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Genetic analysis of candidate genes for allergic eczema in Icelandic horses

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Abstract

Genetisk analys av kandidatgener för allergiskt eksem hos Islandshästen

Genetic analysis of candidate genes for allergic eczema in Icelandic horses

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Allergy is a condition, which many studies have focused on. Its causes are many and complex. The environments as well as several genes interact in the development of allergy. Allergic eczema (AE) is a skin allergy present in many species. The Icelandic horse was used to study two candidate genes for AE. FceR1-beta the high affinity receptor for IgE and Spink5 a serine protease inhibitor, were the genes chosen. The aim of the study was to sequence these two genes in cDNA form and compare them in groups of healthy horses and groups of sick horses, in search for single nucleotide polymorphisms (SNPs). By using information on the genes from databases, primers were designed to amplify the specific genes' cDNA by PCR. The genes were then sequenced and healthy horses were grouped together to be compared to sick horses in search for SNPs. In the FceR1-beta cDNA one SNP was found. It showed no clear linkage to AE. The Spink5 cDNA was partly sequenced and no SNP has yet been discovered. The DNA sequencing of Spink5 should be completed and SNPs searched for. The data retrieved from FceR1-beta should be recorded and used in future studies where multiple genes will be analysed.

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Populärvetenskapligbeskrivning

Allergi orsakas av många faktorer. Mer än en gen kan påverka utvecklingen av allergi men även andra variabler så som miljön spelar roll. Allergiers komplexa karaktär har lett till att många studier inom området har genomförts. Flera olika djur, inklusive människan, är drabbade av allergier i olika former. Islandshästen lider i relativt stor utsträckning av allergiskt eksem (AE), som uppträder som kraftigast under sommarhalvåret. Åkomman orsakar hästarna stort lidande och inverkar negativt på ägarens ekonomi då hästens värde sänks kraftigt och omvårdnadskostnaden höjs. Det är av intresse ur avelsynpunkt att försöka finna gener som kan ligga bakom AE. Två gener (FcεR1-beta och Spink5) som misstänks kunna leda till AE har undersökts. Målet i studien har varit att sekvenera de båda generna. Material för att kunna genomföra studien samlades in från hästar som delades in i två grupper, friska respektive sjuka individer. Proverna behandlades sedan för att den kodande delan av DNA:t skulle kunna utvinnas. Proverna sekvenserades så att den genetiska koden (som består av baserna A, T, G och C) gick att avläsa. Efter det jämfördes de friska hästarnas koder med de sjuka hästarnas. Genen FcεR1-beta skilde sig på en bas mellan olika individer. Det gick inte att se ett tydligt mönster där de sjuka och friska hästarna grupperade sig efter den basen. För att kunna koppla genen till AE skulle de sjuka hästarnas kod skilja sig från de friska, detta var inte fallet här. Undersökning av genen Spink5 påbörjades men har ännu inte slutförts. I den del av Spink5s kod som sekvenserats har ingen skillnad mellan några individer påträffats. Ingen direkt koppling mellan de studerade generna och AE har ännu hittats. Informationen från genen FcεR1-beta bör sparas för framtida studier där flera gener jämförs samtidigt. Spink5 bör sekvenseras klart för att den ska gå att jämföra mellan sjuka och friska hästar.

1 Introduction

This study is part of a larger project which is set to investigate allergic eczema in the Icelandic horse.

1.1 Allergic eczema

The Icelandic horse is one of several known horse breeds that suffer from allergic eczema or summer eczema. The number of local names, e.g. Queensland's itch, Mexican itch, kasen (Japan), dhobie itch and Spanish itch express the world-wide distribution of this disease (Sundquist *et al.* 2005). The amount of horses born outside of Iceland that contract AE is estimated to be around 6%. In sharp contrast to this stand the fact that almost 30% of Icelandic horses exported from the island contracts AE. (Ericson *et al.* 2004) This is a result of the exported horse's immune system reacting to new allergens present outside of Iceland.

The condition is painful for the horse and requires nursing, attention and poses an economical challenge to the owner of the horse. The mild form of AE is characterised by swelling of the skin and an itching mane and tail root. More severe forms of the disorder can result in oozing wounds across the body. The horse becomes restless by the itching which can lead to malnutrition (Sundqvist *et al.* 2005).

Allergic eczema is most probably a multifactorial disease, which means that many factors contribute to it. (Kawano *et al*., Osmola *et al.*, Quiros *et al.*, and Raap *et al*. 2005)
The environment, epigenetics and genetics all play a part. (Vercelli D, 2004) The fact that as many as 30% of the exported horses but only 6% of the horses born outside of Iceland develop eczema suggest that not only the genes decide the outcome, but the environment plays a big part as well. A recent study of AE in Icelandic horses shows that it is a heritable disorder and the study estimates to which extent it is inherited (Lindberg L, 2006).
This has already been shown in many studies on skin allergies in humans. The genetics behind this phenotype needs to be explored. Allergic eczema is like other similar diseases such as asthma, psoriasis, and atopy caused by several genes' interactions (Kawano *et al*., Osmola *et al.*, Quiros *et al.*, and Raap *et al*. 2005). Allergic condition has been researched in many species. It is not uncommon that an individual exhibit more than one disease or that the clinical signs of these diseases partly overlap. The affected horses in this study all have allergic eczema, but a small fraction of these individuals are also diagnosed with atopic dermatitis. It is often the horses that have the most severe type of skin allergy that are atopic. Atopic dermatitis can lead to asthma, food allergy and allergic rhinitis (Leung *et al*. 2004, Stiehm ER, 2006). The definition atopic dermatitis refers to a condition that is caused by allergen specific IgE. Allergic eczema is a condition caused by insect hypersensitivty.

1.2 How the genes in this study were selected

Allergic eczema is caused by the body's reaction to allergens, called immediate hypersensitivity. The *adaptive immune system* produces IgE-antibodies in response to foreign substances, or allergens. The chain reaction that follow, leads in the case of allergic individuals, to release of histamine, which causes the symptoms of allergy. (Abbas et al. 2000 p. 432) Before the foreign substance reaches the adaptive immune system it is confronted by the body's first barrier of defence; the skin, or epithelial and mucosal surfaces (Ibid. p. 4). Recent studies have shown that a defect skin barrier can increase the absorption of foreign substances, and thereby increase the risk of eczema. (Leung *et al.* 2004 , Walley *et al.* 2001)

Several candidate genes were researched in order to find appropriate candidate genes for this study. Criteria searched for included multiple studies confirming linkage to allergy, or if possible skin allergy, and conserved chromosomal regions between species. The genes selected for this study have been chosen to represent each step in the body's defence barriers. FcεR1-beta[1] is the gene coding for the high affinity beta-receptor of IgE. Studies on this gene have linked it to asthma (Palmer *et al*.1999), atopic dermatitis (Cox *et al*. 1998) and elevated IgE levels (Hizawa *et al.* 2000). Spink5[2] is active in epithelial and mucosal surfaces. It encodes a serine proteinase inhibitor and might have a protective role against allergens that are serine proteinases. (Leung *et al*. 2004)
A defect in Spink5 causes Netherton's syndrome, a condition similar to eczema. Spink5 has also been associated to atopic dermatitis (Walley *et al.* 2001).

1.3 Single Nucleotide Polymorphisms

A single nucleotide polymorphism is a base variation at a single nucleotide position. SNPs occur approximately once in every 100 to 300 bases in humans. (Baxevanis 2005 p.27) Often a certain SNP can be present in a group of the population and the SNP can be used as a marker to identify different haplotypes[3] in the population. The change in nucleotides can be either synonymous or non-synonymous. A synonymous change is when the new nucleotide does not affect the amino acid produced. A non-synonymous change referes to when the amino acid produced is altered.

1.4 Aim of study

These are the main objectives of this study:

- To collect samples from groups of sick and healthy Icelandic horses for future disease association analysis.

- To sequence and investigate by comparing groups of healty and sick individuals if FcεR1-beta is linked to allergic eczema in Icelandic horses.

- To clone, sequence and investigate if Spink5 is linked to allergic eczema in Icelandic horses.

1.5 To whom might this study be of interest?

The condition causes much pain and discomfort to the affected horse. Research that might lead to advancement in treatment of the condition will be of value to the affected horses, the concerned owners and veterinarians. A horse that develops AE diminishes in economical value. The owner will lose money if the horse is sold, and the horse will cost a large amount of money to nurse. A plethora of different alternative medications exist that are claimed to be effective against AE, though few studies on them exist. Owners, veterinarians and breeders would therefor find a study of AE in horses useful. A long term breeding program that aims to diminish AE in the Icelandic horse would be useful. Horse breeds such as the Shetland pony and the Gotlandic russ also suffer from AE along with other breeds. The findings of the AE study might also be applied in human medicine. AE is a complex disease with many genes interacting. It is a time consuming area to research, and all studies are of importance as they can be used in the bigger picture of understanding the development of allergies.

[1] Also named MS4A2
[2] Also named serine protease inhibitor, kazal-type 5 precursor
[3] A haplotype is a version of a segment of nucleotides. Several haplotyes can exist within a population.

2 Materials and methods

2.1 The overall work plan

To be able to sequence and test if the two selected candidate genes have a connection to the prevalence of allergic eczema in the Icelandic horse, 30 samples were collected. The samples were taken from groups of horses categorised as being “without allergic eczema” (healthy) and from horses “with allergic eczema” (sick). The horses were put into categories from 1-4 where 1 is healthy, 2 carries a mild form of the allergy and so on. Groups 2-4 make up the group of sick horses, group 1 represents the healthy horses. This is standard clinical classification of AE in horses. The candidate genes in sick and healthy horses will be compared. A difference might, amongst others, be in the form of an SNP (a Single Nucleotide Polymorphism) or by an insertion, or deletion. A change in the nucleotides can be either synonymous or non-synonymous. If the change in nucleotides leads to a different amino acid being produced, the change is non-synonymous. If the same amino acid is produced with the changed nucleotide the change is refered to as being synonymous. Non-synonymous SNPs might produce a different protein which may lead to alter effects in the body. Synonymous SNPs can function as "tags" which gives clues as to what haplotype[4] the studied sample belongs to.

The following sections will describe how the material was collected and which methods were used in the process of searching for SNP’s in the genes Fcεr1-beta and Spink5 in the Icelandic horse. The genes are candidate genes for allergic eczema.

2.2 The horse material

2.2.1 Administrative preparations

Before the work of collecting the sample could begin an ethical application was made to the Swedish Animal Welfare Agency for approval of the study and the methods of collecting skin and blood samples were determined. For every sample taken a form of consent and an ID-sheet were filled in by the owner of the horse participating in the study. The veterinarian who took the samples recorded details concerning the horse’s degree of disease.

2.2.2 Types of samples collected

FcεR1-beta is expressed in blood whilst Spink5 is expressed in skin. It was therefore necessary to collect samples both in form of whole blood and in form of skin biopsies. Blood samples intended to yield cDNA were collected in 10ml citrate tubes (yellow caps). Additional blood samples intended to yield DNA were collected in 10ml EDTA tubes (purple caps). Skin samples (biopsies) were put in sterile Rnase-free 1.7ml tubes directly frozen in liquid nitrogen. RNA degrades quickly. To keep the samples from being rendered non-useable due to this, it was of utmost importance to keep the samples in an Rnase-free environment and to stabilise them as soon as possible. The samples could be stabilised in several ways. Lymphocytes could be separated and stabilised in RNA*later*® solution. The lymphocytes containing the RNA could also be stabilised by freezing them in liquid nitrogen and store at -70^{o}C. Blood samples for DNA extraction could be stored in the EDTA tubes in a cold room since DNA is much more stable than RNA.

[4] A haplotype is a version of a segment of nucleotides. Several haplotyes can exist within a population.

2.3 RNA extraction

Depending on if the samples to be processed were from blood or skin, different kits and protocols needed to be followed.

2.3.1 Extraction of RNA from whole blood

2.3.1.1 Isolation of lymphocytes using Ficoll

Lymphocytes containing total RNA were separated from the whole blood using the Ficoll paque PLUS protocol (Ficoll paque PLUS). PBS (see "Appendix of solutions") supplied by user. The resulting pellet could be RNA stabilized either by directly freezing it for storage at -70^{o}C or it could be suspended in RNA*later*®. Pellet suspended in RNA*later*® needed to be washed before used in the following steps. Adding approximately one times the amount of PBS to the RNA*later*® solution did this. After gently mixing the solutions, the tube was centrifuged at 3000xg for 5-10 minutes or as long as it took the pellet to form properly. Supernatant was then removed and the wash step was repeated. The pellet was after this ready to be used.

2.3.1.2 Bürker chamber

It was necessary to know the concentration of lymphocytes to be used in the procedure. The Bürker chamber contains two chambers each consisting of 9 A-squares. Each A-square is in its turn made up of 16 B-squares. By using the Bürker chamber it was possible to make an approximation of the number of cells in the solution. 0.5ml of PBS was added to the pellet to create a lymphocyte solution. A mixture was made containing 0.5ml Tryptan blue and 0.5ml lymphocyte solution. 10µl of the solution containing the lymphocytes and Tryptan blue was added to the checked glass of the Bürker chamber and the cover glass was pressed against the drop. To count the cells the non blue cells were counted (viable cells) in every B-square in the four centred A-squares. An average amount of cells per A-square was estimated. This number was then multiplied with the dilution factor (here 2x) and finally multiplied with the constant 10^4. Assuming that the average number of cells per A-square was 30 gives us the equation 30*2*10^4 = 6*10^5 cells. This cell count was performed prior to the Kit for total RNA extraction.

2.3.1.3 Isolation of total RNA

The Qiagen Mini Rneasy kit's Spin protocol for Isolation of Total RNA from Animal Cells (Rneasy Mini kit protocol) was used for lymphocyte samples extracted from blood. Frozen lymphocytes could be used directly with the Qiagen Rneasy kit whereas the lymphocytes in RNA*later*®needed to be centrifuged down and collected as a pellet and then washed in PBS solution. The Qiagen Rneasy kit protocol should be executed quickly to prevent RNA degradation. The total RNA should be frozen instantly in liquid nitrogen or on dry ice and then stored at -70^{o}C.

2.3.2 These were the steps used to extract total RNA from skin:

The Qiagen Micro Rneasy kit's Fibrous Tissue protocol (Rneasy Micro kit) was used for skin biopsies. Proteinase-K supplied by user.

2.4 Verifying RNA purity.

Before flash freezing the total RNA an aliquot of a small amount of the RNA could be made for use in analysis of the quality of the sample. Three methods could be used for analysis. They require different amount of time and deliver varying amount of information. The sample could be run in nanodrop, which presented the concentration of RNA present in the sample and how pure it was of contamination by genomic DNA. A second method was to run the RNA sample in a PCR with primers situated within exons amplifying over an intron. If a longer fragment was amplified along with the shorter exon fragment, genomic DNA contamination was verified. The third and most reliable analysis, but also the most time consuming and expensive method was making use of the bioanalyzer. Running the sample in the bioanalyzer resulted in data presenting the quality of RNA in the sample and also to which extent it might have degraded.

2.5 First strand cDNA synthesis

To minimise the risk of DNA contamination a DNase step was added. This step was not included in the Rneasy Mini kit. Ambion's DNA-*free* kit was used on the total RNA product before the step of cDNA synthesis was begun. The RNA sample (about 20µl) was put into a PCR plate. The sample was mixed with 1µl rDNase buffer and 2µl buffer I according to the protocol. The samples were incubated at 37^{o}C for 30 min. 2µl re-suspended DNase inactivation reagent was added and mixed several times during a 2 minute period. The plate was then centrifuged at 2000xg for 5 minutes. The supernatant contained the RNA and was pipetted into an RNase-free 1.7ml tube. The sample was then ready for cDNA synthesis.

The RNA samples were incubated at 65^{o}C for 10 minutes. They were then chilled on ice. 11µl bulk or 5µl bulk solution was added to the RNA depending on the amount of RNA used[5] as well as 1µl DTT solution, 0.5ul pd(N)6 primer and 0.5ul Not-I-d(T)18 primer *(the mixture of primers will synthesis cDNA as well a amplify it).* The solution was mixed gently and incubated for 1 hour at 37^{o}C. The yield of cDNA was measured with nanodrop. The samples were now ready to be stored at e.g. –20^{o}C.

2.6 Designing primers

2.6.1 Fcεr1-beta

The source sequence is as earlier discussed in cDNA form, which means that the template needed in order to create the primers needed to be in exon form. The first gene to be amplified and searched for SNP's in horse was FcεR1-beta. NCBI held one record of a partial horse mRNA sequence of this gene (AJ318332). Primers were based on this sequence. The sequence to be amplified has a length of approximately 800bp. Two pairs of primers (see "Appendix of primers" and "Appendix of exons") were deigned to amplify this sequence.

2.6.2 Spink5

The second gene to be amplified was Spink5. There was no horse specific data on this gene. The template for primer design was in this case constructed from studying orthologous genes, that is the same gene but in other species, in this case mouse, dog and human. The sequences from these three animals were aligned using ClustalW (Tools and Databases: ClustalW). To amplify the CDS (see "Appendix of sequences") of Spink5, which is 3190bp long (in human and dog), eight primer pairs were sketched out. Out of these eight pairs three were selected to

[5] 11µl bulk for 20µl blood derived RNA or 5µl bulk for 8µl skin biopsy derived RNA

start with, to see whether the conserved primers designed with help of the ClustalW alignment would work or not. Together the three pairs (see "Appendix of exons") would amplify a CDS around 1500bp, unique to horse. The exon/intron configuration of both genes were studied using Ensembl's database (Tools and Databases: Ensembl). Conserved regions were studied using UCSC's database (Tools and Databases: USCS). When designing the primers the following checkpoints were followed:

- It was important to make sure that the sequence at the 3' end was conserved. That is, when designing a primer from an alignment, to make sure that no deviations were in the vicinity of the 3' zone. Any deviations, i.e. where the codes differs in between the species, should be spaced at least 4-5bp from the 3' end. This is because the primers need to anneal completely at the 3' end to be able to prime DNA synthesis.

- The GC content of the primer should be around 50%. The Tm of the primer pair should be as similar as possible.

- The primer pairs should not be able to anneal to each other in so-called "primer dimers" nor should they form "hairpins" or "loops". To check for "primer dimers", "hairpins" and "loops" as well as calculating product length and annealing temperature the software Oligo3 was used.

2.7 PCR

2.7.1 Mix

Before the samples were amplified in a PCR machine a PCR-mix needed to be prepared. The following mix (Table 1) could be used for both genes' cDNA. The concentration of the cDNA used in the mix varied as indicated, but the volume was kept constant.

Forward Primer	0.85 µl
Reverse Primer	0.85 µl
Salt ($MgCl_{25}$)	3 µl
PCR Buffer	2 µl
dNTP (10mM)	0.6 µl
TAQ Gold	0.3 µl
DNA*	1.25 µl
H_2O	15.65µl
Total	25 µl

Table 1. *DNA concentration used for FcεR1-beta was ~20µl/ng, for Spink5 ~100µl/ng.

2.7.2 Program

A PCR amplifies a specific segment of nucleotides by running cycles of different temperatures in a certain order. To optimise the program gradient[6] PCR programs and touch down[7] programs were run. A PCR program (see "Appendix of PCR program") was optimised to work with the designed primers.

[6] A program that varies the temperature over the plate as the program runs its cycles
[7] A program that starts slightly above recommended Tm of the primers and ends well below

2.8 Agarose gel and PCR fragment purification

To visualise the PCR product a gel (see "Appendix of solutions") was run with wells containing the PCR product. The gel was then stained with ethidium bromide and studied under UV-light. A 100bp ladder was used to estimate the fragment length. The wells showing a band with the correct length in accordance with the size ladder were selected for purification. Purification was performed either by PCR purification or gel extraction. The protocol used for the gel extraction was the E.Z.N.A Gel extraction Kit. (Gel extraction kit E.Z.N.A)

2.9 Sequencing

The PCR-products were sequenced using MegaBace sequencing kit ([1] Amersham Biosciences, Uppsala, Sweden) and electrophoresed MegaBace 1000 capillary instrument(ibid). The sequences were analysed using the Sequencher 3.1.1 software (Gene Codes, Ann Arbor, MI).

3 Results

3.1 Creation of a bloodbank

A total of 33 samples were collected. 5 of these were skinbiopsies used to create cDNA from skin. The remaining 27 samples were used to create cDNA from blood. 3 samples were discarded because of poor quality. A bloodbank has also been established containing samples from 25 Icelandic horses categorized in groups 1-4 (healthy to sick) for extraction of DNA.

3.2 Cell concentration

The concentration of cells was estimated in the first samples of lymphocytes extracted.[8] This was done with the Bürker chamber. The cell concentration was found to be $5.8*10^5$ cells/ml.[9] This information was later used in the protocol for RNA extraction. The value was assumed to be approximately the same for all samples.

3.3 RNA yield

The Qiagen Rneasy mini kit protocol for animal cells was used and samples were diluted so that they would not have a concentration higher than $5*10^6$cells/ml.The concentration of the extracted RNA was measured by using the nanodrop instrument. This method proved insufficient as it gave a false result of RNA yield when the sample was contaminated with DNA. To find out if the RNA was contaminated with DNA a small amount was run in a PCR. Some samples showed that there was a significant presence of DNA. To further analyse these samples they were run in bioanalyzer instrument. The result of the run showed that samples that had not been RNA-stabilised within a couple of hours had a very poor RNA content, (Figure 1). These samples were discarded.

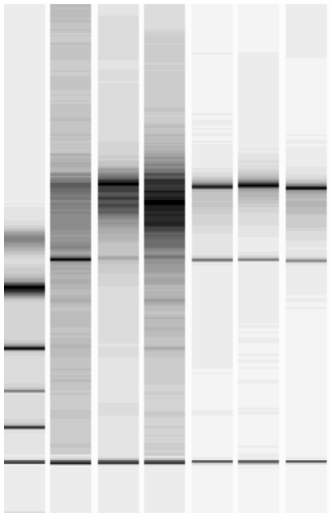

Figure 1. Bioanalyzer. The first sample from the left is the ladder. (In this run the ladder did not correspond to the samples.) It shows six peaks. The peaks that define a good RNA sample are the 18S and 28S ribosomal RNA peaks. (The two top-most.) The three first samples do not show good peaks, this is an indication of degraded RNA. The three last samples show good peaks at 18S and 28S (the two top-most lines) which indicates a good RNA content. (Bioanalyzer FAQ)

Blood samples that had been stabilised within two hours yielded an RNA concentration that varied from 11ng/µl to 80ng/µl in 20µl RNA. This was sufficient for cDNA synthesis. Since the samples processed with the Qiagen Rneasy Mini kit showed a tendency to contain a

[8] Samples used ID: "G" and "S"
[9] average 36 cells per A square, dilution 5 constant 10^4

certain amount of DNA an extra Dnase step was performed after RNA extraction. This proved to be a good method to ensure a good cDNA yield. Skin samples yielded in average 14µl RNA with an RNA concentration of 500ng/ul.

3.4 cDNA concentrations

The cDNA concentrations were found to be about 4000ng/µl for RNA that had been extracted from blood or skin, no matter what the starting RNA concentration had been (range "blood" 11ng/µl – 80ng/µl, "skin" ~500 ng/µl). The yield was approximately 30µl cDNA per sample processed. This was later diluted and adjusted to fit the PCR. For cDNA intended for the FcεR1-B gene a dilution of 20ng/µl was found efficient for the PCR mix (see Table 1). The Spink5 gene required a higher concentration of cDNA of approximately 100ng/µl to work efficiently with the PCR. The results of the samples are presented in two tables in the Appendix of cDNA (Table 3 and Table 4). Table 3 contains the cDNA samples from blood used to amplify FcεR1-beta. Table 4 contains cDNA from the skin biopsies used to amplify Spink5.

3.5 Gel extraction of PCR products

The PCR products were run on a 2% agarose gel at 90V together with a 100bp ladder. The samples that formed a clear stripe of the correct length (around 500bp) were purified for sequencing. Results showed that the gel extraction purification gave better sequences than the PCR purification.

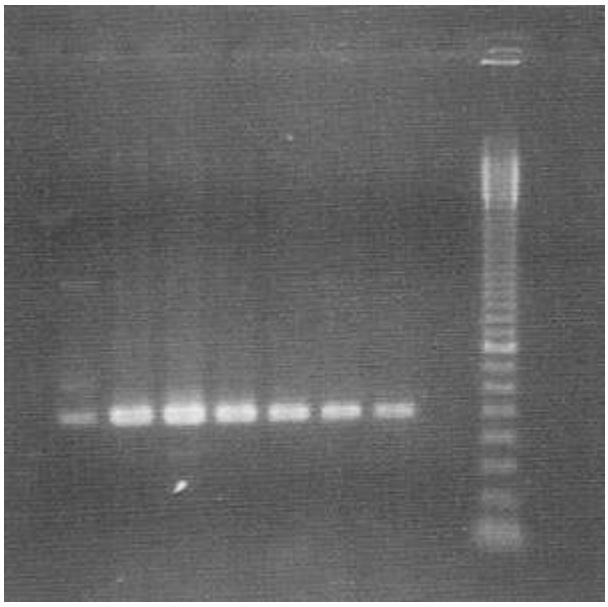

Figure 2. Gel electrophoresis of FcεR1-beta, first primer pair.

3.6 Sequences

3.6.1 FcεR1-Beta SNP

The purified PCR products from five healthy individuals and from five sick individuals were sequenced. The cDNA sequence for FcεR1-Beta showed one synonymous C/T SNP at position 6th exon +30 of the cDNA sequence (see "Appendix of sequences"). The C/T SNP distribution between the groups “Sick” and “Healthy” was as follows:

ID Sick		ID Healthy	
S	CT	4	CC
F	CT	1A	CT
G	CT	2A	CT
3	CT	3A	TT
6	TT	5A	CC
C 0.4	T 0.6	C 0.6	T 0.4

Table 2. The table gives the two groups’ distribution of genotypes. The first column in the two groups contains the individuals ID. The second column contains the genotype. The row under the genotypes gives a calculation of the relative C/T distribution in the groups.

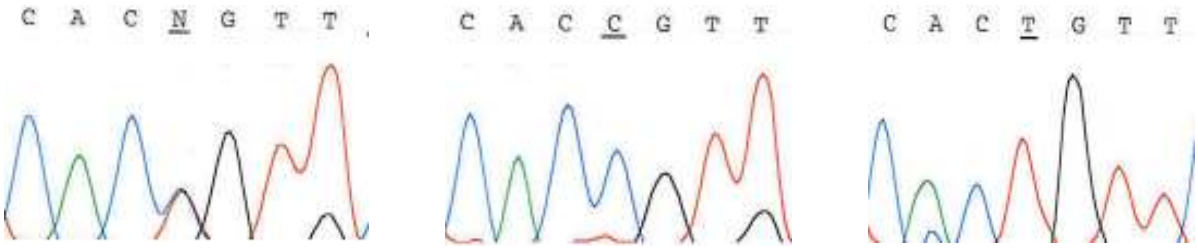

Figure 3. The SNP in exon 6 +30 bases in FcεR1-beta. Here seen as the fourth nucleotide from left in each picture. From left to right: heterozygous CT, homozygous CC and homozygous TT.

3.6.2 Spink5 new sequence

The Spink5 sequence retrieved was 1245bp long. This is about a third of the total Spink5 cDNA. The two first primer pairs did not work efficiently. The third primer pair proved highly efficient. The third primer pair produced a reliable sequence. The sequence was blasted and found to be most similar with the Spink5 gene of *Homo sapiens* (DQ149929). Two healthy individuals and two sick individuals were sequenced. (see "Appendix of cDNA") No SNP was found in this part of the cDNA.

4 Discussion

The SNP in exon 6 + 30 bases in FcεR1-beta is in itself synonymous, which means that the same amino acid will be produced. Information about different haplotypes[10] can be gained from studying the SNP. It can be seen that there is no clear differentiation amongst the C/T distribution between the two groups. Both the sick group and healthy group contain CT and TT individuals. There is no CC individual in the sick group. More samples need to be sequenced in order to find out if the CC genotype is exclusive to the healthy group. No direct association to AE can be drawn from this data. This observation should however be noted for future studies, as AE is a multifactorial disease that is caused by interaction of several gene.

New sequence was retrieved from the Spink5 gene in horse. In the segment amplified no SNP could be identified. The gene can not be considered irrelevant to AE studies at this point. More sequence needs to be retrieved from the gene. When the cDNA of the gene is fully sequenced comparative analysis between the sick and healthy groups can be performed. It is however better to make the comparative analysis between the two groups using genomic DNA. This is because the skin biopsies needed for the Spink5 cDNA are difficult to collect. When Spink5 is sequenced in cDNA form, 3' and 5' race as well as chromosome walk can be used to sequence the genomic DNA.

The definition of AE is not an easy definition to make. The disease takes different forms during different periods of the year, depending on the treatment given to the horse or in what area it lives. This might lead to errors when diagnosing horses. It is therefor important that care is taken to account for the different seasons, environments and treatment the test subjects have been exposed to when collecting and classifying samples. This study has begun the collection of samples from Icelandic horses categorized in groups 1-4, ranging from healthy to sick. The number of samples collected need to be increased so that statistically sound conclusions can be drawn from the research performed.

[10] A haplotype is a version of a DNA sequence. Many different haplotypes may exist within a population.

5 Conclusion

FcεR1-beta was successfully sequenced in cDNA form in the Icelandic horse. No direct linkage to allergic eczema could be concluded from the one SNP that was identified. The variations found should not be disregarded but saved for future analysis. They may be of use when more genes have been searched for linkage to allergic eczema, since the allergy is caused by interactions of several genes. It would also be relevant to repeat the scan of the gene when more material from more individuals is available.

Spink5 was positively identified with the conserved primers. The segment amplified was compared in two healthy and two sick individuals. It contained no SNP. When blasted, the partial sequence of the gene was found most similar to that of humans. This should be kept in mind when constructing primers for the remaining part of the Spink5 gene in horse.

6 Future studies

The continuation of this study should focus on finishing sequencing Spink5 and develop a genomic sequence. The genomic sequence of Spink5 could then be used as a template for designing primers intended to amplify the gene. The sick and healthy groups can then be compared using the DNA already collected. When more material is available more samples should also be sequenced with FcεR1-beta's primer pair II whose product contains the 6^{th} exon +30C/T SNP, so that more data will become available.

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Tools and Databases

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Accession numers by site http://www.ncbi.mlm.nih.gov
Partial mRNA of high affinity Fc beta receptor, *Equus caballus*: AJ318332
Spink5 *Homo sapiens*: DQ149929

Accession numbers refer to the published sequence at NCBI.
PMID refers to pub med. ID at NCBI's database.

Appendix of primers

FcεR1-beta
Primers based on partial mRNA sequence available at NCBI August 2005.

Accession number: AJ318332 (901bp)

In 5' -> 3' order.

F1: CACCAGTGGATAGATCAGTT 55.3 5'pos 1
R1: TGCTCGTCTTCAGGTTG 3'pos 479
Product's length 495bp optimal annealing 51.7 degrees

F2: TATCTGCTACAAGGAAGCC 5'pos 402
R2: ATAAATTTTGCCTGTAGCC 3'pos 826
Product's length 443bp optimal annealing 51.7 degrees

Spink5
Based on Clustalw consensus formed by *Canis familiaris* CDS *Homo Sapiens* CDS and *Mus musculus* CDS. Available at NCBI August 2005.

Accession number Canis familiaris: NM_001025397 (3192bp)
Accession number Homo sapiens: NM_006846 (3195bp)
Accession number Mus muculus: XM_283487 (3052bp)

Primer pairs 1-3 of total 8.
In 5'-3' order:

SPINK5f1: GCCACAGTGCCAATGC 5' pos 86
SPINK5r1: ACATTCCCCTTCACTTTTTA 3' pos 528
Product's length: 462bp annealing 51.6

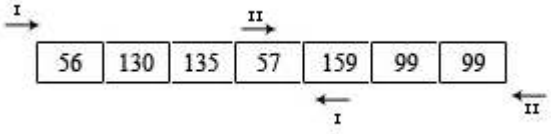
SPINK5f2: TGGCACAGATGGGAAAAC 5' pos 453
SPINK5R2: AGTCTTCCATTCCTCACTTG 3' pos 845
Product's length: 412bp optimal annealing 52.3

SPINK5f3: TGCCATGTGTGCTGAGC 5' pos 736
SPINK5R3: TGACACATGGAACACAAGTT 3' pos 1090
Product's length: 374bp optimal annealing 51.3

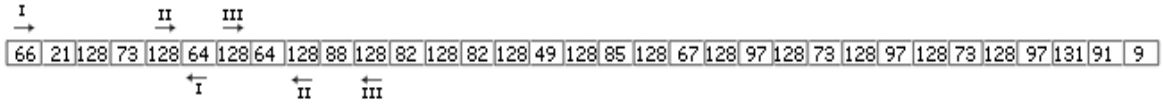
Appendix of exon data

(All data based on human exons.)

Fcε1R-beta's exon and primer data. Each box indicates the presence of an exon and the number inside is its length in bp. The arrows represent the primer pairs (I and II).



Spink5's exon and primer data. Each box indicates the presence of an exon and the number inside is its length in bp. The arrows represent the primer pairs (I, II and III).



Appendix of sequences

DNA sequence of FcεR1-beta:

CCAGTGGATAGATCAGTTAATGAAAAAATGGATCCAGAAAATAGGGCCAGAGCAGAACT
CATTCTCCCAAACCCACAAGGGCCCTCCAGAGTGTCTGAAATTGAACTTGCAGAAGTCTC
TCTCCATGATACCACTTTACTGCGGAAGGGTGCCCAAATCCAACCACACCCTACATGGCT
GACATTTTTGAGGAAGGAGCTGGAATTCCTGGGGGTAACACAAATTCTGATTGGTTTGAC
ATGCCTTTGGTTTGGAACATTTGTCTTCTCCATGCTCACAAAGTCAGACTTTGAGGAAAAC
ATATTTTCATCATTTGAAGCAGGCTACCCATTCTGGGGAGCAATATTTTTTTTTATTTCCG
GATTTTTGTCAATTATGTCTGAAGAGAAACGTGAAATATATCTGCTACAAGGAAGCCTGG
GGGCAAACACTGTCAGCAGCATGGCTGCAGGAACAGGAATCATCATCCTGCTCATCAACC
TGAAGACGAGCATGGCTTTCATCTACAACTGCCAGGACACTTATGAGGAAGACTTCTGCC
TTATCGTTTCTTTTTCCACTGAAATCGTGGCAGTGACCCTGTTTATCAC**N**GTTCTGGGGTTT
TGCAGTGCTGTGTCACTCACAATCTACGGAGTGGGAGAAATATTCGAAAGAAGTAAGATT
CCAGAAGATCGTCTTTATGAAGAATTAAACATATATTCACCAATATACAGTGAGTTGGAA
GAGAGGGGAGATATCTTCTCCCAGTGACTCATAAGAATGCTGTGCCCAGAACATTCTGAT
T<u>C</u>ACAGCAAAGGGTTCAGAAAGCAAGCTCTTTGTTTAAGGGGCTACAGGCAAAA

Bold: SNP C/T exon 6+30
(<u>Underlined</u>: This nucleotide is a "G" in the published mRNA sequence, but in this study it was found to be a "C" in all subjects sequenced.)

DNA sequence of Spink5:

Primer pair I
CAGTGCCAATGCCTCTTGCTCCACGATACTCCCATTTTGTTCTTTACTACATCACACTTTCTACAG
TGCTGGTGGTGTTTACTTTCCTCTCTTTTTATTCACCACGTTATATGGTCCTTAAAACAGCACAGA
TACTCAGAGGAGGAAAATAGGAAACTGTCTGATTAATATGCTAACTTGATAATCCTCCCTCATATA
ATGGAATTGACAGCATATAATTATACACACATTATTTTCACAAAGTTGCTTACTCGGGTCATGTTT
GAAATTCACAGTACACTTCTTATTTTTTATAAAGTGGGTACTGGAAAACAGGAAAGTTTGACTCAT
CTGAAAGTACAAAGAAATTAAAATCAGAGTATGAAAGTATATGAATGATTTCAATAGCTTGCTCTT
CTCTGAAATTCAGGAWKTSGTCCTTTAAAGTTATCAGATGTTTAAAAAGTGAAGGGG
Primer pair II
CAGATGGGAAAACAGAACTTAGTGGGTGAAGAAGTGATTTAAGAAGAGCTTCWTGGCAGAAGG
AACAGCTTATGCAAAGGCCCTGAGGCCGCAGGGAACATTGCTTATCCAGTGGATAGGAAGCCA
GGGCTCTGAGGGGGAGAGTGGCTAGGGTCAGACCTGGCAGGGCTCTGTTCACCATGGTGGGA
GTTTAAATTTTGTTCTGAGAGCAGTGGGAGGCCTTTGGAGGGTTTTGATCGGGGAGAGAGGCTG
TCATTTCACTGTGGCTGCTGAATAGAGAATAGACTGGAAGAGACAAGACGAGGCAGGACACAG
CTTGTTAGACTGGTGGATTGTCCAGTCAAGGGAAGGCAAGTGAGGAATGG
Primer pair III
CMCATGGAACACAAGTTGCCATGCATTTTCCCATCTGGACCACGAATAGGGTCATTTTCT
CTGGTACAGAACAGTATTCCATTCTTTGCTTGATTCTGATATTCACTGCACATTTTCTCAAT
TTCTGTTTTAACTTTAGCTTTTTCTTCAGCCTCTCTCCGGGTTTTATCTTCTTTATTTTTTTCT
TCTATAAAACGCCGTTTGAAAACTTCAGCACACAGGGCACATTTGTTGCCATGCATCCTG
CCATCAGGGCCACGGACTGGGTCACTCTCCCGGGTACAAAACAGTCTTCCATTCCTCACT
TGGTTTTCGTATTCCTTGCAAAAATCCTTTTCAGCACTTCGCCGAATTCTGGTCTCACCTTC
TTGTTTGGCATTTTCTTCAGCTTCTTTTAAGAATAGCTCAGCACACATGGCC
Italic: Low quality sequence

Appendix of PCR program

The following PCR programs were found to be correct for the designed primers mentioned in the Appendix of Primers. (Numbers preceded by "*" indicates program used for Spink5 primers, without "*" indicates program used for Fcεr1-beta primers)

Taq activation
95^{o}C for 10 min

(cycle 5 times)
denaturation
94^{o}C for 30 s
annealing
57^{o}C (*55^{o}C) for 30 s
extension
72^{o}C for 30 sec

(cycle 35 times)
94^{o}C for 30 sec
55^{o}C (*53^{o}C) for 30 s
72^{o}C for 30 s

final extension
72^{o}C for 10 min
end 4^{o}C forever.

Appendix of solutions

1xPBS
Dissolve the following in 800ml distilled water:

8g NaCl
0.2g KCl
1.44g Na_2HPO_4
0.24 KH_2PO_4

10xTBE
Add the following to 800ml distilled water:

108g Tris bae
55g boric acid
9.3g EDTA

10x LB
Dissolve in 6.25ml water
.025g Xylene cyanol
.025g Bromophenol Blue
1.25ml 10% SDS
1.25ml glycerol

2% Agarose gel:

To 50ml 10% TBE add 0.8g Seakem agarose powder. Heat to boiling point. Use in chamber or keep at 60 degrees celcius.

Appendix of cDNA

cDNA samples from blood used to amplify FcεR1-beta in horse.

ID blood	Group (1-4)	Concentration	Successful primer/s	EDTA
1	2	~20ng/µl	I	LYM
2	3 atopic	~20ng/µl	I	X
3	3 atopic	~20ng/µl	I, II	X
4	1	~20ng/µl	I, II	LYM
5	2	~20ng/µl		X
6	2	~20ng/µl	I, II	X
7	2	~20ng/µl		X
8	2	~20ng/µl	I, II	X
F	2 born sweden	~20ng/µl	I, II	X
S	3	~20ng/µl	I, II	X
G	3	~20ng/µl	I, II	X
1A	1	~20ng/µl	I, II	X
2A	1	~20ng/µl	I, II	X
3A	1	~20ng/µl	I, II	X
4A	1	~20ng/µl	I, II	X
5A	1	~20ng/µl	I, II	X
6A	1	~20ng/µl		X
7A	1	~20ng/µl		X
8A	2	~20ng/µl		X
9A	1	~20ng/µl		X
10A	2	~20ng/µl		X
Mick	1	~20ng/µl	I, II	LYM

Table 3. The samples collected from blood. Their stock solutions (1:1) all have a concentration of approximately 4000ng/µl

cDNA samples from skin biopsies used to amplify Spink5 in horse

ID skin	Group (1-4)	Concentration	Successful primer/s	EDTA
Test	1	~100ng/µl	I, III	
D	3	~100ng/µl	III	X
L	2	~100ng/µl	II, III	LYM
P	1	~100ng/µl	III	X

Table 4. The samples collected from skin biopsies. Their stock solution (1:1) all have a concentration of approximately 4000ng/µl

EDTA marked with X indicates that sample was also collected in blood-form. LYM indicates that lymphocytes have been extracted and are saved in stable form.