G244E in the canine factor IX gene leads to severe haemophilia B in Rhodesian Ridgebacks

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Abstract
Haemophilia B in Rhodesian Ridgebacks is currently the most important canine haemophilia in Germany. The aim of this study was to define the underlying genetic defect. Genetic studies were performed including six phenotypically affected male dogs (factor IX activity: approximately 1%), four suspected carriers (factor IX activity 48–69%, one confirmed by affected offspring), and 12 healthy dogs. Comparison of the entire coding region of the canine factor IX DNA sequences and exon–intron junctions from affected dogs with the wild type canine factor IX DNA revealed a G–A missense mutation in exon 7. This mutation results in a glycine (GGA) to glutamic acid (GAA) exchange in the catalytic domain of the haemophilic factor IX. All affected dogs were hemizygous for the detected mutation and carriers were heterozygous, whereas none of the Rhodesian Ridgebacks with normal factor IX activity showed the mutation. No further alterations in the sequences between affected dogs and the healthy control group could be observed. None of the Rhodesian Ridgebacks with undefined haemophilia B status (n = 30) and no individual of three other dog breeds (Doberman Pinscher: n = 20; German Wirehaired Pointer: n = 20; Labrador: n = 25) showed the presence of the mutation. Amino acid sequence alignment and protein structural modelling analysis indicate that the detected mutation causes a relevant functional defect. The results of this study suggest that the detected mutation is responsible for a severe form of haemophilia B in Rhodesian Ridgebacks.

Introduction
Haemophilia B is a condition affecting the blood clotting process by lack of biologically active coagulation factor IX. Factor IX is a 56 kDa plasma glycoprotein that is required for activation of factor X in the coagulation cascade. Canine haemophilia B represents a naturally occurring hereditary coagulopathy with a range in severity of clinical signs and coagulant activity in different breed variants. It has been reported in many breeds. In Rhodesian Ridgebacks, a severe form of haemophilia B with pronounced clinical signs was first described in Lutze et al. (2000). Currently it is the clinically most important canine haemophilia form in Germany.

Different mutations in the gene encoding factor IX have been identified in different dog breeds, including Lhasa Apso, Labrador, Pit Bull Terrier, Airedale Terrier, and German Wirehaired Pointer resulting in clinical symptoms typical for haemophilia B (Evans et al., 1989; Mauser et al., 1996; Brooks et al., 1997; Gu et al., 1999; Brooks et al., 2003; Table 1). To the author’s knowledge, the genetic background of haemophilia B in Rhodesian Ridgebacks, is unknown. Therefore the aim of this study was to investigate whether a genetic defect could be identified as underlying cause of haemophilia B in Rhodesian Ridgeback dogs. A genetic test would help to diagnose diseased dogs and identify genetically affected and carrier dogs.

Materials and methods

Study design
The study included six phenotypically affected male dogs, four suspected carriers, and 12 healthy dogs. From all dogs with known or suspected haemophilia B status, factor IX activity was measured to verify the status for haemophilia B. From all these dogs, DNA was isolated from EDTA blood, and the coding region of canine factor IX was amplified and sequenced. The obtained DNA sequence was compared with the wild type canine factor IX DNA (Gene Bank Accession Number: NM_001003323). In addition, a TaqMan genotyping assay specific for the detected mutation, was performed in all these dogs. In order to prove the functional relevance of the detected mutation, amino acid alignment studies as well as protein structure modelling analyses were conducted. To investigate, if the mutation is just a single nucleotide polymorphism (SNP) unrelated to haemophilia B, additional
TaqMan genotyping assays specific for the mutation were performed in DNA samples of 30 Rhodesian Ridgebacks with undefined haemophilia B status and samples of three other dog breeds (Doberman Pinscher: \(n = 20\); German Wire haired Pointer: \(n = 20\); Labrador: \(n = 25\)).

Animals and sample material

Rhodesian Ridgebacks with known status of haemophilia B were presented as patients or for haemophilia diagnosis to the Small Animal Clinic, University of Veterinary Medicine Hannover. The six phenotypically affected male Rhodesian Ridgebacks had all a known bleeding history and substantially reduced factor IX activities of approximately 1% (Table 2). Clinical signs mainly included severe bleeding after minor surgeries and injuries and occasional spontaneous bleeding. Among the four bitches with suspected carrier status due to moderately reduced factor IX activity (48–69%), one was confirmed by affected littermates. Twelve healthy Rhodesian Ridgeback dogs had a factor IX activity within the reference range and no hint for an excessive bleeding within their history.

Blood sample collection

Citrated blood for factor IX activity measurements was obtained from a cephalic or saphenous vein using sterile disposable needles (1.1/230 mm) and only slight pressure was used to raise the vein. Citrated blood was collected into plastic tubes containing one part (1 mL) 0.11 mol/L sodium citrate solution for nine parts (9 mL) of blood and immediately mixed by careful rocking. Platelet poor citrated plasma for factor IX measurements was gained by centrifugation twice for 10 min at 2000 \(g\). The final supernatant was then collected and stored in aliquots at \(-70^\circ C\).

In addition, 5 mL of EDTA blood were collected for genetic tests.

Table 1

<table>
<thead>
<tr>
<th>Breed</th>
<th>Mutation in canine factor IX gene</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixed-breed, originally Cairn terrier and Beagle (Chapel Hill Colony)</td>
<td>(G \rightarrow A) transition at nucleotide 1477 in the region encoding the catalytic domain (substitution of glutamic acid for glycine)</td>
<td>Evans et al. (1989)</td>
</tr>
<tr>
<td>Lhasa Apso</td>
<td>Deletion including nucleotides 772–776 and a (C \rightarrow T) transition at nucleotide 777</td>
<td>Mauser et al. (1996)</td>
</tr>
<tr>
<td>Labrador</td>
<td>Complete deletion of factor IX gene</td>
<td>Brooks et al. (1997)</td>
</tr>
<tr>
<td>Pit Bull Terrier</td>
<td>Large deletion mutation spanning the entire 5’ region of the factor IX gene extending to exon 6</td>
<td>Gu et al. (1999)</td>
</tr>
<tr>
<td>Airedale Terrier</td>
<td>5 kb insertion in exon 8, which with exon 7 encodes the factor IX catalytic region</td>
<td>Gu et al. (1999)</td>
</tr>
<tr>
<td>German Wirehaired Pointer</td>
<td>Large insertion mutation in intron 5 of the factor IX gene</td>
<td>Brooks et al. (2003)</td>
</tr>
</tbody>
</table>

Table 2

<table>
<thead>
<tr>
<th>Dog No</th>
<th>Internal number</th>
<th>Signalement, pedigree</th>
<th>Clinical signs(^b)</th>
<th>Factor IX activity</th>
<th>Sequence analysis(^c)</th>
<th>TaqMan(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P 1549</td>
<td>Male, 3 months</td>
<td>+</td>
<td>1</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>2</td>
<td>P 1551</td>
<td>Male, 2 years, 5 months</td>
<td>+</td>
<td>&lt;1</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>3</td>
<td>P 1554</td>
<td>Male, 1 year, 2 months</td>
<td>+</td>
<td>3</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>4</td>
<td>P 1615</td>
<td>Male, 6 months (brother of No. 4, son of No. 10)</td>
<td>+</td>
<td>1</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>5</td>
<td>P 1621</td>
<td>Male, 6 month (brother of No. 4, son of No. 10)</td>
<td>+</td>
<td>1</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>6</td>
<td>P 1867</td>
<td>Male, 2 years, 8 months</td>
<td>+</td>
<td>1</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>7</td>
<td>N 983</td>
<td>Female, 2 years, 2 months</td>
<td>–</td>
<td>48</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>8</td>
<td>P 1520</td>
<td>Female, 2 years, 7 months</td>
<td>–</td>
<td>62</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>9</td>
<td>P 1552</td>
<td>Female, 5 months</td>
<td>–</td>
<td>65</td>
<td>c</td>
<td>c</td>
</tr>
<tr>
<td>10</td>
<td>P 1616(^e)</td>
<td>Female, 3 years, 2 months</td>
<td>–</td>
<td>65</td>
<td>c</td>
<td>c</td>
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<tr>
<td>11</td>
<td>N 909</td>
<td>Female, 1 year, 7 months</td>
<td>–</td>
<td>84</td>
<td>n</td>
<td>n</td>
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<tr>
<td>12</td>
<td>N 910</td>
<td>Female, 1 year, 6 months</td>
<td>–</td>
<td>92</td>
<td>n</td>
<td>n</td>
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<td>13</td>
<td>N 988</td>
<td>Male, 3 years, 4 months</td>
<td>–</td>
<td>104</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>14</td>
<td>N 969</td>
<td>Male, 5 years, 1 month</td>
<td>–</td>
<td>100</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>15</td>
<td>156814</td>
<td>Male, 7 years, 11 months</td>
<td>–</td>
<td>154</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>16</td>
<td>156854</td>
<td>Female, 2 months</td>
<td>–</td>
<td>109</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>17</td>
<td>P 1519</td>
<td>Female, 4 months</td>
<td>–</td>
<td>77</td>
<td>n</td>
<td>n</td>
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<tr>
<td>18</td>
<td>105960</td>
<td>Male, 2 years, 2 months</td>
<td>–</td>
<td>127</td>
<td>n</td>
<td>n</td>
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<tr>
<td>19</td>
<td>D 17109</td>
<td>Male castr., 2 years, 6 months</td>
<td>–</td>
<td>110</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>20</td>
<td>P 1943</td>
<td>Male, 1 year, 1 month</td>
<td>–</td>
<td>87</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>21</td>
<td>158120</td>
<td>Male castr., 1 year, 9 months</td>
<td>–</td>
<td>98</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>22</td>
<td>148756</td>
<td>Male, 7 years, 4 months</td>
<td>–</td>
<td>150</td>
<td>n</td>
<td>n</td>
</tr>
</tbody>
</table>

\(^a\) Confirmed carrier, mother of Nos. 4 and 5.

\(^b\) Severe bleeding after minor surgeries and injuries, occasional spontaneous bleeding.

\(^c\) n = Normal, c = carrier, a = affected.

\(^d\) Citrated blood for factor IX activity measurements was obtained from a cephalic or saphenous vein using sterile disposable needles (1.1 × 30 mm) and only slight pressure was used to raise the vein. Citrated blood was collected into plastic tubes containing one part (1 mL) 0.11 mol/L sodium citrate solution for nine parts (9 mL) of blood and immediately mixed by careful rocking. Platelet poor citrated plasma for factor IX measurements was gained by centrifugation twice for 10 min at 2000 g. The final supernatant was then collected and stored in aliquots at \(-70^\circ C\). In addition, 5 mL of EDTA blood were collected for genetic tests.

Factor IX activity measurements

Coagulation factor IX activities were automatically measured using a routine coagulometric test, which was optimised for canine sample material and is based on commercial human deficient plasma (Mischke, 2001). Measurements were performed in an AMAX Destiny coagulation analyser (Trinity Biotech). A mixture of 20 \(\mu\)L of diluted citrated plasma (1:40 diluted with imidazole buffer, Siemens Healthcare Diagnostics), 20 \(\mu\)L of factor IX deficient plasma (Siemens Healthcare Diagnostics), and 20 \(\mu\)L activating reagent (PTT reagent, Diagnostica Stago) was incubated, and exactly after 3 min 20 \(\mu\)L 25 mmol/L \(\text{CaCl}_2\) solution (Diagnostica Stago) were added as starting reagent. A calibration curve was prepared with different dilutions of a canine pooled plasma corresponding to 200% (1:20), 150% (1:26.7), 125% (1:32), 100% (1:40), 75% (1:53.3), 50% (1:80), 25% (1:160), 10% (1:400), 5% (1:800), and 1% (1:4000) which were used instead of the diluted sample. Canine pooled plasma was prepared by mixing identical citrated plasma aliquots from 100 clinically healthy dogs with unremarkable haematological and biochemical profile and its factor IX activity was defined as 100%. All results are mean values of measurements in duplicate. The reference range for this test is 75–140%.

DNA isolation and amplification

Genomic DNA was extracted from 200 \(\mu\)L EDTA whole blood using a commercially available kit Bioprint 15 DNA Blood Kit (Qagen) following manufacturers’ instructions. The canine factor IX cDNA sequence was utilised to search the dog genome database at NCBI\(^1\) and the eight exons of the canine factor IX gene were

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determined. Nine pairs of oligonucleotide primers (sequences and PCR conditions available on request) were used to amplify the coding region and the exon–intron boundaries of the canine factor IX gene using genomic DNA of the dogs. Target sequences were amplified in 50 μL reaction mixtures under standard reaction conditions containing approximately 100 ng genomic DNA and 0.2 μmol/L of each primer.

DNA sequencing

PCR products were purified prior to sequencing using MinEflue PCR Purification Kit (Qiagen). Sequencing was done by cycle sequencing using DyeDeoxy Terminators (Applied Biosystems) in an automated sequencer ABI 3130 Genetic Analyzer (Applied Biosystems). Sequence comparison was performed between different groups of Rhodesian Ridgebacks with defined haemophilia B status using BLAD ClustalW alignment.2

Amino acid sequence alignment and domain analysis

Alignment of the primary structure of the protease domain of canine factor IX with those of human factor IX, bovine trypsin (TRYP), porcine elastase (ELAS), bovine chymotrypsin (CHYM) and porcine kalilkrein (KALL) was determined. The three active site residues (H258, D306 and S402) of this domain are shown in red (small diamonds) as well as disulfide bonds (in grey).

Rapid screening of the missense mutation

TagMan genotyping assay specific for the mutation was designed using File Builder Software Version 3.1 (Applied Biosystems). The region flanking the SNP was amplified in the presence of two allele-specific fluorescent probes. One probe labelled with VIC dye detected the wild type allele sequence and one probe labelled with FAM dye detected the mutant allele sequence. PCR amplification and allelic discrimination was performed using Rotor-Gene 6000 (Corbett).

![Fig. 1.](image1.png)

Fig. 1. Amino acid sequence alignment of the protease domain of canine factor IX (CFIX) with human factor IX (HFIX), bovine trypsin (TRYP), porcine elastase (ELAS), bovine chymotrypsin (CHYM) and porcine kalilkrein (KALL).
Structural protein modelling

Structural protein modelling applied CPHmodels\(^3\) (Lund et al., 2002) to generate a structure model and used PyMol\(^4\) (DeLano, 2002) for graphical analysis.

Results

Identification and rapid screening of the missense mutation

Comparison of the entire coding region of the canine factor IX DNA sequence and of the exon–intron junctions of six haemophilic dogs with severe factor IX deficiency (Nos. 1–6) with the wild type canine factor IX DNA revealed a G–A missense mutation at nucleotide 752 in exon 7 of the haemophilic factor IX gene. This mutation results in a glycine (GGA) to glutamic acid (GAA) exchange in the catalytic domain of the haemophilic factor IX. All affected dogs were hemizygous for the detected mutation in exon 7 (Table 2). Four dogs (Nos. 7–10), which were suspected or proven carriers, were found to be heterozygous for the G–A mutation and none of the healthy Rhodesian Ridgebacks was affected by the mutation. No further alterations in the sequences between affected dogs and the healthy control group could be observed.

In all cases of Rhodesian Ridgebacks with defined haemophilia B status, the result of TaqMan genotyping assay confirmed the haemophilia B status and thereby the results of the sequencing analysis. Analyses with TaqMan genotyping assay did not reveal the presence of the G–A missense mutation in exon 7 of canine factor IX DNA in 30 further Rhodesian Ridgebacks with undefined haemophilia B status and 65 animals of three other dog breeds.

Amino acid sequence alignment and domain analysis

Amino acid sequence alignment of the heavy chain of canine factor IX with the corresponding region of several other serine proteases (human factor IX, bovine trypsin, porcine elastase, bovine chymotrypsin, porcine kallikrein). Fig. 1 demonstrates that the mutation in canine factor IX affects one of the highly conserved

\(^3\) http://www.cbs.dtu.dk/services/CPHmodels/.
\(^4\) http://pymol.sourceforge.net/.

Fig. 3. Comparison of structure of mutated canine factor IX and wildtype factor IX. Illustration of the affected residues of factor IX from the inside; yellow colour indicates wild type, blue indicates mutated protein. Spherical shape: disulfide bridge; ribbon shape: mutated amino acid. The view includes the active site. (i) Inside view of wild type protein. Note, that residue 244 (ribbon shape) is rather small. (ii) Inside view of mutated protein. Residue 244 (mutated) is significantly bigger than in the wild type version of the protein. (iii) Overlay of: (i) and (ii). Note how change of residue 244 leads to a change in overall protein geometry. Especially the disulfide bridge is affected.
amino acids, substituting a negatively charged residue (glutamic acid) for an amino acid that is uncharged and has no side chain (glycine). Domain analysis shows, that mutation of amino acid 244 from G to E is located in the trypsin domain of factor IX (Fig. 2). It also is directly next to a disulfide bridge (residues 243 and 259).

Structural protein modelling

Structure analysis shows that a change in conformation is most likely occurring within the inner part of the protein located close to the active centre due to the fact that the mutation to glutamic acid leads to a long side chain that needs more space and disturbs the structure even more due to its charge (Figs. 3 and 4). The mutation of residue 244 leads to a change in the structure of the pocket-shaped active site of the trypsin domain. The structural proximity is evident: The mutation lies directly adjacent to a disulfide bridge stabilizing the active site and causes, due to the changed structure of the mutated amino acid, a change in the geometry of the disulfide bridge and thus also in the active site.

Discussion

The results of the present study reveal with sufficient certainty that the detected G–A missense mutation in exon 7 is the responsible mutation for severe haemophilia B in Rhodesian Ridgebacks. The presence of the mutation was confirmed by two different techniques in 22 dogs with defined haemophilia B status. In addition, a cohort of samples from different breeds did not show the observed mutation. The latter result suggests strongly that the detected mutation cannot be a common and non-important mutation.

Amino acid sequence alignment and protein structural modelling analysis demonstrates that in haemophilic Rhodesian Ridgebacks a non-conserved amino acid substitution caused by the mutation leads to a major change in structure and activity. Based on the structure analysis of the protein created by protein modelling, the detected mutation most likely results in an activity reduction of factor IX, well in accordance with the low residual factor IX activity assessed in the functional coagulometric test of approximately 1% and the correspondent severe clinical signs. This further supports the hypothesis that the detected mutation is responsible for the haemophilia B in Rhodesian Ridgebacks. In addition, a similar change in the canine DNA resulted in a similar clinical condition (Evans et al., 1989; Table 1).

Factor IX activities measured in the haemophilic dogs were lower than in the previous report on one haemophilic Rhodesian Ridgeback (4%) (Lutze et al., 2000). Based on clinical criteria...
applied to humans, the residual factor IX activity corresponds to a severe (<1%) or moderately severe (1–5%) haemophilia severity degree (Rizza, 1977) and, in general, dogs seem to have a more severe clinic at a defined degree of factor activity reduction due to their discrepant behaviour (Mischke et al., 1996; Lutze et al., 2000). In addition, the major structure abnormality of the factor IX molecule is well in accordance with the fact that a heterologous antibody against the human factor IX, which cross-reacted with the normal canine factor IX molecule, showed reduced affinity to bind at the respective domain in the defect factor IX molecule in affected Rhodesian Ridgebacks (Lutze et al., 2000). In the cited study, the haemophilic Rhodesian Ridgeback dog (factor IX activity: 4%) had an antigen concentration of 25% and a carrier (factor IX activity: 56%) an antigen concentration of 53% of a canine pool plasma. The fact that an ELISA with a heterologous antibody was used in this study may have enhanced this phenomenon.

Without a known genetic background, diagnosis of female carriers of a defect is difficult. Apart from analyses on littermates it is mainly based on factor measurements. The present study demonstrates that factor IX activity in carriers confirmed by genetic analyses (maximum value 69%) can nearly reach the lower limit of the reference range (75%). It is therefore likely that under less optimal conditions (e.g., sample shipment to the laboratory, less optimally calibrated test), carrier detection based on factor IX activity measurements is unreliable. The developed TaqMan genotyping assay specific for the detected mutation is essential for effective breeding hygiene programmes. Although none of the 30 unselected Rhodesian Ridgebacks was tested positive for the mutation, investigation of larger numbers of dogs of this breed seems valuable to define the actual prevalence of the disease.

Conclusions

A G–A missense mutation in exon 7 of the canine factor IX gene was identified as the likely mutation responsible for severe haemophilia B in Rhodesian Ridgebacks. This mutation results in a glycine (GGA) to glutamic acid (GAA) exchange in the catalytic domain of the haemophilic factor IX.

Conflict of interest statement

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

References