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Original Research Paper

GENETIC MONITORING FOR SEVERE COMBINED IMMUNODEFICIENCY CARRIERS IN HORSES IN SLOVENIA

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Summary: Severe combined immunodeficiency disease (SCID) is an autosomal recessive trait affecting purebred and mixed breed Arabian horses. Similar diseases exist in humans, dogs and mice. First immunodeficiency with characteristics of SCID in Arabian horses was reported around 1960 in Australia. The disease was described as a syndrome in 1973. The disease prevents the production of functional lymphocytes in affected individuals, which leads to a complete loss of humoral and cellular immune response. The defect is a result of a genetic mutation (5 bp deletion) in a gene encoding DNA dependent protein kinase C (DNA-PKc). As SCID is recessive autosomal disease, clinical signs are present only in offspring from mating between two heterozygous carriers of the SCID mutation. Heterozygous carriers are asymptomatic, but can be detected by genetic testing.

To identify the normal and mutant versions of the DNA-PKc gene, blood samples were collected from all Arabian horses in Slovenia and DNA extracted from the samples. The specific sequence of DNA-PKc including 5 bp deletion causing SCID was amplified using polymerase chain reaction (PCR) and the size of amplified DNA band was determined on a 4% agarose gel.

After successful amplification and interpretation of the DNA sequences, only negative results were found. We can therefore conclude that at present time there are no carriers of the mutation in DNA - PKc in Slovenia.

Key words: horse diseases; severe combined immunodeficiency-diagnosis-genetics; genetics, population; polymerase chain reaction; horses; mutation

Introduction

Severe combined immunodeficiency disease (SCID) is an inherited defect affecting pure or mixed breed Arabian horses (reviewed in (1-3). Affected individuals have a deficiency in both the number and function of B and T lymphocytes, resulting in an incompetent immune system. Heterozygous carriers appear normal but could transfer mutations to their offspring. In homozygous offspring, disease has 100% mortality due to immunodeficiency (2-4). Genetic cause of a SCID is a 5-base pair deletion within the catalytic subunit of DNA dependent protein kinase (DNA-PKc). Mutation causes a frameshift at codon 3155 of the transcript, resulting in 967 amino acid deletion from the C-terminus that includes entire phosphatidylinositol 3-kinase domain, thus making DNA-PKC functionally inactive (5, 6). It has been established that DNA-PKc deficiency produces an incomplete block in V(D)J recombination - the lymphocyte-specific process that is necessary for the expression of antigen receptors on B and T cells. This defect in V(D)J recombination blocks differentiation of B and T lymphocytes, resulting in profound immunodeficiency (3, 6, 7). Due to immunodeficiency, affected foals are highly susceptible to secondary infections which usually, without any treatment, cause death during the first month of age (2). The origin of the mutation in DNA-PKc gene and subsequently of SCID is unknown. Although the origin of the gene mutation has not been found, it must have initially occurred in a popular stallion used extensively for breeding to enable the spread of the mutation through the population of Arab horses. The disease is normally occurring in Arab horses, but there is also one report of affected Appaloosa foal which

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had an Arab stallion in fifth generation in maternal line (8). Affected foals are clinically normal at birth, but develop signs of infection during the first two months of life (2, 9). They have a deficiency both in numbers and function of B and T lymphocytes. First clinical signs usually occur between two days and six weeks of age. They include elevated temperature and increase in heart and respiratory rates. Foals are unthrifty, lethargic, easily tire but still nurse and eat solid feed. Bilateral nasal discharge, coughing and dyspnea due to respiratory infection often occur. Chronic diarrhea is present in some foals as well as alopecia and dermatitis. Lymphopenia (<1000/μl) and failure of immunoglobulin (IgM) synthesis are constant findings together with the absence of skin hypersensitivity (8). Total white blood cells count may be low, normal or increased (9).

Percentage of affected foals and heterozygous carriers differ between countries. For example, it was reported that 2.3% of 257 foals from 9 USA states were affected and 25.7% of adult Arab horses were carriers of the mutation (10). In another study in Australia, the percentage of homozygous affected foals was higher (8.3 percent of 204 tested; (11) while in U.K., only 2.8 % heterozygous carriers out of 106 tested animals were identified (12). In Brazil, 1.5 % carriers out of 205 tested horses were found (13).

In Slovenia, 63 Arabian and 19 part-bred Arab horses are registered, but were not yet tested for the presence of mutation in DNA-PKc gene. Most of them were imported from Poland, Hungary and Tunisia.

An accurate diagnosis of SCID is not only important because of the grave prognosis but it also provides information that both parents must be mutation carriers and should not be used for further interbreeding. Horses heterozygous for the SCID trait appear healthy, and in the past, the only way to identify heterozygous horses was by use of progeny testing (14). However, identification of mutation enabled development of genetic testing that make identification of carriers easier.

Material and methods

Animals

All pure breed Arabian horses (128) in Slovenia were involved in the present study. None of the animals showed any clinical signs of SCID or any other disease. 3 ml of blood was collected from vena jugularis and stored in tubes containing EDTA.

DNA extraction

Blood was centrifuged for 5 minutes at 3000 rpm to separate blood cells from plasma. Plasma was removed and blood cells were used for DNA extraction using WIZARD genomic DNA isolation kit (Promega, Madison, WI, USA) according to the manufacturer's instructions.

PCR analyses

Primers amplifying 136 bp long fragment of normal (without 5 bp deletion in affected animals) horse DNA-PKc gene were chosen based on Genbank sequence of horse DNA-PKc. Sequence of the primers was:

5'-primer: 5'- GTTGGTCTTGTCATTGAGCTG-3'

3'-primer: 5'- GCATCCGGATATCTGTTTGTC-3'

PCR amplification was performed in 0.2 ml thin walled tubes with following conditions:

initial denaturation for 5 minutes at 95°C followed by 30 cycles of denaturation at 95°C for 30 seconds, primer annealing at 48°C for 30 seconds and extension at 72°C for 1 minute, followed by final extension at 72°C for 7 minutes.

Amplification products were electrophoresed on specific 4% agarose gel for separation of small fragments (Promega) containing Ethidum bromide together with the 100 bp DNA ladder (Promega). Electrophoresis was performed under constant voltage 120V for 30 to 45 minutes. Amplified bands were visualized on UV transilluminator and captured onto computer using Hybaid GelGrab system (Hybaid, Wallisellen, Switzerland).

Results

After successful amplification using polymerase chain reaction, only DNA bands of expected size were observed on the agarose gel (Figure 1, representative sample) in all samples.

Careful examination of amplified DNA bands did not reveal any difference between the lengths of the bands or especially double bands that would indicate the presence of 5 base pair deletion and thus heterozygosity. The results therefore show that there are currently no heterozygous carriers of SCID among Arabian horses in Slovenia.

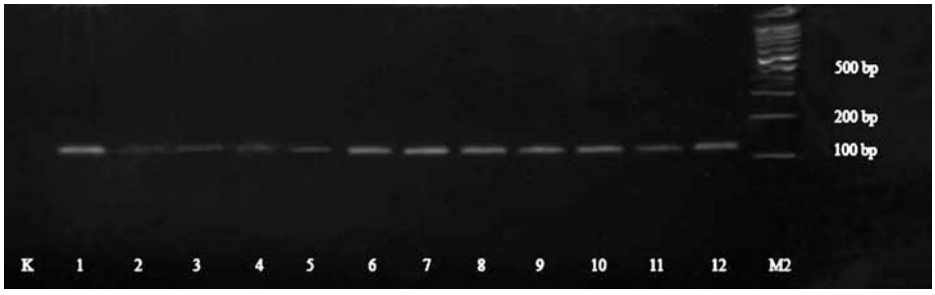

Figure 1: The results of DNA amplification with polymerase chain reaction. The picture shows a representative sample of 12 DNA samples from different horses.

Legend: M1 - DNA ladder (10 base pairs), K - control (no DNA), 1-12 - amplified DNK-PKc fragments; M2 - DNA ladder (100 base pairs).

Discussion

Even though hereditary diseases present only a small proportion of pathologies, they could present a significant problem with extensive economic losses. Inherited immunodeficiency diseases are important for the breeders, because animals without cellular and humoral immunity are unable to fight against foreign substances invading their bodies and consequently usually die due to secondary infections with opportunistic microorganisms (2, 3).

Studying genetic problems underlying certain hereditary diseases is important for several reasons. Firstly, knowing genetic causes of such conditions could help us understand basic physiological processes and how their malfunction leads to the development of the disease. Furthermore, understanding genetic causes of hereditary diseases usually also helps in development of diagnostic methods that could enable detection of healthy carriers of such diseases. Therefore, development of such diagnostic tests is extremely important for the breeders as detection of healthy carriers and their subsequent elimination from breeding programs could help with keeping populations free of certain hereditary diseases or even extermination diseases in certain populations and/or geographical areas. In last 20 years, development of molecular biology led to the development of ever simpler methods for mutation detection and unless the mutations that cause certain genetic disease are very heterogeneous, relatively simple and cost effective methods could be employed. PCR is extremely valuable tool that enables specific amplification of large quantities of DNA that can be used for subsequent analyses.

In the present study, all horses of Arab origin in Slovenia were tested for the presence of mutation in DNA-PKc gene that causes severe combined immunodeficiency syndrome. SCID is caused by a mutation in DNA-PKc which causes a defect in V(D)J recombination in lymphocytes (5, 6). Consequently, lymphocytes are unable to make antibodies against specific pathogens and therefore, affected animals do not have efficient immune system to defend against infections. In all known cases of SCID, disease was caused by a 5 bp deletion in DNA-PKc gene that causes a premature termination of the transcript (3, 10, 12). We employed a new simple method for detecting SCID mutations by using specific primers that amplified 136 bp long stretch of DNA-PKc gene that includes the deletion site. As there are no restriction sites within that 5 bp region to perform a restriction fragment length polymorphism (RFLP) analysis, we choose primers to amplify a fairly short fragment of DNA. Subsequently we used a high concentration (4%) of special agarose for detection of small fragments. According to manufacturer instructions as well as to our own experiences with this particular type of agarose gel, it is possible to detect differences of only 2 - 3 bp in short DNA fragments. Therefore, this method should be sufficient to detect carriers of the mutation in animals included in our study, as 5 bp difference between DNA fragments amplified from wild type and mutant allele should result in double band on the gel. In the present study, we did not find any mutation carriers, showing that epizootiological situation in Slovenia in regard to SCID is good. The origin of the disease is not known, however, the proportion of carriers differ between different countries. The highest percentage was reported in Arab horses in Australia and US (10, 11). Currently, most of Slovenian population of Arab horses can be traced to Hungary, Poland and Tunisia and although we do not have the data for the presence of SCID carriers in those countries, it is likely that occurrence of SCID in those countries is low, what would account for good situation in Slovenia.

SCID is a deadly disease and foals invariably die

within first months of age (2). Disease can be transmitted by natural mating, embriotransfer and artificial insemination. Because of simplicity and widespread use of new methods in animal breeding such as embriotransfer and artificial insemination, SCID as well as other inherited disease could spread easily between the countries. Therefore, it is even more important today than in the past to determine the presence of the carriers of genetic diseases. Classical methods such as selection against recessive genes are inefficient in complete elimination of disease as decrease in the frequency of the recessive gene causes an increasing proportion of recessive genes that are hidden from the effects of selection by occurring in heterozygous animals (12). Therefore, breeding programs for elimination of disease are much more efficient if carriers could be detected by testing their genome. With such methods, all the carriers could be detected and eliminated from breeding programs. Because SCID is not a sex-linked disease and the foal can inherit the defective gene from dam or sire, animals of both sexes must be tested. To prevent loss of some extremely important qualities of a horse, which is at the same time a carrier of the defective gene, some horses could still be used for the control breeding, but mating of two heterozygous animals should be avoided to prevent occurrence of disease with clinical signs. However, the mating of such animals should be carefully planned and recorded to prevent the spread of mutation in subsequent generations as 25% of offspring will inherit the mutation from their parent.

In some countries, SCID testing is already required for breeding Arab horses. In Slovenia, due to the absence of disease, small population of Arabian horses as well as little information about disease and cost the testing presents, the owners are generally not interested in testing their horses if tests would be commercial. However, due to favorable conditions at present, it would be desirable to introduce obligatory testing of all Arab horses imported to Slovenia for breeding purposes to prevent the introduction of SCID in Slovenian population of Arab horses.

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HUDO KOMBINIRANO POMANJKANJE IMUNOSTI PRI KONJIH V SLOVENIJI

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Povzetek: Hudo kombinirano pomanjkanje imunosti (severe combined immunodeficiency disease; SCID) je avtosomalna recesivna bolezen, ki se pojavlja pri arabskih konjih in križancih z njimi. Podobne bolezni obstajajo tudi pri ljudeh, psih in miših. Prvi primer bolezni z značilnostmi SCID je bil opisan leta 1960 v Avstraliji, leta 1973 pa je bila bolezen prvič natančno opisana in opredeljena kot genetski sindrom. Genetska napaka pri prizadetih živalih prepreči nastajanje delujočih limfocitov, zaradi česar so živali brez tkivne in celične imunske obrambe. Vzrok bolezni je mutacija (izbris petih baznih parov) v genu za od DNK odvisne proteinske kinaze C (DNK-PKc). Ker je SCID recesivna bolezen, se klinični znaki pojavijo samo pri homozigotnih potomcih heterozigotnih prenašalcev mutacije. Heterozigotni nosilci mutacije so popolnoma brez kliničnih znakov, zaradi česar jih lahko ugotovimo le z ugotavljanjem mutacije v genomu.
V predstavljeni raziskavi smo odvzeli kri vsem konjem arabske pasme v Sloveniji in iz nje osamili DNK. Z metodo verižne reakcije s polimerazo smo pomnožili del gena za DNK-PKc, ki je vključeval mutacijo (mesto izbrisa 5 baznih parov). Velikost pomnoženih verig DNK smo pregledali na 4% agaroznem gelu za ločevanje kratkih verig DNK. V vseh pregledanih vzorcih smo ugotovili le verige DNK pričakovane dolžine, iz česar lahko sklepamo, da trenutno v Sloveniji ni heterozigotnih prenašalcev mutacije v genu za DNA-PKc.

Ključne besede: konj, bolezni; genetika, populacijska; huda kombinirana imunska pomanjkljivost - diagnostika - genetika; verižna reakcija s polimerazo; konj