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Case Report
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Genetic Abnormalities in a Calf with Congenital Increased Muscular Tonus

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A 2-week-old female calf was referred to the Clinic for Ruminants at the Vetsuisse Faculty, University of Berne, Switzerland, with difficulty standing and muscle tremors since birth. Prior treatment by the private veterinarian with selenium, calcium, magnesium, and vitamins had not led to any improvement. The general status at arrival to the clinic was slightly reduced, though the calf was alert and attentive, and tachycardia (152/min) and tachypnea (80/min) were noticeable. Examination of the skin revealed an infected lesion on the fetlock of the left forelimb and several superficial lesions. Gastrointestinal, respiratory, and urinary tracts were without important abnormalities. The musculoskeletal system was normally developed, no atrophy was noticed, but the calf remained in a recumbent position unless lifted up and helped to stand. When standing, it showed tremor, ataxia, and could only move backward with hypermetria in the hind limbs and tip-toe-standing of the front limbs (Fig 1). Consciousness was normal but the calf was unable to orientate itself in its surroundings. Cranial nerve examination showed no deficits. Muscle tone was generally increased in the limbs. No painful reaction was noticed upon palpation of the limbs. The spinal reflexes were generally reduced. Sensibility was normal in the neck and shoulder area, but reduced in the limbs. The head and neck could be moved in all directions and the ears were symmetrical and loose. The clinical signs were localized in the peripheral nervous or musculoskeletal system.

Based on the clinical signs of weakness, stiffness, trembling, and inability to stand up on physical examination, white muscle disease was considered a possible differential diagnosis, as Switzerland is known for its selenium-poor soils and high prevalence of selenium deficiency in calves.[1,2] A blood chemistry profile revealed no relevant abnormalities, muscle enzyme activities were within normal limits. Further differential diagnoses associated with increased muscle tone, such as spastic paresis[3] and tetanus,[4] or with abnormal footing, as deforming ankylosis of the coffin joint,[5] were considered unlikely based on the clinical findings, and no further diagnostic steps were undertaken. To exclude a bone lesion in the left fetlock, a radiographic examination of the joint was conducted which revealed no abnormality. A complete blood count was within normal limits. A lumbar puncture was not conducted as the problem of the calf had been localized in the peripheral nervous or musculoskeletal system.

Abbreviations:

bp	base pair
MYBPC1	myosin binding protein C slow type
SNP	single nucleotide polymorphism

The calf was treated with antibiotics (procaine-penicillin 30,000 IU/kg SID s.c.[a]) because of the deep lesion on the left fetlock. In addition, the calf was treated with vitamins of the B-complex[b] (5 mg/kg thiamine, 2.5 mg/kg pyridoxinhydrochloride SID i.m.) and selenium[c] (sodium selenite 0.25 mg/kg, alpha-tocopherol 5 mg/kg s.c. once).

The calf was assisted to stand several times a day, and feed and water was always provided for the calf to consume in recumbent position. The movements of the calf did not improve with treatment, however, it always had a good appetite, and was bright and alert. The owner did not wish any further examinations such as electromyography, or nerve or muscle biopsies.

Based on the calf's history of clinical signs since birth and lack of improvement despite treatment, on the clinical presentation and lack of specific findings matching the most common musculoskeletal diseases, congenital disease was suspected and veterinary geneticists were contacted during the calf's stay at the clinic in order to investigate possible genetic causes for the disorder observed in this calf.

The calf was of especially high breeding value for the owner, who insisted to take the calf home for further care. The calf was released from the clinic under the agreement that it would be brought back for further examinations or necropsy if it did not improve. Information was gathered approximately weekly by telephone conversation. A month after returning home, the

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Fig 1. Affected calf at 3 weeks of age. Notice (**A**) the tip-toe-stance of the front legs with straight position of the carpus, and (**B**) the tendency to move backward and the instability on the hind legs.

calf could stand up on its own and could even walk a few steps forward instead of only backward according to the owner. Unfortunately the owner suddenly decided to slaughter the calf without notice to the clinic 5 weeks after discharge.

During recent years, substantial progress has been made in the field of molecular genetics.[6,7] Many domesticated animal genomes, including the cow genome, have been sequenced.[8,9] Recently the 1000 bull genomes project has been initiated and allows participants worldwide to access sequence data from a large number of cattle control genomes.[10] As there was no knowledge of a genetic disease with similar clinical signs in cattle, we assumed a spontaneous *de novo* mutation and therefore sequenced the entire genome of the affected animal. Because of the strong effect of the mutation, we hypothesized that most likely a mutation affecting the coding sequence of a gene would be responsible for the disease. Therefore, genomic DNA was isolated from blood using the Nucleon Bacc2 kit,[d] and a fragment library with a 300 base pairs (bp) insert size was prepared. For whole genome resequencing, one lane of Illumina HiSeq2500 paired-end reads (2 × 100 bp) was collected corresponding to roughly 15× coverage of the genome.[e;11] The obtained sequence reads were mapped to the current version of the Bos taurus reference sequence (UMD3.1/bosTau6) as described before.[11] The whole genome sequence of the affected calf has been deposited under accession number PRJEB7707 at the European Bioinformatics Institute short read archive. The data were checked for deviations (variants) like single nucleotide polymorphisms (SNP), short insertions and deletions from the reference sequence as described before.[11] A total of 67,310 sequence variants were detected across the whole exome, including untranslated regions and 10 bp of flanking introns, of the affected animal. Subsequently, these variants were compared to 46 control cattle genomes that had been sequenced in our laboratory in the course of other ongoing studies to exclude sequence variants present in these controls as being causative. Thereby the number of candidate variants was reduced to a total of 360. In a second filtering step, the remaining list of candidate variants were further checked for presence in the recent sequence variant database containing 1,147 already sequenced cattle genomes of the ongoing 1,000 bull genome project.[10] Thereby, the number of private DNA variants present in the affected calf only was reduced to 18 (Table S1).

Following the assumption that the calf was a carrier of a newly occurred mutation, we expected the causal mutation to be present in the calf and absent in its parents. Therefore, we screened the sire, dam, and the affected offspring for the 18 remaining candidate variants by Sanger sequencing. For this procedure, DNA of the parents was isolated from blood of the dam and semen of the sire, which was used for artificial insemination, using the Nucleon Bacc2 kit[d], and variant flanking primers were designed with Primer3 software[f] after masking of repetitive sequences with RepeatMasker.[g] PCR products were amplified using AmpliTaq Gold360 Master Mix[h] and directly sequenced on an ABI3730 capillary sequencer[i] after treatment with exonuclease I[j] and shrimp alkaline phosphatase.[k] The sequence data was analyzed with Sequencher 5.1 software[l] and sequence variants which were present in the parents were excluded. Other possible scenarios such as a dominant inheritance with incomplete penetrance or mosaicism in one of the parents would have permitted the mutation to be present in one of the parents also. For 14 of the 18 remaining variants, one parent was genotyped as heterozygous like the sequenced calf (Table S1), but no obvious functional candidate gene was affected. Therefore, these variants were considered as less likely to have been causative. In addition, 3 variants were shown to be called false positive as genotyping did not confirm their presence. Finally one single variant was left: a SNP replacing a thymine by a guanine on bovine chromosome 5 at bp-position 65,787,153. It was clearly identified as a *de novo* mutation as it was absent in both parents, but present in the calf (Fig 2A). Interestingly, this SNP situated in exon 13 of the myosin binding protein C slow type (*MYBPC1*) gene at position 885 of the open reading frame (c.885T>G) is predicted to lead to an amino acid exchange from leucine to arginine of the encoded MYBPC1 protein sequence at position 295 (p.Leu295Arg). Leucine is an nonpolar (hydrophobic) amino acid with a molecular weight of 131, whereas

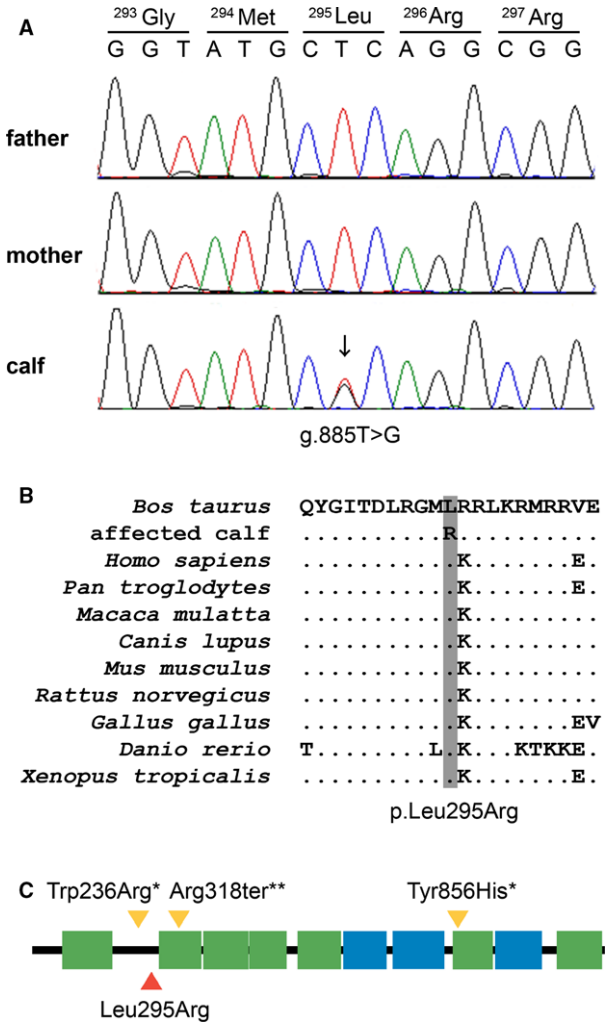

Fig 2. A de novo missense mutation in *MYBPC1* is associated with the disease phenotype. (**A**) Electropherograms of the *MYBPC1* c.885T>G mutation. (**B**) Multiple sequence alignment of the MYBPC1 protein in the region of the p.Leu295Arg mutation. Note the perfect conservation of the leucine at position 295 in all known MYBPC1 homologs. (**C**) Localization of known human and bovine mutations affecting the MYBPC1 protein. The protein consists of seven immunoglobulin C2 repeats (displayed in green) and three fibronectin type-III repeats (blue). The positions of the published human mutations are marked with yellow triangles. The mutations, which cause dominant distal arthrogryposis type 1 are labeled with one star (*) and the mutation, that causes the recessive lethal congenital contractural syndrome type 4 is labeled with two stars (**). The mutation found in the presented calf is shown below with a red triangle.

arginine is positively charged (basic) with a molecular mass of 174. These physical differences are predicted to have an impact on the protein folding (increased length of a beta-strand) using Phyre2 prediction software.[12] The affected residue is conserved among vertebrates (Fig 2B). The functional effects of the amino acid exchange were calculated by PolyPhen-2[m] and SWIFT,[n] they were predicted to be probably damaging and not tolerated, respectively.

Myosin binding protein C slow type consists of two repetitive domains: 3 fibronectin type-III repeats and 7 immunoglobulin C2 repeats (Fig 2C), and it is specifically expressed in skeletal muscle.[13] It has both structural and regulatory roles in muscle function, providing thick filament stability and modulating contractility through interactions with myosin and actin (OMIM 160794). In humans, two mutations in *MYBPC1* have been found to cause autosomal dominant distal arthrogryposis type 1, a condition characterized by contractures in the hands and feet.[14] Both mutations are SNPs resulting in amino acid exchanges (p.Trp236Arg and p.Tyr856His respectively; Fig 2C). Another mutation, a SNP which introduces a premature stop codon (p.Arg318ter) has been associated with the recessive lethal congenital contractural syndrome type 4 in humans.[15] This syndrome is the most severe and neonatally lethal form of arthrogryposis. Interestingly, the mutation detected in the presented calf affected the same segment of the MYBPC1 protein as the human p.Trp236Arg mutation (Fig 2C). More remarkably, the calf's phenotype resembled the human distal arthrogryposis type 1 phenotype. Similar to the contractures in hands and feet in humans, the affected calf was not able to fully stretch its extremities. In addition, it was not able to stand; it walked backward only and showed reduced sensibility mainly in its hind limbs, although the interpretation of the neurological examination was difficult because of the contractures. In summary, the detected *de novo* missense variant in the *MYBPC1* gene, an apparent functional candidate gene which is associated with similar phenotypes in humans, strongly suggests this mutation as being causative for the observed phenotype.

Currently, the responsible gene mutation of nearly every fifth rare disease in domestic animals has been determined.[16] This has been done during the past 25 years either by targeted analysis of individual candidate genes or labor- and resource-intensive so-called positional cloning approaches, like linkage mapping or genome wide association studies. To this purpose, a series of cases showing an identical phenotype is usually needed. The advent of next-generation sequencing technology, in combination with the establishment of a reference genome sequence for domestic animal species, as for the bovine genome in 2009, has changed prospects enormously.[8–10] Today, studying the molecular etiology of single cases is also feasible eg, in cattle[10,11,17] as it is successfully performed in man since nearly 5 years now.[18]

In conclusion, we successfully identified the likely causative mutation for a rare disease with only 1 case by taking advantage of the most recent technologies. However, providing functional proof of causality for putative causative mutations remains a limiting factor. This study is an example of how current molecular genetic methods can provide a new tool to diagnose and explain previously unexplainable diseases in cattle. As progress in this field is rapid and techniques are becoming cheaper, these tools will become affordable for routinely diagnosing rare diseases in animals.

Footnotes

[a] Procacillin® ad.us.vet, MSD Animal Health GmbH, Lucerne, Switzerland
[b] Corébral® ad.us.vet, Vétoquinol AG, Ittigen, Switzerland
[c] Selen-E Vetag® ad.us.vet., MSD Animal Health GmbH, Lucerne, Switzerland
[d] Nucleon BACC2 Genomic DNA Extraction Kit, GE Healthcare, Uppsala, Sweden
[e] Illumina HiSeq2500, Illumina, San Diego, CA, USA
[f] Homepage Primer3 (2014) Available at: http://bioinfo.ut.ee/primer3-0.4.0. Accessed September 22, 2014.
[g] Homepage Repeat Masker Server (2014) Available at: http://www.repeatmasker.org. Accessed September 22, 2014
[h] AmpliTaq Gold360 Master Mix, LifeTechnologies, Zug, Switzerland
[i] ABI3730 capillary sequencer, LifeTechnologies, Zug, Switzerland
[j] Exonuclease I, Roche, Basel, Switzerland
[k] Shrimp alkaline phosphatase, New England BioLabs, Ipswich, MA USA
[l] Sequencher 5.1 software, Gene Codes Corporation, Ann Harbor, MI, USA
[m] Homepage PolyPhen2 (2014) Available at: http://genetics.bwh.harvard.edu/pph2. Accessed September 17, 2014
[n] Homepage SWIFT (2015) Available at: http://sift.jcvi.org/. Accessed March 24, 2015

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Off-label Antimicrobial Declaration: Authors declare no off-label use of antimicrobials.

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Supporting Information

Additional Supporting Information may be found online in Supporting Information:

Table S1. Private exonic sequence variants of the affected calf.

Video S1. 2-week old calf with increased muscular tonus, tremor and ataxia. Notice that the calf can only move backwards with hypermetria in the hind-limbs and tip-toe-standing of the front limbs.