

## Allelic variations in coding regions of the vitamin D receptor gene in dairy cows and potential susceptibility to periparturient hypocalcaemia

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Periparturient hypocalcaemia (milk fever) is a disorder of Ca metabolism in dairy cattle primarily affecting multiparous cows. The major reasons for the rapid decrease of blood Ca concentration after calving are the prompt increase of Ca secretion into the colostrum and the delayed activation of Ca regulation mechanisms including calcitriol, a metabolite of vitamin D. In man, vitamin D receptor (VDR) gene polymorphisms are reported to be associated with disturbances of Ca metabolism, whereas data confirming the same in dairy cows are still missing. Moreover, polymorphisms that only affect non-coding regions are sometimes difficult to ascribe to a specific disorder as pathways and unequivocal links remain elusive. Therefore, the idea of the present study was to investigate in a small group of dairy cows with documented clinical records whether polymorphisms in the coding regions of the VDR gene existed and whether these potentially found variations were correlated with the incidence of periparturient hypocalcaemia. For this purpose, blood DNA was isolated from 26 dairy cows in their 4th to 6th lactation, out of which 17 had experienced hypocalcaemia at least once, whereas 9 cows had never undergone periparturient hypocalcaemia in their lifetime. The 10 VDR exons and small parts of adjacent introns were sequenced and compared with the *Bos taurus* VDR sequence published on NCBI based on the DNA of one Hereford cow. In total, 8 sequence alterations were detected in the fragments, which were primarily heterozygous. However, only 4 of them were really located on exons thereby potentially causing changes of the encoded amino acid of the VDR protein, but were not correlated with the incidence of periparturient hypocalcaemia. Certainly, this lack of statistical correlation could be due to the small number of animals included; anyhow, it was not encouraging enough to initiate a larger study with hundreds of cows and document blood Ca levels post partum for at least four lactations.

**Keywords:** Vitamin D receptor, polymorphism, dairy cow, hypocalcaemia.

Periparturient hypocalcaemia (parturient paresis, milk fever) is a common disorder in dairy cows during the first days of lactation. A physiological drop in blood Ca concentration is generally observed in this period due to an increased Ca requirement for milk synthesis at the onset of lactation and approximately 0–10% of cows exhibit clinical symptoms of milk fever (DeGaris & Lean, 2008). Multiparous high-performance cows are predominantly affected, rather

than heifers, but why some high-producing cows are affected and others of the same herd are not, remains elusive.

The primary sources for the maintenance of blood Ca are diet and bone. Intestinal Ca absorption and bone Ca resorbing processes are vastly enhanced under the influence of Ca-regulating hormones to compensate for the soaring demand of milk secretion (Horst et al. 1994). Parathyroid hormone (PTH) is secreted by the parathyroid glands at low ionized Ca concentrations in the blood and stimulates Ca release from the bone and renal Ca absorption. Moreover, it favours synthesis of the active form of vitamin D, 1,25-dihydroxycholecalciferol [1,25(OH)<sub>2</sub>D<sub>3</sub>, calcitriol].

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Calcitriol is bound to the nuclear vitamin D receptor (VDR), which activates a variety of genes leading to increased intestinal and renal Ca absorption or even to Ca release from bone. Hence, the signal cascade of regulation of Ca in blood is well known, but a conclusive understanding of the pathogenesis of milk fever is still missing.

As circulating levels of PTH have been shown to be similar in normo- and hypocalcaemic cows (Yamagashi & Naito 2006), VDR, the key component in the cascade of calcitriol effects, may be responsible or at least involved in delayed or impaired regulation of Ca metabolism. VDR is expressed in tissue such as gut (Yamagashi & Naito 2006), kidney and bone well known as 'classical' target organs of calcitriol, but was also found in brain, skin, genitals, endothelial cells (Merke et al. 1989) and cells belonging to the immune system such as monocytes and T cells (Holick, 1995). After binding of the ligand to its receptor, the calcitriol-VDR complex attaches to the nuclear transcription factor retinoid-X receptor (RXR) forming a heterodimer. By binding to the vitamin D response element (VDRE) this heterodimer activates gene expression of several genes of which some are involved in Ca homeostasis, e.g. epithelial Ca channels TRPV5 and 6, intracellular calbindins, basolateral pumps PMCA1b and sodium-calcium exchanger NCX1 (Hoenderop et al. 2005).

In man, polymorphisms in the VDR gene are associated with altered bone-mineral density (BMD)/osteoporosis susceptibility (Wood & Fleet 1998), can cause a decrease in [<sup>3</sup>H] 1,25(OH)<sub>2</sub>D<sub>3</sub> binding affinity (Malloy et al. 1997), can lead to functional changes in the VDR protein (Whitfield et al. 2001), and lower VDR mRNA levels (Fang et al. 2005). The functions of VDR (e.g. DNA or hormone binding) are impaired according to the domain of the VDR in which the alteration is located (Haussler et al. 1997). When Bezerra et al. (2008) reported that an increased Ca content in human breast milk is also correlated with mutations of VDR, the question came up whether VDR gene variations could be involved in milk fever pathogenesis. However, corresponding data were missing for dairy cows. It is known that there are at least 138 single nucleotide polymorphisms (SNPs) in the bovine VDR gene (NCBI 2011, last updated in January 2011), but they are located mainly on introns and regulatory regions with minor to no effect on the VDR protein and their relevance in terms of hypocalcaemia susceptibility is unexplored.

Hence, the present study sought to test whether it would be a promising approach to screen herds of dairy cows for the existence of a polymorphism in the coding regions of the VDR gene and to correlate potentially found variations with the incidence of periparturient hypocalcaemia.

## Materials and Methods

All experimental procedures were approved by the authorities of the county Thuringen in accordance with the German animal welfare law [paragraph 8 (7)].

## Blood collection

Animals for the present study were chosen from one dairy farm in Gotha/Germany (mean annual milk yield about 9600 kg; 900–1000 lactating cows with about 200 cows in their first lactation; hybrids of Holstein Friesian and German Black Pied Cattle) to exclude the influence of food quality and other living conditions on the susceptibility to hypocalcaemia. Only 26 cows matched the inclusion criteria which were: 'long-serving'/at best in their 5th or 6th lactation (two were only in the 4th lactation; this was the postulate most difficult to realize as most cows are usually culled before their 4th lactation), unrelated to each other (just two cows had the same father but belonged to different groups), and with documented levels of ionized blood Ca 24 h post partum. Hypocalcaemia was defined as ionized Ca levels 24 h after calving <0.75 mmol/l (measured by CRT 1–14, NOVA biomedical®, Waltham MA, USA). According to this, only nine cows had never developed hypocalcaemia before (= 'group incidence of hypocalcaemia: never'), whereas 17 cows had experienced hypocalcaemia or even periparturient paresis at least once (= group 'incidence of hypocalcaemia: yes'). Blood samples for genomic analysis were obtained from the *vena coccygea* in EDTA tubes at time points that were not related to parturition. The tubes were stored at room temperature and DNA was isolated within 48 h. DNA was isolated from leucocytes using Puregene Core Kit A according to the manufacturer's instruction (Qiagen GmbH, Hilden, Germany).

## Primer design

The bovine VDR gene has been identified to be approximately 55 kb in length, containing 10 exons. For primer design, the predicted *Bos taurus* chromosome 5, reference assembly (based on Btau\_3.1), whole genome shotgun sequence (NCBI reference sequence NC\_007303.2; gi 119951957; based on breed Hereford) was used. Eight primer pairs (Table 1) were designed to amplify the 10 exons (pair 5/6 spanning exons 5 and 6, pair 8/9 spanning exons 8 and 9) thereby mostly also including small parts of introns. Owing to technical limitations some primers missed to cover exiguous parts of the exon.

## PCR, gel electrophoresis and sequencing

Genotyping was performed using PCR in a thermal cycler (Eppendorf; Hamburg, Germany) with the following program: 95 °C for 5 min., 40 PCR-cycles: 95 °C for 10 s, annealing at corresponding primer temperature (Table 1) for 30 s, elongation at 72 °C for 60 s, last elongation step at 72 °C for 10 min.

Separation of PCR fragments by length and charge was performed using an ethidium bromide stained 2% agarose gel (100 V, 30 min). Length of the fragments was identified using TrackIt™ 100 bp DNA Ladder (Invitrogen GmbH; Karlsruhe, Germany). Images were acquired using

**Table 1.** PCR fragments and comprising exons, appendant primer pairs (sense and antisense) and sequences, melting temperature ( $T_m$ ) for each primer and annealing temperatures ( $T_a$ ) used in PCR reaction

	Exon	Primer	Sequence	$T_m$ (°C)	$T_a$ (°C)
Fragment 1	1	Pr1 fwd	5'-agcgcggagcagcgtgc	66.8	62
		Pr1 rev	5'-cccatgtatggtcacctgcgag	62.6	
Fragment 2	2	Pr2 fwd	5'-caacacagggctgtacataaagc	57.8	58
		Pr2 rev	5'-aggccatagatgcctagacctc	57.2	
Fragment 3	3	Pr3 fwd	5'-gctcgaattgtgtagcttc	56.9	56
		Pr3 rev	5'-ctctaccattgatgatcagagc	55.0	
Fragment 4	4	Pr4 fwd	5'-gtcacaggctctctcatgca	55.6	58
		Pr4 rev	5'-ctgagacttcacgctattctcaca	57.0	
Fragment 5/6	5+6	Pr5/6 fwd	5'-cctgactgatgaagaagtgcag	55.9	56
		Pr5/6 rev	5'-ggatcactgtacctcctccag	57.2	
Fragment 7	7	Pr7 fwd	5'-gacacaatggagccaaccag	57.7	56
		Pr7 rev	5'-gacactgccagtggaagttc	55.0	
Fragment 8/9	8+9	Pr8/9 fwd	5'-acctcaccctgaggaccagatc	63.0	60
		Pr8/9 rev	5'-ccatctgcattgtctcccag	60.5	
Fragment 10	10	Pr10 fwd	5'-gagacgaggtgggacaggac	58.3	58
		Pr10 rev	5'-gtttggcaacagatctctctga	59.2	

the GeneGenius Gel imaging system (Syngene Cambridge, UK).

Purification of the PCR-products was conducted using 1  $\mu$ l ExoSAP-IT® with 10- $\mu$ l sample (USB Corporation; Cleveland OH, USA) according to the ExoSAP-IT Clean-up protocol. The eight PCR-products, comprising the ten VDR-exons, were sequenced separately in sense and anti-sense direction by the corresponding primer using GenomeLab™ DTCS-Quick Start Mastermix (Beckman Coulter Inc., Fullerton CA, USA) and template-dna in the thermo cycler (Eppendorf, Hamburg, Germany). Purification of the sequencing-products was conducted on a microplate (96 well) according to Agencourt® CleanSEQ® protocol using GenomeLab™ DTCS-Quick Start Kit. After adding GenomeLab™ Separation Gel-LPA I the samples were sequenced in a CEQ™ 8000 Genetic Analysis System (all Beckman Coulter Inc, Fullerton CA, USA) using the following program: 30 cycles: 94 °C for 20 s, 55 °C for 20 s, 60 °C for 2 min. The resulting sequences were analysed using the CEQ™ 8000 Genetic Analysis System (Beckman Coulter, Inc) and compared with the published sequence (NCBI. NC\_007303.4, *Bos taurus* breed Hereford chromosome 5, Btau\_4.2, whole genome shotgun sequence) using the software PhyDE® (<http://www.phyde.de>).

### Statistics

Frequencies of sequence aberrations from the published sequence were identified and integrated in contingency tables. Correlations were calculated using Fisher's exact probability test for 2  $\times$  2 contingency tables, or Freeman-Halton's extension of Fisher's exact probability test for 2  $\times$  3 contingency tables, respectively.  $P$ -values <0.05 were considered statistically significant (Freeman 1951; Agresti 1992; Bender & Lange 2001).

## Results

### Sequencing

In fragments 3, 4, 5/6, and 8/9 no alterations to the *Bos taurus* sequence published on NCBI were found (data not shown). Fragments 1, 2, 7 and 10 showed homo- or heterozygous single base variations, considered 'allelic variations' (Table 2), either in an intron segment (4 of 8) or in an exon segment (4 of 8). The four exon alterations caused potential changes in the amino acid sequence due to changes in codons (conservative: potential switch from alanine to valine in fragment 7 (Fig. 1); non-conservative: the potential change from leucine to methionine in fragment 7 and from asparagine to tyrosine in fragment 10) or could lead to production of a truncated protein (Lys 428 Stop, fragment 10).

The C-G alteration found in fragment 2 had already been described and published on NCBI (Reference SNP Cluster Report rs110759021).

### Statistical analysis

There was no statistically evident correlation between the detected VDR gene allelic variations and the incidence of hypocalcaemia ( $P > 0.05$ ).

## Discussion

Hypocalcaemia, especially subclinical hypocalcaemia, can still be considered as one of the most cost-intensive diseases in dairy cows and many factors have been discussed as possible causes.

VDR gene polymorphisms were already shown to influence Ca homeostasis in humans, and for milk fever prevention, strategies including vitamin D supplementation have been tested successfully. Hence, we thought that a possible explanation for the incapacity of some dairy cows

**Table 2.** Allelic variations found in 26 dairy cows

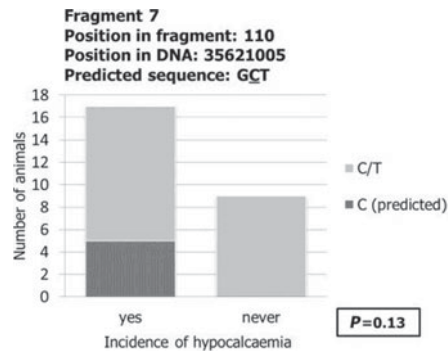
Fragment number†	1	2	7	7	7	10	10	10		
Position in fragment	147	344	106	110	336	212	365	389		
Position in DNA NC_007303.4	35575667	35597615	35621001	35621005	35621232	35630878	35631031	35631055		
Position in mRNA XM_613129	–	–	813	817	–	–	1284	1308		
Published sequence (AS‡)	GTCTG	GTGGG	CTG (Leu)	GCT (Ala)	TTTGA	CGCCC	AAG (Lys)	AAC (Asn)		
Mutation	GTT/CTG	GTG/CGG	C/ATG	GC/TT	TTT/AGA	CGC/ACC	A/TAG	A/TAC		
Sequence determined	GTTTG, GTCTG and heterozygous	GTGGG, GTCCG and heterozygous	CTG and heterozygous	GCT and heterozygous	TTTGA and heterozygous	CGCCC and heterozygous	AAG and heterozygous	AAC and heterozygous		
Potential amino acid change	–	–	Leu 271 Met§	Ala 272 Val	–	–	Lys 428 Stop	Asn 436 Tyr		
Eartag	Incidence of hypocalcaemia		Lactation		Sequencing results					
63 143	Yes	5	T	G	C/A	C	T/A	C/A	A	A/T
63 655	Yes	4	T	G	C	C	T	C	A	A
86 045	Yes	6	C/T	G/C	C/A	C	T/A	C/A	A	A/T
86 321	Yes	6	C/T	C	C/A	C	T/A	C/A	A/T	A/T
60 698	Yes	6	C/T	G/C	C/A	C	T/A	C/A	A	A/T
60 105	Yes	6	C	C	C/A	C/T	T/A	C	A	A/T
63 139¶	Yes	5	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
63 183	Yes	5	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
63 663	Yes	4	T	G/C	C/A	C/T	T/A	C/A	A	A/T
86 334	Yes	5	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
86 374	Yes	6	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
42 038	Yes	6	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
60 854	Yes	6	T	G	C/A	C/T	T/A	C/A	A	A/T
63 018	Yes	6	T	C	C/A	C/T	T/A	C/A	A	A/T
63 023	Yes	5	C/T	G/C	C/A	C/T	T/A	C	A	A/T
60 852	Yes	5	C/T	C	C/A	C/T	T/A	C/A	A	A/T
63 168	Yes	5	C/T	G/C	C/A	C/T	T/A	C	A	A/T
63 455	Never	5	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
86 145	Never	6	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
86 235	Never	5	T	G	C/A	C/T	T/A	C/A	A	A/T
63 030	Never	5	T	G	C/A	C/T	T/A	C/A	A	A/T
86 588	Never	5	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
63 439¶	Never	5	C	C	C/A	C/T	T/A	C	A	A/T
86 463	Never	6	T	G	C/A	C/T	T/A	C	A	A/T
63 511	Never	5	C/T	G/C	C/A	C/T	T/A	C/A	A	A/T
63 537	Never	5	C/T	G/C	C/A	C/T	T/A	C	A	A/T
<i>P</i> -values		<i>P</i> >0.99		<i>P</i> =0.73	<i>P</i> >0.99		<i>P</i> =0.13	<i>P</i> >0.99	<i>P</i> =0.66	<i>P</i> >0.99

† Fragment numbers match the comprising exons

‡ Amino acids (AS): leucine (Leu), alanine (Ala), lysine (Lys), asparagine (Asn), methionine (Met), valine (Val), stop codon (Stop), tyrosine (Tyr)

§ Published amino acid (Leu), number of amino acid (271), potential amino acid change (Met)

¶ Same father



**Fig. 1.** Frequencies of allelic variations in the two groups ('incidence of hypocalcaemia: yes' and 'incidence of hypocalcaemia: never') found in fragment 7. In position 110 (35621005 of DNA) the predicted base was C (underlined), however, most cows (~80%) showed C and T in this position (light grey). Only five cows were homozygous for C (dark grey) and had all shown hypocalcaemia at least once. In group 'never' only heterozygotes (C and T) were seen. Statistical calculations did not show a significant correlation ( $P=0.13$ ).

to maintain Ca homeostasis after parturition could be an impairment within the functionality of the VDR.

Of course, gene alterations changing the amino acid sequence thereby impairing the protein's functionality are not the only possibilities of how VDR effects can be influenced. A total decrease of VDR in tissue at the onset of lactation has been described for cows, independent of the incidence of hypocalcaemia (Liesegang et al. 2007). Moreover, Wood & Fleet (1998) have described a decreasing vitamin D sensitivity with age and, according to this, an age-dependent VDR decline has been described in man (Ebeling et al. 1992), rats and cows (Horst et al. 1990; Liang et al. 1994) which might explain the finding that heifers rarely develop hypocalcaemia. On the other hand, there are also publications reporting contradictory findings, namely that an age-related VDR decline correlated with decreasing Ca uptake in the elderly could not be observed (Kinyamu et al. 1997) and that a VDR decline with age could not be confirmed (Lee et al. 2003; Liesegang et al. 2008). Hence, the search for genetic aberrations is just one approach in the wide field of VDR-focused research.

Nevertheless, if genetic factors for milk fever became evident, this could be of great importance for breeding selection. And DNA chips to test for SNPs have already been introduced into the evaluation of genetic breeding values of dairy cows (VanRaden et al. 2009).

Many studies have investigated the relation between VDR gene polymorphisms in man and individual irregularities in Ca homeostasis such as fracture risk (Fang et al. 2003), changed VDR expression in the small intestine (Arai et al. 2001) and altered incidence of osteoporosis (Uitterlinden & Ralston, 2006). The SNPs used for disease screening in man (*Apal*, *BsmI*, *TaqI*, *FokI*) are supposedly non-functional and are, therefore, used as markers in association studies (Uitterlinden et al. 2002). Their correlation with diseases

can be explained by linkage disequilibrium (Uitterlinden et al. 2004). Functional SNPs in exons would probably alter the protein structure with or without impact on the function. However, genetic predispositions such as variations in the VDR gene with a subsequently impaired VDR protein structure could not be proven yet.

In this study, we found 8 single-base aberrations in the VDR gene of 26 dairy cows compared with the published sequence. Most of the sequence variations found affect only one allele, the only 2 homozygous variations are located on introns. After all, 4 out of the 8 single-base alterations are located on exons and cause a potential change in the amino acid sequence on one allele. But as no significant correlation with the incidence of hypocalcaemia has been detected, tests for the functionality of these sequence alterations were considered dispensable. Nevertheless, these 4 alterations found in coding regions should be tested in a greater number of cows to decide whether they can be referred to as SNPs.

Although sequencing of non-coding regions was beyond the scope of this initial approach, in future studies the entire gene including introns and regulatory regions could possibly be targeted. Or all the known SNPs listed on the NCBI web page (dbSNP) could be checked; firstly, to detect further sequence alterations, and secondly, because SNPs in intron regions of the human VDR gene seem to be associated with Ca-homeostasis-related diseases as well. Nonetheless, to examine whether these are correlated with the history of hypocalcaemia, huge numbers of cows receiving the same diet would need to be clinically monitored for years (several lactations) and blood samples drawn frequently to test for subclinical hypocalcaemia.

This precondition constitutes a limitation of the present study and led to the small number of animals included, which may also account for the lack of statistical significance. If more cows could have been included in the study, especially in group 'incidence of hypocalcaemia: never', the working hypothesis of this study could have proven to be a promising approach to initiate further studies. For example, fragment 7 position 110 would have turned significant if 5 more cows could have been included that had never shown hypocalcaemia and were all found to exhibit the heterozygous C/T variant.

Nevertheless, all we can conclude so far is that VDR exon screening is probably not helpful in predicting susceptibility to periparturient hypocalcaemia.

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