Molecular diagnosis and control strategies for the relevant genetic diseases of cattle

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ABSTRACT

The availability of the bovine genome sequence and the use of DNA markers have widened our understanding of a number of hereditary diseases in cattle, leading to the development of techniques for their early diagnosis. By isolating DNA from nucleated cell samples, followed by in vitro amplification techniques and digestion with restriction enzymes, it is now possible to detect the presence of lethal or mutant alleles for a specific phenotype. Such techniques are already being used for the study of genetic diseases of dairy cattle such as BLAD (Bovine leukocyte adhesion deficiency), CVM (Complex vertebral malformation), DUMPS (Deficiency of uridine-monophosphate synthase) and citrullinemia, among others, where the disorder is caused by the presence of a recessive allele in homozygosis. Although the frequency of these alleles in the population is usually very low, it can be easily increased if heterozygotic (carrier) sires are used during large-scale stockbreeding, ultimately resulting in significant economic losses; on the other hand, certifying the absence of such mutations in sires increases their value. Since there is a worldwide tendency towards the implementation of monitoring programs for hereditary diseases in cattle, it is important to update the technical personnel involved in cattle breeding (researchers and veterinary doctors) as well as farmers on the use and importance of DNA molecular markers in animal health.

Keywords: genetic bovine disease, molecular diagnosis

Diagnóstico molecular y estrategias para el control de enfermedades hereditarias importantes en ganadería bovina. El conocimiento del genoma bovino y la utilización de marcadores de ADN han permitido conocer el origen de algunas enfermedades hereditarias y desarrollar técnicas de diagnóstico precoz. Mediante el aislamiento de ADN a partir de muestras nucleadas y técnicas de amplificación in vitro y digestión con enzimas de restricción se puede diagnosticar si un animal es portador de un gen letal o mutante para determinadas características. En la actualidad es posible estudiar enfermedades hereditarias del ganado bovino lechero como la deficiencia de adhesión leucocitaria bovina (BLAD), la malformación vertebral compleja (CVM), la deficiencia de la enzima uridina-monofosfato sintasa (DUMPs) y citrullinemia, entre otras, cuyos orígenes están relacionados con la presencia de alelos recesivos. Si bien la frecuencia de estos genes mutantes es muy baja, debe considerarse que la utilización a gran escala de reproductores portadores puede incrementarla y generar importantes pérdidas económicas; por otra parte, el conocimiento de que un reproductor está libre de tales mutaciones, incrementa su valor agregado. Es por ello, la tendencia mundial a la implementación de programas de vigilancia para enfermedades genéticas, de aquí la importancia de divulgar tanto entre investigadores y médicos como entre los productores la utilización e importancia de los marcadores moleculares de ADN en la sanidad animal.

Palabras clave: enfermedad genética bovina, diagnóstico molecular

Introduction

Genetic diseases, caused by the vertical transmission of defective genes to the offspring, can lead to significant losses in agricultural yield during animal husbandry. The technological advances that have taken place in the fields of molecular genetics and bioinformatics during the last decades have enabled the identification in dairy cattle of the genes responsible for a number of important genetic diseases with a monogenic origin. In most cases, the defective allele carries a mutation that results in the synthesis of a non-functional protein variant, leading to developmental or metabolic disorders [1]. When this allele is recessive, heterozygotic (carrier) animals have a normal phenotype but can pass the genetic defect to their offspring; carrier individuals within the bovine livestock, therefore, imply the silent transmission of genetic defects and hence an increase in the chances of appearance of genetic defects among calves, with its associated economic implications.

From the viewpoint of farmers and breeders, genetic disorders constitute an insidious threat whose consequences often become evident only after several generations of crossing, when short-term, low cost solutions are no longer possible. The extensive use of individual and genealogic registries attests to the attention paid by breeders to this problem. Occasionally, a phenotypically normal elite sire carrying a recessive version of a defective gene has been used in large-scale breeding programs, with detrimental consequences amplified by the effects of the increased consanguinity of the progeny.

The changes or alterations underlying many hereditary diseases are currently known, making it possible not only to understand their development, but to diag-

nose their presence before the appearance of actual symptoms. New methodological approaches and molecular technologies that have been used in animal science for a number of years have endowed current geneticists with the necessary tools for the eradication of specific genetic disorders from animal populations [2], aided by the use of diagnostic methods based on molecular genetics in order to perform directed crossings for the obtention of disease-free calves.

The present work tries to review some theoretical aspects concerning the most relevant genetic diseases of bovine livestock, which are gaining an increased importance in the current situation of an ever greater demand for food among the human population, coupled to an increased need for sustainable and dependable sources of alimentation.

**Congenital defects**

Congenital defects can lead to abortions or have consequences after birth; although uncommon, they could be present in most cattle breeds. They may be physical or functional anomalies with environmental or genetic causes. Although congenital defects arising from environmental conditions are relatively easy to eliminate, those with a genetic origin constitute a much more complicated problem that poses, understandably, a much greater challenge for its correction [3].

**Environmental causes**

The appearance of congenital defects with an environmental source can be corrected by simply controlling the environmental factors that are known to have an influence on these disorders, such as the diet [4]. The economic losses caused by these defects are often smaller than those with a genetic origin.

Some of the telltale signs indicating an environmental factor as causative effect for a genetic anomaly:

1. Temporal coincidence with an environmental factor, and disappearance when the factor is eliminated
2. Occurrence of the same defect in groups of genetically unrelated animals
3. The symptomatology is similar to that of a well-known defect with an environmental origin

**Genetic causes**

Caused by the inheritance of an absent or otherwise non-functional (mutated or translocated) gene. The appearance of a genetic disorder due to the mutation of a single gene is not common, and most monogenic disorders are caused by a recessive allele [4]. In that case, a cross between two carrier parents produces a genetically healthy (normal homozygote) animal in only 25% of the cases, a carrier in 50% of the cases, and a recessive homozygote in the remaining 25% of the cases.

The main conditions indicating the presence of a genetic disorder are:

1. The disorder is present only in a group of genetically related animals
2. Similar symptomatology to other genetic disorders previously studied by crossing tests. They entail the use of karyotyping studies and molecular markers for a full identification of the underlying defect.

**Strategies for genetic diagnosis**

The strategies for the diagnosis of genetic defects can be split in two groups:

1. **Direct**: Based on the use of a molecular marker that directly identifies a mutation in a gene associated to a specific disease
2. **Indirect**: The diagnosis does not require the previous knowledge of the specific gene causing the disorder. It is based on the identification of a molecular marker showing a pattern of inheritance compatible with that of the disorder, followed by the examination of nearby genes (which will show genetic linkage to the marker) for the possible cause. These markers are often repetitive sequences (microsatellites) that are studied for co-segregation with the disorder.

**Bovine leukocyte adhesion deficiency**

Bovine granulocytopathy, also known as bovine leukocyte adhesion deficiency (BLAD), was first described in 1983 by Hagemoser et al. for a Friesian Holstein heifer [5]. The disease was characterized as a state of increased sensitivity to infectious agents during the first two years of life, with altered neutrophil function resulting in the inability of the animal to initiate an inflammatory response in spite of high neutrophil counts. Later, Nagahata et al. [6] described in Japan, in 1987, a similar disease characterized by a granulocytic syndrome affecting Holstein Friesian calves and heifers originating from the U.S. These authors used pedigree analysis to classify this syndrome as a genetic disorder with single autosomal recessive inheritance.

In 1990, Kehrli et al. [7] defined the molecular basis of bovine granulocytopathy as a deficiency in the Mac-1 glycoprotein complex (CD11b/CD18). Among the clinical signs and laboratory findings typical of the disease are recurrent infections of the soft tissues such as granulomatous and ulcerative stomatitis, enteritis, pneumonitis and periodontitis; defective scattering, death before sexual maturity, leukocytosis with marked predominance of neutrophils, persistent and progressive neutrophilia, moderate lymphocytosis and functionally abnormal neutrophils with altered motility as well as decreased phagocytic and oxidative capacity. The necropsy of affected animals reveals large numbers of neutrophils in sinusoid capillaries and blood vessels, in contrast to low numbers of extravascular neutrophils in tissues undergoing an inflammatory response, which are typically overloaded with microorganisms. The most frequently described histopathological lesions are necrotic enteritis, lymphoid hyperplasia and histiocytosis at the lymph nodes; the histological examination often reveals macroscopic lesions from pneumonia or pulmonary abscesses. Laryngitis and tracheitis, as well as myeloid hyperplasia at the bone marrow, have also been observed as a result of this disorder [8].

BLAD is lethal for the Holstein breed [9], where it leads to death of the animals at 2 to 8 months of age, with unspecific symptomatology. The affected individuals are phenotypically normal at birth, but start to suffer episodes of high fever, chronic diarrhea, impaired scattering, gingivitis and generalized infections after a few weeks. The infections are usually recal-

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citrate to antibiotic treatment, and the animal eventually dies. A similar disease is also found among Irish Setter dogs and in humans, where it is called leukocyte adhesion deficiency (LAD) and is caused by genetic defects affecting the LFA-1, p150, 95 and Mac-1 glycoproteins, also known as the CD11/CD18 beta-integrins by the World Health Organization. The affected children develop recurrent bacterial infections with persistent leukocytoses, and die at an early age if not treated by bone marrow transplantation.

The defective allele of the b subunit of the integrins from bovine CD18 has been identified and sequenced [10]. When compared to the sequence of the normal gene, this allele has two point mutations, one of them silent and the other resulting in a change from aspartic acid to glycine at position 128, in the highly conserved extracellular domain. This mutation affects the functioning of a protein receptor in leukocytes, and subsequently influencing the inflammatory and the immune responses against infections.

The presence of a localized infection in healthy animals usually attracts leukocytes to the infected tissue, where they target and kill the invading microorganism. This attraction is mediated by the appearance, on the walls of the surrounding blood vessels, of a number of specific molecules which are induced by the infection. Leukocytes anchor to these walls through interactions with these molecules using specific surface receptors, and then migrate through the vascular endothelium in order to reach the affected area. At a molecular scale, BLAD-associated mutations result in modifications of these specific receptors at the leukocyte surface, eliminating or decreasing the ability of the affected leukocyte to attach to the vascular endothelium of vessels in order to reach the infected tissue [11]; the net effect is that the affected animal is not able to control common bacterial infections, which then become recurrent or persistent. BLAD is a recessive autosomal disease, and can therefore be silently transmitted from carrier parents to the progeny.

The diagnosis of BLAD is usually based on the sequence of the cDNA from bovine CD18 (GenBank® accession number M81233), performing a PCR with the primers described by Tammen et al. [12] (5'GTC AGGAGATTGTCGTTCA-3' and 5'-GAGGTCA TCCACATTGAGT-3'), which introduce a new restriction site into the resulting 101 bp amplicon. Other authors digest the same fragment with restriction enzymes into the resulting 108 bp fragment, amplifying a 108 bp fragment from CD18 which was later sequenced and digested with Fnu4HI. The results allowed the assignment of a specific restriction pattern to the mutant genotype, and evidenced that the defect responsible for BLAD is not circumscribed to the Holstein breed.

**Complex Vertebral Malformation**

Complex Vertebral Malformation (CVM) was first described in Denmark in October 2000 for Holstein calves. The gene and the mutation responsible for the disease were later identified in 2001 by researchers at the Danish Agricultural Sciences Institute, and the source of the mutation was traced to the elite American sire Carlin-M Ivanhoe Bell. This sire is a BLAD carrier that has been extensively used in stockbreeding programs worldwide, increasing the mortality of the resulting Holstein calves [14]. Its father, Penstate Ivanhoe Star (USA 1441440, born January 20th, 1963), has recently also been characterized as a carrier [15].

The genetic defect responsible for CVM is inherited as a recessive autosomal trait [16-18], and calves carrying one copy of the defective allele may be phenotypically normal, without detectable developmental disorders. Carrier animals are usually notated with the code CV in pedigree analyses or lineage registers and non-carrier, healthy individuals are notated as TV; this convention is in the process of becoming an international standard [19].

The typical symptoms of CVM are malformations of the cervical and thoracic segments of the vertebrae column (fusion of the last two cervical vertebrae and disturbances in the 3 first thoracic vertebrae, respectively) that lead to mild scoliosis, mild symmetric bilateral contractions of carpal joints and shortening of the neck and anterior limbs with medial rotation of the latter. Occasionally, tarsal lengthening at both anterior limbs may be observed as well. An examination of the hearts of animals afflicted with CVM reveals a hypertrophy of the right side of the heart (where the pulmonary artery and aorta originate) with upper intraventricular defects in approximately 50% of the cases. CVM can also have an impact on pregnancy rates, with a large number of abortions around day 159 of pregnancy and significant numbers of prematurely born calves, which are usually dead [16, 17, 20-23]. Additionally, there are cases of intrauterine mortality that decrease the success rate of artificial insemination, causing further economic damage [24].

Although most cases of CVM result in growth disorders and vertebral malformations, the diagnosis of this disease is complicated by the wide range of symptoms under which it may appear, as well as the occurrence of phenotypically silent cases and vertebral phenocopies (e.g. BVDV). A presumptive diagnosis of CVM is reached, for most cases, through a combination of autopsy and pedigree analysis [25].

CVM is caused by a single G to T transversion at position 559 of the gene for bovine solute carrier family 35 member 3 (SLC35A3), located at chromosome 3 [26]. This gene codes for a UDP-N-acetylgalactosamine transporter, where the mutation replaces valine 180 by a phenylalanine residue [27]. The mutation can be identified, at the molecular level, by allele-specific PCR, by PCR-RFLP, or by PCR-PIRA [28].

The method is based on the introduction of a restriction site in the resulting amplicon, using allele-specific forward primers that anneal to the region spanning nucleotides 537 to 554 of the target gene and include either a Pat I or an EcoT22 site depending on whether the wild-type or the mutant allele has been amplified.
In herds where this mutation is known to be present, due to its simplicity, repeatability and low cost. PCR-SSCP can also be employed for setting a warning threshold when the frequencies of the mutated allele rise above 20 to 30% of the individuals.

The practical effect of this diagnostic tool is the identification of carriers, which is highly dependent on the exact nucleotide sequence of the molecule, allowing therefore the detection of mutations through the detection of changes in electrophoretic mobility for each allele [30]. The use of PCR-RFLP analysis for the detection of CVM carriers is based on the fact that the electrophoretic mobility of a single-stranded molecule of DNA depends not only on its molecular weight, but also its conformation. The conformation of single-stranded DNA, in turn, is determined by the base sequence, which may be read from 5’ to 3’ as well as from 3’ to 5’. The presence of the mutation in homozygosis compromises the growth and survival of the carrier calf, having clinical signs of intoxication due to hyperammonemia, as well as progressively worsening neurological symptoms that lead to death 1 to 2 weeks after birth [36]. Limmack Kriss King, one of the most widely used sires for stockbreeding programs in Holstein cattle, has been identified as heterozygotic carrier for CVM, and carrier (DP) animals was performed by digestion of the resulting fragment with Ava I.

Bovine citrullinemia

Citrullinemia is a recessive autosomal disorder of the metabolism of urea, originated by a deficiency in the activity of arginine-succinate synthase (ASS). This disorder was first identified in humans and later in dogs and Friesian calves. When present in homozygosis, the affected calves can not excrete ammonia and have clinical signs of intoxication due to hyperammonemia, as well as progressively worsening neurological symptoms that lead to death 1 to 2 weeks after birth [36]. Limmack Kriss King, one of the most widely used sires for stockbreeding programs in Holstein cattle, has been identified as heterozygotic carrier for citrullinemia.

The gene coding for ASS has been mapped to chromosome BTA11 in the bovine genome. Since citrullinemia is caused by the presence of a point mutation in this gene, it can be directly diagnosed by PCR-RFLP [37]. At a molecular level, the disease is caused by a cytosine to thymine transition at codon 86 from exon 5 of the ASS gene that can be amplified by PCR and verified by digesting the resulting amplicon with Ava I [15, 39].

Conclusions

Recessive autosomal genetic diseases are found at very low frequencies in cattle, and yet they have a disproportionate economic impact on agriculture. This situation has arisen from the massive dissemination of hereditary defects (some of which are outlined in this review) through the extensive use of elite sires that have turned out to be phenotypically silent carriers.
for a number of previously unknown genetic disorders, coupled to the intensive use of artificial insemination techniques.

Currently this situation appears to be under control, due in no small amount to the interest shown by associations of breeders for specialized genotypes (particularly for the Holstein breed) in different countries. However, it is still recommended to monitor young sires in order to eradicate these recessive alleles from bovine herds to the extent compatible with the maintenance of good agricultural traits. This requires the deployment of rigorous control systems in order to ensure that the appearance of recessive autosomal genetic disorders in cattle remains a manageable health problem for the foreseeable future.

Under very specific circumstances a breeder may need to obtain offspring from an elite sire which is, however, a known carrier for a genetic disease. In such cases, the crossing strategy must be designed beforehand, using healthy homozygotic partners whenever possible and closely monitoring the progeny, which has to be genotyped in order to guarantee that only healthy, dominant homozygotes are used as sires in future. The breeder may even choose to screen all the embryos obtained from carrier parents for added safety, transferring only those which have not inherited the mutant allele. Still, it is not recommended to use carrier sires in massive insemination campaigns, given the inherent costs of screening and genotyping the progeny.

It should be noticed that a number of genetic improvement programs in different countries have used male Holstein with females from other breeds, including local genotypes. Such a strategy requires a proper analysis of risks and benefits due to the possible propagation of Holstein-specific genetic defects, necessitating therefore the implementation of a genetic monitoring program in order to prevent the genetic erosion of local breeds. Likewise, massive artificial insemination campaigns must be closely followed in order to diagnose and confirm the presence of genetic disorders in those individuals with clinical symptoms suggestive of recessive autosomal defects.

The methods of analysis based on the detection of molecular markers are powerful tools for the control of the genetic makeup of bovine herds. The transfer of these techniques to the arsenal of stockbreeders and animal health personnel, therefore, constitutes a necessary requisite for the development of animal farming in animal biotechnology.

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