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# Resistance against strongylid nematodes in two high prevalence Equine Recurrent Airway Obstruction families has a genetic basis

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**Summary**

A recent study showed increased resistance against strongylid nematodes in offspring of a stallion affected by recurrent airway obstruction (RAO) compared with unrelated pasture mates. Resistance against strongylid nematodes was associated with RAO affection. Hypothesis: Resistance against strongylid nematodes has a genetic basis. The genetic variants influencing strongylid resistance also influence RAO susceptibility. Faecal samples from the half-sibling offspring of two RAO-affected Warmblood stallions – 98 offspring from the first family (family 1) and 79 from the second family (family 2) – were analysed using a combined sedimentation-flotation method. The phenotype was defined as a binary trait - either positive or negative for egg shedding. The influence of non-genetic factors on egg shedding was analysed using SAS, the mode of inheritance was investigated using PAP and iBay, and the association between shedding of strongyle eggs and RAO was estimated by odds ratios. Previously established genotypes for 315 microsatellite markers were used for QTL analyses using GRID QTL. The inheritance of "strongylid egg shedding" is influenced by major genes on ECA15 and ECA20. Shedding of strongylid eggs is associated with RAO in family 1 but not in family 2. Conclusions: The status of "shedding of strongyle eggs" has a genetic background. The results were inconclusive as to whether "egg shedding" and RAO share common genetic components. Our results suggest that it may be possible to select for resistance against strongylid nematodes.

**Keywords**: horse / strongylid nematodes / recurrent airway obstruction / resistance / major gene

**Resistenz gegen kleine Strongyliden in zwei Warmblut-Familien mit erhöhter Prävalenz rezidivierender Atemwegsobstruktion hat eine genetische Grundlage**

Eine kürzlich erschienene Arbeit zeigte eine erhöhte Resistenz gegen kleine Strongyliden bei Nachkommen eines Hengstes mit rezidivierender Atemwegsobstruktion (Recurrent Airway Obstruction (RAO)) im Vergleich mit ihren nichtverwandten Weidekameraden. Die Resistenz gegen kleine Strongyliden war mit RAO assoziiert. Hypothese: Resistenz gegen kleine Strongyliden hat eine genetische Basis. Die genetischen Varianten welche die Resistenz gegen kleine Strongyliden beeinflussen, beeinflussen auch die RAO-Anfälligkeit. Material und Methoden: Kotproben von Nachkommen zweier RAO-betroffenen Warmbluthengste – 98 Nachkommen der ersten Familie (Familie 1) und 79 Nachkommen der zweiten Familie (Familie 2) – wurden mittels kombinierter Sedimentations-Flotations-Verfahren untersucht. Der Phänotyp wurde als binäres Merkmal definiert, entweder positiv oder negativ für die Eiausscheidung. Der Einfluss von nicht-genetischen Faktoren wurde mittels SAS analysiert, der Erbgang wurde mittels PAP und iBay untersucht und die Assoziation zwischen Ausscheidung von Strongylideneier und RAO wurde mittels Odds Ratio geschätzt. Die früher determinierten Genotypen für 315 Mikrosatteliten wurden für die QTL Analyse (GRID QTL) gebraucht. Die Vererbung der Ausscheidung von Eiern kleiner Strongyliden wird durch Gene auf ECA15 und ECA20 beeinflusst. Die Ausscheidung von Eiern kleiner Strongyliden ist mit RAO in Familie 1 assoziiert aber nicht in Familie 2. Der Status "Ausscheidung von Strongyliedeneier" hat einen genetischen Hintergrund. Die Resultate sind als ersten Hinweis auf einen gemeinsamen genetischen Hintergrund für Eiausscheidung und RAO zu werten. Unsere Resultate deuten darauf hin, dass es möglich sein könnte für eine Resistenz gegen kleine Strongyliden zu selektionieren.

**Schlüsselwörter**: Pferd / kleine Strongyliden / rezidivierende Atemwegsobstruktion / Resistenz / Gen

## Introduction

Since the hygiene hypothesis was postulated by *Strachan* (1989), the investigative interest in associations between parasite, especially helminth infection and development of hypersensitivities has greatly increased. The most common hypersensitivity-associated disease in horses is recurrent airway obstruction (RAO). RAO shares many characteristic features with human asthma (*Robinson* 2001): e.g. airway inflammation, mucus accumulation, reversible airway obstruction and bronchial hyperresponsiveness (*Martin* 2001, *Gerber* at al. 2004).

RAO is strongly influenced by environmental factors, mainly allergens in hay dust, but a genetic predisposition for this disease has also been demonstrated (*Marti* et al. 1991, *Ramseyer* et al. 2007, *Gerber* et al. 2009). The prevalence and clinical manifestation of RAO was found to be increased in two large half-sib sire families (referred to here as family 1 and family 2) in comparison with a control group (*Ramseyer* et al. 2007). In family 1, *Jost* and co-workers (2007) demonstrated association and linkage of RAO with microsatellite markers on ECA13 near the interleukin 4 receptor chain (IL4R) gene. Polymorphismus in human IL4R are associated with phenotypes related to asthma and atopy (*Hershey* et al. 1997, *Ober* et al. 2000). Moreover, IL4R is associated with parasitic defence in humans and animals (*Scales* et al. 2007). *Neuhaus* and co-workers (2008) observed that offspring of the RAO-affected sire with

the IL4R association (family 1) were more resistant against strongylid nematodes compared to a matched control group. Helminthiasis seems to play a beneficial, even protective role in the context of autoimmune diseases and allergies (da *Rocha Fa* 2006, *McKay* 2006). The classical hygiene hypothesis postulates that infections and unhygienic contact may confer protection against the development of allergic diseases. Another possible explanation for the inverse relationship between resistance to certain parasitic diseases and asthma is that genetic determinants which confer protection against detrimental worm burdens are the same determinants involved in atopic asthma (*Barnes* et al. 2005). This alternative hypothesis could also explain the observation that individuals with a history of atopy appear to be more resistant to helminthic parasites (*Lynch* et al. 1998).

The family of strongylids contains both the large strongyles (Strongylinae) which are nowadays rarely found in horses in Switzerland (*Meier* and *Hertzberg* 2005) and the small strongyles (Cyathostominae) which are the most important parasitic pathogens of grazing horses (*Love* and *Duncan* 1992). Although many horses harbour large burdens of cyathostomes they rarely develop clinical signs (*Love* et al. 1999).

*Neuhaus* and co-workers (2008) observed that offspring from family 1 had a decreased risk of severe infestation with strongylids as compared to unrelated, RAO-unaffected pasture mates, matched for age, breed, stable, pasture and deworming management. Furthermore, within the offspring from family 1, the RAO-affected individuals had a >5-fold reduction in shedding of parasite eggs compared to unaffected offspring. This raised the question whether there is a relationship between resistance to helminth infections and susceptibility to RAO in horses in general. An investigation in family 2 found no significant difference in parasite infestation level between the offspring and the matched control group (*Bründler* et al. 2011). However, *Bründler* et al. (2011) did observe that unrelated RAO-affected Warmblood horses shed fewer strongylid eggs than their matched controls.

To our knowledge, a possible genetic basis for the status of patent nematode infection and respective "egg shedding" in horses has not yet been investigated. Therefore, the main goal of this study was to elucidate the role of genetics in the status of "egg shedding" in family 1 and 2 by investigating the mode of inheritance and performing genetic association and linkage analysis with microsatellite markers. In addition, we investigated whether "parasite egg shedding" and RAO share a common genetic basis in family 1 and 2.

## Materials and Methods

### *Animals*

The study was approved by the Animal Experimentation Committee of the Canton of Berne, Switzerland (authorisation number BE 33/07). Two half-sibling Warmblood families have been investigated with respect to RAO (*Ramseyer* et al. 2007). On the basis of a standardised questionnaire, the RAO status of all horses was classified using the Horse Owner Assessed Respiratory Signs Index (HOARSI), as described in detail by *Ramseyer* and co-workers (2007). Briefly, owner supplied information on coughing, nasal discharge, performance and breathing pattern was used to assign the HOARSI classification which has four grades: unaffected (1), mild (2), moderate (3) and severe (4) clinical signs. The classification refers to the period when the horses showed the most severe clinical signs.

In the present study, 98 offspring from family 1 and 79 offspring from family 2 were included. Family 1 comprised 43 females and 55 males ranging in age from 9 to 17 years with an average of 13.5 years, while family 2 comprised 46 females and 33 males ranging in age from 7 to 15 years with an average of 12.5 years. Thirty-four (35%) descendants of family 1 were considered unaffected (HOARSI 1), 21 (21%) were classified as HOARSI 2, 29 (30%) as HOARSI 3 and 14 (14%) as HOARSI 4. Nineteen (24%) descendants of family 2 were considered unaffected (HOARSI 1), 25 (32%) were classified as HOARSI 2, 25 (32%) as HOARSI 3 and 10 (12%) as HOARSI 4. All 177 individuals had been previously genotyped with 315 microsatellite markers (*Swinburne* et al. 2009).

### *Faecal sample collection*

A sample was collected from all 177 horses and information was gathered through a questionnaire. The horses were located in 160 stables. All owners were asked what anthelminthic treatment specifically was used as the last deworming treatment. Further, information was collected on pasture management, such as removal of manure from pasture, other species on the pasture, or mowing of the pasture, geographic region and altitude. The faecal samples were only collected after the egg reappearance period (ERP), defined as the period when horses are expected to reach a maximum positive egg count after treatment (*von Samson-Himmelstjerna* 2006). Since different anthelminthics were used by different owners, a maximum egg reappearance period of 90 days was chosen to include long-active agents such as Moxidectin (*von Samson-Himmelstjerna* 2006). Horses were excluded when this protocol was not adhered to. Samples collected from the stable floor had to be fresh and uncontaminated by other faeces or mud. If no faeces were readily available, the faecal samples were recovered rectally.

### *Coprology*

Each sample was examined by only one person without knowledge of the health status of the horses and the family they belonged to. Samples were analysed separately by a combined sedimentation-flotation method (*Bauer* 2006). The results were expressed as a binary trait: "egg shedding", positive or negative.

### *Statistics*

The four HOARSI categories were combined into two classes, where HOARSI 1 was considered unaffected and HOARSI 2, 3 and 4 affected. A possible association between HOARSI and "egg shedding" was investigated by calculating odds ratios (http://statpages.org/ctab2x2.html)[1]. For the joint ana-

lysis of both families PROC GLIMMIX of the SAS package[2] was used to estimate non-genetic effects on the probability of egg shedding in a linear model. The effects entering the model were sire, gender, HOARSI, age, removal of manure from pasture, geographic region, altitude (≤ 500m and >500m above sea level), sharing pasture with other species (livestock) and mowing of the pasture. Segregation analyses were performed using PAP[3] together with NPSOL[4] and iBay1.44[5]. Linkage between egg shedding and the microsatellite markers were calculated using GRID QTL (http://www.gridqtl.org.uk/ *Seaton* et al. 2006). A single QTL model with mowing as a fixed effect was fitted at 1 Mb steps along the chromosomes. A QTL was considered present if the F statistic exceeded a chromosome-wide significance level of P=0.05. All analyses were performed with each family separately and also with both families combined. F statistics were also compared with a genome-wide significance level at P=0.05.

## Results

*Coprology; association between "egg shedding" and HOARSI*

In the majority of the faecal samples, no "egg shedding" was detected. The percentage of offspring "shedding eggs" was significantly lower (P<0.01) in family 1 (12%) than in family 2 (34%). Shedding of strongylideggs and HOARSI were associated in family 1 but not in family 2 (Table 1).

*Non-genetic effects on "egg shedding"*

The effects on "egg shedding" of gender, HOARSI, age, removal of manure from pasture, geographic region, altitude, sharing pasture with other species, and mowing of the pasture were not significant (P>0.05). However, the effect of the sire on "egg shedding" was highly significant (P=0.01). Mowing of the pasture became significant (P=0.02) when sharing pasture with other species HOARSI, geographic region and altitude were all removed from the model. After removing all non-significant effects the final model comprised only sire (P<0.01) and mowing of the pasture (P=0.02).

*Segregation analyses*

Segregation analyses were performed for both sire families jointly and for each family separately. Using PAP with NPSOL, in all three analyses a general genetic model explained our data much better than an environmental model (P<0.01). In all three cases the general genetic model could not be differentiated from the mixed inheritance model (P>0.05). Also the major gene model and the polygenic model could not be differentiated from the mixed inheritance model (P>0.05). Using iBay mixed inheritance models fitted the data in all three cases better, with higher Bayes factors (BF), than the other models. Parameter estimates were averaged over four to seven runs with 10 chains per run. The estimates of the frequency of the deleterious allele "a" and the penetrances are given in Table 2, together with the percentages of the total

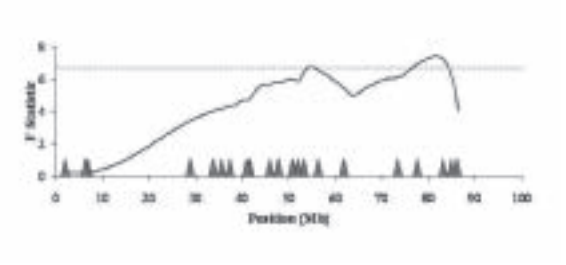

**Fig 1** F statistics of the QTL analyses on ECA15 in family 1. The broken horizontal line indicates the chromosome-wide significance threshold at P<0.05. The positions of informative markers are shown with grey triangles.

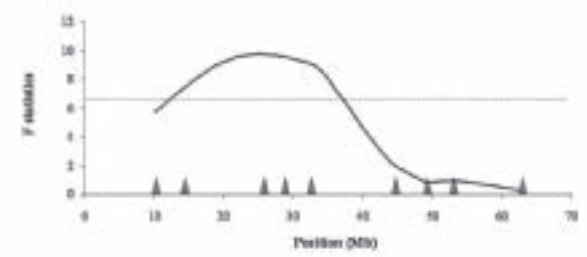

**Fig 2** F statistics of the QTL analyses on ECA20 in family 2. The broken horizontal line indicates the chromosome-wide significance threshold at P<0.05. The positions of informative markers are shown with grey triangles.

**Table 1** Odds ratios (OR), confidence intervals (CI) and two-sided P-values for the association between presence of eggs and HOARSI in the two half-sibling sire families. The percentage of the offspring shedding eggs is given in the column ES for the two families.

| | ES% | OR | 95% CI | $P_{2\text{-sided}}$ |
|---|---|---|---|---|
| Family 1 | 12 | 0.257 | 0.074-0.901 | 0.045 |
| Family 2 | 34 | 1.235 | 0.413-3.665 | 0.785 |

Table 2 Parameter estimates for the separate and joint analyses of families 1 and 2

| | Family 1 & Family 2 | Family 1 | Family 2 |
|---|---|---|---|
| Frequency of allele a | 0.50 | 0.54 | 0.50 |
| Penetrance for AA | 0.03 | 0.01 | 0.11 |
| Penetrance for Aa | 0.14 | 0.10 | 0.33 |
| Penetrance for aa | 0.42 | 0.45 | 0.64 |
| Major gene[1] | 0.48 | 0.50 | 0.33 |
| Polygenic component[2] | 0.39 | 0.42 | 0.60 |

[1] Part of the total variance explained by the major gene, [2] Part of the total variance explained by the polygenic component

variance explained by the major gene and the polygenic component.

*QTL analyses*

QTL analyses using mowing as a fixed effect resulted in significant signals ($P<0.05$, chromosome wide) on ECA15 (Fig 1) for family 1 and on ECA20 (Fig 2) for family 2. The joint analysis of both families gave a significant signal on ECA20 but not on ECA15 and revealed an additional signal on ECA11 ($P<0.05$). None of the signals reached genome wide significance.

## Discussion

Our results suggest the presence of more than one major gene influencing strongylid egg shedding in horses. Regions harbouring one or more major genes for egg shedding were identified on ECA15 in family 1 and on ECA20 in family 2. Egg shedding was not only influenced by two different chromosome regions in the two families, but it was significantly less frequent in family 1 than in family 2. In addition, egg shedding was significantly associated with RAO in family 1 but not in family 2. Egg shedding among the offspring in family 2 (with 34% positive faecal samples) was almost three times as frequent as in family 1 (12%). This disparity could be explained by different frequencies of the unfavourable allele of the putative major genes which are located on different chromosomes in the two families. The association of egg shedding with RAO in family 1 could also be an artefact as the two families were collected to investigate RAO and therefore are not random samples of the population. Other explanations for this association such as linkage between egg shedding and RAO or pleiotropy were evaluated but our data did not support one hypothesis or another. A larger, random sample would be needed to further investigate these hypotheses.

Our investigation was a field study in which natural infestation was examined, rather than a controlled clinical study in which each horse would be exposed to an equal infection pressure. However, post-mortem surveys of naturally occurring gastrointestinal cyathostome burdens of horses have indicated that virtually all grazing horses are infected (*Ogbourne* 1976, *Reinemeyer* et al. 1984, *Krecek* et al. 1989). For this reason, we assumed that the horses in our study were exposed to a similar infection pressure. Geographic region and altitude had no effect on egg shedding supporting our assumption of a similar infection pressure across Switzerland. Gender also had no effect as the sex ratio did not significantly differ in the two groups in which eggs were present or absent. As expected in a study of horses aged over 7 years, age had no effect. High faecal egg counts and clinical cyathostominosis are more frequently seen in young horses (*Love* and *Duncan* 1992, *Giles* et al. 1985). HOARSI had no apparent effect on egg shedding status, which may be due to the combined analysis of families 1 and 2. The significant effect of mowing on egg shedding could be explained by the fact that mowing reduces the number of infectious strongylid larvae on the grass (*Ardeleanu* et al. 2008). The sire had by far the largest effect on the probability of egg shedding, clearly indicating a genetic component. The large effect of the sire could possibly obscure the effects of other factors.

In both families, the segregation analyses showed the presence of a major gene. However, the results also suggested that the gene influencing egg shedding status is not the same in the two families as the penetrance and the percentage of the variance explained by the major gene differed. Furthermore there must be other genes associated with egg shedding besides these major genes as the polygenic component explained a considerable part of the variance in both families (Table 2).

The separate QTL analysis of both families gave a significant chromosome wide signal on ECA 15 for family 1 and on ECA20 for family 2. This result suggests that for egg shedding, as for RAO (*Gerber* et al. 2009), different major genes play a role in the two families. However, the signal on ECA11 suggests an overlap of the genetic background in the two families.

There are several candidate genes on ECA15, such as the interleukin 1 receptor genes or the interleukin 18 receptor accessory protein gene, all involved in the development of asthma (*Nakae* et al. 2003, *Ishikawa* et al. 2006) and in the defence against parasites (*Titus* et al. 1991, *Sasaki* et al. 2005).

The genes of the major histocompatibility complex (MHC) class I, II and III in horses are located on ECA20. The MHC plays an important role in the pathogenesis of allergic diseases (*Blaser* 2008) and in the defence against helminths (*Paterson* et al. 1998). The MHC also has been implicated with nematode resistance in sheep (*Davies* et al. 2006).

In conclusion, our results point at the presence of major genes on ECA15 and ECA20 affecting strongylid egg shedding and possibly RAO as well. This needs to be confirmed in the sample of the general equine population. It should be possible to select for resistance against patent strongylid infection, although a possible antagonistic relation between the infection with strongylids and RAO must be considered.

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