy*. 2008;38:374-381.
- Bulone V, Krogstad-Johnsen T, Smestad-Paulsen B. Separation of horse dander allergen proteins by two-dimensional electrophoresis-molecular characterisation and identification of Equ c 2.0101 and Equ c 2.0102 as lipocalin proteins. *Eur J Biochem*. 1998;253:202-211.
- Goubran Botros H, Rabillon J, Grégoire C, David B, Dandeu JP. Thiophilic adsorption chromatography: purification of Equ c2 and Equ c3, two horse allergens from horse sweat. *J Chromatogr B Biomed Sci Appl*. 1998;710:57-65.
- Ho JX, Holowachuk EW, Norton EJ, Twigg PD, Carter DC. X-ray and primary structure of horse serum albumin (*Equus caballus*) at 0.27-nm resolution. *Eur J Biochem*. 1993;215:205-212.
- Fjeldsgaard BE, Paulsen BS. Comparison of IgE-binding antigens in horse dander and a mixture of horse hair and skin scrapings. *Allergy*. 1993;48:535-541.
- Goubran Botros H, Poncet P, Rabillon J, Fontaine T, Laval J-K, David B. Biochemical characterization and surfactant properties of horse allergens. *Eur J Biochem*. 2001;268:3126-3136.
- Vance SJ, McDonald RE, Cooper A, Kennedy MW, Smith BO. Resonance assignments for latherin, a natural surfactant protein from horse sweat. *Biomol NMR Assign*. 2014;8:213-216.
- Vance SJ, McDonald RE, Cooper A, Smith BO, Kennedy MW. The structure of latherin, a surfactant allergen protein from horse sweat and saliva. *J R Soc Interface*. 2013;10:20130453.
- Martini M, Swiontek K, Antonicelli L, et al. Lysozyme, a new allergen in donkey's milk. *Clin Exp Allergy*. 2018;48:1521-1523.
- McDonald RE, Fleming RI, Beeley JG, et al. Latherin: a surfactant protein of horse sweat and saliva. *PLoS ONE*. 2009;4:e5726.
- Bingle CD, LeClair EE, Havard S, Bingle L, Gillingham P, Craven CJ. Phylogenetic and evolutionary analysis of the PLUNC gene family. *Protein Sci*. 2004;13:422-430.
- Chapman MD, Vailes LD, Ichikawa K. Immunoassays for indoor allergens. *Clin Rev Allergy Immunol*. 2000;18:285-300.
- Emenius G, Larsson PH, Wickman M, Härfast B. Dispersion of horse allergen in the ambient air, detected with sandwich ELISA. *Allergy*. 2001;56:771-774.
- Emenius G, Merritt A-S, Härfast B. Dispersion of horse allergen from stables and areas with horses into homes. *Int Arch Allergy Immunol*. 2009;150:335-342.
- Binnmyr J. Improved diagnosis and vaccination of pet allergy: from molecular mechanisms to clinic. (Inst för klinisk neurovetenskap/Dept of Clinical Neuroscience, Karolinska Institutet, 2016).
- Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet*. 1986;1:307-310.
- R Core Team. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing; 2016.
- Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. *J Stat Softw*. 2015;67:48.
- Hothorn T, Bretz F, Westfall P. Simultaneous inference in general parametric models. *Biom J*. 2008;50:346-363.
- van Milligen FJ, Vroom TM, Aalberse RC. Presence of *Felis domesticus* allergen I in the cat's salivary and lacrimal glands. *Int Arch Allergy Appl Immunol*. 1990;92:375-378.
- Saarelainen S, Taivainen A, Rytönen-Nissinen M, et al. Assessment of recombinant dog allergens Can f 1 and Can f 2 for the diagnosis of dog allergy. *Clin Exp Allergy*. 2004;34:1576-1582.
- Polovic N, Wadén K, Binnmyr J, et al. Dog saliva – an important source of dog allergens. *Allergy*. 2013;68:585-592.
- Jalil-Colome J, de Andrade AD, Birnbaum J, et al. Sex difference in Fel d 1 allergen production. *J Allergy Clin Immunol*. 1996;98:165-168.
- Zielonka TM, Charpin D, Berbis P, Luciani P, Casanova D, Vervloet D. Effects of castration and testosterone on Fel d I production by sebaceous glands of male cats: I—immunological assessment. *Clin Exp Allergy*. 1994;24:1169-1173.
- (15) Working with male rodents may increase risk of allergy to laboratory animals. *ResearchGate*. https://www.researchgate.net/publication/11772506_Working_with_male_rodents_may_increase_risk_of_allergy_to_laboratory_animals. Accessed June 13, 2018.

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