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Original scientific paper

PRP GENE POLYMORPHISM IN THE RED DEER (*Cervus elaphus L.*)

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Transmissible spongiform encephalopathy (TSE) belongs to the group of contagious disease that affects the nervous system with fatal outcome. Chronic wasting disease belongs to this group and is characteristic for animals from the deer family. In this study polymorphisms in eleven codons located in exon 3 of the PrP gene has been investigated red deer (*Cervus elaphus L.*) population. Exon 3 regions, 628 bp long, were amplified by the PCR method from genomic DNA. Sequencing was performed by applying the Sanger dideoxi method. Results showed presence of eight different genotypes and eight different haplotypes. Susceptible genotypes were not found at a high frequency.

Keywords: PrP, transmissible spongiform encephalopathy, Deer (*Cervus elaphus L.*)

INTRODUCTION

Transmissible spongiform encephalopathy in the deer (*Cervus elaphus L.*) population is represented by chronic wasting disease (CWD). This disease is known in the deer population for more than 30 years. First published records about chronic wasting disease originate from the eighties of the 20th century WILLIAMS and YOUNG (1980; 1982). Before these publications, biologists working on symptoms had already recognized it as a syndrome shortening life expectancy. Few years later, the disease had been recognized as contagious WILLIAMS (2005). Chronic wasting disease appears in two different groups of the deer family, in deer (*Cervus elaphus L*) and elk (*Alces*), both in captivity and the wilderness. Research on possible genetic impacts on the occurrence of the disease in deer has however, been done at a lower intensity then

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in case compare to sheep and goat. In one of first studies of the deer family *PrP* gene its DNA sequence has been published together with the derived sequences of amino acids, which were compared with sequences of other animals. It was found that the disease shows more often in animals that are homozygous at 132nd codon for methionine (M132M), O'ROURKE *et al*. (1999), compared with heterozygotes with amino acid combination methionine and leucine (M132L), or homozygote combination leucine (L132L). Although the natural occurrence of the disease was found in all three genotypes, it was hypothesized that the incubation period could be elongated in animals with M132L and L132L genotypes. If this is correct, it is possible to achieve selective advantages in captive animals with benefits regarding the control of chronic wasting disease by breeding for a longer incubation period. This is underpinned by experimental studies were the pathogen was cerebrally inoculated and animals with M132L genotype showed a longer incubation period compared to M132M. This difference was however, not significant. Anyway, genotype effects on the distribution of the pathogen in brain and lymphoid tissue have not been established up to now.

In deer, four additional polymorphisms have been discovered, located in codons 95 (Q95H), JOHNSON *et al*. (1996), 96 (G96S), O'ROURKE *et al*. (2004), 116 (A116G), JOHNSON *et al*. (1996) and 225 (S225F) WILLIAMS, (2005). Recent studies showed that all genotypes are CWD susceptible. The disease has been found in all major genotypes in white tailed deer (*Odocoileus virginianus*) in study conducted in Wisconsin and Nebraska (USA), and only a few animals, which were homozygotes on codon S96S showed a higher frequency of the disease than statistically expected JOHNSON *et al*. (2003); O'ROURKE *et al*. (2004). A study conducted in captive black tailed deer (*Odocoileus hemionus*) showed that heterozygotes with serine/phenylalanine on codon 225 (S225F) could be infected by oral ingestion of the pathogen, but the incubation period was extended compared with homozygotes on this codon (S225S). In an endemic area for this disease in Wyoming and Colorado (USA), it was found that the genotype (S225F) had a lowered frequency among diseased animals in from a population of 296 CWD positive animals, including one with S225F genotype and 295 carrying genotype S225S. Statistically 22 animals carrying genotype S225F were expected. Beside the polymorphisms on codons 132 and 225, which were found to have impact on disease development or an extended incubation period, other mutations have been found on codons G59S, T98A, P168S, Q226E and silent mutations on codons 15, 21, 78 and 136 WILLIAMS (2005).

In the deer family, the influence of the genotype on the incubation period of chronic wasting disease deterioration and its pathogenesis requires further research. Although apparently no single genotype can provide absolute immunity to the disease, genetics can have an impact on the development of strategies to control this disease. The aim of this study was to investigate polymorphisms that may be associated with the occurrence of transmissible spongiform encephalopathies in red deer.

MATERIAL AND METHODS

Research has been conducted on a deer population from the Republic of Croatia. Samples from 20 animals were taken from the earlobe during the regular hunting season, transported to our laboratory and stored at -70^0C until DNA isolation. For isolation, a small amount of tissue was taken from the earlobe, sample and processed according to the protocol as provided by the manufacturer of the kit, which has been used for isolation *FastDNA MP Biomedicals*. To measure the concentration of extracted DNA the absorbance at 260 nm and 280nm was measured, using a spectrophotometer (Genova, JENWAY, UK). The samples were

diluted 10x with diH2O (deionized water). Purity of DNA was checked by calculating the A260A280 ratio.
The quality of the resulting DNA was checked by gel electrophoresis on a 1% agarose gel, for 45 minutes at 120 V. Using the following primers PELETTO *et al*. (2009):

Forward 5' – ATTTTGCAGATAAGTCATC – 3'
Reverse 5' – AGAAGATAATGAAAACAGGAAG – 3',

Fragments of exon 3 PrP gene 771 bp long, has been amplified (EMBL –CDS: ACO53399). Conditions for polymerase chain reaction were optimized by using temperature gradient PCR (iCycler, BIORAD, Germany). PCRs were performed in 20μL of mixture with 38 cycles.

Conditions for PCR were:
1. Initial DNA denaturation for 2 minutes at 95^0C
2. DNA denaturation 45 sec at 95^0C
3. Annealing initials 30 sec at 54^0C
4. Polymerization for 1 minute at 72^0C (steps 2, 3 and 4 were performed in 38 cycles)
5. Final polymerization for 5 minutes at 72^0C.

PCR products were analyzed by electrophoresis on 0,8% agarose gels. Sequencing was performed by using the same primers as for PCR on an ABI PRISM® 3100 – Avant Genetic Analyser. Sequences were analyzed using

BioEdit software,version 7.0.9.0. HALL (1999). Sequences were aligned with the help of the Clustal program, version 2.2.10. LARKIN *et al*. (2007). First, we estimated alleles for the SSHL("scrapie susceptible haplotype locus") using the Bayesian approach. Furthermore, we estimated genotype and allele frequencies for SSHL and other loci (codons) showing polymorphism. All calculations were done by PROC Haplotype and PROC Allele implemented in SAS/Genetics 9.1.3. (SAS Institute, Cary, NC).
Genotypes were designed using the symbol for amino acids according to IUPAC (International Union of Pure and Applied Chemistry).

RESULTS

Table 1. Genotype frequency

Genotype – codon											Frequency (%)	n
26	78	95	96	98	116	132	136	138	206	225		
KK[1]	QQ	QQ	GG	TT	AA	MM	AA^2	SS	QE	VV	5.00	1
KK	QQ	QQ	GG	TT	AA	MM	A^2A^2	SS	QE	VV	10.00	2
KK	QQ	QQ	GG	TT	AA	ML	A^2A^2	SS	QE	VV	5.00	1
KK	QQ	QQ	GG	TT	AA	MM	A^2A^2	SS	EE	VV	10.00	2
KK	QQ	QQ	GG	TT	AA	MM	AA^2	SS	QE	VV	35.00	7
KK	QQ	QQ	GG	TT	AA	MM	AA	SS	QE	VV	15.00	3
KK	QQ	QQ	GG	TT	AA	MM	AA	SS	QQ	VV	15.00	3
KK	QQ	QQ	GG	TT	AA	MM	AA	SS	QQ	VV	5.00	1

In the population under study eight genotypes has been found (Table 1). The most frequent genotype was KQQGTAMASQV/KQQGTAMA2SEV with 35% frequency, while the genotypes KQQGTAMASQV/K1QQGTAMA[2]SEV, KQQGTAMA[2]SQV/KQQGTAMA[2]SEV and KQQGTAMASQV/KQQGTAMASQV had the lowest observed frequencies of 5%, respectively. This is equivalent to a single occurrence of the genotype.

Table 2.Haplotype frequency

Haplotype	n	Frequency (%)
K[1]QQGTAMASQV	1	2.50
KQQGTAMA[2]SQV	3	7.50
KQQGTAMA[2]SEV	14	35.00
KQQGTAMASQV	14	35.00
KQQGTAMASEV	3	7.50
KQQGTALA[2]SEV	1	2.50
KQQGAAMA[2]SEV	3	7.50
KQQGAAMASQV	1	2.50

[1]Silent mutation, AAA → AAG; K → K; [2]GCC→GCT; A→A

The most frequent haplotypes (Table 2) were KQQGTAMA2SEV and KQQGTAMASQV with frequencies of 35%. The lowest frequencies were observed for the following haplotypes: KQQGAAMASQV, KQQGTALA2SEV and K1QQGTAMASQV, which were 2.5% in all cases.

Table 3. Polymorphism and frequency of SNPs

Locus	Allele	n	Frequency (%)
26	K_{26} AAG	39	97.50
	K_{26} AAA	1	2.50
78	Q_{78} CAG	39	97.50
	Q_{78} CAA	1	2.50
95	Q_{95} CAA	40	100.00
96	G_{96} GGT	40	100.00
98	T_{98} ACC	36	90.00
	A_{98} GCC	4	10.00
116	A_{116} GCA	40	100.00
132	M_{132} ATG	39	97.50
	L_{132} CTG	1	2.50
136	A_{136} GCC	21	52.50
	A_{136} GCT	19	47.50
138	S_{138} AGC	40	100.00
206	Q_{206} CAG	19	47.50
	E_{206} GAG	21	52.50
225	V_{225} GTA	40	100.00

In this study eleven loci of the PrP gene were analyzed, and polymorphisms has been found on six of them (26, 78, 98, 132, 136, and 206, see Table 3). On the 26th locus there was a silent mutation for lysine. Occurrence of silent mutations was recorded on 78 and 136 loci responsible for glutamine and alanine synthesis. Mutations in all other polymorphic loci result in amino acid changes. These mutations had a maximum frequency at the locus 206, where alleles encoding for glutamine Q (47.5%) and glutamic acid E (52.5%) were found.

DISSCUSION

Chronic wasting disease belongs to the group of transmissible spongiform encephalopathies and is present in populations of the deer family (Cervidae). The disease occurs in animals in the wild as well as in animals on farms. Working long incubation disease is difficult to establish in animals in the wild, as a result of diseases of the nervous system, and the general weakness of the animals become easy prey to predators. Sick animals with long incubation period are hard to observe, since they are eradicated by predators during early life because of their weakness.

There is no indication that this disease exists in populations of the deer family and other wild ruminants in European countries. Just in case, the European Union conducts active surveillance to identify those potential outbreaks of disease in populations of deer.

In this sample from the population of deer in Croatia polymorphisms in codons 26, 78, 95, 96, 98, 116, 132, 136, 138, 206 and 225 were investigated. On the 26th codon a silent mutation for lysine (AAG → AAA) was found. The allelic frequency for AAA was 2.50%. No literature references indicating an association between these mutations with the risk of developing chronic wasting disease were found.

At codon 78 a silent mutation CAG → CAA was determined. This mutation has previously been observed WILLIAMS (2005), and is associated with the occurrence of the disease and by an extended incubation period. In some species of deer mutations at codon 95 of Q95H were observed. In our sample such mutations have not been found. Instead all animals were homozygous Q95Q.

At codon 96 no mutations were found although a polymorphism has been reported in the literature. All the genotyped animals proved to be homozygous G96G, whereas the heterozygous G96S variant was not found though it is associated with resistance to illness or the extension of the incubation period WILLIAMS (2005), JOHNSON *et al*. (2006), which should enhance its probability to be detected in a random sample from the living population.

Codon 116 was also monomorphic, A116A, an allelic variant of this codon, represent genetic resistance to the disease (DELWYN *et al*.,2008, PELETTO, 2009).

The most important role for the prevalence of chronic wasting disease in the deer has the codon 132 polymorphism, with the substitution of methionine for leucine. This substitution and the appearance of leucine in the protein offer some protection from the disease. WILLIAMS (2005), SEABURY *et al*. (2007), PERUCCHINI *et al*. (2008). The results showed that this substitution occurred in the study population at a frequency of 1% (M132L).

Mutations at codon 98 included an amino acid change from threonine to alanine. This mutation has previously been reported in the literature and has not been marked as important for occurrence of chronic wasting disease.

At codon 116 a change in the amino acids glycine to alanine, as observed in other studies (JOHNSON *et al*., 2003), has not been detected. The study population was monomorphic for the homozygous variant with GCA (A).

Codon 132 is often associated with the occurrence of the diseases. Variants M132L or L132L are considered to provide more resistance to the disease, while the less resistant variant is M132M. In the analyzed population a high frequency of the methionine variant (M) was found at a 97.50% frequency, while the frequency synthesizing leucine (L) was only 2.50%.

Codon 136 had a variant A136A. At this codon a silent mutation A → A, was found, which also may have an impact when it comes to animal resistance to disease. WILLIAMS (2005). At this codon silent mutation is present GCC → GCT (A → A). This mutation has already been documented in the literature WILLIAMS (2005). In the study population the frequencies were 52.50% for GCC and 47.50% for GCT. There are no data on the effect of these mutations on the occurrence or absence of disease in deer. At codon 138 serine was found at 100% frequency. There was no polymorphism at this codon. In previous research, there were no polymorphisms at codon 206 of PrP gene in deer reported. In our population under study a polymorphism at this codon was found: the amino acid glutamine (Q) (CAG) with a frequency of 47.50% and glutamic acid (E) GAG) with a frequency of 52.50%. Since this new polymorphism has not been previously reported in the literature its association with the genetic aspects of chronic wasting disease is unknown at the moment.

The codon 225 polymorphism is associated with the occurrence of chronic wasting disease (JEWELL *et al*. (2005). In animals which are resistant to the disease, or who have prolonged incubation time, there is the heterozygosity for phenylalanine and serine. Animals that are homozygous for serine are more susceptible to developing it. In the analyzed samples, valine was found at a frequency 100%. The valine variant belongs to the so-called wild type which has no impact on chronic wasting disease.

CONCLUSIONS

This is first conducted study on deer (*Cervus elaphus L*.) population in Croatia reflected on genetic aspects of CWD and PrP gene sequencing. These populations seems to be were similar to other populations in the world, with respect to its genetic characterization at the PrP locus and the lack possibilities for easy standardized genotyping of PrP variants remains a fundamental problem which should be solved in order to assess to genetic risk for CWD more precisely for any population.

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POLIMORFIZAM PRP GENA KOD CRVENIH JELENA (*Cervus elaphus L.*)

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Izvod

Prenosive spongiformne encefalopatije (PSE) pripadaju grupi zaraznih bolesti koje napadaju nervni sistem i koje za posljedicu imaju smrtni ishod. Hronična razorna bolest propadanja pripada ovoj grupi bolesti i karakteristična je za životinje iz porodice jelena. U ovoj studiji ispitivan je polimorfizam PrP gena na jedanaest kodona smještenih na egzonu 3. Regija egzona 3, dugačka 628 bp je umnožena pomoću PCR metode iz genomske DNK. Sekvenciranje je provedeno pomoću Sangerove dideoksi metode, Rezultati su pokazali prisustvo osam različitih genotipova i osam različitih haplotipova. Genotipovi koji su sumnjivi na razvoj bolesti nisu ustanovljeni u velikoj frekvenciji.

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