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Brief Communication

PCR Screening for Carriers of Hereditary Citrullinaemia in Danish Holstein-Friesian Bulls

Bovine citrullinaemia is a hereditary metabolic disease caused by impairment of the urea cycle. It was first described in Friesian cattle in Australia (*Harper et al.* 1986), where it has become widely distributed due to the use of carrier bulls for artificial insemination (*Healy et al.* 1990). The molecular basis for the disease is a single base mutation (cytosine converted to thymine in the mutant) resulting in a nonsense codon and thus causing premature chain termination (*Dennis et al.* 1989). Therefore, the normal metabolism of citrulline to argininosuccinic acid by the enzyme argininosuccinate synthetase (ASAS) is impaired; the result is an elevated citrulline concentration in plasma and cerebrospinal fluid and hyperammonaemia (*Healy et al.* 1990).

The clinical symptoms of bovine citrullinaemia are those of a severe progressive dysfunction of the higher central nervous system leading ad mortem with the first 6 days of life (*Healy et al.* 1990). The clinical symptoms and histopathological changes found in bovine citrullinaemia do not seem pathognomonic (*Harper et al.* 1986 & 1988, *Healy et al.* 1990). A specific diagnosis is based on either a markedly elevated plasma citrulline concentration without elevation in argininosuccinic acid concentration (*Harper et al.* 1986) or by identification of the mutant allele using restriction enzyme analysis (*Dennis et al.* 1989). Using the PCR (Polymerase Chain Reaction), the specific amplification of a fragment of the ASAS gene including the mutant site is possible. Further, it is possible to analyze this fragment by restriction enzyme cleavage and thus to distinguish between the normal and the mutant allele. In human medicine, the PCR method has already provided rapid, non-radioactive diagnostic tests for several diseases e.g., sickle cell anaemia (*Kazazian* 1989).

Based on the Australian experiences, we investigated Danish Holstein-Friesian bulls for this particular mutation of the ASAS gene. Semen samples of 72 young bulls from 5 breeding stations were obtained. All the bulls were first to third generation of imported American Holstein-Friesian belonging to mainly 2 lines, which either had the bull Elevation or Arli Chief in the pedigree.

DNA from an Australian Friesian heterozygote (ASAS/asas) and a homozygous normal (ASAS/ASAS) for ASAS deficiency was generously provided by J. A. Dennis, Elizabeth Macarthur Agricultural Institute, Australia. DNA from sperm of Danish Holstein-Friesian bulls was isolated by washing the spermatozoa from 1 straw of each bull 4 times in 1 ml of 0.9 % NaCl. After draining the washed spermatozoa they were resuspended in 200 µl of 200 mmol/l NaOH supplemented with 300 µg of dithiothreitol (Sigma), and heated at 100°C for 10 min. After neutralizing with 200 µl of 200 mmol/l Tris-HCl (Sigma) the sample was centrifuged for 15 min at 13,000 rpm. Approximately 100 ng, which corresponds to a 0.1–2 µl aliquot of the supernatant, was used for PCR.

The primers for amplification of the region

of the bovine ASAS gene were as described previously by *Dennis et al.* (1989): sense primer 5'-GTGTTCATTGAGGACATC-3' and antisence primer 5'-CCGTGAGACA-CATACTTG-3'. Both primers were synthesized by Genosys Inc. (Houston, Texas, USA). The PCR was performed essentially as described by *Saiki et al.* (1988). In brief, approximately 100 ng of the sample DNA was incubated in a final volume of 25 µl containing 50 mmol/l KCl, 10 mmol/l Tris/HCl pH 8.4, 1.5 mmol/l $MgCl_2$, 0.01 % gelatine, 5 picomol of each primer, 200 mmol/l of each dNTP and 0.625 IU Taq polymerase (Perkin-Elmer) at 94°C for 3 min, followed by 30–35 cycles of 1) 58°C for 1 min, 2) 72°C for 2 min and 3) 94°C for 30 s. Products of the amplification were digested with *Ava*II under conditions recommended by the supplier (Amersham). PCR-products and restriction enzyme digests were analyzed by gel electrophoresis on 4 % Nusieve 3:1 agarose gels.

PCR using template DNA of the obligate heterozygote (ASAS/asas), of the homozygote normal control (ASAS/ASAS) and of 72 breeding bulls with unknown genotype on the ASAS locus produced the expected 177 bp DNA fragment (Fig. 1, lanes U). The sizes of the PCR products and the corresponding DNA fragments after restriction enzyme cleavage are estimated using the sizes of DNA fragments from marker DNA in lane M-1 (*Pst*I-digested DNA of phage lambda) and M-2 (*Msp*I digested DNA of plasmid pBR322) shown at the left and right margin respectively. *Ava*II-digestion of this fragment from the normal control animal and all the breeding bulls resulted in cleavage into 2 DNA fragments as illustrated in Fig. 1, lanes C, #1, #3 and #4. Generally no band was present at 177 bp after *Ava*II-digestion, but sometimes a faint band indicating an incomplete digest could be observed. Contrary to these findings, the obligate heterozygote analyzed as a positive control showed the expected 3 bands, the most intense one at 177 bp indicative of the mutant allele without an *Ava*II site (Fig. 1, lane C of #2). It is concluded that all Danish Holstein-Friesian bulls investigated here have a normal genotype (ASAS/ASAS) at the ASAS locus.

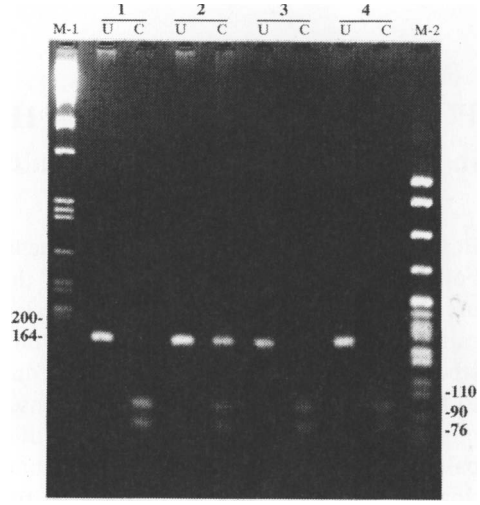

Figure 1. PCR-products of bovine genomic DNA using primers for the bovine argininosuccinate gene. DNA (100 nanogram) was amplified from a normal homozygote control (#1), a heterozygote control (#2) and two bulls showing the normal homozygous genotype (#3 and #4) and analyzed without (lanes U - uncut) and with (C - cut) digestions with *Ava*II. Sizes (in basepairs) of marker DNA fragments flanking the PCR-products or *Ava*II digests are shown at the left and right margins respectively. Marker DNA in lane M-1 is *Pst*I-digested DNA of phage lambda and in lane M-2 *Msp*I digested DNA of plasmid pBR322.

Although deficiency of the enzyme argininosuccinate synthetase could occur due to several inherited defects, including other mutations than the particular one analyzed for

in this study, the present results show that we can examine whether the latter mutation occurs in the Danish breeding population. This can be effective done by the PCR method using the primers described by *Dennis et al.* (1989). In this way, breeding bulls of unknown status as well as imported animals can be genotyped by submitting a semen sample for DNA analysis. It might be advantageous if such investigations could be combined with genotyping for other traits influencing production e.g., kappa-casein genotype (*Medrano & Aguilar-Cordova* 1990).

Clinical cases of bovine citrullinaemia have never been recorded in Denmark, but because of the rapid progression of this disease leading ad mortem and the general difficulties in identification of specific metabolic disorders, cases are easily overlooked, as happens with other congenital lethal enzyme defects. To increase the likelihood of detecting hereditary diseases in general a surveillance system has been implemented in Danish cattle (*Nielsen et al.* 1990).

Healy et al. (1991) reported that the widely used bull Linmack Kriss King (LKK) was a heterozygous of bovine citrullinaemia. The bull Athol Famous Prefect, which is a grandson of LKK, has been used in Denmark. However, the bovine citrullinaemia mutant sequence could not be demonstrated in Athol Famous Prefect (*P. Healy,* personal communication).

Because of the economic significance of hereditary diseases even time consuming and expensive procedures such as testmatings have proven worthwhile. The alternative use of PCR technique is cheap, and many animals can be screened within a short time. Because of the rapidly expanding knowledge of the bovine genome and of bovine genetic diseases, it is expected that this technique will become of increasing importance in identification of hereditary bovine diseases.

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