

Evaluation of the Inheritance of the Complex Vertebral Malformation Syndrome by Breeding Studies

By J. S. Agerholm¹, O. Andersen³, M. B. Almskou³, C. Bendixen⁴, J. Arnbjerg², G. P. Aamand³, U. S. Nielsen³, F. Panitz⁴, and A.H. Petersen⁴

¹Laboratory of Pathology, Department of Veterinary Pathobiology and ²Department of Clinical Studies, Radiology, The Royal Veterinary and Agricultural University, Frederiksberg, ³Danish Cattle, Skejby, and ⁴Institute of Agricultural Sciences, Department of Animal Breeding and Genetics, Tjele, Denmark.

Agerholm JS, Andersen O, Almskou MB, Bendixen C, Arnbjerg J, Aamand GP, Nielsen US, Panitz F, Petersen AH: Evaluation of the inheritance of the complex vertebral malformation syndrome by breeding studies. Acta vet. scand. 2004, 45, 133-137. – To investigate the congenital complex vertebral malformation syndrome (CVM) in Holstein calves, two breeding studies were performed including 262 and 363 cows, respectively. Cows were selected from the Danish Cattle Database based on pedigree and insemination records. Selected cows were progeny of sires with an established heterozygous CVM genotype and pregnant after insemination with semen from another sire with heterozygous CVM genotype. Following calving the breeders should state, if the calf was normal and was requested to submit dead calves for necropsy. In both studies, significantly fewer CVM affected calves than expected were obtained; a finding probably reflecting extensive intrauterine mortality in CVM affected foetuses. The findings illustrate increased intrauterine mortality as a major potential bias in observational studies of inherited disorders.

Complex vertebral malformation; CVM; abortion; congenital; Holstein; cattle.

Introduction

Complex vertebral malformation (CVM) is a lethal syndrome of Holstein cattle, which in premature and mature calves is characterised by congenital growth retardation, malformed vertebrae, and tetramelic arthrogryposis (Agerholm *et al.* 2001). Genomic studies have shown, that the syndrome is autosomal recessively inherited (Bendixen *et al.* 2002). Statistical analyses of breeding data have demonstrated reduced maternal fertility if carriers of CVM are mated; an observation probably due to extensive intrauterine mortality in CVM affected foetuses (Nielsen *et al.* 2003).

Breeding studies are often used to evaluate the inheritance of congenital disorders in domestic animals. In cattle, selection of relevant individuals from the general population for such studies is often possible, if the genotype of sires used for artificial insemination is known. Such an approach was unsuccessfully applied on the CVM syndrome shortly after the identification of the disorder. In the present study, the results of 2 breeding studies are reported illustrating the severe effects of intrauterine mortality on the segregation patterns seen in premature and mature progeny.

Materials and methods

Two breeding studies (A and B) were performed. The design was largely similar in both studies. Based on pedigree and insemination records cows or heifers which were progeny of a sire with heterozygous genotype for CVM, and which had been inseminated with semen from another carrier were selected from the Danish Cattle Database. Using this design, a ratio of 1:7 between CVM affected and unaffected calves with equal numbers of affected males and females was expected.

Animals with estimated calving date between May 15th 2000 and July 11th 2000 were included in study A, while study B included calvings from April 8th 2001 to May 21st 2001. The genotypes of the sires were partly determined by previously performed necropsies of affected progeny (Agerholm et al. 2001) partly by genotyping.

The breeding data were based on the recorded pregnancy status of the cows on the day of data extraction which was done within 8 weeks prior to expected calving date.

Questionnaire

Prior to the expected calving date, the breeders received a questionnaire and a description of CVM. Following calving the breeder should state, if the calf was aborted, was stillborn or died within the first 7 days following parturition, or if it was alive. In addition, the farmer should state, if the calf showed signs of CVM. The farmer was requested to submit stillborn calves and calves dying within the first 7 days for necropsy, whether they seemed malformed or not. If calves showed no signs of CVM 7 days old, they were considered to be normal.

Selection of animals, study A

Two-hundred-and-eighty-six cows being progeny of the bull T Burma (DK230104) and already inseminated with semen from the sire

KOL Nixon (DK234042) were selected. Both sires were carriers of CVM. The cows were selected consecutively starting with cows having an estimated calving date of May 15th 2000.

Selection of animals, study B

In this study 382 cows and heifers with estimated calving date from April 8th 2001 and onwards were selected consecutively. The animals were progeny of 16 CVM heterozygous sires and had been inseminated with semen from one of 13 carriers. However, approximately 81% of the calvings were the result of insemination with semen from the sires T Klassy (DK 236398), Etazon Lord Lily (NLD780180664), or T Laluffe (DK237454) on cows being daughters of NJY Hubert (DK18382), VE Thor (DK 227511), or T Burma (DK230104).

Laboratory examination

Necropsy was performed on all calves submitted and included radiological examination of the vertebral column. Radiographs were taken of a specimen of the entire vertebral column following removal of the arcus (except caudal vertebrae) and the spinal cord to obtain optimal radiographs of the vertebral bodies. From precolostral calves specimens of lung and spleen were examined for bovine virus diarrhoea virus (BVDV) and pleural effusions for antibodies against BVDV (Agerholm et al. 1997). Muscle tissue was sampled for genotyping.

Genotyping method

DNA was purified from 0.25-0.5 cm³ muscle tissue using a method 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 (PerkinElmer Life Sciences). This genotyping method is based on an initial PCR amplifi-

Table 1. Results from breeding studies A and B, including 262 and 363 cows, respectively showing the ratio between phenotypically normal calves and calves having complex vertebral malformation (CVM).

	Study	No. of calves examined by breeder	No. of calves examined by necropsy	Total
Stillborn, normal	A	14	8	22
	B	6	19	25
Died day 1-7, normal	A	0	3	3
	B	0	1	1
CVM affected	A	5	10	15
	B	3	8	11
Alive 7 days old, normal	A	226	0	226
	B	343	2	345
Total	A	245*	21	266
	B	352#	30	382

* Including 4 twins
Including 19 twins

cation of the locus containing the single-base mutation followed by a specific template-directed single-base extension at the mutation site. Fluorescent signals revealing the nature of the base at the mutation site was detected by fluorescence polarization in a Victor² (PerkinElmer Wallac).

Primers for PCR amplification of the CVM locus:

SLC_F: 5'-GGC CCT CAG ATT CTC
AAG AGC-3'

SLC_R: 5'-CGA TGA AAA AGG AAC
CAA AAG GG-3'

Primer for template-directed single-base extension:

SLC_upper: 5'-GGC TCA CAA TTT GTA
GGT CTC ATG GCA-3'

Statistical analyses

The null-hypotheses: no differences in the observed and the expected frequencies in each study and in the combined study were tested by means of Chi-square tests. Twin calves were assumed to be independent individuals, as the impact from monozygotic twins was considered minimal.

Results

Of the 286 cows included in study A, 24 cows had aborted without examination of the foetus before the questionnaire was received. The remaining 262 cows gave birth to 15 CVM affected calves and 251 calves apparently normal calves (Table 1). The sex of affected calves was recorded in 14 cases of which 8 were males and 6 were females. The expected segregation ratio between affected and non-affected calves at calving was 33.25 to 232.75. However, significantly fewer defective calves than expected were found ($\chi^2 = 11.45$, $p < 0.001$). The difference between the observed numbers of males and females and the expected 1:1 ratio was not statistically significant ($p > 0.05$). One CVM affected calf was aborted prior to gestation day 260, while 14 cases were delivered after day 260.

In study B, 382 cows were included. However, 19 cows aborted without examination of the foetus. The remaining 363 animals gave birth to 382 calves of which 11 cases were classified as CVM affected (Table 1). Of these, 7 were females and 4 were males. All were delivered after gestation day 260.

The expected ratio between CVM affected calves and normal calves in study B was 47.75 to 334.25. As in study A the number of CVM affected calves at calving was significantly lower than expected ($p < 0.0001$), while the difference between the observed and expected ratios of affected males and females was statistically non-significant ($p > 0.05$).

Due to low quality of the routine abortion registrations in Danish Cattle Database, it was impossible to obtain detailed information on the 43 cows that had an unregistered abortion. Consequently, these cows were excluded.

Statistical analysis of the segregation ratios demonstrated that the observed ratios did not differ between the studies ($p > 0.05$).

In general, lesions in CVM affected calves were similar to those previously reported (Agerholm *et al.* 2001) consisting of growth retardation, arthrogryposis and vertebral, costal, and cardiac malformations. Additional malformations were found in few cases.

Malformations were found in 8 necropsied calves not affected by CVM. One calf had malformed thoracic vertebrae, cerebellar hypoplasia and multiple renal cysts and was categorised as CVM unaffected based on genotyping. The calf had precolostral antibodies against BVDV. Except for this calf, accordance between necropsy findings and genotyping results was found.

Another calf had Arnold-Chiari malformation associated with lumbar spina bifida and bilateral posterior arthrogryposis, while an other had tetralogy of Fallot and bilateral abdominal cryptorchidism. Additional cases included 2 calves having an interventricular septal defect, one calf having a serous cyst attached to the hepatic surface, and 2 cases of freemartinism.

Discussion

The study demonstrates that significantly fewer CVM affected calves than expected were re-

peatedly obtained. In study A, 45.1% of the expected CVM affected calves was found, while only 23.0% was obtained in study B. As the inherited basis for CVM has been determined (Bendixen *et al.* 2002), it is most likely, that the prevalence of affected calves was reduced due to a high rate of intrauterine mortality. Statistical analyses of breeding data have revealed an extensive foetal mortality between gestations days 56 and 260, while losses prior to day 56 was found non-significant (Nielsen *et al.* 2003). Another possible explanation for the unobserved CVM cases could be an incomplete penetrance of the CVM genotype. However, this seems less likely, as extensive genotyping investigations have failed to identify viable CVM affected cases (Bendixen, personal observation).

The study was based on cows in late gestation. Therefore, cows with a registered abortion/reinsemination or dead/slaughtered animals were not selected except for those with an unregistered event. Retrospectively, it was impossible to evaluate the reproduction data in detail due to poor quality of the data related to early pregnancy. To obtain detailed information on the reproductive consequences of CVM, a longitudinal study based on close monitoring of the pregnant animals throughout the entire gestation period should be performed.

This study clearly demonstrates some of the problems related to breeding studies. Selecting animals from the general population based on pedigree information and pregnancy status is a fast and economically attractive method compared to controlled breeding trials. However, it is a major prerequisite that prenatal losses are negligible, that the genotype is expressed at the time of examination, and that progeny is available for examination. In this study, the first of these preconditions was not fulfilled leading to inconclusive results regarding the inheritance of CVM.

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References

- Agerholm JS, Willadsen CM, Nielsen TK, Giese SB, Holm E, Jensen L, Agger JF: Diagnostic studies of abortion in Danish dairy herds. *J. Vet. Med. A* 1997, 44, 551-558.
- Agerholm JS, Bendixen C, Andersen O, Arnhjerg J: Complex vertebral malformation in Holstein calves. *J. Vet. Diagn. Invest.* 2001, 13, 283-289.
- Bendixen C, Svendsen S, Jensen H, Panitz F, Aasberg A, Holm LE, Horn P, Høj A, Thomsen B, Jeppesen M, Nielsen VH, Jonker M: Genetic test for the identification of carriers of complex vertebral malformations in cattle. World Intellectual Property Organization, 2002. Publication No. WO 02/40709 A2.
- Nielsen US, Aamand GP, Andersen O, Bendixen C, Nielsen VH, Agerholm JS: Effects of complex vertebral malformation on fertility traits in Holstein cattle. *Livestock Prod. Sci.* 2003, 79, 233-238.

Sammendrag

Undersøgelse af arvelighed af complex vertebral malformation syndrom ved avlsstudier.

Med henblik på at undersøge det medfødte syndrom complex vertebral malformation (CVM) hos kalve af Holstein racen blev to avlsstudier med henholdsvis 262 og 363 køer gennemført. Køerne blev udvalgt fra den danske kvægdatabase på baggrund af deres afstamning og insemineringsregistreringer. Udvalgte køer var afkom af tyre med en kendt heterozygotisk CVM status og desuden drægtige efter inseminering med sæd fra en tyr med kendt heterozygotisk CVM status. Efter kælvning skulle besætningsejeren oplyse om den fødte kalv var normal og blev desuden anmodet om at indsende døde kalve til obduktion. I begge studier fandtes et signifikant lavere antal CVM afficerede kalve end forventet; et fund der sandsynligvis skyldtes høj intrauterin mortalitet hos CVM afficerede fostre. Undersøgelsen viser betydningen af intrauterin mortalitet som en væsentlig potentiel fejlkilde ved studier af arvelige sygdomme.

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Reprints may be obtained from: Dr. J.S. Agerholm, Department of Veterinary Pathobiology, The Royal Veterinary and Agricultural University, Bülowsvej 17, DK-1870 Frederiksberg C, Denmark. E-mail: jager@kvl.dk.