Novel Variations at Three Different Positions of Prion Protein Coding Gene in Ethiopian Sheep Breeds and the Resistance/Susceptible Status to Classical and Atypical Scrapie

Eden Yitna Teferedegn
Ege University https://orcid.org/0000-0003-2526-1672
Yalcin Yaman
Bandirma Sheep research institute.
Cemal Un (cemaluen@gmail.com)
https://orcid.org/0000-0002-4248-9671

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Abstract

Background: Classical Scrapie susceptibility in sheep has been linked to three polymorphisms at positions 136, 154, and 171 in the *PRNP* gene whereas atypical scrapie susceptibility is related to a polymorphism at position 141. Many other variants over the length of the prion protein coding gene were reported. Since infectious prion protein itself seems to be polymorphic, the identified novel *PRNP* gene variations may play a crucial role in fighting against the emergence of a new form of scrapie disease. Many studies conducted around the world to identify disease resistant status and new variants of *PRNP* gene in different breeds. However, such in-depth studies have never addressed the African continent's sheep breeds. Therefore, genotyping native Ethiopian sheep breeds *PRNP* gene provides essential information to the current knowledge. This study aimed to identify potential novel variations in the Ethiopian sheep *PRNP* gene, thereby determine the uniqueness of the native breeds and predict scrapie status of sheep population based on the genotypes distribution.

Results : Five novel variants were identified in the *PRNP* gene of native Ethiopian sheep. Four non-synonymous heterozygous substitutions at H99Q (CAC–>CAA), H99L (CAC–>CTA), A116E (GCA–>GAA), A116T (GCA–>ACA), and one synonymous N103N (AAC–>AAT) variants were detected. In addition to the novel variations, polymorphisms at 126,127,138,142,146,231, and 237 positions were also identified. The haplotype ARR was observed only in Menz and Afar breeds with frequencies 0.02 and 0.05 respectively. However, neither ARR/ARR nor VRQ/VRQ genotypes were identified in all of the breeds.

Conclusion: Two of the novel variations at position 99 and 103 that are placed closer to the cleavage site and variant at 116 spotted in the palindrome region along with variants at position 127 in Glycine repeat domain may influence the conformational flexibility of prion protein. The low frequency of ARR haplotype and the sole variant 141L demonstrated that Ethiopian sheep are susceptible to classical scrapie and resistant to atypical scrapie. This study provides a valuable dataset that can be potentially integrated into selective breeding strategies against scrapie during inbreeding, crossbreeding and help to take precautious measures.

Background

Prion diseases are a collective name for infectious neurodegenerative diseases caused by the misfolding of Prion protein [1].The misfolded protein (PrP^{Sc}) has a different structural dynamic than cellular prion (PrP^{c}). Specific motifs of prion protein were identified in relation to the conversion of PrP^{c} to PrP^{Sc} [2].

Though the exact underlined disease mechanism is not yet known, transmission and susceptibility of prion diseases have genetic bases. Previous studies identified three polymorphisms (A136V, R154H, and Q171R/H) related to scrapie resistance/susceptibility status in sheep PRNP (prion protein coding gene). ARQ represents susceptible widespread wild type allele; ARR and VRQ are the most resistant and highly susceptible genotypes respectively [7–10]. In atypical scrapie susceptibility is higher in individuals with AHQ, AHQ/ARQ and ARR genotypes along with homozygosity for phenylalanine at position 141 [11]. An

earlier study linked genotypes such as $AC_{151}RQ$ to prolonged incubation period after scrapie exposure [10]. Together with that, several studies reported polymorphisms such as G126A, G126G, G127G, G127V, G127A, and S138S [3–6] in sheep prion protein with or without direct effect to susceptibility to scrapie.

In the past several years there were efforts to genetically characterize local breeds of many countries in identifying new variants and determining resistance/susceptible haplotypes against scrapie. Based on the findings, measures were taken to control and reduce transmission of transmissible spongiform encephalopathy horizontally and vertically [12–14]. However, there are limited studies that addressed Sub-Saharan African countries such as Ethiopia where livestock is the main economic source and a large proportion of the population depends on livestock products. On the other hand, the ever-increasing animal product demand enhances crossbreeding programs for the last few decades through the importation of exotic animals and the distribution of crossbred F1 in different parts of Ethiopia. Such practices are the potential factors in changing the genetic structure of the population and may introduce new disease susceptible phenotypes [15, 16].

Taking in to account the public health, economic and scientific merits genotyping native Ethiopian sheep breeds PRNP gene provides essential information to the current knowledge [17]. This study aimed to identify novel variations in the Ethiopian sheep PRNP gene, thereby determine the uniqueness of the native breeds and predict scrapie status of sheep population based on the genotypes distribution.

Methods

Animal selection

Whole blood was taken from genetically unrelated 97 female native sheep breeds (Washera N = 39, Menz N = 35, and Afar N = 23) that are concentrated in the respective regional breeding center. Washera sheep breed is localized in west-east Gojam and AgewAwi zones of Amhara region (11°00'0.00" N 36°39'59.99" E). This breed is commonly known by its short fat tail, short-hair and large body size. They are reared for commercial mutton production. Menz sheep breeds are one of the most common sheep breeds in Ethiopia distributed in Menz, North Shewa zones of Amhara region (10°15'00.0" N39°30'00.0"E). Their characteristic feature is a fatty short tail, well developed wooly undercoat with unique spiral horns. They are adapted to a cold environment and known for the production of meat and wool. Afar sheep named after Afar region. They are well adapted to harsh environments. They are a good source of fatty meat. Afar breeds from Amibara Woreda at a geographic coordinates 1033254, 0629012 were included in the study. Information on the phenotypes of the breeds was taken from Ethiopian Biodiversity booklet, 2018.

DNA extraction and Polymerase Chain Reaction

Genomic DNA was isolated from the EDTA treated blood using a commercial kit (Geneaid). PCR (Polymerase chain reaction) was carried out to amplify the coding region of the PRNP using forward (TCTGCAAGAAGCGACCAAAAC) and reverse (CACAGGAGGGGAAGAAAAGAGG) primers (NM_001009481.1). PCR mixture containing 200 μM of each dNTP, 2 mmol MgCl2, 5 pmol of each primer,

0,05 U Taq polymerase, 10 X PCR buffer (Thermo Fisher Scientific Inc., USA), 10–50 ng of genomic DNA and ddH2O to a final volume of 12,5 ul was used for PCR reaction. 2.5 ul of PCR product was used for further analysis.

Sequencing and bioinformatics

After incubation with 1 U Exo-SAP, a chain termination reaction was performed with BigDye™ terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific Inc., USA). At the final stage, all samples were purified by ethanol precipitation method and sequenced by Applied Biosystems 3500 genetic analyzer (Thermo Fisher Scientific Inc, USA). Chromatograms were checked with FinchTV and aligned using Mega v7.0 software. Hardy-Weinberg equilibrium state for the multilocus allele was calculated using Popgene 32.

Results

In the present study, novel variants were detected in the three native Ethiopian sheep *PRNP* protein-coding gene. Heterozygosity at nucleotide 296 and 297 resulted in two heterozygous amino acid substitution at position 99 i.e. H99Q (CAC–>CAA) and H99L (CAC–>CTA) **Fig 1**. Similarly, new substitutions at the already recognized polymorphic sites were identified i.e. A116T (GCA–>ACA) and A116E (GCA–>GAA). A synonymous substitution at the previously reported polymorphic site was also identified N103N (AAC–>AAT). The variant N103N was not detected in Afar breed. The new variants are localized in the main functional domains of prion protein **Fig 2**. Additional polymorphisms at different positions i.e. G126A (heterozygous and homozygous), G127G, G127V (heterozygous), G127A (heterozygous), S138S, I142I (heterozygous), N146S (heterozygous and homozygous), R231R and L237L were identified. Most of those polymorphic sites were heterozygous for the specified loci **Fig 3.** Potential resistant variants M112T, M137T, R151C and N176K were not detected in the studied breeds.

In the current study, the highly susceptible haplotypes i.e. haplotypes categorized under groups 4 and 5 were not detected. Instead, ARQ was the dominant allele in all breeds. The haplotype ARR was observed only in Menz and Afar breeds with frequencies 0.02 and 0.05 respectively. ARQ/ARQ and ARH/ARH genotypes appeared in Menz breeds with 0.29 and 0.17 frequencies respectively while ARQ/ARQ genotype was dominant in Washera (0.68) and Afar (0.78) breeds. Neither the highly susceptible, VRQ, nor highly resistant, ARR, genotypes were observed in this study. In all of the samples analyzed; homozygous Leucine at position 141 was detected **Table 1**. The population under study was not in Hardy-Weinberg proportion (χ_6^2: 35.27 27.58 $p<0.05$).

Discussion

In the current study, novel amino acid substitutions at a novel and previously identified polymorphic site were detected in the native Ethiopian sheep PRNP protein-coding gene. These substitutions (H99L, N103N, A116T, and A116E) are spotted in a PK resistant region of the prion protein. Substitutions at amino acid 99 and 103 positions are localized more close to the signal peptide cleavage sites. Variants

A116T and A116E are placed in hydrophobic palindrome region (A_{116}GAAAAG) which was described as the critical motif in the process of conversion of PrP^{c} to PrP^{Sc} and its propagation [18]. Substitution with a different variant having different physicochemical properties might influence the conformational plasticity of prion protein and my further could tempt the emergence of new scrapie disease form.

Similar to previous studies, additional amino acid polymorphic sites i.e. G126A, G126A(heterozygous), G127G, G127V(heterozygous), G127A(heterozygous), S138S, I142IT, N146S, N146S(heterozygous), R231R, and L237L were identified [3–6]. In the present study, the variant at position 127 was in particular highly polymorphic. Amino acid variants at positions 126 and 127 are localized in the highly conserved Glycine repeat motif GAVVGGLGGYMLG which is reported to antagonize prion disease development by blocking amyloidal fibril formation [2]. Recent work on the Ethiopian goat PRNP protein-coding gene reported polymorphism at this position and implied its importance in the normal cellular function of prion protein [19].The synergetic effect of alterations in palindrome motif PrP (113–120) and Glycine repeat regions PrP(124–128) may strongly affect cellular prion protein conformational flexibility.

The possibility of infectious disease transmission from animal to animal, animal to human and vice-versa makes genetic characterization of local breeds undeniably crucial especially in understanding and preventing transmittable disease [20]. So far, there is strong evidence that scrapie susceptibility and prolonged incubation period are linked to different PRNP genotypes. Known polymorphic alleles at position 136A/V, 154R/H, and 176R/H/Q are implicated in classical scrapie disease susceptibility [10]. Accordingly, the highest resistant genotype is ARR/ARR while VRQ/VRQ is the highest susceptible genotype. In atypical scrapie, AHQ, ARQ and ARR genotypes along with homozygosity for phenylalanine at position 141 are susceptible haplotypes. ARR genotype which is central protective haplotype in classical scrapie is not protective in atypical scrapie [11, 21].

In the current study, a significant proportion of the population's alleles under study were less resistant to classical and atypical scrapie (fall under scrapie resistance category groups 2 and 3). The haplotype, $A_{136}L_{141}R_{154}Q_{171}$, was predominant in the population under study. In the present study, the frequency of the ARQ allele was lower in Menz breed than the other two breeds. On the contrary, ARQ is the highest frequent allele in Afar breed. The relative allele frequency variation among breeds in this study might be directly related to the geographic barriers favoring inbreeding and later results in genetic distinction among breeds.

In countries such as Canada where scrapie was once apparently high, the resistant haplotype, ARR, became dominant over the previously reported VRQ and ARQ haplotype [7, 22]. Similarly, in India and China where scrapie case was never been epidemic, the predominant allele was ARQ [23, 24]. Earlier works on sheep PRNP polymorphism from Israel, Iran, and Turkey revealed that ARQ was the dominant haplotype [25–29]. Atypical scrapie associated haplotype, ALRQ, was also prominent in the above listed countries where scrapie infection was not epidemic [25, 28]. Studies from North African countries, Tunisia and Algeria reported ARQ, ARR, AHQ, ARH, and VRQ as major alleles. Those studies also identified identical polymorphisms which were reported in Spanish and Italian sheep and this similarity can be

considered as a piece of evidence that some of Mediterranean surrounding countries' breeds had similar genetic background. If the transboundary infection had happened during the times when scrapie was epidemic in European countries, it is sound to expect a high frequent ARR and low frequent VRQ in Algeria and Tunisia [30, 31].

Earlier case-control studies linked specific alleles to survival rate after scrapie infection along with ARQ genotype. T_{112}ARQ, AT_{137}RQ, AC_{151}RQ, and ARQK_{176} haplotypes were identified as potential protective variants [11, 32, 33]. Accordingly, the detected homozygous Methionine at position 112&137, Arginine at 151, and Asparagine at 176 in all the analyzed samples potentiates the susceptibility of the breeds to scrapie.

Despite there have been cross-breeding practices especially community-based breeding programs in small ruminants in recent years [15, 16], in the present study frequency of homozygous genotypes were relatively dominant over heterozygous genotypes.

Conclusion

Two of the novel variations at position 99 and 103 that are placed closer to the cleavage site and variant at 116 which is spotted in the hydrophobic palindrome region along with variants at position 127 in Glycine repeat domain may influence the conformational flexibility of prion protein and /or introduce new form of scrapie disease. The predominant ARQ/ARQ genotype is a potential cue to assume that there was no natural selection against classical scrapie at least in the current study area. Considering the wild type variant 141L, Ethiopian native sheep breads are resistant to atypical scrapie. However, because of the identified disease susceptible genotypes and the spontaneity of atypical scrapie, the native Ethiopian sheep breeds are prone to contract scrapie. Therefore, these findings provide a valuable dataset that can be potentially integrated into selective breeding strategies against scrapie during inbreeding, crossbreeding and help to take precocious measures.

Declaration

Ethics approval and Consent to Participate- Animals were treated with great care and sample was taken according to the institute guideline. Ethical approval is deemed unnecessary according to the Ethiopıan National Research Ethics Review Guideline/EFDRE ministry of science and Technology Sep 2014 5th ed. Article 8.3.5.1, 10.2 and 10.5.1. Consent was granted to take blood samples from the respective regional state livestock development and promotion offices NS/AR/U-01/42/2010 and NS/AR/U-01/41/2010.Genetic material export permit was assured from Ethiopian Biodiversity Institute, Ref. No.EBI71/943/2018.

Consent for publication-Not applicable

Availability of data and materials-The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interest-The authors declare no conflict of interest.

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Authors Contribution- ET involved in data collection, performed laboratory activities and wrote the manuscript. YY engaged in PCR and sequence optimization. CU formulated concept and organized the study. All authors have read and approved the manuscript.

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Abbreviations

PRNP gene: prion protein coding gene;PrP^{c}: cellular prion; PrP^{Sc}: Scrapie form of prion; A: Alanine; C: Cysteine; D: Aspartic acid; E: Glutamate; G: Glycine; H: Histidine; I: Isoleucine; K: Lysine; L: Leucine, M: Methionine; N: Asparagine; P: Proline ; Q: Glutamine R: Arginine; S: Serine; T: Threonine; V: Valine; Y:Tyrosine.

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Tables

Table 1 Scrapie associated allele and genotype frequencies of Menz, Washera Afar sheeps' *PRNP* gene

Overall Frequency	Menz	Washera	Afar	
Allele				
ARQ	0.62	0.46	0.68	0.78
ARH	0.29	0.41	0.26	0.17
AHQ	0.07	0.11	0.06	–
ARR	0.02	0.02	–	0.05
Genotype				
ARQ/ARQ	0.50	0.29	0.62	0.61
ARQ/ARH	0.17	0.17	0.07	0.31
ARH/ARH	0.20	0.31	0.20	–
ARQ/AHQ	0.06	0.14	0.05	–
ARH/AHQ	0.02	0.03	0.03	–
AHQ/AHQ	0.02	0.03	0.03	–
ARQ/ARR	0.02	0.03	–	0.04
ARH/ARR	0.01	–	–	0.04

Figures

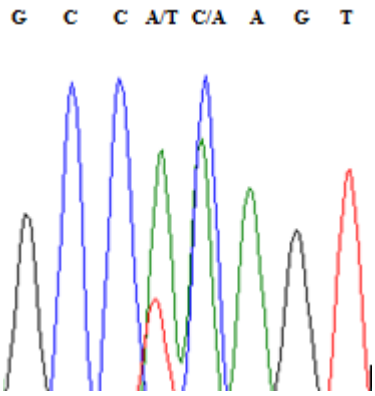

Figure 1

Heterozygosity at nucleotide 296 and 297 resulted in two heterozygous amino acid substitutions at position 99. Such Variants were seen in 3 animals from Afar, 2 from Washera and 1 from Menz.

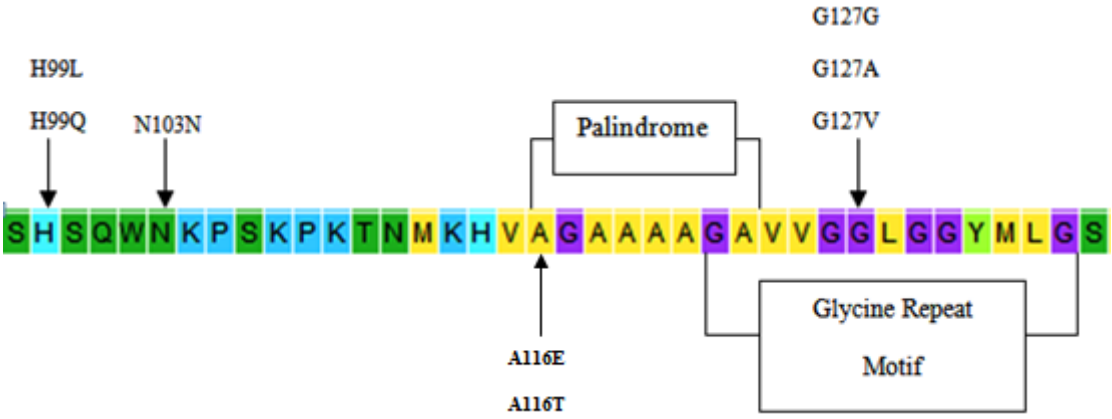

Figure 2

Polymorphisms at cleavage site, palindrome and highly conserved hydrophobic region of PrP.

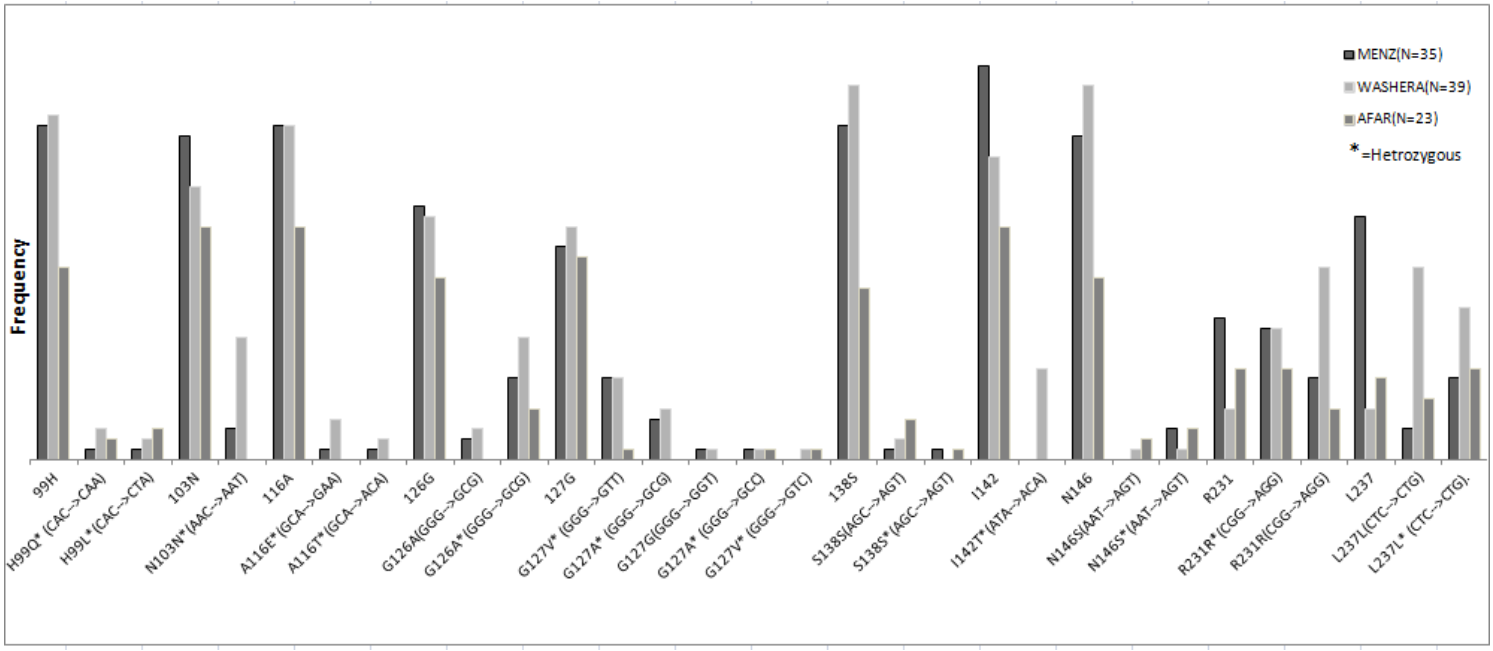

Figure 3

Amino acid substitutions over the length of the sequenced region.