Chapter 4

Congenital myasthenic syndrome of Brahman cattle in South Africa

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Abstract

A congenital myasthenic syndrome in Brahman cattle is caused by a homozygous 20 base pair deletion (470del20) in the gene coding for the epsilon subunit of the acetylcholine receptor at the neuromuscular junction. It causes a progressive muscle weakness, starting either at birth or within the first month. A PCR-based DNA test, using blood or semen stored on FTA paper, was developed and validated; the test makes it possible to differentiate rapidly and accurately between homozygous wild-type, heterozygous and homozygous affected animals. Preliminary testing of Brahman cattle in South Africa has revealed several carrier animals, some of them influential animals in the breeding population.

Introduction

Congenital myasthenic syndromes in human beings are a group of heterogeneous disorders of neuromuscular transmission, arising from presynaptic, synaptic or postsynaptic defects. Postsynaptic disorders, resulting from mutations in the genes coding for the various subunits of the nicotinic acetylcholine receptor (nAChR), are most frequently described, with the epsilon subunit being most commonly affected (Engel and Ohno, 2002). The syndromes, which are also referred to as congenital myasthenia gravis, have been reported in dogs (Johnson et al., 1975; Flagstad, 1982; Miller et al., 1983; Wallace and Palmer, 1984) and cats (Indrieri et al., 1983), but their genetic basis remains unknown. Very few of the many inherited defects recorded in domestic animals have been characterised at the molecular level (Nicholas, 1999; Fyfe, 2002).

A suspected congenital myasthenic syndrome was recently reported for the first time in cattle (Thompson, 1998), and the underlying genetic defect was later identified as a homozygous 20-base pair (bp) deletion in the gene (bov*CHRNE*) coding for the ε-subunit of the nAChR at the neuromuscular junction (Kraner et al., 2002). This mutation (470del20) is predicted to result in a non-functional allele and the inability to produce functional adult-type nAChR, thus explaining the severe muscle weakness observed. It appears to be inherited in an simple recessive manner and heterozygotes are clinically normal. Two bulls whose semen had been widely used for artificial insemination were identified as carriers. This paper describes the clinical signs in the cases observed so far, the development of a screening test for the mutation and the preliminary results of further screening.

Materials and methods

Clinical syndrome

Clinical signs were originally observed in four closely related red Brahman calves and have been described in detail by Thompson (1998). Using the recorded pedigrees of

the affected calves, closely related animals were traced and their owners were questioned about the occurrence of similar clinical signs in their calves.

When the mutation responsible for the condition had been identified and a screening test had been developed (as described below) it was possible to test calves that were reported to be showing signs of muscle weakness. The clinical signs and post-mortem findings in a homozygous affected calf are described.

Screening test

Blood samples were collected by venepuncture from 26 relatives (first, second or third generation offspring) of the known carrier bulls, 14 unrelated red Brahmans and eight cattle of other breeds, into Vacutainer tubes containing EDTA as anticoagulant. An aliquot of 100 μl of blood was transferred to FTA paper (Whatman Bioscience), allowed to air dry, and stored at room temperature until analysed. A 2 mm punch was removed from the stored sample, placed in a 200 μl PCR tube, and washed three times with 200 μl of FTA reagent (Whatman Bioscience) and twice with 200 μl of TRIS-EDTA buffer. The punches were allowed to air dry for one hour before being processed by PCR.

A specific primer pair was used to amplify a 211-base pair segment of exon 5 of the bovine *CHRNE* gene. The sequence of the forward primer was 5′-CCAACTCTCGGTTTCCTGGA-3′ and that of the reverse primer was 5′-GGGCCTGCGAACAAGTAAGT-3′. The 5′ end of the forward primer was labelled with 6-FAM (Applied Biosystems). The PCR was carried out on a GeneAmp 9700 PCR System (Applied Biosystems) in a total volume of 25 μl, containing 2 pmol of the forward and reverse primers, 200 μM of each dNTP, 1.5mM $MgCl_2$, 50 mM KCl, 15 mM Tris-HCl (pH 8.0) buffer and 0.1 U AmpliTaq Gold polymerase (Applied Biosystems). The parameters of the PCR were as follows: denaturation at 95°C for 10 minutes, followed by 33 cycles of 95°C for 30 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 45 seconds, followed by a final extension step at 72°C for 10 minutes.

The PCR product was diluted 10-fold in water and 1 μl was mixed with 10 μl of HiDi Formamide (Applied Biosystems) and 0.25 μl of Genescan size standard. The PCR fragments were sized by capillary electrophoresis on an ABI 310 Genetic Analyser (Applied Biosystems). Further analysis was carried out using STRand software (Board of Regents, University of California) on a personal computer.

For verification, 30 aliquots of the same blood samples were screened independently at the Institute for Human Genetics, University Hospital Bonn, Germany. The DNA was extracted from 100 μl of whole blood by lysing the erythrocytes with sodium chloride-EDTA, and then lysing the leucocytes with sodium hydroxide and heat. Exon 5 was amplified by PCR, and the fragments were separated by polyacrylamide-gel electrophoresis as described by Kraner et al. (2002). The results were confirmed by sequencing one of each different sized fragment using an ABI 377 Genetic Analyser (Applied Biosystems).

The screening test was also adapted for processing fresh or frozen semen. A 1:10 dilution of semen was made with a semen-processing diluent (Schulman et al., 2002). Aliquots of 100 μl of semen were placed on separate pieces of FTA paper and allowed to dry overnight. A 2 mm punch was removed from the stored FTA paper containing each semen sample, placed in a 200 μl PCR tube, and 200 μl of FTA purification reagent (Whatman Bioscience), 20 μl of 1M dithiothreitol (Sigma-Aldrich) and 5 μl of proteinase K (Roche Diagnostics) (20 mg/ml) were added to each tube. The tubes were incubated at 56°C for one hour in a GeneAmp 9700 PCR System (Schulman et al., 2002). The punch was again washed twice with 200 μl of FTA purification reagent and twice with 200 μl of TRIS-EDTA buffer. The punches were allowed to air dry for at least one hour, and then processed and analysed as described for the blood samples.

Further screening

After the screening test had been validated, further testing was carried out. The 125 animals, other than unweaned calves, in the herd from which the original affected calves had come, were bled and tested, and 105 Brahman cattle attending a national show were also tested. Several farmers requested that some of their breeding cattle and stored semen should be tested. In addition, three Brahman herds reported having weak

calves; these calves, their dams and sires, and several other animals in these herds were tested. In an attempt to detect additional carriers among influential breeding animals, stored frozen semen from 68 Brahman bulls was tested; the selection of these samples depended upon the availability of stored semen and the willingness of the owners to have their animals tested.

Results

Clinical syndrome

One further affected calf was found, and confirmed to be homozygous for the 470del20 mutation. The owner reported that the heifer calf had been weak at birth and unable to rise. When helped to its feet it could stand and walk for 30 to 45 minutes before collapsing, and it could suck from its dam. The severity of its condition had fluctuated and then gradually deteriorated. However, it died at three months of age as a result of an abomasal and omasal volvulus, before it could be examined clinically. A post-mortem examination was carried out and the animal was found to be in fair body condition, without obvious muscle atrophy. It was markedly dehydrated and had a mild, acute to subacute bronchopneumonia, with some plant material in the bronchioli and moderate cerebral congestion. Its abomasum and omasum were twisted through 360˚ and distended, with foul-smelling, red-brown, watery contents. Histologically the muscles and peripheral nerves appeared normal. Its dam and sire were both tested, and confirmed to be carriers of the 470del20 mutation.

The owner of another herd, in which carrier animals were found, also reported having had several weak calves in previous years; they had apparently been normal at birth, but became progressively weaker after four to seven days of age. They had been destroyed after several weeks of gradual deterioration.

Screening test

Of the 26 related red Brahmans initially tested, eight were identified as heterozygous for the 470del20 mutation, and 18 were homozygous for the wild-type allele. All the 22 unrelated cattle were homozygous wild-type. Of the 30 samples tested at both laboratories, eight were carriers and 22 were homozygous wild-type. There was complete agreement between the laboratories in the results obtained. After computer analysis, the output of the PCR fragment sizing identified cattle homozygous for the wild-type allele by a single peak at 211 bp, heterozygotes by two peaks at 191 bp and 211 bp, and homozygous affected calves by a single peak at 191 bp.

Further screening

Of the 125 cattle tested in the herd in which the condition was originally detected, 15 (12%) were found to be carriers of the mutation; these nine cows and six bulls were all first, second, third or fourth generation descendants of one of the known carrier bulls. The screening of semen resulted in the detection of four carrier bulls, which had been widely used for artificial insemination, and three further carrier animals were detected by the screening of blood samples.

Testing animals in one of the three herds that had reported having weak calves, resulted in the detection of the affected three-month-old heifer calf described above. Testing the other two herds with weak calves showed that the mutation had not been the cause of the problem, although a single carrier was detected in one of the herds.

Of the 183 animals tested that were not selected because they were related to known carriers or because they were part of a herd reporting weak calves, three were found to be carriers.

Discussion

The full range of clinical signs of congenital myasthenic syndrome in cattle is not yet known and requires further investigation. In the cases so far observed, the calves have either been weak at birth or become weak during the first month of life. In humans, the myasthenic symptoms appear from birth or early childhood, but some cases may develop only in later life (Engel and Ohno, 2002). The reason for the survival of patients homozygous for *CHRNE* mutations is thought to be the persistent expression of the *CHRNG* gene coding for the foetal type γ-subunit (Engel and Ohno, 2002); the AChR ε-subunit normally replaces the γ-subunit during the perinatal period.

The cases observed have survived for several months, although they required assistance to gain access to their mothers' teats. Because most Brahman cattle in South Africa are kept under extensive conditions, it is likely that many cases go unobserved and die as a result of starvation or predation. The abomasal volvulus in one affected calf is unlikely to have been related to the underlying myasthenic syndrome, because nAChR containing the ε-subunit occurs only at the neuromuscular junction of skeletal muscle and not in the gastrointestinal tract (Utkin et al., 2000).

Perinatal calf mortality is an important cause of economic loss in the beef cattle industry (Wikse et al., 1994). Although many important risk factors have been identified, numerous cases remain undiagnosed. The "dummy calf" syndrome, most commonly observed in Brahman cattle, is significantly affected by the sire (Kim et al., 1988), indicating a possible genetic component in its aetiology. Investigation into the possible involvement of the 470del20 mutation in cases of idiopathic perinatal calf mortality and the "dummy calf" syndrome is therefore warranted. Congenital myasthenic syndromes have also been found in association with arthrogryposis multiplex congenita in humans (Brownlow et al., 2001), and it may therefore be interesting to screen calves affected by arthrogryposis for the 470del20 mutation.

As in humans heterozygous for *CHRNE* mutations, carriers of the 470del20 mutation appear to be clinically unaffected, and some have been top performers at shows and in performance evaluations.

Although the prevalence of the mutation is unknown, the discovery of three carriers amongst 183 randomly selected animals suggests a prevalence of 1–2%. Theoretically, with unselected mating and in a population of unlimited size, this would result in approximately six affected calves per 100,000 conceived. However, within certain herds the prevalence of carriers can be much higher; in the herd described above it was 12%, despite the farmer having discontinued the use of the carrier bulls eight years previously. It was also shown that there were carriers amongst stud bulls being used for artificial insemination.

An examination of the recorded pedigrees, extending back for up to 12 generations, has revealed no ancestors common to all the known carriers. However, it is unlikely that the 470del20 mutation occurred independently more than once within the Brahman population. The bov*CHRNE* sequence adjacent to the 470del20 mutation does not show any obvious features of a mutation hot-spot. In human patients, more than 50 *CHRNE* mutations have been found, most of them only once. Only one human mutation was detected in patients from several different families, most of which were of Gypsy origin and therefore probably distantly related (Abicht et al., 1999). A more plausible explanation for the frequency and distribution of the 470del20 mutation in South African Brahmans, would be a founder effect resulting from imported ancestors. The mutation is therefore also likely to be present in Brahman populations elsewhere in the world.

The validation of this screening test provides a rapid, inexpensive and accurate method for identifying carriers of the bov*CHRNE* 470del20 mutation responsible for this congenital myasthenic syndrome. It can therefore be used to investigate the prevalence and the clinical and economic importance of the mutation, and to implement a screening programme if it should be deemed necessary, as it has been for generalised glycogenosis in Brahman cattle in Australia (Dennis et al., 2002).

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