PRION
2018, VOL. 12, NOS. 3–4, 245–252
https://doi.org/10.1080/19336896.2018.1513316

RESEARCH PAPER

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The first report of polymorphisms and genetic characteristics of the prion protein gene (*PRNP*) in horses

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ABSTRACT
Prion diseases have a wide host range, but prion-infected cases have never been reported in horses. Genetic polymorphisms that can directly impact the structural stability of horse prion protein have not been investigated thus far. In addition, we noticed that previous studies focusing on horse-specific amino acids and secondary structure predictions of prion protein were performed for limited parts of the protein. In this study, we found genetic polymorphisms in the horse prion protein gene (*PRNP*) in 201 Thoroughbred horses. The identified polymorphism was assessed to determine whether this polymorphism impedes stability of protein using PolyPhen-2, PROVEAN and PANTHER. In addition, we evaluated horse-specific amino acids in horse and mouse prion proteins using same methods. We found only one single nucleotide polymorphism (SNP) in the horse prion protein, and three annotation tools predicted that the SNP is benign. In addition, horse-specific amino acids showed different effects on horse and mouse prion proteins, respectively.

Abbreviations: *PRNP*: prion protein gene; SNP: single nucleotide polymorphism; CJD: Creutzfeldt-Jakob disease; CWD: chronic wasting disease; TME: transmissible mink encephalopathy; FSE: feline spongiform encephalopathy; MD: molecular dynamics; ER: endoplasmic reticulum; GPI: glycosylphosphatidylinositol; NMR: nuclear magnetic resonance; ORF: open reading frame; GWAS: genome-wide association study; NAPA: non-adaptive prion amplification; HMM: hidden Markov model; NCBI: National Center for Biotechnology Information

ARTICLE HISTORY
Received 7 May 2018
Revised 11 July 2018
Accepted 7 August 2018

KEYWORDS
Horses; prion; *PRNP*; prion protein; polymorphism; single nucleotide polymorphism; SNP

Introduction

Prion diseases are irreversible neurodegenerative conditions [1] and have a wide range of host variability, encompassing Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and goats, chronic wasting disease (CWD) in deer, transmissible mink encephalopathy (TME) in mink, and feline spongiform encephalopathy (FSE) in cats, cheetahs and pumas; however, prion disease in horse has never been reported thus far, even though the life span of the horse is approximately 30 years [2–8].

Previous studies noted that the stability of horse prion protein is related to disease progression. Thus, several studies have focused on identifying the horse-specific structure of prion protein gene (*PRNP*), which can contribute to the stability of horse prion protein. Two horse-specific structures have been reported, called the β2-α2 loop and salt bridges. Horse-specific amino acids S169, Y224 and F227 are composed of the horse-specific β2-α2 loop and denote remarkable stability among species [9]. In addition, a molecular dynamics (MD) study found 4 salt bridges in horse prion protein and confirmed that the salt bridges bestow upon the most durable structure of horse prion protein that can withstand harsh conditions [10]. Another factor that can contribute to protein stability is genetic polymorphisms. Recent studies have reported disease associated genetic polymorphisms. In humans, more than 20 single nucleotide polymorphisms (SNPs) and mutations on *PRNP* gene are associated with disease susceptibility [6,11–13]. In addition, two insertion/deletion polymorphisms affect BSE susceptibility in cattle [14–17]. According to the haplotypes of *PRNP* codons 136, 154 and 171, sheep are subdivided into 5 levels of scrapie risk groups from R1 to R5 [18,19]. Goats have been reported to have SNPs at *PRNP* codons 142, 143, 146, 154, 171, 211 and 222 that are related to the vulnerability to scrapie [20,21]. In deer and elk, genotype distributions of *PRNP* codons 95, 96, 132 and 225 showed correlations with the number of CWD-positive animals [22–27]. In recent studies, genetic polymorphisms of prion gene family members have been reported in cattle, goats and chickens [28–31]. However, polymorphisms of

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the horse *PRNP* gene have not been investigated thus far. Because the horse is generally believed to be a prion-resistant animal, an investigation of the polymorphisms in the horse *PRNP* gene is important.

In this study, we investigated genotype and allele distributions of a horse *PRNP* gene polymorphism by DNA sequencing in 201 Thoroughbred horses. In addition, we also evaluated the impact of the identified SNP and horse-specific amino acids through PolyPhen-2, PROVEAN and PANTHER. Furthermore, we compared amino acid sequences of prion protein among species using ClustalW2 and Wasabi.

Results

Identification and analysis of polymorphisms in the horse PRNP gene

To investigate the genotype and allele frequencies of horse *PRNP* gene polymorphisms, we performed PCR to amplify the horse *PRNP* gene. The amplified horse *PRNP* gene was sequenced in 201 Thoroughbred horses by using an ABI 3730 automatic direct sequencer. The DNA sequences in the open reading frame (ORF) region of the horse *PRNP* gene sequenced in 201 Thoroughbred horses are identical to the *PRNP* gene of *Equus caballus* registered in GenBank (ACG59276.1). Interestingly, we found only one non-synonymous SNP (c.525A> C, N175K) in the ORF of the horse *PRNP* gene (Figures 1 and 2). Of the 201 Thoroughbred samples, 110 (54.7%) were homozygous for the A allele, 11 (5.5%) were homozygous for the C allele, and 80 (39.8%) were heterozygous at codon 175, with an allele frequency of 0.746:0.254 A:C. Next, to evaluate whether the SNP location was important, we drew a schematic map of horse prion protein and marked N175K on it. Interestingly, N175K did not impede the components of the two horse-specific structure, the β2-α2 loop (residues 167–174) and four salt bridges (E198-R158-H189, R158-D204, E213-H179 and D180-R166) (Figure 3).

Predicting the impact of the non-synonymous SNP on horse prion protein

To estimate the impact of the non-synonymous SNP (N175K) on horse prion protein, we utilized PolyPhen-2, PROVEAN and PANTHER. PolyPhen-2 predicted that N175K was ‘Benign’ with the score 0.008. PROVEAN estimated N175K to be ‘Neutral’ with the score −0.757. PANTHER evaluated N175K as ‘Probably benign’ with the score 176 (Table 1).

Evaluation of the effect of horse-specific amino acids

We tried to investigate the effect of horse-specific amino acids. For this, we performed a sequence alignment among species to find horse-specific amino acids. The horse-specific amino acids were determined by ClustW2 and Wasabi based progressive alignment construction and phylogeny-aware methods, respectively. The horse-specific amino acids were as follows: 169S, 224Y, 227F, 228Q and 243V (Figure 4a,4b). First, we substituted horse-specific amino acids with interspecific conserved amino acids sequentially and estimated the substitution effect on horse prion protein using PolyPhen-2, PROVEAN and PANTHER. Notably, the substitution effect did not seem to be significant on horse prion protein (Table 2). Second, we measured the D166S (previous referred as D167S) substitution effect in mouse prion protein, which was performed using the same design as a previous *in vivo* mutagenesis

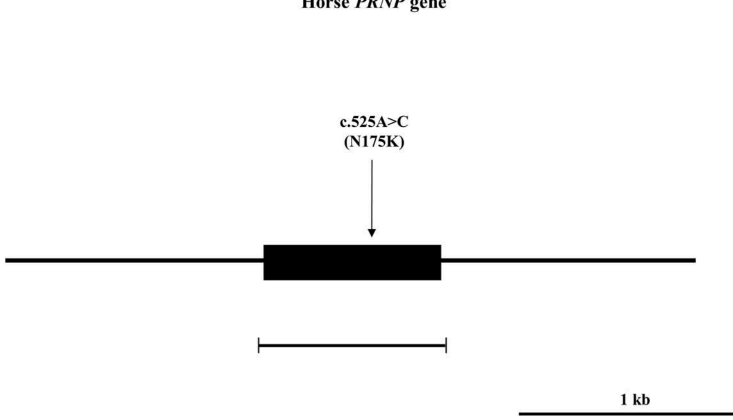

Figure 1. Gene map and polymorphism identified in the horse prion protein gene (*PRNP*) on chromosome 22. The open reading frame (ORF) is indicated by a shaded block. Horizontal bars with edges indicate the regions sequenced. Arrows indicate the polymorphism found in this study.

c.525A>C
(N175K)

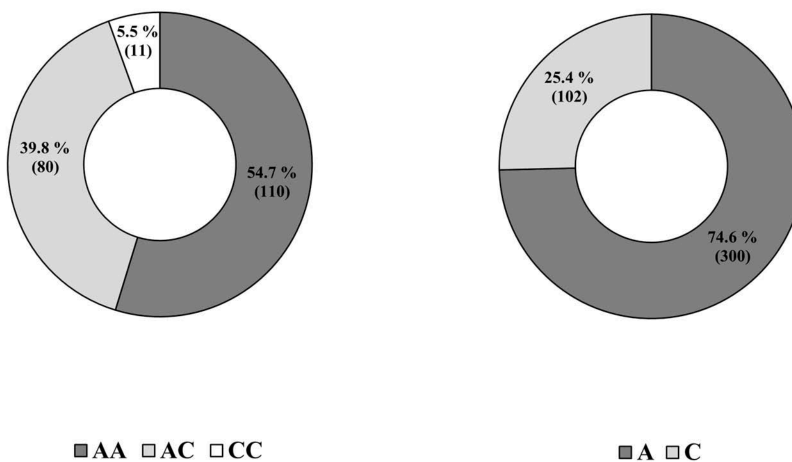

Figure 2. Pie chart for the genotype and allele frequencies of c.525A> C (N175K) in horse *PRNP* gene. Left pie chart indicates the genotype frequency of c.525A> C (N175K) in the horse *PRNP* gene. Right pie chart indicates the allele frequency of c.525A> C (N175K) in the horse *PRNP* gene.

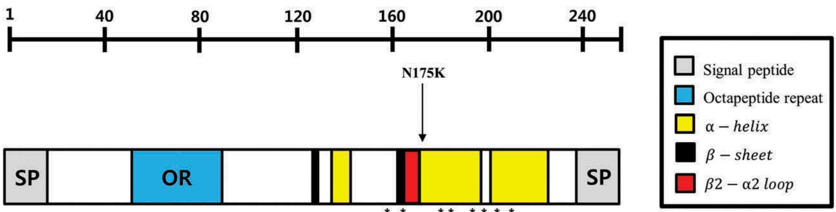

Figure 3. Evaluation of the SNP location on a schematic map of horse prion protein. The protein sequence of horse prion protein was determined by automatic direct sequencing of 201 Thoroughbred horses in this study. Each structure was determined by methods described in the 'Materials and Methods' section. Arrows indicate the polymorphism found in this study. The N-terminal endoplasmic reticulum (ER) signal peptide is located on residues 1–19 and the C-terminal glycosylphosphatidylinositol (GPI) signal peptide is located on 231–255. The α-helix is located on residues 145–158, 175–196 and 202–230 and the β-sheet is located on 130–133 and 163–166. The β2-α2 loop is located on residues 167–174. Components of four salt bridges (E198-R158-H189, R158-D204, E213-H179 and D180-R166) are marked by asterisks.

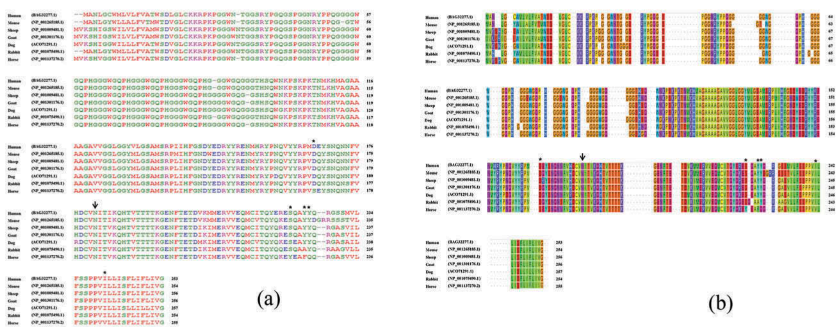

Figure 4. Comparison of amino acid sequences of prion protein in human, mouse, sheep, goat, dog, rabbit and horse. Prion protein sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI), including those of human (*Homo sapiens*, BAG32277.1), mouse (*Mus musculus*, NP_001265185.1), sheep (*Ovis aries*, NP_001009481.1), goat (*Capra hircus*, NP_001301176.1), dog (*Canis lupus familiaris*, ACO71291.1), rabbit (*Oryctolagus cuniculus*, NP_001075490.1) and horse (*Equus caballus*, NP_001137270.2). A. Protein sequences were aligned using ClustalW2 based on progressive alignment methods. Colors indicate the chemical properties of amino acids; blue: acidic; red: small and hydrophobic; magenta: basic; green: hydroxyl, sulfhydryl, amine and glycine. The arrow indicates the polymorphism. Asterisks indicate horse-specific residues. B. Protein sequences were aligned using Wasabi based on phylogeny-aware methods. Colors were followed 'Taylor color'. The arrow indicates the polymorphism. Asterisks indicate horse-specific residues.

Table 1. Prediction of protein functional alterations induced by the non-synonymous single nucleotide polymorphism (SNP) in horse prion protein.

Variation	Method	Score	Prediction
N175K	PolyPhen-2	0.008	Benign
	PROVEAN	-0.757	Neutral
	PANTHER	176	Probably benign

Table 2. Measurement of the effect of substitutions from horse-specific amino acids to interspecific conserved amino acids on horse prion protein.

Residues	Horse specific	Interspecific conserved	Methods	Score	Prediction
169	S	D	PolyPhen-2	0.000	Benign
			PROVEAN	0.515	Neutral
			PANTHER	2	Probably benign
224	Y	S	PolyPhen-2	0.005	Benign
			PROVEAN	0.977	Neutral
			PANTHER	2	Probably benign
227	F	Y	PolyPhen-2	0.000	Benign
			PROVEAN	0.908	Neutral
			PANTHER	2	Probably benign
228	Q	Y	PolyPhen-2	0.000	Benign
			PROVEAN	0.861	Neutral
			PANTHER	2	Probably benign
243	V	I	PolyPhen-2	NA	Unknown
			PROVEAN	0.068	Neutral
			PANTHER	2	Probably benign

* NA, Not available

study [32]. PolyPhen-2 and PANTHER predicted that the substitution effect was 'Probably damaging' and 'Possibly damaging', respectively (Table 3).

Discussion

Since prion-infected cases have never been reported in horse, there are many efforts to identify factors that contribute to the resistance of prion disease. Previous studies concentrated on investigating the structural stability of horse prion protein, which participates in prion disease resistance. Thus, we assumed that genetic polymorphism, one of the factors that can be involved in protein stability, could be a factor for the resistance of prion disease in the horse. To identify genetic polymorphisms in the horse *PRNP* gene, we performed automatic direct sequencing in 201 Thoroughbred horses. Interestingly, we found only one non-synonymous SNP (N175K) in the ORF of the horse *PRNP* gene. It is a curious result, because polymorphisms in the *PRNP* gene have a decisive effect on prion diseases susceptibility, unlike other polygenic diseases. This potent propensity was proven by a genome-wide association study (GWAS) that demonstrated that the *PRNP* gene has an extraordinarily high *P*-value [33]. In addition, previously reported prion disease-susceptible animals have numerous prion disease-associated SNPs in their genes. Thus, very the low polymorphic feature of the horse *PRNP* gene is worth noting. Furthermore, since previous studies indicated that Thoroughbred, which considered very inbred status, has several SNPs in the other gene, thus we presumed that very low polymorphic *PRNP* gene is not caused by inbred status of Thoroughbred [34,35]. However, study just in inbred species may skew data and limit interpretation. Thus, since outbred wild horses did not inhabit in the South Korea, for exact confirmation, further comparison study of horse *PRNP* gene is needed in other countries that have wild horses.

Table 3. Measurement of the effect of substitutions from mouse amino acids to horse-specific amino acid on mouse prion protein.

Residue	Mouse amino acid	Horse amino acid	Method	Score	Prediction
166	D	S	PolyPhen-2	0.991	Probably damaging
			PROVEAN	−1.005	Neutral
			PANTHER	220	Possibly damaging

Because prion disease-infected cases have not been reported in horse, a case-control study could not be performed in this animal. Thus, we devised two ways to evaluate N175K. First, we drew a schematic map that marked horse-specific structures and interpreted the importance of the N175K location. Second, we performed analyzes using PolyPhen-2, PROVEAN and PANTHER to determine the impact of this SNP on horse prion protein. As illustrated in Figure 3, N175K does not impede two horse-specific extraordinary stable structures, the β2-α2 loop and four salt bridges. In addition, the PolyPhen-2, PROVEAN and PANTHER predictions all suggested that N175K is benign (Table 1). Horse-specific amino acid, S169 participates in composing the highly ordered solution structure of the β2-α2 loop. Since this structure contributes to the stability of horse prion protein, the β2-α2 loop with 169S has been considered to be one factor of resistance to prion disease in horse [9]. In addition, four salt bridges, identified by a previous MD study, take part in inhibiting the formation of the β-intermediate state against changes in pH and temperature. Because the β-intermediate state is postulated to be the middle stage of conformational change from an α-helix to β-sheet, the salt bridges have also been considered to be one factor of resistance to prion disease in horse [10]. An important finding was that although N175K polymorphism has approximately 25% of minor allele frequencies, N175K did not impede these two horse-specific structures, and the SNP was predicted

to be benign. Because previous study has reported that horse prion protein has more stable structure than other species, horse prion protein which contains N175K may have stable structure. However, since stability of prion protein did not necessarily correlate with prion disease susceptibility, careful interpretation is needed in this study. Indeed, although G114V, D178N, T183A and E200K found in inherited prion disease were predicted as deleterious by *in silico* analysis, V180I were predicted as 'benign' in previous study [36]. In addition, although PolyPhen-2, PROVEAN and PANTHER can evaluate non-synonymous SNPs based on sequence homology, the alteration of protein structure, and the similarity of previous reported pathogenic mutations, those *in silico* tools cannot estimate all sides of pathogenicity of non-synonymous SNPs. To evaluate impact of N175K, conventional approach in transgenic mouse model expressing horse prion protein with N175K is necessary in the future.

Next, we thought that the partial horse-specific residues and the SNP could not account for all of the prion disease-resistance in horse. Although prion disease-resistance in horse has been reaffirmed through a recent non-adaptive prion amplification (NAPA) study of horse prion protein transgenic mice [37,38], however, a previous study reported that a mouse that contains mouse prion protein with a horse-specific amino acid (166S) develops a spontaneous form of prion disease [32]. Thus, the impact of horse-specific amino acids were elusive. We investigated the effects of horse-specific amino acids by substitution from horse-specific amino acids to interspecies conserved amino acids on horse prion protein. For this, we aligned the prion protein sequences of seven species and found horse-specific amino acids. After substituting horse-specific amino acids with interspecific conserved amino acids sequentially in horse prion protein, we measured the substitution effect using PolyPhen-2, PROVEAN and PANTHER. Interestingly, the substitution effects of horse-specific amino acids are quite low among all horse-specific residues (Table 2). Next, we assessed horse-specific amino acid D166S (previous referred as D167S) effects on mouse prion protein by using the same experimental design that was previous performed in an *in vivo* transgenic mouse study [25]. Notably, two programs predicted that D166S has a deleterious effect on mouse prion protein. These results suggest that the effect of amino acid substitutions may be different according to species because of the differences in protein sequence, and there is severe deficiency of functional studies that use mutagenesis and ignore interspecific sequence differences. For analysis of species-specific amino acids, it is necessary to analyze the whole structure of proteins. However, prediction results, 'deleterious', are not easily interpretable because the programs provide only comprehensive results based internal algorithms. Because *in silico* analysis provides just preliminary data of protein alteration impact, to interpret this prediction, introduction of structural mimic or electronic mimic of 166S in transgenic mouse model will helpful to understand the mechanism of 166S induced toxicity. In addition, since *PRNP* knock-out mouse showed relatively normal phenotype, additional functional analysis is needed in paralogs of prion protein such as prion-like protein, Doppel or shadow of prion protein, Shadoo. Furthermore, to thoroughly estimate impact of horse-specific amino acids of prion protein, *in vivo* experiment using simultaneous substitution of whole horse-specific amino acids is more judicious in the future study.

Collectively, we suggest two major points in this study. First, horse has only one benign SNP in the *PRNP* gene, which does not impede the horse-specific structure and is considered to participate in prion disease resistance. Second, we assessed the impact of horse-specific amino acids and identified a severe deficiency of mutagenesis studies. We hope that these two new results will provide a promising approach to understanding the mechanisms of prion disease.

Materials and methods

Ethics statement

Whole blood of 201 Thoroughbred horses was provided by the Seoul Race Park in the Republic of Korea. All experimental procedures performed in the present study were approved according to the recommendations of the Guiding Principle for Animal Care and Use Committee of Chonbuk National University (IACUC number: CBNU 2016–65).

Genomic DNA extraction

Genomic DNA was extracted from 200 μl of whole blood using the blood genomic DNA Isolation kit (Qiagen, Valencia, California, USA) following the manufacturer's instructions.

Genetic analysis

The horse *PRNP* gene was amplified from the genomic DNA using forward and reverse gene-specific primers. The sequences of the primers were as follows: Horse PRNP-F (AGAAGTGCAGAGTGTGACATGC) and Horse PRNP-R (CAAGCGTATTAGCCTACGGGTG). Polymerase chain reaction (PCR) was performed using GoTaq® DNA

Polymerase (Promega, Fitchburg, Wisconsin, USA). The PCR mixture contained 20 pmol of each primer, 5 µl of 10 ×*Taq* DNA polymerase buffer, 1 µl of 10 mM dNTP mixture and 2.5 units of *Taq* DNA polymerase. The PCR conditions were 94°C for 2 min to denature; 35 cycles of 94°C for 45 sec, 59°C for 45 sec, and 72°C for 1 min 30 sec; and then 1 cycle of 72°C for 10 min to extend the reaction. PCR was performed by an S-1000 Thermal Cycler (Bio-Rad, Hercules, California, USA). The PCR products were obtained by the PCR Purification Kit (Thermo Fisher Scientific, Bridgewater, New Jersey, USA) and directly sequenced with an ABI 3730 automatic sequencer (ABI, Foster City, California, USA). Sequencing results were read by Finch TV software (Geospiza Inc, Seattle, USA) and genotyping was performed.

Schematic map of horse prion protein

Amino acid sequences of horse prion protein were determined by automatic direct sequencing with genomic DNA samples of 201 Thoroughbred horses in this study. The N-terminal endoplasmic reticulum (ER) signal peptide (residues 1–19) was predicted by the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The C-terminal glycosylphosphatidylinositol (GPI) signal peptide was marked according to a previous study based on the interspecific homology of the GPI signal peptide sequence (residues 231–255). The secondary structure (α-helix: 145–158, 175–196 and 202–230; β-sheet: 130–133 and 163–166) and β2-α2 loop (residues 167–174) of horse prion protein were determined by a previous nuclear magnetic resonance (NMR) study of horse [9]. Four salt bridges (E198-R158-H189, R158-D204, E213-H179 and D180-R166) were confirmed by a previous MD study of horse [10].

Assessment of protein functional alterations

Possible impacts on horse prion protein induced by the substitution of amino acids were predicted by PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml), PROVEAN (http://provean.jcvi.org/seq_submit.php) and PANTHER (http://www.pantherdb.org/) [39–41]. PolyPhen-2 provides a position-specific, independent count (PSIC) score (score ranges from 0.0 to 1.0). The prediction results can be presented as three types: 'benign', 'possibly damaging' and 'probably damaging'. PROVEAN is a sequence-based predictor that calculates the impact score of protein sequence polymorphisms on protein function. If the final score is below −2.5, protein variants are predicted to be 'neutral'; otherwise, if the final score is above −2.5, protein variants are predicted to be 'deleterious'. PANTHER utilizes a hidden Markov model (HMM) based on statistical modeling methods and multiple sequence alignments to perform evolutionary analysis of coding missense SNPs. PANTHER predicts SNPs as two types: 'deleterious' (score < −3) or 'neutral' (score > −3).

Sequence alignment and secondary structure prediction of horse prion protein

The sequence alignments were carried out using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalo) and Wasabi (http://wasabiapp.org/). ClustalW2 and Wasabi aligned interspecies prion protein sequences based on progressive alignment construction and phylogeny-aware methods, respectively [42,43]. The assays were performed for prion protein sequences from human, mouse, sheep, goat, dog, rabbit and horse. Prion protein sequences were obtained from GenBank at the National Center for Biotechnology Information (NCBI), including those of human (*Homo sapiens*, BAG32277.1), mouse (*Mus musculus*, NP_001265185.1), sheep (*Ovis aries*, NP_001009481.1), goat (*Capra hircus*, NP_001301176.1), dog (*Canis lupus familiaris*, ACO71291.1), rabbit (*Oryctolagus cuniculus*, NP_001075490.1) and horse (*Equus caballus*, NP_001137270.2).

Acknowledgments

We wish to thank DVM Young-Bak Lim and Min-Hyun Lee for providing blood samples of Thoroughbred horses.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This research was supported by the Basic Science Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2015R1D1A1A010599). This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2017R1A6A1A03015876). Y.C. Kim was supported by the BK21 Plus program in the Department of Bioactive Material Sciences.

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