Ankara Üniv Vet Fak Derg, **66**, 261-265, 2019
DOI: 10.33988/auvfd.437314

Analysis of prion protein coding gene polymorphisms in Palestinian native sheep breeds

Osama ALSAYED[a], Sedef ERKUNT ALAK[b], Cemal ÜN[c]✉

Ege University, Faculty of Science, Department of Biology, İzmir, Turkey.
[a]ORCID: 0000-0001-8328-8270; [b]ORCID: 0000-0001-8563-1239; [c]ORCID: 0000-0002-4248-9671.

✉Corresponding author: cemaluen@gmail.com
Received date: 11.09.2018- Accepted date: 03.05.2019

Abstract: Prion protein coding gene (*PRNP)* is the genetic locus correlated with the greatest impact on classical scrapie susceptibility in sheep. At codons 136, 154, and 171 of *PRNP* alanine/arginine/glutamine (ARQ) and valine/arginine/glutamine (VRQ) haplotypes, in turn, are related to susceptibility to classical scrapie while alanine/arginine/arginine ARR haplotype is correlated with resistance. The aim of the present study was to genotype the Palestinian native sheep breeds for detection of genetic resistance. A total of 38 healthy sheep from Awassi and Assaf breeds were randomly sampled. Genomic DNA was isolated from blood samples. After PCR amplification and DNA sequencing, ARQ, ARR, ARH, AHQ, ARL and VRQ alleles and ARR/ARQ, ARQ/ARQ, ARQ/ARL, ARH/ARQ, ARH/ARL, AHQ/ARQ and ARQ/VRQ genotypes were detected in *PRNP* gene. ARQ allele was found as a predominant allele in this study with the frequency of 0.76 for Awassi and Assaf breeds while the uncommon allele ARL was identified at low frequencies in both breeds. In addition, two different polymorphisms were recognized (V12I and L23H) at different codons of *PRNP*. Results have indicated that most of the genotypes belong to risk group 3. The careful dissemination of ARR/ARR sheep is suggested to increase resistant allele frequencies in Assaf and Awassi breeds.
Keywords: Assaf, Awassi, Palestinian, *PRNP*, scrapie.

Filistin yerli koyun ırklarında prion protein kodlama geni polimorfizmlerinin analizi

Özet: Prion protein kodlama geni (PRNP) koyunlarda klasik scrapie duyarlılığıyla ilişkilendirilen genetik lokustur. Genin 136, 154 ve 171. kodonlarındaki ARQ ve VRQ allellerinin scrapie duyarlılığıyla, ARR allelinin ise scrapie dirençliliğiyle ilgili olduğu bilinmektedir. Bu çalışmanın amacı, scrapieye karşı genetik direncin belirlenmesi için Filistinli yerli koyun ırklarının genotiplendirilmesidir. Çalışmada, İvesi ve Assaf ırklarından, 38 sağlıklı ve rastgele seçilmiş koyun örnekleri kullanılmıştır. Koyun kan örneklerinden DNA izole edilmiş, PZR amplifikasyonu yapılmış ve sekans analizi gerçekleştirilmiştir. Prion protein kodlama geninde, ARQ, ARR, ARH, AHQ, ARL ve VRQ allelleri ve ARR/ARQ, ARQ/ARQ, ARQ/ARL, ARH/ARQ, ARH/ARL, AHQ/ARQ ve ARQ/VRQ genotipleri belirlenmiştir. Her iki ırkta da baskın olan ARQ alleli 0.76 ile en yüksek frekansta bulunurken, az görülen ARL alleli ise her iki ırkta da düşük frekanslarda belirlenmiştir. Ayrıca *PRNP* geninin farklı kodonlarında iki farklı polimorfizm (V12I ve L23H) tanımlanmıştır. Sonuçlar genotiplerin çoğunluğunun 3.risk grubuna dahil olduğunu göstermektedir. Assaf ve İvesi ırklarında dirençli allel sıklığını arttırmak için ARR/ARR koyunlarının dikkatli bir şekilde yaygınlaştırılması önerilmektedir.
Anahtar sözcükler: Assaf, Filistin, İvesi, *PRNP*, scrapie.

Introduction

Scrapie is a contagious prion disease in sheep which affects the central nervous system (24). The aggregation of the misfolded pathological form (PrP^{Sc}) of the normal cellular prion protein (PrP^{C}) causes the disease (15). It was the first diagnosed prion disease (17) to be regarded as a small ruminant disease in 1732 where the first examination carried out by veterinarians in Britain, Germany and France (27).

Prion protein coding gene (*PRNP)* is the genetic locus correlated with the greatest impact on classical scrapie susceptibility in sheep (6). The prion protein which is conserved in mammals is encoded by *PRNP* (31). While the normal prion protein is regarded as PrP^{C}, the misfolded pathological form is considered PrP^{Sc} (18, 30).

Even any genetic or posttranslational differences could not be found between PrP^{C} and PrP^{Sc}, the dissimilarities in C-terminal globular domain configuration and biochemical hallmarks such as protease resistance and solubility have been detected (4). The developmental preservation of the essential structure of prion protein (PrP) through mammals, additionally its

gene expression level in brain and localization in privatised membrane domains offer that the normal cellular protein (PrP^C) has a significant role in signaling, cell adhesion and differentiation (14,19).

In sheep, goats and cattle, the *PRNP* is found on chromosome 13 (3). The sheep *PRNP* has three exons (52, 98, and 4028 nucleotides in length) separated by two introns (2421 and 14,031 nucleotides in length) (20). In spite of the fact that scrapie is considering a contagious disease, the polymorphisms in *PRNP* strongly influence the susceptibility of scrapie in sheep. At codons 136, 154, and 171 of *PRNP* alanine/arginine/glutamine (ARQ) and valine/arginine/glutamine (VRQ) haplotypes in turn are related to susceptibility to classical scrapie while alanine/arginine/arginine *ARR* haplotype is correlated with resistance (16).

In Palestine, sheep were distributed across the breeds of Awassi (52.9%), Assaf (35.7%), crossbreed (11%) and other breeds (0.4%). The native Awassi sheep are used for meat, milk and wool production. The Assaf sheep which is a result of crossbreeding of local Awassi and Swiss East Friesian breeds in occupied Palestinian territories in 1955, produce high quality of milk and meat in comparison to Awassi (26). In 1993 the first diagnosis of scrapie in Assaf breed was detected in the north of Palestine and the whole flock was destroyed for precaution. After that in 1996, 2002 and 2005 scrapie cases were determined in different areas of Palestine and caused the cull of Assaf flocks.

According to our knowledge, there is only one research in the literature about ovine *PRNP* in Palestinian. Gootwine et al. (13) were genotyped only codons 136, 154 and 171 in Assaf sheep breed to find out the genetic resistance to scrapie. According to their results, ARR/ARR genotype could not be detected whereas ARR/ARQ, ARR/ARH, ARQ/ARQ were observed with frequencies of 0.177, 0.097, 0.613 respectively (13).

The aim of this study was to genotype the Palestinian native sheep breeds (Assaf and Awassi) by polymerase chain reaction (PCR) and DNA sequencing for detection of genetic resistance and possible additional polymorphisms and for comparing allele frequencies in addition to the previous study.

Materials and methods

Samples and DNA isolation: In this study, 38 clinically healthy sheep of native Palestinian sheep breeds from four towns were randomly sampled. 17 sheep of Awassi breed from four flocks and 21 sheep of Assaf breed from five flocks were randomly chosen. Blood samples were collected by Palestinian Veterinary Services (Gaza, Palestine) into tubes containing EDTA. Genomic DNAs were isolated from blood samples manually by using Qiagen DNeasy blood and tissue kit.

PCR amplification and sequencing: The Primer3web tool (http://bioinfo.ut.ee/primer3/) (25) has used for the design of primers for PCR amplification of prion protein coding gene (Gen-Bank accession number M31313 from nucleotide 24 – 912). The sequences 5'-CGTGGGCATTTGATGCTGACAC- 3' as a forward primer and 5'-GCTGCAGGTAGACACTCCCTC- 3' as a reverse primer were chosen. In PCR amplification, 30 µl final volume prepared which was including 6 µl 5X Master Mix, 5 µl (50-100 ng) genomic DNA and 0.3 µl (10 µM) of each primer. The PCR amplification was carried out in thermal cycler with a denaturation step 94°C for 3 min and 35 cycles of 94°C for 45 s, 57°C for 45 s, 72°C for 1 min; and a final extension at 72°C for 5 min. The PCR products were conducted on a 1.5% agarose gel for electrophoresis and were visualized with a long wavelength UV transilluminator. The PCR products (889 bp) sequenced after purification and sequences were analysed by using MEGA 6 program (28) for detection of polymorphisms at codons 136, 154, 171.

Statistical analysis: According to results, the gene and genotype frequencies were figured out by using the formulas; (23)

$$Xij = nij / n \text{ and } x\hat{}i = 2nii + nij / 2n$$

where Xij indicates the genotypic frequency of AiAj; nij and nii show the number of individuals for homozygous (AiAi) and heterozygous (AiAj) genotypes, ; xˆi indicates the gene frequency of Ai and n shows the total number of individuals.

For assessing the potential deviations from Hardy – Weinberg equilibrium $\chi 2$ tests were conducted for each breed. The susceptibility to scrapie was evaluated by the grouping system which is proposed by Tongue et al. (29) according to each breed's genotypic distribution.

Results

The allele frequencies for 6 alleles (ARQ, ARR, ARH, AHQ, VRQ and ARL) in Awassi and Assaf breeds are shown in Table 1. ARQ allele which is associated with susceptibility to scrapie was found as predominant allele in two breeds, with the frequencies of 0.764 and 0.761 for Awassi and Assaf, respectively. The most resistant allele ARR was only detected in Awassi breed with the frequency of 0.205. AHQ allele which is also related to low resistance to scrapie was the lack in Awassi breed and observed at low frequency (0.071) in Assaf breed. Besides ARQ and AHQ alleles, ARH allele that is regarded as low resistance to scrapie as well as the lack in Awassi breed and detected at low frequency (0.095) in Assaf breed. The high susceptible allele VRQ was discovered with a frequency 0.023 in Assaf breed whereas was not detected in Awassi breed. In addition, ARL allele was determined at low frequencies of 0.031 and 0.050 in Awassi and Assaf respectively.

Table 1. Allele frequencies of *PRNP* in Palestinian sheep breeds.
Tablo 1. Filistin koyun ırklarında *PRNP* allel frekansı.

Allele	Awassi Breed	Assaf Breed
ARQ	0.764	0.761
ARR	0.205	0.000
ARH	0.000	0.095
AHQ	0.000	0.071
VRQ	0.000	0.023
ARL	0.031	0.050

Table 2. Genotype frequencies of *PRNP* in Palestinian sheep breeds.
Tablo 2. Filistin koyun ırklarında *PRNP* genotip frekansı.

Risk group	Genotype	Awassi	Assaf
2	ARR/ARQ	0.412	0.000
3	ARQ/ARQ	0.529	0.571
3	ARQ/ARL	0.059	0.048
3	ARH/ARQ	0.000	0.142
3	ARH/ARL	0.000	0.048
3	AHQ/ARQ	0.000	0.142
5	ARQ/VRQ	0.000	0.048

Table 3. Additional polymorphisms in *PRNP* of Palestinian sheep breeds.
Tablo 3. Filistin koyun ırklarında *PRNP* ek polimorfizmleri.

Breed	Sample	Haplotype	Polymorphism	Homozygote/ Heterozygote
Awassi	1	ARQ/ARQ	L23H	Homozygote
Awassi	4	ARQ/ARQ	L23H	Homozygote
Awassi	9	ARR/ARQ	L23H	Heterozygote
Awassi	10	ARR/ARQ	L23H	Heterozygote
Awassi	12	ARR/ARQ	L23H	Heterozygote
Awassi	15	ARR/ARQ	L23H	Heterozygote
Awassi	16	ARQ/ARQ	V12I	Homozygote
Awassi	16	ARQ/ARQ	L23H	Homozygote
Awassi	17	ARR/ARQ	L23H	Heterozygote
Assaf	18	ARQ/ARQ	L23H	Homozygote
Assaf	21	ARQ/ARQ	L23H	Homozygote
Assaf	24	ARQ/ARQ	L23H	Homozygote
Assaf	25	ARQ/ARQ	L23H	Homozygote
Assaf	26	ARH/ARL	L23H	Heterozygote
Assaf	28	AHQ/ARQ	L23H	Heterozygote
Assaf	29	ARQ/ARQ	L23H	Homozygote
Assaf	30	ARQ/ARQ	V12I	Homozygote
Assaf	30	ARQ/ARQ	L23H	Homozygote
Assaf	31	ARQ/ARQ	V12I	Homozygote
Assaf	31	ARQ/ARQ	L23H	Homozygote
Assaf	36	AHQ/ARQ	L23H	Heterozygote
Assaf	37	ARH/ARQ	L23H	Heterozygote

In *PRNP* gene the polymorphisms were detected which consist of seven genotypes (ARR/ARQ, ARQ/ARQ, ARQ/ARL, ARH/ARQ, ARH/ARL, AHQ/ARQ and ARQ/VRQ) (Table 2). The genotypes pertain to the second and fifth risk group. The ARQ/ARQ which is low resistance to scrapie was the dominant genotype in both Assaf and Awassi breeds and the high susceptible genotype ARQ/VRQ was observed only in one Assaf sheep.

Two additional polymorphisms were detected (V12I and L23H) moreover to determined polymorphisms at codons 136, 154 and 171 (Table 3). L23H polymorphism was detected in 8 and 11 sheep samples in Awassi and Assaf breeds, respectively, whereas V12I polymorphism detected in 1 and 2 sheep samples in Awassi and Assaf breeds, respectively. At codon 12, ATT instead of GTT caused V (Valine) to I (Isoleucine) alteration as well as at codon 23 CAC instead of CTC caused L (leucine) to H (Histidine) alteration.

According to the results of statistical analysis, P value was found greater than 0.05 for both breeds. Also $\chi 2$ was found 1.37 (degree of freedom 3) and 9.4 (degree of freedom 10) for Awassi and Assaf breeds respectively. These results implicate that the populations used in this study were in Hardy-Weinberg equilibrium.

Discussion and Conclusion

According to results in Awassi and Assaf breeds ARQ allele was discovered as a dominant allele in this study with a frequency of 0.76 which is compatible with the results of many previous studies performed for different sheep breeds in Turkey (21, 22), Iran (10), Greece (8), Italy (29) Portugal (11), Spain (1), Germany (7) and Britain (2) and also in previous study in Palestinian. In addition, ARQ is estimated to be wild-type allele of the *PRNP* and this study supports this prediction.

The association between Arginine amino acid at codon 136 and resistance to scrapie has shown before (9, 20). In spite of the fact that the significance of amino acid residue is not completely recognized for codon 154, H has a positive relation with resistance to scrapie at this position (9) while R amino acid was predominance at codon 154 in this study. R, Q, H and L were detected at codon 171 as different amino acid residues. The high frequency of Q and the low frequency of H amino acids are compatible with previously reported *PRNP* polymorphisms (5, 12).

In this study, ARR allele in Assaf breed was not detected. This is a major point of interest and may be a good explanation for some clinical scrapie cases that were detected in Assaf sheep previously (13). In comparison to the results of Gootwine et al. study in Awassi breed, ARR allele was detected with a higher frequency which is 0.205 and this can be the result of breeding programs. Also in

this study ARH and AHQ alleles could not be observed where in a previous study they were found with frequencies of 0.123 and 0.029 respectively in Awassi breed. However, ARL allele which is identified as uncommon in sheep populations and was observed in Iranian Zandi sheep previously (10) was also detected in this study in both breeds.

The highest resistance genotype ARR/ARR and the highest susceptibility genotype VRQ/VRQ were absent in Awassi and Assaf breeds matching with the results of the previous study in Awassi breed. The genotypes ARR/ARQ (risk group 2) and ARQ/ARQ (risk group 3) were detected with frequencies of 0.411 and 0,529, respectively. When these results were compared with the previous study, an increase in the frequency of the genotype in risk group 2 and a decrease in the frequency of the genotype in risk group 3 observed and this suggests the rising of resistant genotypes in Awassi breed (13). Also, the frequency differences shown by the results of the previous studies with the results obtained in this work reveal the importance of repeating these studies. The relation between additional polymorphisms (L23H,V12I) and the susceptibility to scrapie is yet to be investigated.

In conclusion, the results of this study demonstrate the significance of implementing appropriate breeding programs for increasing the genetic resistance against classical scrapie. According to low ARR allele frequency in Assaf breed, inbreeding has to be avoided in breeding programs. Also, insertion of sheep from different flocks with high ARR allele frequency for increasing resistance should be done carefully to avoid atypical scrapie and losses of genetic variations in breeds.

Acknowledgement

This study is the master thesis of the first author and was supported by Scientific Research Projects of Ege University with a project number 2016-FEN-013. The summary of this research was presented in 10th International Animal Science Conference, 26-28 October 2018, Antalya, Turkey.

References

1. **Acin C, Martin-Burriel I, Goldmann W, et al.** (2004): *Prion protein gene polymorphisms in healthy and scrapie-affected Spanish sheep,* J Gen Virol, **85**, 2103-2110.
2. **Arnold M, Meek C, Webb C, et al.** (2002): *Assessing the efficacy of a ram-genotyping programme to reduce susceptibility to scrapie in Great Britain*, Prev Vet Med, **56**, 227–249.
3. **Castiglioni B, Comincini S, Drisaldi B, et al**. (1998): *Comparative mapping of the prion gene (PRNP) locus in cattle, sheep and human with PCR-generated probes,* Mamm Genome, **9**, 853-855.
4. **Cohen FE, Prusiner SB** (1998): *Pathologic conformations of prion proteins,* Annu Rev Biochem, **67**, 793-819.
5. **DeSilva U, Guo X, Kupfer D, et al.** (2003): *Allelic variants of ovine prion protein gene (PRNP) in Oklahoma sheep,* Cytogenet Genome Res, **102**, 89-94.
6. **Diaz C, ViteZica Z, Rupp R, et al.** (2005): *Polygenic variation and transmission factors involved in the resistance/susceptibility to scrapie in a Romanov flock,* J Gen Virol, **86**, 849-857.
7. **Drogemüller C, De Vries F, Hamann H, et al**. (2004): *Breeding German sheep for resistance to scrapie,* Vet Res, **154**, 257-260.
8. **Ekateriniadou L, Kanata E, Panagiotidis C, et al.** (2007): *PrP genotypes in scrapie-affected sheep in Greece—The contribution of the AHQ 1 polymorphism,* Small Rumin Res, **73**, 142-149.
9. **Elsen J, Amigues Y, Schelcher F, et al.** (2014): *Genetic susceptibility and transmission factors in scrapie: detailed analysis of an epidemic in a closed flock of Romanov,* Arch Virol, **144**, 431-445.
10. **Frootan F, Nikbakht G, Ögentürk N, et al.** (2012): *Prion Protein Coding Gene (PRNP) Variability in Sheep from Turkey and Iran,* Biochem Genet, **50**, 277–284.
11. **Gama L, Carolino M, Santos-Silva M, et al.** (2006): *Prion protein genetic polymorohisms and breeding strategies in Portuguese breeds of sheep.* Livest Sci, **99**, 175-184.
12. **Gombojav A, Ishiguro N, Horiuchi M, et al.** (2003): *Amino acid polymorphisms of PrP gene in Mongolian sheep,* J Vet Med Sci, **65**, 75-81.
13. **Gootwine E, Abdulkhaliq A, Jawasreh KIZ, et al.** (2008): *Screening for allele frquency at the PrP locus in (PRNP) Awassi and Assaf populations in Israel, the Palestinian Authority and Jordan,* Small Rumin Res, **77**, 83-88.
14. **Hetz C, Maundrell K, Soto C** (2003): *Is loss of function of the prion protein the cause of prion disorders?* Trends Mol Med, **9**, 237–243.
15. **Hornlimann B, Riesner D, Kretzschmar H** (2006): *Prions in Human and Animals,* Walter de Gruyter, Berlin, Germany.
16. **Hunter N** (1997): *PrP genetics in sheep and the implications for scrapie and BSE,* Trends Microbiol, **5**, 8.
17. **Johnson RT** (2005): *Prion diseases,* Lancet Neurol, **4**, 635-642.
18. **Kupfer L, Hinrichs W, Groschup MH** (2009): *Prion Protein Misfolding,* Curr Mol Med, **9**, 826-835.
19. **Martins VR, Linden R, Prado Marco AM, et al.** (1998): *Cellular prion protein: on the road for functions,* FEBS Lett, **512**, 25-28.
20. **Mead S** (2006): *Prion disease genetics,* Eur J Hum Genet, **14**, 273-281.
21. **Meydan H, Yüceer B, Degirmenci R, et al.** (2012): *Prion protein gene polymorphism and genetic risk evaluation for scrapie in all Turkish native sheep breeds,* Virus genes, **45(1)**, 169-175.
22. **Meydan H, Özkan MM, Yildiz MA, et al.** (2013): *Novel polymorphisms in ovine prion protein gene,* Anim Genet, **44(5)**, 588-591.
23. **Nei M** (1987): *Molecular Evolutionary Genetics*. Columbia University Press, New York, USA.
24. **Prusiner SB** (1982): *Novel Proteinaceous Infectious Particles Cause Scrapie,* Science, **216**, 136-144.

25. **Rozen S, Skaletsky H** (2000): *Primer3 on the WWW for general users and for biologist programmers*. In: Krawetz S, Misener S (eds) Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ, pp 365-386.
26. **Rummel T, Valle Z´arate A, Gootwine E** (2005): *The Worldwide Gene Flow of the Improved Awassi and Assaf Breeds of Sheep from Israel.* Grauer, Stuttgart and Beuren, Germany.
27. **Schneider K, Fangerau H, Michaelsen B, et al.** (2008): *The early history of the transmissible spongiform encephalopathies exemplified by scrapie*, Brain Res Bull, **77**, 343-355.
28. **Tamura K, Stecher G, Peterson D, et al.** (2013): *MEGA6: Molecular Evolutionary Genetics Analysis version 6.0.* Mol Biol Evol, **30**, 2725-2729.
29. **Tongue SC, Wilesmith JW, Cook CJ** (2004): *Frequencies of prion protein (PrP) genotypes and distribution of ages in 15 scrapie-affected flocks in Great Britain*, Vet Rec, **154**, 9–16.
30. **Watts JC, Prusiner SB** (2009): *Experimental Models of Inherited PrP Prion Diseases*, Cold Spring Harb Perspect Med. doi: 10.1101/cshperspect.a027151.
31. **Wopfner F, Weidenhofer G, Schneider R, et al.** (1999): *Analysis of 27 mammalian and 9 avian PrPs reveals high conservation of flexible regions of the prion protein*, J Mol Biol, **289**, 1163–1178.