Exclusion Of The Bovine *Factor XI* Gene As Genetic Background Of Bovine Neonatal Pancytopenia In Calves

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Introduction

Recently, several reports about haemorrhagic diathesis in cattle have been published (Shimada *et al.* 2007, Braun *et al.* 2008, Friedrich *et al.* 2009, Kappe *et al.* 2010). Specifically, an increasing number of cases of bovine neonatal pancytopenia (BNP) have been observed in Germany and many other European countries. However, the background of BNP still remains an open question. While on the one hand immunmodulating external noxes are discussed, genetic factors also have to be considered given the examples of monogenic effects for blood clotting disorders. Prominent candidate genes of bleeding disorders are coagulation factors like factor XI, whose deficiency was verified in Holstein cattle in the USA (Kociba *et al.* 1969), in Canada (Gentry *et al.* 1975), Britain (Brush *et al.* 1987), Japan (Ghanem *et al.* 2005), in the Czech Republic (Čítek *et al.* 2008) and in Turkey (Meydan *et al.* 2009).

Factor XI (plasma thromboplastin precursor), a zymogene of a plasma serine protease, is involved in the early stage of the intrinsic pathway of blood coagulation. The activated form of factor XI is one of the responsible factors for the conversion of factor X into its activated form, which converts prothrombin to thrombin. This reaction transforms soluble fibrinogen into an insoluble fibrin clot (Brush *et al.* 1987).

Marron *et al.* (2004) identified the molecular basis of the factor XI deficiency in Holstein cattle as an insertion of a 76-bp segment within exon 12 of the *factor XI* gene on chromosome 27. The outcome is a truncated protein as a result of the presence of a stop codon. In consequence, the mature factor XI protein would be lacking the functional protease domain encoded by the last three exons (13-15). Because factor XI deficiency is known in Holstein cattle, we sequenced all 15 exons of the *factor XI* gene to test for mutations causing factor XI deficiency as a potential genetic background of BNP.

Material and methods

Calves treatment and phenotype. The calves ($N_{healthy} = 294$, $N_{affected\ calves} = 7$, born from May 2007 to July 2009) belonged to a F_2 resource population established from an experimental cross between Charolais and German Holstein (SEGFAM resource population, Kühn *et al.* 2002) at the FBN in Dummerstorf. After birth, all calves were removed from

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their mothers within 30 minutes. The calves were housed in a barn with straw bedding and fed a colostrum diet, which was replaced by a milk replacer at day five.

The affected calves died within 2 weeks after birth at an average of three days after displaying the first symptoms. They were characterised by fever (40°C), haemorrhages in skin and subcutis (especially in exposed segments, e.g., scapula), various organs (e.g. rumen, abomasum, small intestine, heart) and from injection or tagging sites together with petechiae on visible mucous membranes and haemorrhagic faeces. The symptoms were identical to the clinical picture described in Friedrich *et al.* 2009 and Kappe *et al.* 2010. All deceased calves were submitted to a pathologic section in a routine veterinary diagnostic laboratory (LALLF Rostock). Examinations of the carcasses and blood revealed a haemorrhagic diathesis as a result of a thrombocytopenia. In addition, the partial thromboplastin time was prolonged.

Analysis of *factor XI* gene variation. The animals selected for resequencing comprised three affected calves (AC) and 13 healthy cattle ($N_{\text{related to AC}} = 6$, $N_{\text{unrelated to AC}} = 7$). Genomic DNA was extracted either from blood or tissue samples (ear, spleen) via phenol extraction. The sequencing of the *factor XI* exon sequences was carried out either with the 3130 Genetic Analyzer (Applied Biosystems Inc, Foster City, CA, USA) or the MegaBACE 1000 (GE Healthcare, Munich, Germany). To analyse and compare the exon sequences, BioEdit Sequence Alignment Editor (Version 7.0.5.2) was used. In addition to this comparison, the obtained exon sequences were matched against Btau_4.0 (*NC_007328.3*). Furthermore, with the help of the Basic Local Alignment Search Tool (BLAST, NCBI), the bovine protein sequence of factor XI (*NP_001008665.1*) was aligned with the orthologs of other vertebrate species.

Pedigree analysis. For all calves, a full four generation pedigree was available for genetic analysis. A binomial test was performed to test whether there was a significant concordance of affected and unaffected individuals in the two groups of calves originating from the common F_1 sire A or not originating from that sire:

$$P(X_1 = a) = \binom{n}{a} p_A^{\,a} (1 - p_A)^{n-a}$$

where $P(X_1 = a)$ is the probability of observing calves with BNP descending from sire A assuming a random distribution of cases, n is the total number of calves showing BNP, a is the number of calves from sire A showing BNP, p_A is the probability that a random calf is a descendent of sire A.

Results and Discussion

Sequencing of *factor XI* gene. The sequencing of all 15 exons revealed single nucleotide polymorphisms (SNPs) in exon 7, 12 and 14. But there was no 76-bp insertion in exon 12 as described by Marron *et al.* 2004. Instead, we found 4 SNPs within the 176-bp segment of this exon. Three of the SNPs induce an amino acid exchange (Figure 1). In exon 7 (160 bp) and in exon 14 (140 bp) the investigation exhibited 2 SNPs, respectively. One of the SNPs in exon 7 results in an amino acid exchange (Figure 1).

None of the amino acid exchanges was specific for the affected calves. Apparently, the results indicate a high variability within the *factor XI* gene. This is supported by the fact that the amino acid sequence of the bovine *factor XI* gene differs in many positions from the orthologs of other species, e.g., exon 7, 12 and, 14 (Figure 2). Therefore, an intact and functional factor XI can be generated from different primary protein sequences without a phenotypically apparent impact on the phenotype.

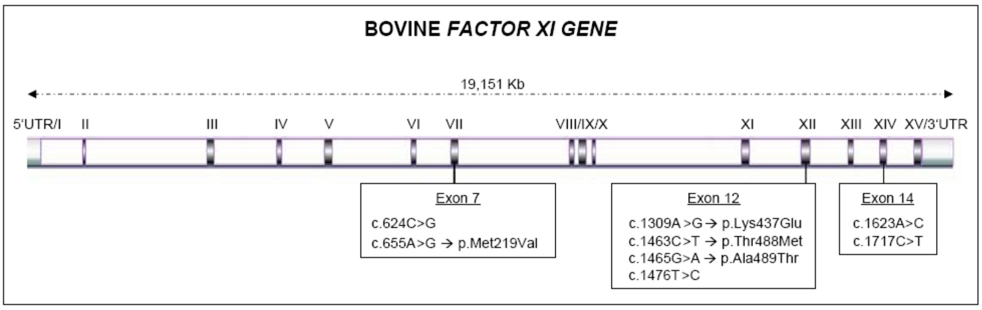
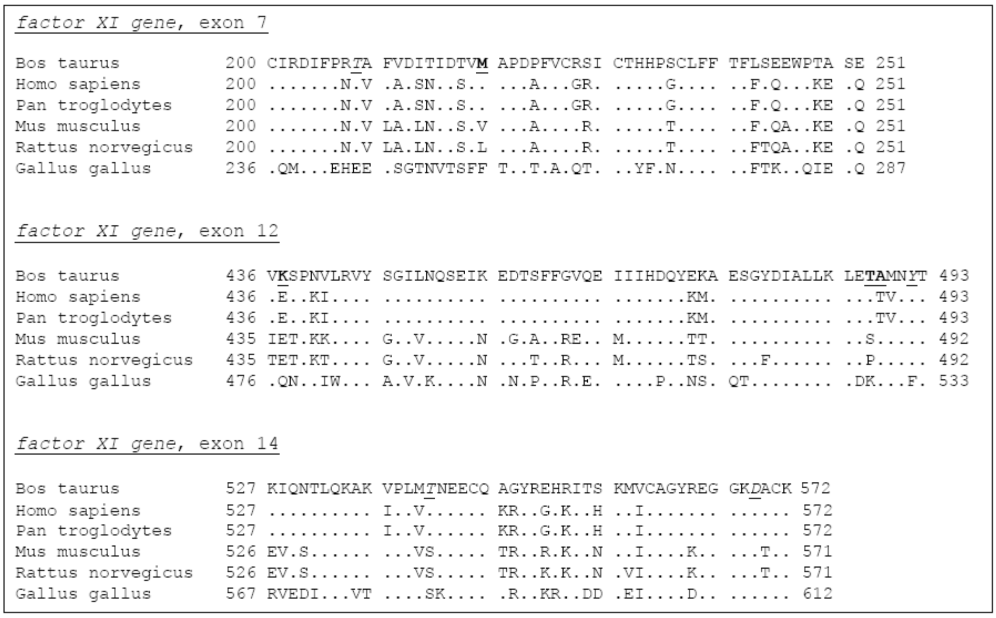


Figure 1: Structure of the bovine *factor XI* gene with detected SNPs and amino acid exchanges. Exons are shown as rectangles labelled by exon numbers in roman numerals. The 5'UTR and the 3'UTR are marked in grey, coding sequences are darker coloured.



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factor XI gene, exon 7

Bos taurus          200 CIRDIFPRTA FVDITIDTVM APDPFVCRSI CTHHPSCLFF TFLSEEWPTA SE 251
Homo sapiens        200 .......N.V .A.SN..S.. ...A...GR. .....G.... ..F.Q...KE .Q 251
Pan troglodytes     200 .......N.V .A.SN..S.. ...A...GR. .....G.... ..F.Q...KE .Q 251
Mus musculus        200 .......N.V LA.LN..S.V ...A....R. .....T.... ..F.QA..KE .Q 251
Rattus norvegicus   200 .......N.V LA.LN..S.L ...A....R. .....T.... ..FTQA..KE .Q 251
Gallus gallus       236 .QM...EHEE .SGTNVTSFF T..T.A.QT. ..YF.N.... ..FTK..QIE .Q 287


factor XI gene, exon 12

Bos taurus          436 VKSPNVLRVY SGILNQSEIK EDTSFFGVQE IIIHDQYEKA ESGYDIALLK LETAMNYT 493
Homo sapiens        436 .E..KI.... .......... .......... .......KM. .......... ...TV... 493
Pan troglodytes     436 .E..KI.... .......... .......... .......KM. .......... ...TV... 493
Mus musculus        435 IET.KK.... G..V.....N .G.A..RE.. M......TT. .......... ..S..... 492
Rattus norvegicus   435 TET.KT.... G..V.....N ...T..R... M......TS. ...F...... ..P..... 492
Gallus gallus       476 .QN..IW... A.V.K....N .N.P..R.E. ....P..NS. QT........ .DK...F. 533


factor XI gene, exon 14

Bos taurus          527 KIQNTLQKAK VPLMTNEECQ AGYREHRITS KMVCAGYREG GKDACK 572
Homo sapiens        527 .......... I..V...... KR..G.K..H ..I....... ...... 572
Pan troglodytes     527 .......... I..V...... KR..G.K..H ..I....... ...... 572
Mus musculus        526 EV.S...... ...VS..... TR..R.K..N ..I....K.. ...T.. 571
Rattus norvegicus   526 EV.S...... ...VS..... TR..K.K..N .VI....K.. ...T.. 571
Gallus gallus       567 RVEDI...VT ....SK.... .R..KR..DD .EI....D.. ...... 612
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Figure 2: Amino acid sequence alignments of the factor XI orthologs of cattle (*NP_001008665.1*), human (*NP_000119.1*), monkey (*XP_001165847.1*), mouse (*NP_082342.1*), rat (*XP_224872.4*) and fowl (*XP_420678.2*). A dot indicates the same amino acid. Positions with detected SNPs are underlined in the bovine sequence: bold letters stand for an amino acid exchange; italic font mark the SNP with no impact on the resulting amino acid.

Clustering of cases in specific pedigrees. There was a clustering of cases of BNP in the offspring of a F_1 sire line. All calves affected with BNP belonged to the SEGFAM resource population (Kühn *et al.* 2002). One calf was a F_2-offspring, while the other six calves were backcrosses generated by mating F_2 females to two different purebred German Holstein sires. Further pedigree analysis revealed that all individuals could be traced back to a single F_1 male ancestor. Statistical analysis revealed that the restriction of affected calves to the progeny of sire A was statistically significant. The likelihood of observing exclusively descendents of sire A as affected was p=0.003.

Conclusion

Although no indication on a simple mode of inheritance of the phenotype was observed, our data suggest that there may be a genetic component involved in the development of the BNP. Currently, several environmental factors e.g., vaccination of the mothers (Friedrich *et al.* 2009) or virus infections (e.g., circovirus, Kappe *et al.* 2010) are discussed, however, without clear conclusive evidence for a single causal agent. Our results indicate that epidemiological studies should consider at least an interference of genotype with environmental noxes.

References

Braun, U., Schmid, T., Muggli, E. *et al.* (2008). *Schweiz. Arch. Tierheilk.*, 150 (11): 553-557

Brush, P.J., Anderson, P.H., and Gunning, R.F. (1987). *Vet. Rec.*, 121 (1): 14-17

Čítek, J., Řehout, V., Hanusová, L. *et al.* (2008). *J. Sci. Food Agr.*, 88: 2069-2072

Friedrich, A., Rademacher, G., Weber, B.K. *et al.* (2009). *Tierärztl. Umschau*, 64: 423-431

Ghanem, M.E., Nishibori, M., Nakao, T. *et al.* (2005). *J. Vet. Med. Sci.*, 67 (7):713-715

Gentry, P.A., Crane, S., and Lotz, F. (1975). *Can. Vet. J.*, 16 (6): 160-163

Kappe, E.C., Halami, M. Y., Schade, B. *et al.* (2010). *Berl. Munch. Tierartzl. Wochenschr.*, 123 (1-2): 31-41

Kociba, G.J., Ratnoff, O.D., Loeb, W.F. *et al.* (1969). *J. Lab. Clin. Med.*, 74 (1): 37-41

Kühn, C., Bellmann, O., Voigt, J. *et al.* (2002). *Arch. Anim. Breed.*, 45: 317-330

Marron, B.M., Robinson, J.L., Gentry, P.A. *et al.* (2004). *Anim. Genet.*, 35 (6): 454-456

Meydan, H., Yildiz, M.A., Özdil, F. *et al.*(2009). *Acta Vet. Scand.*, 51 (1): 5

Shimada, A., Onozato, T., Hoshi, E. *et al.* (2007). *J. Vet. Med. Sci.*, 69 (12): 1317-1319