Morphological variation of “complex vertebral malformation” in Holstein calves

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Abstract. A study was performed to investigate the morphological expression of the inherited syndrome “complex vertebral malformation” (CVM) in Holstein calves. A total of 107 late-term aborted, premature, or neonatal calves suspected of having CVM were necropsied and retrospectively analyzed for the causal mutation in the gene \textit{SLC35A3}. Sixty-two calves were homozygous affected, 16 were heterozygous, and 29 were homozygous normal. Calves affected by CVM were growth retarded. Vertebral lesions identified by radiography were present in 61 cases, of which 58 also had costal malformation. Malformation of the head, primarily in the form of dysplasia or palatoschisis, was present in 15 cases. Bilateral symmetric flexion of the carpal and metacarpophalangeal joints was present in all cases, whereas posterior arthrogryposis was found in 54 cases. Interventricular septal defects occurred in 33 calves, often in combination with other cardiac malformations. A wide spectrum of additional malformations was found. Other congenital syndromes were in most cases distinguishable from CVM on a morphological basis. However, a calf with a prenatal infection with bovine virus diarrhea virus constituted a phenocopy. The study demonstrated that the morphological expression of CVM is wide, but certain aspects, i.e., growth retardation, vertebral malformation, and symmetric arthrogryposis, are almost constant findings. However, cases without vertebral defects and phenocopies constitute a diagnostic problem. A presumptive diagnosis of CVM can in most cases be based on necropsy findings combined with information on descent and paternal CVM genotype, whereas a definitive diagnosis requires genotyping.

Introduction

“Complex vertebral malformation” (CVM) is the designation for a bovine congenital syndrome caused by a mutation in the gene \textit{SLC35A3} coding an uridine-diphosphate-N-acetylglucosamine transporter. A single-base transversion of guanine to thymine has been located in the abnormal allele at position 559 (Bendixen C, Svendsen S, Jensen H, et al.: 2002, Genetic test for the identification of carriers of complex vertebral malformations in cattle. World Intellectual Property Organization. Publication No. PCT/WO 02/40709 A2. http://ipdl.wipo.int). If present in both copies of the allele, this mutation is lethal. Approximately 80% of homozygous affected fetuses are aborted before gestation day 260.\textsuperscript{17} Most studies have been performed on calves delivered after gestation day 260, whereas reports regarding aborted fetuses are almost absent. Most examined calves have been stillborn, but affected calves are occasionally born alive. Euthanasia must be performed because of humanitarian reasons.

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The major morphological changes in CVM-affected calves delivered after gestation day 260 consist of growth retardation, vertebral malformation, and bilateral symmetrical arthrogryposis affecting the carpal and the metacarpophalangeal joints. Additional arthrogryposis of the posterior distal joints is present in some cases. The vertebral malformation is characterized by hemivertebrae, scoliosis, misshaped vertebrae, and ankylosis affecting especially the vertebrae around the cervicothoracic junction. Additional malformations have been recorded in several cases.\textsuperscript{1,6,16,20,23}

Pedigree studies and DNA analyses of semen from sires used for insemination have demonstrated a widely branched familial occurrence of CVM in the Holstein breed. By analyses of archived materials, the mutation in the \textit{SLC35A3} gene has been traced to the US sire Penstate Ivanhoe Star (US1441440) born in 1963 and his widely used son Carlin-M Ivanhoe Bell (US1667366) born in 1974 (Danish Agricultural Advisory Center. The CVM-mutation is not restricted to descendants of the American Holstein Friesian bull Carlin-M Ivanhoe Bell. Press release. November 1, 2001). Through these sires and elite sires genetically related to them, the defect has been disseminated in the Holstein breed worldwide.

CVM was first recognized in Denmark in the fall of 1999, and in the following time period, calves suspected of having this defect were submitted for necropsy. In the present study, the necropsy results are
compared with genotype results obtained retrospectively to establish the phenotypic variation of CVM in premature or full-term calves.

**Materials and methods**

**Animals.** Holstein calves with external lesions indicating the presence of congenital vertebral malformation were submitted for laboratory examination from October 15, 1999, to September 15, 2001. This broad and relatively nonspecific clinical case definition was used to obtain calves with various malformations. Most of the submitted cases were based on the breeders’ recognition of a malformation. Information about the appearance of CVM in Danish Holsteins was given to breeders and veterinarians during the study period. However, submission of calves was restricted neither to breeders nor to calves genetically related to identified heterozygous sires. Malformed calves obtained in 2 breeding studies as well as calves previously reported were included in this study. The calves were submitted either directly from the breeders to the laboratory or through a local veterinary surgeon. If alive, calves were euthanized by intravenous administration of pentobarbital sodi-

**Laboratory examination.** Necropsy was performed on all calves. Histopathology was performed on relevant tissues if autolysis was not excessive and if necessary to establish a diagnosis. Specimens were fixed by immersion in 10% neutral buffered formalin, processed routinely, sectioned at 2–3 μm, and stained with hematoxylin and eosin.

All calves obtained as part of the breeding studies and calves with lesions indicating a viral etiology, i.e., cerebellar hypoplasia, were examined for bovine virus diarrhea virus (BVDV) and antibodies. Samples of lung and spleen were examined by cell culture virus isolation and antigen capture enzyme-linked immunosorbent assay (ELISA), whereas pleural effusions were examined for antibodies against BVDV by an ELISA technique. Bone specimens were radioographed after necropsy. These included the vertebral column after removal of the arch (except for the caudal vertebrae) and, when relevant, other parts of the skeleton.

**Genotyping.** All calves were genotyped with regard to the mutation in the SLC35A3 gene. Muscle samples were taken at necropsy and stored at −20°C until analysis. After thawing, DNA was purified from 0.25 to 0.5 cm³ muscle tissue by a technique based on Proteinase K digestion, salt precipitation, filtration, and isopropanol precipitation. Genotyping of the CVM locus was performed in a template-directed single-base extension assay, using the AcycloPrime-FP SNP detection kit. In brief, this method is based on an initial polymerase chain reaction amplification of the region in SLC35A3 containing the guanine to thymine transversion using the primers SLCL-F (5'-GGC CCT CAG ATT CTC AAG AGC-3') and SLCL-R (5'-CGA TGA AAA AGG AAC CAA AAG GG-3'). This was followed by a specific template-directed single-base extension at the transversion site using primer SLClupper (5'-GGC TCA CAA TTT GTA GGT CTC ATG GCA-3'). Fluorescent signals revealing the base at position 559 were detected by fluorescence polarization in a multilabel plate reader (Victor2).

**Statistical methods.** Sex ratios were analyzed by chi-square test, whereas other statistical analyses were performed by the General Linear Model Procedures. To evaluate fetal growth, body weights of normal full-term Holstein calves in Denmark (43.5 kg for males and 41.5 for females) were compared with body weights of affected calves delivered between gestation days 269 and 289. Normal gestation length for Holsteins in Denmark is 279 days, with a standard deviation of 5 days.

**Results**

A total of 107 Holstein calves suspected of having CVM were submitted for necropsy. All calves were retrospectively genotyped: 62 being homozygous for thymine, 16 heterozygous, and 29 homozygous for guanine at position 559.

**Findings in homozygous affected calves.** The sex ratio between affected calves was 30 males to 32 females ($\chi^2 = 0.0645, P > 0.5$). The gestation period was established in 58 cases based on breeding records. One calf was aborted at gestation day 159 (excluded from all calculations), whereas the others were delivered between gestation days 223 and 288. The length of the gestation periods in males and females was not statistically different ($P = 0.1033$). The mean gestation period for all affected calves was slightly reduced ($\bar{x} = 269.8$ days, SEM = 1.65 days).

The body weight was established in 31 cases delivered between gestation days 269 and 289. Affected calves were growth retarded, with a mean body weight of $26.1 \pm 4.7$ kg for males and $24.2 \pm 5.2$ kg for females. Most cases had a compact appearance of the neck and thorax because of reduced length of the cervical and thoracic parts of the vertebral column. The anterior part of the thorax protruded dorsally because of increased height of the anterior thoracic spinous processes. A considerable variation in the length of the neck was observed, varying from cases with a normal length ($n = 3$) to cases with a severe reduction (Fig. 1). Lesions in the head were present in several cases. Lesions usually consisted of subcutaneous edema and circulatory changes due to dystocia. However, malformations occurred in 15 cases and consisted of a broad neurocranium in combination with a slight dysplasia of the splanchnocranium and protrusion of the tongue ($n = 5$), brachygnathia superior ($n = 2$), straight course of the bridge of the nose ($n = 1$), bilateral ventrocaudal displacement of the ears ($n = 2$), palatoschisis ($n = 4$), and a linear epithelial defect located in the anterior midline of palatum durum ($n = 1$) (Fig. 2).

Vertebral lesions were found in 61 cases and were absent in 1 case. Lesions were only recognizable by radiography in 1 case. The course of the vertebral column was irregular because of malformation of multi-
ple vertebral bodies, i.e., adjacent hemivertebrae and butterfly vertebrae that were irregularly aligned. The extent of vertebral malformations varied considerably between cases from 2 affected vertebrae to multiple malformed vertebrae as previously reported.1 Lesions occurred in all parts of the vertebral column, except the sacrum. Scoliosis and ankylosis were common findings in affected areas. Thoracic spinous processes were often misshaped with lateral deviation of the proximal part, increased height, and with synostosis between adjacent processes. Dystocia-related vertebral epiphyseal fracture was present in 3 cases.

Costal malformations were observed in 58 cases. Ribs originating from malformed vertebra radiated from the vertebral column, with nonparallel intercostal areas increasing in width peripherally. Adjacent ribs often had synostosis of the proximal part and in some cases also affecting the mid part. The costal lesions varied extensively between cases both with regard to the number and location of malformed ribs and the degree of synostosis. The number of ribs varied from 10 to 13, representing both unilateral and bilateral reductions. Ribs originating from unaffected parts of the vertebral column appeared normal.

Lesions affecting the anterior limbs were present in all cases. Lesions were bilateral and consisted of a 10–45° flexure of the carpal joints and a 30–90° flexure of the metacarpophalangeal joint. In addition, the phalanges were rotated, usually laterally, causing a medial deviation of the tips of the contracted digits. Lesions were usually bilaterally symmetric. However, 1 case had bilateral arthrogryposis with lateral rotation of the left anterior phalanges and medial rotation of the right anterior phalanges.

Posterior limb involvement was recorded in 54 cases. In all cases, lesions were restricted to the tarsal and the metatarsophalangeal joints and were bilaterally symmetric. Tarsal lesions consisted of either flexion or extension occurring in an almost equal number of cases. The degree of flexion varied from slight to severe. In most cases, flexure of the metatarsophalangeal joint with medial rotation of the phalanges was present. However, 2 cases had extension of the metatarsophalangeal joint, and 1 case had lateral rotation of the digits.

Diffuse congenital atelectasis was present in 55 calves, 2 calves had partial atelectasis, and in 6 cases the lungs were fully aerated. Cardiac malformations were observed in 33 cases. In all cases, a defect of the upper part of the interventricular septum adjacent to the aortic ostium was present, with diameters ranging from 0.4 to 3.0 cm. In addition, dextroposition of the aorta varying from slight to complete displacement (n = 20) and transposition of the aorta and truncus pulmonalis (n = 2) were recorded. Secondary lesions consisting of eccentric right ventricular hypertrophy (n = 26), left ventricular hypertrophy (n = 8), and dilatation of truncus pulmonalis (n = 7) were frequent additional findings.

Intestinal lesions were diagnosed in 3 calves. Focal stenosis of the ascending colon 2 cm aborally to the ileal orifice with prestenotic intestinal dilatation, focal rectal atresia, and abomasal eventration through a ventral abdominal wall defect were found once in each of 3 calves. Curdled colostrum was present in the abomasum in 3 calves.

The hepatic diaphragmatic surface was often irregular because of multiple round to oval elevated areas (Fig. 3). Single (n = 2) or multiple (n = 1) serous or serohemorrhagic cysts were found attached to the capsule on the diaphragmatic surface. An analogous cyst was observed in the peritoneum of another calf. The
abdominal organs were often closely apposed because of reduced columnar length. The amount of abdominal and subcutaneous fat was within normal range.

Central nervous system lesions were restricted to 6 cases having slight caudal displacement of the brain. Brain weight was measured in 9 other calves with a cerebellar weight to total brain weight ratio of 0.087 (minimum 0.076, maximum 0.109). Spinal cord compression was not evident.

Additional malformations were found in 2 cases. One calf had a unilateral dermoid, whereas the other had a 4–5-mm ventral midline epithelial defect extending from the ventral labial commissure of the vulva to 10 cm caudal to the umbilicus.

Findings in heterozygous/homozygous normal calves. Sixteen calves were heterozygous for the guanine to thymine substitution, whereas 29 were homozygous normal. Ten cases either had dystocia-related lesions or bacterial infection without any malformation. A wide range of malformations was observed in the other calves: interventricular septal defect \((n = 3)\), tetralogy of Fallot \((n = 1)\), congenital hepatic cyst \((n = 1)\), prognathism \((n = 1)\), hydranencephaly \((n = 1)\), growth retardation \((n = 1)\), anterior arthrogryposis \((n = 4)\), bilateral tibial hemimelia and femoral hypoplasia \((n = 1)\), facial dysplasia and brain malformation \((n = 1)\), and generalized edema, tetralogy of Fallot, pulmonal hypoplasia, bilateral hydronephrosis, tetramelic arthrogryposis, and hydranencephaly \((n = 1)\).

Vertebral malformation was found in 20 cases. These included atlanto-occipital fusion \((n = 2)\), caudodirecto-urogenital syndrome \((n = 4)\), sacral spina bifida and Arnold–Chiari malformation \((n = 3)\), scoliosis affecting cervical, thoracic, lumbar, or caudal vertebrae, mostly with additional malformations, i.e., palatoschisis and arthrogryposis \((n = 7)\) and thoracic lordo-scoliosis with duplication of the lumbar, sacral, and caudal vertebrae and spinal cord segments \((n = 1)\).

Three cases shared several features with homozygous affected calves, making differentiation based on morphology difficult: a growth-retarded calf delivered at gestation day 264 was initially diagnosed as CVM with concurrent BVDV-associated teratogenic lesions. Lesions were characterized by bilateral symmetric flexion of the carpal and the metacarpophalangeal joints with lateral phalangeal rotation, bilateral symmetric extension of the tarsal joints, and flexion of the metatarsophalangeal joint, hemivertebrae in thoracic vertebrae 5 and 8, brachygnathia inferior, polycystic kidneys, and cerebellar hypoplasia. The calf tested positive for precolostral BVDV antibodies and was retrospectively genotyped as heterozygous for the guanine to thymine transversion. Two other calves genotyped as homozygous normal shared several features with CVM including growth retardation, vertebral malformation, and bilateral symmetric arthrogryposis of the distal joints in combination with multiorgan malformations.

**Discussion**

The present study demonstrates that growth retardation and bilateral flexure of the carpal and metacarpophalangeal joints with rotation of the digits were constant findings in premature, stillborn, and neonatal CVM-affected calves. Vertebral malformation and posterior arthrogryposis were common but not constant findings. The severity of each type of lesion varied considerably between cases, and it is notable that vertebral lesions were absent in 1 case. In addition to cardiac malformation, a wide range of lesions was recorded at least once. Based on the number of CVM-affected calves reported until now, it is uncertain whether all these lesions are part of the syndrome or whether some lesions occurred by coincidence. However, the association with CVM is indicated by the occurrence of nonvertebral lesions in humans affected by multiple vertebral segmentation defects and may be explained by abnormal function of the Notch signaling pathway, which is important for normal embryogenesis.

A wide range of other malformations than CVM was diagnosed. Most of these could be differentiated from CVM based on morphology. The differentiation was retrospectively confirmed by genotyping. Because some cases shared one or more features with CVM, and because the variation of CVM became obvious during the study, genotyping was necessary to definitively differentiate CVM from other malformations. In accordance with other studies, this study demonstrates that certain malformations cannot be discriminated from CVM based on morphology.
in the calf with BVDV-associated malformations were similar to those expected in a CVM-affected calf with concurrent BVDV infection. Such calves are expected to occur in Holstein populations with endemic BVDV infection and constitute a confounding factor for CVM diagnosis. Other teratogens may cause similar problems.

The age of affected fetuses varied from 159 to 288 days. However, most cases were born at term or within the last 2 weeks of the gestation period. A previous study based on breeding registrations has shown that 45% of CVM-affected fetuses are aborted before gestation day 150, whereas 77% are lost before gestation day 260. The high frequency of abortions due to CVM was not reflected in the cases submitted in this study. This was probably because of difficulties in recognizing external lesions in aborted fetuses, which was a prerequisite for submission. In the 159-day-old fetus, lesions were restricted to the vertebral column and not recognized until opening of the thorax during routine diagnostic examination. The finding of a statistically normal gestation period was most likely because of an age-related bias in the submission of calves. The dominance of late-term or full-term calves in the study may indicate that fetuses, which survive certain critical developmental stages, are viable until the end of the gestation period. Experimental studies are needed to evaluate the development of CVM-affected fetuses and to investigate whether fetuses are aborted evenly throughout the gestation period or at certain developmental stages.

During embryonic development, vertebral primordia (somites) are formed by subdivision of the unsegmented paraxial mesoderm. The somites are located laterally to the neural tube and the notochord as paired spheres and consist of 3 parts, of which the sclerotome develops to vertebrae and ribs. Studies in mice have demonstrated that the transmembrane receptor Notch1 is required for normal segmentation and rostrocaudal patterning of somites. In Drosophila, a signaling molecule encoded by the Fringe gene is needed for activation of Notch. The conserved role of this interaction in mammalian species has been supported by expression studies in murine embryos. The function of this signaling molecule depends on adding of N-acetylgalactosamine onto fucose residues. The calves affected by CVM are deprived of this highly specific pathway because the transporter of uridinediphosphate-N-acetylgalactosamine into the Golgi lumen, where the signaling molecule is synthesized, is defective.

Growth retardation and vertebral malformation are central lesions in CVM. Similar lesions have been found in mice experimentally deprived of the effects of Notch (a murine homologue to Drosophila Notch) or the Fringe gene. Furthermore, the vertebral malsegmentation syndromes in humans, spondylocostal dysostosis and Alagille syndrome, are associated with mutations in genes coding for Notch ligands, which are necessary for normal function of the Notch signaling pathway. These findings support the outlined pathogenesis with abnormal Notch function as the terminal event leading to malformation in CVM-affected calves. Abnormal development of dorsal spinal root ganglia and spinal nerve axons has been observed in mice with abnormal Notch function. Although this did not cause arthrogryposis in mice, it may be the cause of arthrogryposis in calves. A wide spectrum of lesions, as observed in CVM-affected calves, has not been reported in mice with abnormal Notch function. The cause of this is unknown but may be associated with a much more diverse genetic background in calves than in inbred mouse strains.

Studies in mice have demonstrated that normal function of the Notch family of transmembrane receptors is important for fetal development and survival. Extensive fetal mortality has been reported in Notch1- or Notch2-deficient murine embryos, whereas Fringe-deficient fetuses survived until birth. However, although severely affected neonates died, less affected mice could survive until adulthood. The cause of this extensive fetal loss has not been established, but widespread necrosis has been observed in mutant embryos. Similar pathogenetic mechanisms might be responsible for the severe fetal mortality seen in cattle. Neonatal mortality in cases of multiple vertebral segmentation defects in both humans and mice has been attributed to respiratory distress due to vertebral and costal malformation. Similar considerations as for humans and mice may also apply for calves.

By combining the necropsy findings and the genotyping results, it can be concluded that growth retardation, bilateral symmetric flexure of the carpal and the metacarpophalangeal joints with rotation of the digits, bilateral symmetric arthrogryposis of tarsal and metatarsophalangeal joints, and vertebral malformation causing shortening of the neck are present in most premature, stillborn, and neonatal CVM-affected calves. However, cases occur that lack one or more of these lesions or have deviating lesions. It must be noted that these lesions are not pathognomonic for CVM.

Fetal diagnostic pathology constitutes a separate problem regarding CVM because several lesions may be difficult to recognize, i.e., slight arthrogryposis. In fetuses, an initial diagnosis of CVM must be based on the presence of vertebral and costal lesions. However, genotyping is necessary to obtain a definitive diagnosis. Information on descent and paternal genotyping
results, which are available in several countries, may be helpful in establishing a presumptive etiological diagnosis in routine diagnostic pathology.

Sources and manufacturers

a. Perkin-Elmer Life Sciences Inc., Boston, MA.

References


