A specific selection programme is required in the autochthonous Cikta Sheep which is endangered by own frequent ARQ prion haplotype?

Endre Kovács*, Susanne Mitro**, Károly Tempfli*, Petra Zenke**, Ákos Maróti-Agóts**, László Sáfár***, Ágnes Bali Papp*, and András Gáspárdy**

Abstract

The authors study the current status of Hungarian Cikta Sheep based on genetic background of scrapie resistance. The aim of this investigation was to estimate the relative frequency of prion haplotypes, -genotypes, and risk categories as well as to reveal the efficiency of scrapie eradication program achieved over the last decade.

The authors confirmed based on larger sample size the previous knowledge, that the resistance against scrapie of Cikta breed is considered as low, and the classification of this breed according to risk category has not been improved. However, the frequent genotype ARQ and risk category 3 can also be considered for breed specificity. The careful use of these genotypes is permitted and will contribute to the maintenance of breed diversity according to other aspects.

Keywords: *scrapie resistance, Cikta, Zaupel, breed conservation*

Zusammenfassung

Ist für das autochthone Cikta-Schaf, das durch einen eigenen, häufig auftretenden ARQ Prion Haplotyp gefährdet ist, ein spezielles Zuchprogramm erforderlich?

Ist ein spezielles Züchtungsprogramm beim autochthonen Cikta Schaf erforderlich, das durch einen eigenen häufig auftretenden ARQ Prion Haplotyp gefährdet ist?

Die Autoren haben den ungarischen Cikta-Schafbestand auf seine genetisch veranlagte Scrapie-Resistenz hin untersucht. Es wurden zum Einen die Prion-Haplo- und Genotypen bestimmt und diese zum Anderen aufgrund der relativen Häufigkeit in entsprechende Risikogruppen eingestuft. Dadurch konnte die Wirksamkeit des seit zehn Jahren laufenden Programms der genetischen Prävention gegen Scrapie nachgewiesen werden. Dabei wurde die frühere Annahme bestätigt, wonach bei größerer Anzahl der Proben die genetische Resistenz gegen Scrapie noch immer als niedrig einzustufen ist. Die besondere Häufigkeit des Haplotyps ARQ und der Risikogruppe 3 sind als rassenspezifisch anzusehen. Zur Erhaltung der Rassenvielfalt muss diese Tatsache akzeptiert werden, um die wertvollen Eigenschaften des Cikta-Schafbestandes zu konservieren.

Schlüsselwörter: *Scrapie-Resistenz; Cikta-Schaf; Zaupel-Schaf; Erhaltungszüchtung*

* Széchenyi István University, Department of Animal Science, Vár tér 2, 9200 Mosonmagyaróvár, Hungary

** University of Veterinary Medicine, Department for Animal Breeding, Nutrition and Laboratory Animal Science, István 2, 1078 Budapest, Hungary

*** Hungarian Sheep- and Goat Breeders' Association, Lőportár 16, 1134 Budapest, Hungary

Contact: gaspardy.andras@univet.hu

1 Introduction

Despite considerable research, the exact lineages of subspecies and the subspecies of *Ovis ammon* are not completely resolved (Teichert and König, 1990; Ludewig, 1997; Wassmuth et al., 2001; Sambraus, 2010). When you ask an interested sheepherder or a lay person for different breeds, everybody will know a few. In the course of our investigation a today derivate of the long forgotten breed named "Zaupelschaf" (Fitzinger, 1860; May, 1868; Bohm, 1878; Adlung, 1912) comes into view. It is assumed, that historically the Zaupel sheep was the most important one within Germany (Korth, 1825).

The scrapie of small ruminants can be found all over the world and it plays an important role in veterinary medicine (Dexler, 1931; Rabenau, 2009). The scrapie as a generally lethal infectious, notifiable disease caused by a prion is manifasted in a degenerative change of the brain substance (Selbitz and Bisping, 1995; Bostedt and Dedié, 1996). The PrPC, which is a cellular protein is located on the surface of neurons. The PrPSc, which is its patogenic isoform) is not broken down at the cell membrane by the enzyme Proteinase K, compared to the normal (PrPC), and they multiply indefinitely, thus destroying all cells (Foster and Hunter, 1998; McCutcheon et al., 2005; Kang et al., 2017). Regulation of European Community No 999/2001 (EC, 2001) lays down rules for the prevention, control and eradication of transmissible spongiform encephalopathies (TSEs) in sheep among others.

According to state regulations, since 2005 suitable brain tissue samples for prion testing have to be taken from slaughtered and fallen animals, and from animals culled in the framework of eradication (infected herds).

Additionally, genotyping for scrapie resistance must also be carried out for the selection programmes including programs of autochthonous breeds.

The number of annual scrapie cases (affected herds) varied between 10 and 15 from 2002 to 2014 in Germany (Balkema-Buschmann et al., 2014). The first known case of scrapie in Hungary was diagnosed in 1964 (Áldásy and Süveges, 1964), since then only sporadically.

Our aims were to determine the prion haplotypes and genotypes against scrapie in the Cikta Sheep as the Hungarian descendant of the Zaupel Sheep (since it came to Hungary with Swabian emigrants in the 18th century; Seibold, 1990; Koppány, 2000; Haller, 2015) and to compare them to the results from 10 years ago in order to demonstrate the effectiveness of a prevention program introduced at that time.

2 Material and method

2.1 The Cikta Sheep

After the declining of the Zaupel Sheep they were displaced in some other regions and stayed there until the 19th century. In parts of Bohemia (Czech) a small stock of so called Sumavska sheep can be found. Also, a few animals reached Southern Hungary with the help of Swabian emigrants from the year 1720 because King Karl III., subsequently Queen Marie Therese and her son, King Josef II. invited German settlers to the depopulated areas liberated from the Turkish conquest.

To this day they are known under the name Cikta Sheep or Hungarian Zaupel (or Swabian Sheep of Counties Tolna and Baranya) with many preserved characteristics like course wool and sexual aseasonality (Figure 1). They are very modest and defy extreme weather conditions. However, a lambing season twice per year (in February and August) was often provoked.

Figure 1
Cikta ram No. 11123605 with shorn mothers in Domoszló (photo Ákos Balássy, 2013)

There was a possibility to introduce the Cikta Sheep to West Hungary since the German shepherds leased Hungarian pastures in order to keep their animals. Informally, they were called "Zibben-Schäfer"(birkás). The term "Zibben" (birka) was already used in the 16th and 17th century, at first for a Bohemian/Moravian breed. This was a variety of the central European Landsheep. In the north of the country, these herds were taken over by local farmers from Moravian shepherds. The term "Zibben" slowly became common in the whole country Hungary. Hence, the separation from the Racka Sheep was completed.

The breed would have fallen into oblivion if the interest in autochthonous breeds of farm animals had not have emerged. Due to a government regulation, Racka and Tsigai (cigája) breeds, as well as the still existing Cikta Sheep were collected within the country and relocated to the breeding institution of Bezzeg-puszta, in 1974. Since that time the state has supported autochthonous breeds in Hungary. The Hungarian Sheep- and Goat Breeders' Association (MJKSZ) re-established the herd booking for Cikta Sheep, and a central raising station for breeding rams developed in 2014.

The Cikta is a breed of smaller body size, its height at withers is about 58 to 60 cm, the ewes weight 35 to 45 kg and the rams 45 to 55 kg. Head and extremities are covered with short, white hairs, claws and horns are waxy-yellow, the skin is unpigmented. Staple length is 20 to 24 cm, the sortiment is B/C and C/D. The shearing weight of ewes per year is 1.5 to

2 kg and of rams is 2.5-3.5 kg with a rendement (wool base) of 65 to 70 %.

2.2 Sampling and analysis

MJKSZ employees collected biological samples (ear cartilage tissue) during the identification of the lambs using TypiFix™ (Agrobiogen GmbH, 2016).

Then the samples were sent to the Agrobiogen GmbH Laboratory for typing. The analysed fragment of the prion protein gene is marked with a primer and afterwards the DNA sequence is multiplied in vitro using PCR. Next, the characteristic parts as a result of mutations in a variant (haplotype) are determined through pyrosequencing. In codon 136 appears alanine (A, more resistant) or valine (V), in codon 154 arginine (R, more resistant) or histidine (H) and in codon 171 arginine (R, more resistant) or glutamine (Q) and histidine (H). For the genotypes the homozygous $A_{136}R_{154}R_{171}$/$A_{136}R_{154}R_{171}$ genotype will be the most resistant, while the homozygous $V_{136}R_{154}Q_{171}$/$V_{136}R_{154}Q_{171}$ genotype is the most susceptible (Baylis et al., 2002). Table 1 shows these grouped into the TSE (Transmissible Spongiform Encephalopathies) risk groups. According to the Hungarian national breeding program all rams should be genotyped, and only R1 to 3 rams are allowed to be bred. Preferably, if possible, R3 rams should be avoided. Animals with VRQ allele may leave the flock only for slaughter.

Table 1

PrP-genotypes of the sheep and their classification into different risk groups (A = alanine; V = valine; R = arginine; H = histidine; Q = glutamine; after Balkema-Buschmann, 2011)

Genotype	Risk category	Degree of resistance/susceptibility
ARR/ARR	R1	Sheep that is genetically most resistant to scrapie
ARR/AHQ ARR/ARH ARR/ARQ	R2	Sheep that are genetically most resistant to scrapie, but will need careful selection when used for further breeding
AHQ/AHQ AHQ/ARH AHQ/ARQ ARH/ARH ARH/ARQ ARQ/ARQ	R3	Sheep that genetically have little resistance to scrapie and will need careful selection when used for further breeding
ARR/VRQ	R4	Sheep that are genetically susceptible to scrapie and should not be used for breeding unless in the context of a controlled breeding program
AHQ/VRQ ARH/VRQ ARQ/VRQ VRQ/VRQ	R5	Sheep that are highly susceptible to scrapie and should not be used for breeding

In years 2013 to 2015 a total of 1145 individual samples (young rams n = 336 and ewes n = 809) from 10 flocks were analysed for current scrapie genotyping. This amount of sampling covered the whole Cikta population regarding the breeding animals and the breeding candidates.

The work of Fésüs et al. (2004; 2008; n = 69), the first investigation of Cikta in this field just before the National program (FVM, 2004) has entered into force served as a control condition, but which was related to one flock only.

The required data was taken from the Microsoft Excel database and statistically evaluated using the Dell statistics program (Dell Inc., 2015). The number of animals and the relative frequency were determined in haplotypes and genotypes of prion gene as well as in the risk groups of scrapie. Using the Chi^2-test the current condition (2013 to 2015) was compared to the former condition. Later on the similarity of males versus females was determined. Furthermore, the current whole- and share (by gender) Cikta Sheep populations were described concerning their genetic equilibrium (Hardy-Weinberg) status.

3 Results

Table 2 shows the distribution of the haplotypes from current years (2013 to 2015), where the most frequent (74.93 %) ARQ haplotype is followed by ARR (14.19 %) and AHQ (10.70 %). The occurrence of ARH and VRQ haplotypes is insignificant. The most resistant haplotype ARR of the current analysis, as well as in the former analysis (2004), shows much lower values than desirable. The Chi^2-test shows no significant difference (p = 0.519) between the current and the former results concerning the haplotypes. It needs to be mentioned, however, that in the former analysis made by Fésüs et al. (2004; 2008), the haplotypes ARH and VRQ were missing.

Only eight of the prion genotypes could be identified (Table 2). The most frequent genotypes were the least favorable ARQ-bearing genotypes which comes from the highest frequency of the ARQ haplotype. These are followed by the more favorable ARR- and AHQ-bearing genotypes. The most sensitive homozygous VRQ/VRQ did not occur. With regards to the genotypes, there were no significant differences (p = 0.083) between the populations over time, despite knowing that the least favourable genotype increased by 10 %.

The Chi^2-test proved that the current population of Cikta Sheep is in complete Hardy-Weinberg genetic equilibrium ($Chi^2 = 0.269$; df = 14; p = 1.000; the expected frequencies are not presented).

Risk groups with their changes can be found in the bottom part of Table 2. It should be highlighted that R4 is missing and R5 is represented by only one individual. Due to the high frequency of ARQ, R3 is present with almost 74 %, and only about 2.5 % are those animals that are best suited for breeding (R1). Between the two evaluations it was statistically proven (p < 0.031) that the Cikta Sheep has changed in terms of risk grouping. By increasing the rate of R1, or by a

Table 2

Prion haplo- and genotypes of Ciktka Sheep, as well as the distribution of scrapie risk groups

Groups	2004 observed, %	2013 - 15 observed, %
Haplotypes: Chi² = 3.235; df = 4; p = 0.519	(n = 138)	(n = 2290)
ARR	20.29 (28)	14.19 (325)
AHQ	9.42 (13)	10.70 (245)
ARH	0.00 (-)	0.13 (3)
ARQ	70.29 (97)	74.93 (1716)
VRQ	0.00 (-)	0.04 (1)
Genotypes: Chi² = 12.564; df = 7; p = 0.083	(n = 69)	(n = 1145)
ARR/ARR	1.45 (1)	2.45 (28)
ARR/AHQ	4.35 (3)	2.79 (32)
ARR/ARH	-	-
ARR/ARQ	33.33 (23)	20.70 (237)
AHQ/AHQ	0.00 (-)	1.31 (15)
AHQ/ARH	-	-
AHQ/ARQ	14.49 (10)	15.98 (183)
ARH/ARH	-	-
ARH/ARQ	0.00 (-)	0.26 (3)
ARQ/ARQ	46.38 (32)	56.42 (646)
ARR/VRQ	-	-
AHQ/VRQ	-	-
ARH/VRQ	-	-
ARQ/VRQ	0.00 (-)	0.09 (1)
VRQ/VRQ	-	-
Risk groups: Chi² = 8.846; df = 3; p < 0.031	(n = 69)*	(n = 1145)
R1	1.45 (1)	2.45 (28)
R2	37.68 (26)	23.49 (269)
R3	60.87 (42)	73.97 (846)
R4	0.00 (-)	0.00 (-)
R5	0.00 (-)	0.09 (1)**

* according to risk group correction because ARQ/ARQ was classified as R4 in the past

**the single VRQ-carrier individual, a ewe was prompt selected out

Table 3

Prion haplo- and genotypes of Cikta Sheep, as well as the distribution of scrapie risk groups according to sex

Groupes	Young rams 2013 - 15 %	Ewes 2013 - 15 %
Haplotypes: Chi² = 2.445; df = 4; p = 0.654	(n = 672)	(n = 1618)
ARR	15.48 (104)	13.66 (221)
AHQ	13.54 (91)	9.52 (154)
ARH	0.15 (1)	0.12 (2)
ARQ	70.83 (476)	76.64 (1240)
VRQ	0.00 (-)	0.06 (1)
Genotypes: Chi² = 7.425; df = 7; p = 0.386	(n = 336)	(n = 809)
ARR/ARR	3.87 (13)	1.85 (15)
ARR/AHQ	3.27 (11)	2.60 (21)
ARR/ARH	-	-
ARR/ARQ	19.94 (67)	21.01 (170)
AHQ/AHQ	2.38 (8)	0.87 (7)
AHQ/ARH	-	-
AHQ/ARQ	19.05 (64)	14.71 (119)
ARH/ARH	-	-
ARH/ARQ	0.30 (1)	0.25 (2)
ARQ/ARQ	51.19 (172)	58.59 (474)
ARR/VRQ	-	-
AHQ/VRQ	-	-
ARH/VRQ	-	-
ARQ/VRQ	0.00 (-)	0.12 (1)
VRQ/VRQ	-	-
Risk groups: Chi2 = 2.325; df = 3; p < 0.508	(n = 336)	(n = 809)
R1	3.87 (13)	1.85 (15)
R2	23.21 (78)	23.61 (191)
R3	73.81 (248)	74.41 (602)
R4	-	-
R5	0.00 (-)	0.12 (1)

remarkable increase in the rate of R3 a doubtful improvement can be seen.

In Table 3 a comparison was made between the observed frequencies for males and females. Although the values appear to be more favourable for males the p-values of Chi^2-test indicate that there are no statistically significant deviations in regards to haplotype-, genotype- and risk group frequencies by gender.

The chi-squared test preformed informs us about an existing genetic equilibrium in both male- and female share populations ($Chi^2 = 1.5947$, df = 9, p < [illegible] and $Chi^2 = 0.1162$, df = 14, p < 1.000, respectively; the expected frequencies are not presented).

4 Discussion

Our investigation and substantial sampling confirms the idea that genetic resistance of the Cikta Sheep population against a scrapie infection must be rated as low. At the same time, it should be noted that the risk classification of the Cikta Sheep (especially concerning haplotype ARR) has not improved. Possible reasons for that are the selection for breeding was restricted to the rams only, and the short period of time which was hindering an intense response

There have been no outbreaks of scrapie in Hungary for the last 10 years. Therefore, yielding an estimate of scrapie risk, especially in extensively kept grazing animals is difficult.

However, it is necessary to exclude the susceptible animals (with VRQ allele) from breeding and all in all, efforts should be made to use rams belonging to the risk group 1 to 2. Although the still frequent haplotype ARQ and risk group 3 must be considered as breed specific. Their use is permitted and the genotypes are also applied as markers for the preservation of breed diversity.

From the research of Baylis et al. (2004) it could be strongly concluded that the scrapie risk of homozygous ARQ/ARQ is greater than that of two VRQ-bearing heterozygotes (ARR/VRQ, AHQ/VRQ; there were no statistically significant differences in either case). Reassuringly it confirmed the considerable lower risk of scrapie in the ARQ/ARQ genotype than in ARQ/VRQ or VRQ/VRQ. This suspicion could lead to a more severe selection condition has been set in the Czech breeding program for resistance to scrapie: a permission for breeding is only given for VRQ carriers (Stepanek and Horin, 2017).

Future plans look to prevent the appearance of scrapie by increasing the use of rams (without haplotype VRQ) and eliminating VRQ-encoding individuals (if it occurs). The modification of AHQ, ARH and ARQ alleles can have a long stay of execution. Our results presented here strongly support the belief that the Cikta Sheep population that is free from VRQ will be largely resistant to the scrapie which can infect occasionally.

Due to the elimination program, the proportion of scrapie-resistant sheep (mostly in intensive breeds; Drögemüller et al., 2001) increased worldwide by today as evidenced by an increase in the ARR haplotype. However, we may encounter even worse and unsatisfactory results in other breeds than ours. For example, in the investigation of Cameron et al. (2014) the Canadian Arcott breed had higher proportions of susceptible sheep and a higher frequency of VRQ alleles (15 % VRQ in a population which consisted of 183 individuals) and a higher rate of disadvantageous R4 and R5 (over 10 % each).

In our study the unselected pool of male candidates showed the same values as the maternal level which is truly natural.

A gradual selection for resistant individuals and, in particular, resistant rams should be associated with a reduction in the possible incidence of scrapie. On the other hand the maintenance of productivity and genetic diversity must also be considered. Álvarez et al. (2007; 2009) concluded that ARR-heterozygotes should be first chosen before the beginning of a selective conservation programme, and not all the individuals of undesirable (particularly unacceptable) risk groups (R4 and R5) should be rejected for breeding to avoid the diminishing diversity of autochthonous sheep breeds.

These are the arguments for continuation of integrated programs such as the National Scrapie Plan for Hungary with a multiple trait conserving selection of rare breed.

Further objectives are to compare again the prion genotypes to other autochthonous Hungarian breeds and other Zaupel relatives survived in Germany (Bayerisches Waldschaf), Czech Republik (Sumavka) Austria (Krainer Steinschaf) and Slovenia (Bovska; Brem et al., 1982; Feldmann et al., 2005) where the frequency of VRQ haplotype equally high was (cit. Fésüs et al., 2004).

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