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Genomic Analysis of the Spring Leg Defect in the Canadian Dorset Sheep Breed

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ABSTRACT: Planned matings were carried out to produce 150 Dorset lambs and validate the hypothesis of a genetic determinism for the spring leg defect in sheep. Two groups of lambs were produced through planned matings; one at risk of developing spring leg and one at low risk of developing the defect. In the high risk group, 23.1% of the lambs developed the defect, versus 3.4% in the low risk group. A total of 192 affected and healthy animals were genotyped with the ovine 600K SNP panel, and association studies were carried out between SNP genotypes and the expression of the defect. Three promising SNPs were detected on chromosome 24, showing polymorphisms significantly associated with the spring leg defect.
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Introduction

Since the early 2000s, purebred sheep breeders have noticed the emergence of a new defect in their herds. Affected animals present with a hyperflexion of one or both hind limbs when moving at a slow pace. Breeders, amongst themselves, quickly started using the term "spring leg" ("*crampage*" in French) to describe this defect since the hind limb of affected animals would lift off the ground abnormally as if the animal had a cramp.

To validate the hypotheses that were brought forward by veterinary neurologists and geneticists, a project was designed, including the use of new technologies, both in veterinary medicine and genomics. The main objective of this study was to identify the causes of the defect through the validation of hypotheses raised in a preliminary project and to identify early detection methods of spring leg on live animals.

Materials and Methods

Animals. Two groups of lambs were produced following planned matings in the Centre d'expertise en production ovine du Québec (CEPOQ) experimental farm. The CEPOQ herd is a closed Dorset herd since its creation in 1998. The "spring leg" condition is quite common in this flock and several cases of the defect are reported every year. Animals from this herd were therefore ideal for this project. Services were planned to produce two groups of animals with either low or high risk of developing the defect. The amount of risk was based on the presence of the defect in rams and ewes themselves, and the presence of ancestors in the pedigree of selected rams and ewes, suspected of transmitting the defect. During a preliminary project, several common ancestors were identified in the pedigree of sick animals, which may have been carriers of one or more gene(s) responsible for the condition. To maximize the number of families and obtain more variation with regards to genotypes, a large number of rams were used to produce lambs from both experimental groups, in four different cohorts - 20 rams in total.

Planned matings produced a total of 150 lambs (72 males and 78 females) assigned to two experimental groups: AT RISK and at LOW RISK of developing the condition. It was not possible to have a control group with no risk of developing the condition, since the CEPOQ herd hadn't introduced any new Dorset lines since its creation in 1998, and the majority of animals were at the least distantly related to one of the problematic ancestors. After weaning, lambs were allocated to test pens by group, weight and sex, and were individually monitored on a weekly basis for growth and clinical signs, over a maximum period of 58 weeks. When observations ended in December 2013, some animals had suspect of very suspect locomotion, but couldn't be confirmed as sick animals. Therefore, four classes were defined based on the status of lambs at the end of the project: normal, suspect, very suspect and affected (confirmed). To carry out Genome-Wide Association Study (GWAS), the statuses of 191 genotyped animals (healthy, sick, suspect and unknown) were used as observations. GWAS was only carried out for 105 healthy and 47 sick Dorset sheep. The 37 sheep classified as suspect and one classified as unknown were excluded from further analyses.

SNP Genotyping. A subset of 192 animals including all rams used in the project, most of the ewes, a subset of sick and healthy lambs from the project, as well as other animals developing the defect during the project, and affected animals outside of the CEPOQ herd, were selected to be genotyped. Blood and ear punches samples were sent on dry ice to Delta Genomics located in Edmonton, Alberta. This laboratory carried out DNA extraction from samples, SNP genotyping using the high density ovine SNP panel (600K) and preliminary genomic analyses including quality

control. Genotypes were then loaded into the Canadian Centre for Swine Improvement (CCSI) database for further validation and genomic analyses.

Statistical Analyses. Association analyses of genetic markers with spring leg observations were performed using the single-locus mixed model of the Golden Helix SNP & Variation Suite (SVS) software. Marker relationships were included as random effects in a mixed model GWAS using the EMMAX (Efficient Mixed-Model Association eXpedited) procedure (Kang et al. (2010)). The EMMA (Efficient Mixed-Model Association) procedure considers genetic relatedness between individuals to avoid inflation in test statistics and spurious associations. EMMAX uses a variance component approach, which is computationally more efficient than EMMA (Kang et al. (2008)). The chromosome-wise false discovery rate (FDR) (Storey (2002)) was also used to account for association tests on thousands of SNPs on the high-density panel.

Results and Discussion

Results of planned matings. Table 1 shows the number of lambs in each experimental group and their status at the end of the project. In total, 23 lambs developed the spring leg defect during the observation period, including 21 in the AT RISK group, and 41 lambs having suspect or very suspect gaits at the end of the project (including 30 in the AT RISK group). On average, animals developed the defect at about 200 days of age, but it varied considerably between individuals (confirmation of the defect occurred between 65 to 258 days of age). The contrast in defect frequency between both experimental groups confirms the hypothesis of a genetic transmission of the defect. In addition, about a quarter of lambs born from an affected ram developed the defect themselves, which suggests a recessive gene or group of genes. The relatively high relationship level within and between groups makes a comparative pedigree analysis quite difficult. Moreover, a longer observation period would be required to confirm whether animals observed as suspect would become affected over time. It is also possible that some healthy animals could also become sick over time.

SNP genotyping results. There are 606,006 SNPs on the ovine high-density SNP panel. SNPs were distributed at an average distance of 4.6 kb from each other. About 23% of SNPs were separated by a distance of less than one kb and 11% were more than 10 kb far apart. Approximately 40% of SNPs were located between one to five kb apart and more than 75% of SNPs were located within in a distance of less than 7 kb from each other. The number (percentage) of SNPs by chromosome found on this high-density SNP panel varies from 60,188 SNPs (9.93%) for chromosome number 1 to 9,377 SNPs (1.55%) for chromosome number 26. There were 43,487 SNPs with unknown chromosomal location found on the panel. SNP maps were obtained from Ensembl Genome Browser (www.ensembl.org). A Hardy-Weinberg Equilibrium (HWE) test of SNPs was also performed. About 97% SNPs were in HWE with a p-value of greater than $10^{-6,}$ indicating that most of the SNPs on the chip probably haven't been subject to natural or artificial selection.

Call rate is the ratio of successfully genotyped SNPs to the total number of SNPs on the panel. From 606,006 SNPs on the panel, a total number (percent) of 30,744 (5%) of SNPs had a call rate of zero, 3,083 (0.5%) of SNPs had a call rate between 0.95 and 0.99 and 572,179 (94%) of SNPs had call rate greater than 0.99. Call rates of samples were also calculated and relatively consistent call rates were observed for all genotyped animals. The average (range) of the samples call rate was 0.9481 (0.9428-0.9483).

Studies such as GWAS need variability in allelic frequencies so that SNPs located on the sheep 600K SNP panel can be associated with different alleles of a QTL or gene affecting different traits under study. Minor allele frequency (MAF) is a criterion that shows the amount of variability of a bi-allelic marker such as SNP. The MAF across SNPs was calculated for the 189 genotyped animals with the sheep 600K SNP panel. Excluding The 30,744 SNPs with a call rate of zero, the average MAF across the remaining 575,262 SNPs was 0.21 for genotyped animals. A total number (percentage) of 46,631 (8%) of SNPs had a MAF equal to zero (SNP is fixed in the genotyped population) and 115,993 (20%) SNPs had a MAF greater than zero but lower than 0.10. There were also 98,379 (17%) SNPs that had a MAF equal to or greater than 0.4, of which 1,268 (0.22%) SNPs had a MAF equal to 0.5. SNPs with MAF less than 0.10 and call frequencies inferior to 0.95 were excluded from analyses. A total number of 162,624 SNPs had MAF less than 0.10 and 30,744 SNPs had call frequency inferior to 0.95. After filtering SNPs for low MAF and call rate, 412,638 remaining SNPs were included in association analyses. The relatively close proximity between SNPs and observed levels of MAF should provide enough information and variability for GWAS.

Association studies. Only three significant SNPs were detected on chromosome 24 (FDR<0.05) after adjusting p-values for multiple tests. No other significant SNPs were discovered at FDR<0.10. The three significant SNPs were oar3_OAR24_1341130, oar3_OAR24_1343567 and oar3_OAR24_1358660 located approximately 18 kb far apart. The three significant SNPs were located in the same haplotype block at a very high LD (r2>0.86). To explore the relationship between spring leg and the frequency of significant markers in healthy and sick animals, the frequency of the three significant SNPs on chromosome 24 were calculated within the healthy and sick animals (Table 2) and it was found that sick animals had a higher frequency of the GG genotype for SNP oar3_OAR24_1341130 and higher frequency of the AA variant for SNPs oar3_OAR24_1343567 and oar3_OAR24_1358660. Since the GG, AA and AA genotypes of oar3_OAR24_1341130, oar3_OAR24_1343567 and oar3_OAR24_1358660 SNPs, respectively, were more frequent in sick animals, the haplotype association chi-square test was performed. From eight possible combinations of haplotypes, only haplotypes

AGA, AGG and GAA were observed in the studied samples with a frequency of 0.03, 0.41 and 0.55. From the three observed haplotypes, haplotypes GAA and AGG were significantly associated with spring leg ($p<0.01$). This corroborates the results of single SNP association tests and opens the door to possibly selecting against the GAA haplotype consisting of the three above-mentioned SNPs to decrease the incidence of the spring leg defect. Table 3 summarizes frequency of the AGG and GAA haplotypes in healthy and affected animals.

Functional analysis. To further explore the region with significant SNPs, an area of about 250 kb on each side of the three significant SNPs on chromosome 24 (from base pair 1,042,158 to 1,574,441) was mapped according to the sheep genome (Oar_v3.1, Ensembl 74), and a total of 34 genes were identified. Due to limited information on the functionality of ovine genes, human orthologues of the 34 sheep genes were obtained using the BioMart software available on the following site: www.ensembl.org (Kasprzyk, 2011). A total number of 39 homologous human genes were then submitted to the Database for Annotation, Visualization and Integrated Discovery (DAVID: http://david.abcc.ncifcrf.gov/). DAVID provides a comprehensive set of free functional annotation tools to investigate biological pathways and understand the biological meaning of the mapped genes (Huang et al. 2009ab). One of the selected genes was NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 10, 22kDa (NDUFB10) which is involved in different pathways that cause diseases such as Parkinson's (Tretter et al. 2004), Alzheimer's and Huntington's (Starr et al. 2008). Parkinson's disease is a degenerative disorder of the central nervous system, in which the most obvious symptoms are movement-related. In sheep, the NDUFB10 gene is located on chromosome 24: between base pairs 1,503,091-1,505,967 and located about 140 kb away from the significant SNPs. It has been reported that NDUFB10 gene is also associated with Huntington's disease, which is a neurodegenerative genetic disorder that affects muscle coordination (Starr et al. 2008). Considering the effects of NDUFB10 gene in humans, it is also possible that a variation on this locus could be associated with the spring leg defect in sheep. This is the case in Parkinson's disease where more than one gene is involved in causing the onset of the disease.

Conclusion

This study confirmed the existence of a genetic component in the spring leg defect. Genomic analyses showed some promising SNPs on chromosome 24 that could be used in selecting against spring leg, but it is necessary to continue genotyping more animals to estimate marker effects in a larger population and perform validation tests. A simple validation could consist of looking at the frequency of the three significant SNPs on an independent dataset of healthy animals. It is also recommended to further explore the regions of the genome that contain significant SNPs as a means of understanding the process involved in causing the spring leg defect and to identify one or more causative mutations leading to the defect. In the medium term, if validation tests confirm these findings, a simple, affordable DNA test based on the three significant SNPs could be developed.

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Table 1. Number and frequency of affected, suspect, very suspect or normal lambs by experimental group

	AT RISK		LOW RISK	
Status	No	%	No	%
AFFECTED	21	23.1	2	3.4
SUSPECT	16	17.6	9	15.2
VERY SUSPECT	14	15.4	2	3.4
NORMAL	40	43.9	46	78.0
	91		59	

Table 2. Frequency of the three significant SNPs on chromosome 24 in affected and healthy animals

Status	Healthy			Affected		
Genotype	AA	AG	GG	AA	AG	GG
oar3_OAR24_1341130	24	63	17	4	18	25
oar3_OAR24_1343567	17	63	24	25	17	5
oar3_OAR24_1358660	20	61	23	29	15	3

Table 3. Frequency of the two significant haplotypes on chromosome 24 in affected and healthy animals.

Haplotype	AGG/AGG	AGG/GAA	GAA/GAA
Healthy	23	60	17
Affected	3	13	25