THEORY AND PRACTICE OF PRION PROTEIN ANALYSIS IN FOOD PRODUCTS

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Abstract: The article presents the results of the research on methods of identification and quantitative determination of prion proteins in biological samples and multicomponent mixtures based on them. Analysis of nucleotide sequence of DNA encoding the PRNP gene of the prion protein, including phylogenetic and comparative analysis of nucleotide sequences of normal and pathogenic prion protein in cattle, was performed. Oligonucleotide primers for amplification of the PRNP gene of pathogenic prion protein were designed and synthesized. The high specificity of the developed test system was confirmed.

Keywords: prion, protein, encephalopathy, safety, quality, PCR, analysis

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INTRODUCTION

Prions (proteinaceous infectious particles) are a special class of purely protein agents, free of nucleic acids, causing severe diseases of central nervous system in human and a number of higher animals [1–3].

Prion protein can exist in two forms: a non-infectious vitally important protein present in the organism of mammals, including human, and an infectious protein, which is a mutation of the normal prion protein causing prion diseases of animals and man.

Prion diseases are a group of transmissive neurodegenerative diseases of animals and humans. The diseases are characterized by prolonged incubation periods, but rapid progression from the moment of clinical onset of the disease. All prion diseases are lethal and there is no efficient methods of treatment so far. In 1997, Stenly B. Prusiner won the Nobel Prize for the outstanding discovery of prions.

Spongiform encephalopathy in cattle was registered in Great Britain, Switzerland, Ireland, Portugal, France, Germany, the Netherlands, Italy, Denmark, and Falkland Islands. The reported cases of disease were caused by the import of infectious animals or diseased meat-and-bone meal tankage produced from the killing products and used for breeding of the young stock in these countries [4, 5].

Prophylaxis of prion diseases is based on prohibition of the infected meat products or other killing products on food market. In this connection, in the Enactment of the Chief State Medical Officer of the Russian Federation no. 15 of 15.12.2000 «On the Measures for Prevention of Creutzfeldt–Jacobs Disease Spreading on the Territory of the Russian Federation», preventing measures aimed at prohibition of import of diseased meat and meat products were defined for the first time.

Taking this into account, improvement and development of new methods for identification of prion proteins in biological material is of scientific and practical interest.

OBJECTS AND METHODS OF THE STUDY

Whole milk, whole beef blood, blood plasma, cheese, beef muscle tissue, stromal fractions, gelatin, and samples of cattle meat were used. Samples of meat and blood were collected from animals having passed the veterinary control; the carcasses were proven fit for human consumption. The following nucleotide sequences corresponding to the PRNP gene of the prion protein deposited in the GenBank database were analyzed: *Equus caballus* (house horse), *Equus asinus* (house donkey), *Sus scrofa* (pig), *Bos taurus* (cow), *Bos javanicus* (Javan bull), *Bubalus bubalis* (buffalo), *Syncerus caffer caffer* (African buffalo), *Capra hircus* (goat), *Ammotragus lervia* (jubate sheep), *Ovis aries* (urial), *Rangifer tarandus granti* (northern deer), *Capreolus capreolus* (roedeer), *Alces alces alces* (elk), *Cervus elaphus nelsoni* (northamerican elk), *Cervus dama* (fallow deer), and *Homo sapiens* (human).

In the work, we used standard, common, and original methods, including the phylogenetic analysis of the protein gene nucleotide sequences, differential amplification of specific sequences and real-time polymerase chain reaction (PCR). The experiments involving PCR were performed following the requirements on determination of pathogenic microorganisms in cattle processing products.

Prior to the studies, independently of the analysis method, primary treatment of the samples was performed. In the case of analysis of soft and easily grinded materials (meat, cheese, etc.), averaged sample of the product weighing 1 g was collected, grinded using a sterile scalpel, scissors, and disposable spatula, and homogenized using a porcelain pistil in a ceramic mortar, with thorough mixing of the content.

For samples of dry particulate materials (gelatin) and liquid or semi-liquid materials (milk, blood, etc.), which require no grinding and are homogeneous, disposable spatula or a pipette was used to introduce 100–150 μL of bulk volume of a sample to an

Eppendorf tube (5–7 mm from the tube bottom). To prevent cross-contamination, the grinding instruments were used once, washed carefully, and sterilized.

For isolation of different protein fractions of animal origin, the samples were pretreated as follows: muscles of different animals were thoroughly freed from fat and connective tissue; weighed amount (3–4 g) of the tissue was cut by a knife on a watch glass. Distilled water was added at the ratio of 1 : 6 (by mass) and extraction was performed on cold at 0°C for 30 min. Then, the sediment was separated by centrifugation at 83 s^{-1} for 5 min. The supernatant was carefully decanted and used for quantitative protein determination.

Liquid samples were prepared by dilution in distilled water, so that the protein content in a gel pocket would not exceed 5 μg per 20 μL solution.

Determination of the total protein in samples was performed according to a technique of total and protein nitrogen fractions determination in meat, meat products, and protein-containing food products by the burning method of Duma [6].

Protein identification was performed by fingerprinting of peptide masses. Proteins were identified by the mass spectrum of amino acid sequences upon hydrolysis with trypsin in polyacrylamide gel.

To perform the mass spectrometry analysis, 0.5–2 μL of sample solution and 0.3 μL of a 20 mg/mL 2,5-dihydroxybenzoic acid solution in 20% acetonitrile aqueous solution with 0.5% TFA (Aldrich) were mixed on a ground steel support. Mass spectra were recorded on an Ultraflex II (Bruker, Germany) tandem MALDI time-of-flight mass spectrometer in the mass range of 700–4500 *m/z* under laser power optimal for the best resolution and registration of trypsin autolysis peaks, which were further used for internal calibration.

Mass spectra were processed with a FlexAnalysis 2.4 (Bruker Daltonics, Germany) software. If needed, fragmentation spectra of individual peptides were registered under tandem mode. Possible amino acid sequences were indexed in successfully fragmented peptides.

The accuracy of the average [MH+] measured mass in the linear mode was 5 Da. The accuracy of the monoisotopic measured masses in the reflecto-mode without internal calibration was 0.01% and after an additional calibration using trypsin autolysis peaks, 0.005%. Accuracy of the monoisotopic measured masses of fragments was 1 Da.

Molecular mass distribution of the proteins in the samples was evaluated by protein electrophoresis according to Laemmli [7].

Proteins were separated in denaturing 12% separating and 4% concentrating polyacrylamide gel supplemented with 0.1% sodium dodecyl sulfate. Electrophoresis was performed in a separating buffer supplemented with 0.1% sodium dodecyl sulfate under 15 mA. Gel was stained with 0.2% Coomasie Brilliant Blue R250 dye, prepared using glacial acetic acid, at high temperature for 7–10 min and then washed three times with distilled water.

Gels were viewed and imaged using a TCP-20M (Vilber Lourmat, United States) UV-transilluminator at the wavelength of 312 nm. Data storage and processing were performed with a DOC-it-LS gel-documenting system.

Gel calibration was performed using a set of protein markers by SibEnzyme containing 12 highly purified recombinant proteins of molecular mass between 10 and 250 kDa. For quantitative evaluation of normal prion protein content, gel was calibrated using human serum albumin protein solutions of known concentration.

Protein concentration in a sample was calculated according to the formula:

$$C = (C_p \cdot C_f)/100,$$

where C_p is the mass fraction of the total protein in a sample, g/100 g, and S_f is the mass fraction of a protein fraction to the total protein content in a samples, g/100 g protein.

In the course of the study, 17 nucleotide sequences of the PRNP prion protein gene deposited in the GenBank were used. To elucidate the differences and search for homologous sequences, NCBI database was used [8, 9]. Nucleotide acid sequences were aligned using the ClustalW software.

For comparative analysis of the DNA nucleotide sequences encoding the PRNP gene, OligoCalc software was used. Phylogenetic tree was designed using the ClustalW software.

Computer-based primer selection analysis for amplification of specific sequences of the pathogenic prion protein was performed using the following software: NCBI Blast2 for determination of homology upon sequencing of relevant primers and Primer3 Output for selection and evaluation of the primers.

Immuno-PCR was performed using the reaction mixtures presented in Table 1.

Table 1. Composition of the PCR reaction mixture

Component	Final concentration	Component content per 25 μL of the mixture
10× PCR buffer	0.1 μM	2.5 μL
10 mM dNTP mixture	0.2 mM	0.5 μL
Primer 1 (50 μL)	1 μM	0.5 μL
Primer 2 (50 μL)	1 μM	0.5 μL
Taq DNA polymerase	1.25 un.	0.25 μL
25 mM $MgCl_2$	1.5 mM	1 μL
DNA template	0.1–1 μg	Varies in function of concentration in a sample
Deionized water	-	Adjusted to 25 μL

RESULTS AND DISCUSSION

Total protein content in the samples is reported in Table 2.

Table 2. Total protein content in the samples

Subject of the study	Mass, mg	Total nitrogen content, %	Coefficient for calculations	Total protein, %	Measurement error, ±δ,%
Whole milk	195.60	0.656	4.64	3.02	0.31
	192.40	0.648			
Gelatin	188.12	15.123	5.55	84.32	0.89
	180.72	15.538			
Whole blood	214.50	3.462	6.25	21.97	1.32
	254.80	3.571			
Cheese	189.70	4.686	4.64	21.46	0.95
	229.60	4.653			
Beef	126.50	3.222	5.62	18.46	1.11
	110.00	3.347			
Water-soluble beef proteins	97.40	1.148	5.62	6.48	0.41
	96.50	1.167			
Salt-soluble beef proteins	136.30	1.520	5.62	8.52	0.53
	142.25	1.528			
Stromal beef proteins	186.80	0.675	5.62	3.78	0.23
	171.20	0.679			

Based on the data presented in Table 2, one may conclude that the total protein content in samples was 84.32 g/100 g for gelatin, 21.97 g/100 g for blood, 21.46 g/100 g for cheese, and 18.46 g/100 g for beef, with 6.48 g/100 g of water-soluble beef proteins, 8.52 g/100 g salt-soluble beef proteins, and 3.78 g/100 g stromal proteins.

We did not succeed to optimize the conditions for separation of cattle whole blood electrophoretic separations, therefore, we analyzed the blood plasma which was obtained by centrifugation at 3000 rpm for 5 min. The supernatant containing light fractions of blood proteins was used for analysis. The results of protein fraction distribution in the samples are presented in Fig. 1 and Table 3.

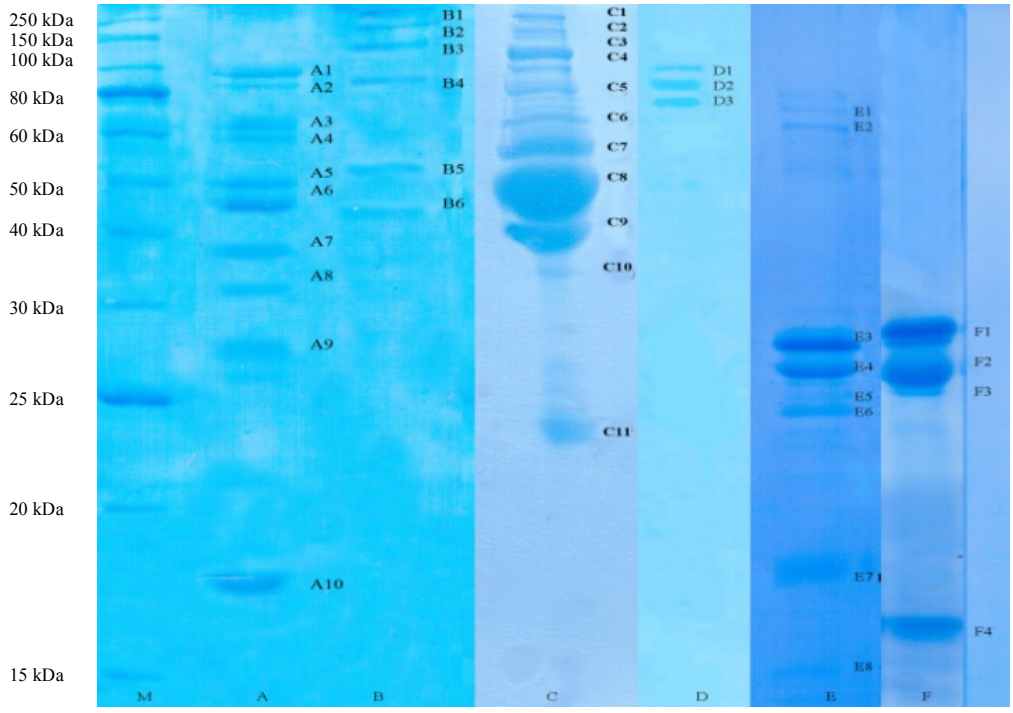

Fig. 1. PAGE in a 12% separating and 4% concentrating gel: M, molecular weight marker; A, beef protein water-soluble fraction; B, beef protein salt-soluble fraction; C, blood plasma; D, gelatin; E, whole milk; F, cheese.

Table 3. Mass fraction of the total protein and fraction distribution of the proteins

Sample	Number of samples	Total protein, g/100 g	Number of protein fractions in a range		
			15–30 kDa	30–40 kDa	40–250 kDa
Beef protein water-soluble fraction	20	6.48	1	2	6
Beef protein salt-soluble fraction	20	8.52	0	0	6
Beef protein stromal fraction	20	3.78	0	0	2
Blood plasma	20	9.73	1	2	8
Gelatin	10	84.32	0	0	3
Whole blood	20	3.02	6	0	2
Cheese	20	21.46	4	0	1

In the course of the study, we found that in fractions of stromal and salt-soluble beef proteins there are no low molecular weight protein fractions, which is in good agreement with the literature data, while in the fraction of water soluble proteins there are two protein fractions with masses from 30 to 40 kDa. The indicated protein mass is within the range of normal prion protein mass.

The obtained electrophoresis diagrams of blood plasma samples indicate the presence of two protein fractions with masses from 30 to 40 kDa.

Electrophoresis separation of industrial samples of gelatin, which is produced by partial hydrolysis of collagen obtained from cattle nails, jacket and skin, strings, and tendons, demonstrated high grade of purity. No low molecular weight protein fractions were observed.

Milk and cheese proteins were also fractionated (Table 4). Analysis of electrophoresis diagrams indicates the presence of traditional milk proteins in all samples. Caseins are characterized by molecular masses of ~22–32 kDa; β-lactoglobulin, ~18 kDa; α-lactalbumin, ~14 kDa; lactoferrin, 80 kDa; and serum albumin, ~66 kDa. No alien protein fractions weighing from 30 to 40 kDa was detected in the samples.

Table 4. Fraction composition of whole milk and cheese proteins

Band number	Molecular weight, kDa	Protein	%, to the total casein content	%, to the total serum protein content	%, to the total protein content
E 1	73.82	lactoferrin	-	17.76	3.39
E 2	68.11	blood serum albumin (SA)	-	17.04	43.24
E 3	29.63	αs1-casein	53.99	-	23.84
E 4	27.83	β-casein	29.77	-	4.14
E 5	26.09	αs2-casein	5.17	-	8.87
E 6	25.28	κ-casein	11.07	-	8.93
E 7	18.62	β-lactoglobulin	-	44.85	4.05
E 8	15.88	α-lactalbumin	-	20.34	3.39
D1	32.11	αs1-casein	37.31	-	37.31
D2	30.34	αs2-casein	11.23	-	11.23
D3	28.03	β-casein	41.50	-	41.50
D4	26.51	κ-casein	9.96	-	9.96

The absence of protein with molecular mass corresponding to that of normal prion protein (30–40 kDa) evidences the low possibility of prion protein presence in samples of whole milk, cheese, and salt-soluble and stromal beef proteins and therefore, the low level of infectiveness of the samples under study. The standard procedure of veterinary control, that is the certificate of fit for human consumption, is sufficient.

Further on, for unambiguous identification of the protein fractions in the above-indicated samples as normal prion proteins, one-dimensional electrophoresis was followed by the protein in-gel cleavage with trypsin and identification by peptide mass fingerprinting.

Samples selected for studies are presented in Table 5.

Table 5. Protein samples for investigation

Sample name	Protein mass, kDa
Beef protein water-soluble fraction	32.38
Blood plasma	34.89

Mass spectrum of amino acid sequences upon in-gel hydrolysis with trypsin was used for protein identification.

Quantitative content of the normal prion protein in samples was estimated by electrophoresis according to Laemmli followed by staining of the gel with Coomasie Brilliant Blue R250 (Table 6).

Table 6. Protein fraction composition of samples under study

Sample name	Molecular weight, kDa	%, to the total protein content	%, to the total protein content in a sample	Mass fraction, %
Beef protein water-soluble fraction	37.42	18.19	10.78	0.74
	32.38		7.41	0.51
Blood plasma	39.17	22.06	21.03	2.05
	34.89		1.03	0.1

Relative content of protein fractions from 30 to 40 kDa in fractions of beef water-soluble proteins was 18.19% to the total amount. Electrophoresis diagrams of blood plasma indicate that the relative content of protein fractions from 30 to 40 kDa in blood plasma was 22.06%. Therefore, our study evidences that the fractions of water-soluble proteins from beef and blood plasma under study indeed are normal prion proteins of the cattle.

Phylogenetic connections between the organisms may be elucidated by comparison of sequences of whole genes or their fragments encoding ribosomal RNA. The data on completely or partially sequenced rRNA genes of different organisms are deposited in international databases and are available in the internet. Today, methods based on determination of ribosomal gene nucleotide sequences are widely used for identification of different infection types [10].

We chose the following PRNP sequences: *Equus caballus* (house horse), *Equus asinus* (house donkey), *Sus scrofa* (pig), *Bos taurus* (cow), *Bos javanicus* (Javan bull), *Bubalus bubalis* (buffalo), *Syncerus caffer caffer* (african buffalo), *Capra hircus* (goat), *Ammotragus lervia* (jubate sheep), *Ovis aries* (urial), *Rangifer tarandus granti* (northern deer), *Capreolus capreolus* (roedeer), *Alces alces alces* (elk), *Cervus elaphus nelsoni* (northamerican elk), *Cervus dama* (fallow deer), and *Homo sapiens* (human) (see Fig. 2).

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gi|27733849 Equus       ATGGTGAAAAGCCACGTAGGCGGCTGGATTCTGGTTCTCTTTGTGGCCAC 50
gi|119514511 Equus      ATGGTGAAAAGCCACGTAGGCGGCTGGATTCTGGTTCTCTTTGTGGCCAC 50
gi|119489983 Sus        ATGGTGAAAAGCCACATAGGTGGCTGGATCCTCGTTCTCTTTGTGGCCGC 50
gi|119489801 Bos        ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGCCAT 50
gi|54125480 Bos         ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGCCAT 50
gi|54125508 Bubalus     ATGGTGAAAAGACACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGTCAT 50
gi|54125464 Syncerus    ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGTCAT 50
gi|119514499 Capra      ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGCCAT 50
gi|119655282 Ammotragus ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGCCAT 50
gi|89160951 Ovis        ATGGTGAAAAGCCACATAGGCAGTTGGATCCTGGTTCTCTTTGTGGCCAT 50
gi|73697718 Rangifer    ATGGTGAAAAGCCACATAGGCAGCTGGATCCTAGTTCTCTTTGTGGCCAT 50
gi|50442265 Rangifer    ATGGTGAAAAGCCACATAGGCAGCTGGATCCTAGTTCTCTTTGTGGCCAT 50
gi|50442321 Capreolus   ATGGTGAAAAGCCACATAGGCAGCTGGATCCTAGTTCTCTTTGTGGCCAT 50
gi|50442307 Alces       ATGGTGAAAAGCCACATAGGCAGCTGGATCCTAGTTCTCTTTGTGGCCAT 50
gi|158714095 Cervus     ATGGTGAAAAGCCACATAGGCAGCTGGATCCTAGTTCTCTTTGTGGCCAT 50
gi|50442285 Cervus      ATGGTGAAAAGCCACATAGGCAGCTGGATCCTAGTTCTCTTTGTGGCCAT 50
gi|308194928 Homo       ATGGCG------AACCTTGGCTGCTGGATGCTGGTTCTCTTTGTGGCCAC 44
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gi|27733849 Equus       ATGGAGTGACGTGGGGCTCTGCAAGAAGCGACCGAAGCCTG---GAGGAT 97
gi|119514511 Equus      ATGGAGTGACGTGGGGCTCTGCAAGAAGCGACCGAAGCCTG---GAGGAT 97
gi|119489983 Sus        ATGGAGTGACATAGGGCTCTGCAAGAAGCGACCAAAGCCTGGCGGAGGAT 100
gi|119489801 Bos        GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|54125480 Bos         GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|54125508 Bubalus     GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|54125464 Syncerus    GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|119514499 Capra      GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGCGGAGGAT 100
gi|119655282 Ammotragus GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGCGGAGGAT 100
gi|89160951 Ovis        GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGCGGAGGAT 100
gi|73697718 Rangifer    GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|50442265 Rangifer    GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|50442321 Capreolus   GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|50442307 Alces       GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|158714095 Cervus     GTGGAGTGACGTCGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|50442285 Cervus      GTGGAGTGACGTGGGCCTCTGCAAGAAGCGACCAAAACCTGGAGGAGGAT 100
gi|308194928 Homo       ATGGAGTGACCTGGGCCTCTGCAAGAAGCGCCCGAAGCCTG---GAGGAT 91
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Fig. 2. *Beginning.* Alignment of the PRNP gene nucleotide sequences.

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gi|27733849 Equus       GGAACACTGGGGGGAGCCGATACCCCGGGCAGGGCAGTCCTGGAGGCAAC 147
gi|119514511 Equus      GGAACACTGGGGGGAGCCGATACCCCGGGCAGGGCAGTCCTGGAGGCAAC 147
gi|119489983 Sus        GGAACACTGGGGGGAGCCGATACCCAGGGCAGGGTAGTCCTGGAGGCAAC 150
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gi|54125480 Bos         GGAACACGGTGGGGAGCCGATACCCAGGACAGGGCAGTCCTGGAGGCAAC 150
gi|54125508 Bubalus     GGAACACTGGGGGGAGCCGATACCCGGGACAGGGCAGTCCTGGAGGCAAC 150
gi|54125464 Syncerus    GGAACACTGGGGGGAGCCGATACCCAGGACAGGGCAGTCCTGGAGGCAAC 150
gi|119514499 Capra      GGAACACTGGGGGGAGCCGATACCCGGGACAGGGCAGTCCTGGAGGCAAC 150
gi|119655282 Ammotragus GGAACACTGGAGGGAGCCGATACCCGGGACAGGGCAGTCCTGGAGGCAAC 150
gi|89160951 Ovis        GGAACACTGGGGGGAGCCGATACCCGGGACAGGGCAGTCCTGGAGGCAAC 150
gi|73697718 Rangifer    GGAACACTGGGGGGAGCCGATACCCGGGACAGGGAAGTCCTGGAGGCAAC 150
gi|50442265 Rangifer    GGAACACTGGGGGGAGCCGATACCCGGGACAGGGAAGTCCTGGAGGCAAC 150
gi|50442321 Capreolus   GGAACACTGGGGGGAGCCGATACCCGGGACAGGGAAGTCCTGGAGGCAAC 150
gi|50442307 Alces       GGAACACTGGGGGGAGCCGATACCCGGGACAGGGAAGTCCTGGAGGCAAC 150
gi|158714095 Cervus     GGAACACTGGGGGGAGCCGATACCCGGGACAGGGAAGTCCTGGAGGCAAC 150
gi|50442285 Cervus      GGAACACTGGGGGGAGCCGATACCCGGGACAGGGAAGTCCTGGAGGCAAC 150
gi|308194928 Homo       GGAACACTGGGGGCAGCCGATACCCGGGGCAGGGCAGCCCTGGAGGCAAC 141
                ******* * .** ********** **.***** ** ***********
gi|27733849 Equus       CGCTACCCACCCCAGGGCGGTGGCGGCTGGGGTCAACCCCATGGTGGTG- 196
gi|119514511 Equus      CGCTACCCACCCCAGGGCGGTGGCGGCTGGGGTCAACCCCATGGTGGTG- 196
gi|119489983 Sus        CGCTATCCACCCCAGGGAGGGGGTGGCTGGGGACAGCCCCACGGAGGTG- 199
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gi|54125508 Bubalus     CGTTATCCATCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGAGGTGG 200
gi|54125464 Syncerus    CGTTATCCATCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGAGGTGG 200
gi|119514499 Capra      CGCTATCCACCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGAGGTG- 199
gi|119655282 Ammotragus CGCTATCCACCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGAGGTG- 199
gi|89160951 Ovis        CGCTATCCACCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGAGGTG- 199
gi|73697718 Rangifer    CGCTATCCACCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGGGGTG- 199
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gi|50442321 Capreolus   CGCTATCCACCTCAGGGAGGGGGTGGCTGGGGTCAGCCCCATGGAGGTG- 199
gi|50442307 Alces       CGCTATCCACCTCAGGGAGGGGGTGGCTGGGGTCAGCCTCATGGAGGTG- 199
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                        * ***** **.** ** **.** ****
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gi|73697718 Rangifer    GGGGTCAGCCCCATGGTGGTGGCTGGGGGCAGCCACATGGTGGTGGAGGC 276
gi|50442265 Rangifer    GGGGTCAGCCCCATGGTGGTGGCTGGGGGCAGCCACATGGTGGTGGAGGC 276
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Fig. 2. *Continued.* Alignment of the PRNP gene nucleotide sequences.

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gi|50442307 Alces       GGGGGCAGCCCCATGGTGGTGGCTGGGGGCAGCCACATGGTGGTGGAGGC 276
gi|158714095 Cervus     GGGGTCAGCCCCATGGTGGTGGCTGGGGACAGCCACATGGTGGTGGAGGC 276
gi|50442285 Cervus      GGGGTCAGCCCCATGGTGGTGGCTGGGGACAGCCACATGGTGGTGGAGGC 276
gi|308194928 Homo       GGGGGCAGCCCCATGGTGGTGGCTGGGGACAGCCTCATGGTGGTG---GC 264
                ****  *****  **  **.**  **  *****.*****  ********  *   **
gi|27733849 Equus       TGGGGTCAAGGTG---GCTCCCATGGTCAGTGGAACAAGCCCAGTAAGCC 320
gi|119514511 Equus      TGGGGTCAAGGTG---GCTCCCATGGTCAGTGGAACAAGCCCAGTAAGCC 320
gi|119489983 Sus        TGGGGTCAAGGTGGTGGCTCCCACGGTCAGTGGAACAAGCCCAGTAAGCC 326
gi|119489801 Bos        TGGGGTCAAGGTG---GTACCCACGGTCAATGGAACAAACCCAGTAAGCC 347
gi|54125480 Bos         TGGGGTCAAGGTG---GTACCCACGGTCAATGGAACAAACCCAGTAAGCC 347
gi|54125508 Bubalus     TGGGGTCAAGGTG---GTACCCACGGTCAATGGAACAAGCCCAGTAAGCC 347
gi|54125464 Syncerus    TGGGGTCAAGGTG---GTACCCACGGTCAATGGAACAAGCCCAGTAAGCC 347
gi|119514499 Capra      TGGGGTCAAGGTG---GTAGCCACAGTCAGTGGAACAAGCCCAGTAAGCC 323
gi|119655282 Ammotragus TGGGGTCAAGGTG---GTAGCCACAGTCAGTGGAACAAGCCCAGTAAGCC 323
gi|89160951 Ovis        TGGGGTCAAGGTG---GTAGCCACAGTCAGTGGAACAAGCCCAGTAAGCC 323
gi|73697718 Rangifer    TGGGGTCAAGGTG---GTACCCACAGTCAGTGGAACAAGCCCAGTAAACC 323
gi|50442265 Rangifer    TGGGGTCAAGGTG---GTACCCACAGTCAGTGGAACAAGCCCAGTAAACC 323
gi|50442321 Capreolus   TGGGGTCAAGGTG---GTACCCACAGTCAGTGGAACAAGCCCAGTAAACC 323
gi|50442307 Alces       TGGGGTCAAGGTG---GTACCCACAGTCAGTGGAACAAGCCCAGTAAACC 323
gi|158714095 Cervus     TGGGGTCAAGGTG---GTACCCACAGTCAGTGGAACAAGCCCAGTAAACC 323
gi|50442285 Cervus      TGGGGTCAAGGTG---GTACCCACAGTCAGTGGAACAAGCCCAGTAAACC 323
gi|308194928 Homo       TGGGGTCAAGGAGGTGGCACCCACAGTCAGTGGAACAAGCCGAGTAAGCC 314
                ***********.*   * : *** .****.********.** *****.**
gi|27733849 Equus       AAAAACCAACATGAAGCATGTGGCAGGAGCTGCGGCAGCTGGGGCAGTGG 370
gi|119514511 Equus      AAAAACCAACATGAAGCATGTGGCAGGAGCTGCGGCAGCTGGGGCAGTGG 370
gi|119489983 Sus        GAAAACCAACATGAAGCATGTGGCAGGCGCCGCTGCAGCTGGGGCAGTGG 376
gi|119489801 Bos        AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 397
gi|54125480 Bos         AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 397
gi|54125508 Bubalus     AAAAACCAACATGAAGCATATGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 397
gi|54125464 Syncerus    AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 397
gi|119514499 Capra      AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 373
gi|119655282 Ammotragus AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 373
gi|89160951 Ovis        AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 373
gi|73697718 Rangifer    AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCCGCTGGAGCAGTGG 373
gi|50442265 Rangifer    AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCCGCTGGAGCAGTGG 373
gi|50442321 Capreolus   AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 373
gi|50442307 Alces       CAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 373
gi|158714095 Cervus     AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 373
gi|50442285 Cervus      AAAAACCAACATGAAGCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGG 373
gi|308194928 Homo       AAAAACCAACATGAAGCACATGGCTGGTGCTGCAGCAGCTGGGGCAGTGG 364
                ****************** .****.** ** ** **.*****.*******
gi|27733849 Equus       TTGGGGGCCTCGGCGGCTACATGCTGGGGAGTGCCATGAGCAGACCCCTC 420
gi|119514511 Equus      TTGGGGGCCTCGGCGGCTACATGCTGGGGAGTGCCATGAGCAGACCCCTC 420
gi|119489983 Sus        TAGGGGGCCTCGGCGGTTACATGCTGGGGAGTGCCATGAGCAGACCCCTG 426
gi|119489801 Bos        TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 447
gi|54125480 Bos         TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 447
gi|54125508 Bubalus     TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 447
gi|54125464 Syncerus    TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 447
gi|119514499 Capra      TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|119655282 Ammotragus TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|89160951 Ovis        TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|73697718 Rangifer    TAGGGGGCCTCAGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|50442265 Rangifer    TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|50442321 Capreolus   TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|50442307 Alces       TAGGGGGCCTTGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|158714095 Cervus     TAGGGGGCCTCGGTGGCTACATGCTGGGAAGTGCCATGAGCAGGCCTCTT 423
gi|50442285 Cervus      TAGGGGGCCTCGGTGGCTACATGCTGGGAAGTGCCATGAATAGGCCTCTT 423
gi|308194928 Homo       TGGGGGGCCTTGGCGGCTACATGCTGGGAAGTGCCATGAGCAGGCCCATC 414
                * ********.* ** **********.**********. **.** .*
gi|27733849 Equus       ATTCATTTTGGCAATGACTATGAGGACCGTTACTATCGTGAAAACATGTA 470
gi|119514511 Equus      ATTCATTTTGGCAATGACTATGAGGACCGTTACTATCGTGAAAACATGTA 470
gi|119489983 Sus        ATACACTTTGGCAGTGACTATGAGGACCGTTACTATCGTGAAAACATGTA 476
gi|119489801 Bos        ATACATTTTGGCAGTGACTATGAGGACCGTTACTATCGTGAAAACATGCA 497
```

Fig. 2. *Continued.* Alignment of the PRNP gene nucleotide sequences.

```
gi|54125480 Bos          ATACATTTTGGCAGTGACTATGAGGACCGTTACTATCGTGAAAACATGCA 497
gi|54125508 Bubalus      ATACATTTTGGTAATGACTATGAGGACCGTTACTATCGTGAAAACATGCA 497
gi|54125464 Syncerus     ATACATTTTGGTAATGACTATGAGGACCGTTACTATCGTGAAAACATGCA 497
gi|119514499 Capra       ATACATTTTGGCAATGACTATGAGGACCGTTACTATCATGAAAACATGTA 473
gi|119655282 Ammotragus  ATACATTTTGGCAATGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|89160951 Ovis         ATACATTTTGGCAATGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|73697718 Rangifer     ATACATTTTGGCAACGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|50442265 Rangifer     ATACATTTTGGCAACGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|50442321 Capreolus    ATACATTTTGGCAACGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|50442307 Alces        ATACATTTTGGCAATGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|158714095 Cervus      ATACATTTTGGCAATGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|50442285 Cervus       ATACATTTTGGCAATGACTATGAGGACCGTTACTATCGTGAAAACATGTA 473
gi|308194928 Homo        ATACATTTCGGCAGTGACTATGAGGACCGTTACTATCGTGAAAACATGCA 464
                         **.** ** ** *.********************.********** *

gi|27733849 Equus        CCGTTACCCCAACCAAGTGTACTACAGGCCGGTAAGTGAGTACAGCAACC 520
gi|119514511 Equus       CCGTTACCCCAACCAAGTGTACTACAGGCCGGTAAATGAGTACAGCAACC 520
gi|119489983 Sus         CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTACAGCAACC 526
gi|119489801 Bos         CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAGTAACC 547
gi|54125480 Bos          CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAGTAACC 547
gi|54125508 Bubalus      CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAGTAACC 547
gi|54125464 Syncerus     CCGTTACCCCAACCAAGTATACTACAGGCCAGTGGATCAGTATAGTAACC 547
gi|119514499 Capra       CCGTTACCCCAACCAAGTGTACTACAGACCAGTGGATCAGTATAGTAACC 523
gi|119655282 Ammotragus  CCGTTACCCCAACCAAGTGTACTACAGACCAGTGGATCAGTATAGTAACC 523
gi|89160951 Ovis         CCGTTACCCCAACCAAGTGTACTACAGACCAGTGGATCAGTATAGTAACC 523
gi|73697718 Rangifer     CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAATAACC 523
gi|50442265 Rangifer     CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAATAACC 523
gi|50442321 Capreolus    CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAATAACC 523
gi|50442307 Alces        CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAATAACC 523
gi|158714095 Cervus      CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAATAACC 523
gi|50442285 Cervus       CCGTTACCCCAACCAAGTGTACTACAGGCCAGTGGATCAGTATAATAACC 523
gi|308194928 Homo        CCGTTACCCCAACCAAGTGTACTACAGGCCCATGGATGAGTACAGCAACC 514
                         ******************.********.**.*...* **** *.****

gi|27733849 Equus        AGAACAACTTTGTGCACGACTGCGTCAACATCACGGTCAAGCAGCACACA 570
gi|119514511 Equus       AGAACAACTTTGTGCACGACTGCGTCAACATCACGGTCAAGCAGCACACG 570
gi|119489983 Sus         AGAACAGTTTTGTGCATGACTGCGTCAACATCACCGTCAAGCAGCACACA 576
gi|119489801 Bos         AGAACAACTTTGTGCATGACTGTGTCAACATCACAGTCAAGGAACACACA 597
gi|54125480 Bos          AGAACAATTTTGTGCATGACTGTGTCAACATCACAGTCAAGGAACACACA 597
gi|54125508 Bubalus      AGAACAACTTTGTGCATGACTGTGTCAACATCACAGTCAAGGAACACACA 597
gi|54125464 Syncerus     AGAACAGCTTTGTGCATGACTGTGTCAACATCACAGTCAAGGAACACACA 597
gi|119514499 Capra       AGAACAACTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|119655282 Ammotragus  AGAACAACTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|89160951 Ovis         AGAACAACTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|73697718 Rangifer     AGAACACCTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|50442265 Rangifer     AGAACACCTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|50442321 Capreolus    AGAACACCTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|50442307 Alces        AGAACACCTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|158714095 Cervus      AGAACACCTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|50442285 Cervus       AGAACACCTTTGTGCATGACTGTGTCAACATCACAGTCAAGCAACACACA 573
gi|308194928 Homo        AGAACAACTTTGTGCACGACTGCGTCAATATCACAATCAAGCAGCACACG 564
                         ******  ******** ***** ***** *****.*****.*.*****.

gi|27733849 Equus        GTCACCACCACCACCAAGGGGGAGAACTTCACCGAGACCGACGTCAAGAT 620
gi|119514511 Equus       GTCACCACCACCACCAAGGGGGAGAACTTCACCGAGACCGACGTCAAGAT 620
gi|119489983 Sus         GTGACCACGACCACCAAGGGGGAGAACTTCACCGAGACGGACGTCAAGAT 626
gi|119489801 Bos         GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGAT 647
gi|54125480 Bos          GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGAT 647
gi|54125508 Bubalus      GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGAT 647
gi|54125464 Syncerus     GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACGTCAAGAT 647
gi|119514499 Capra       GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGAT 623
gi|119655282 Ammotragus  GTCACCACCACCACCAAGGGGGAGAACTTCACTGAAACTGACATCAAGAT 623
gi|89160951 Ovis         GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGAT 623
gi|73697718 Rangifer     GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATTAAGAT 623
gi|50442265 Rangifer     GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATTAAGAT 623
gi|50442321 Capreolus    GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATTAAGAT 623
gi|50442307 Alces        GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATTAAGAT 623
gi|158714095 Cervus      GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGAT 623
gi|50442285 Cervus       GTCACCACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGAT 623
gi|308194928 omo         GTCACCACAACCACCAAGGGGGAGAACTTCACCGAGACCGACGTTAAGAT 614
                         ** ***** ********************** **.** ***.* *****
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Fig. 2. *Continued.* Alignment of the PRNP gene nucleotide sequences.

```
gi|27733849 Equus       CATGGAGCGCGTGGTGGAGCAGATGTGCATCACCCAGTACCAGAAAGAGT 670
gi|119514511 Equus      CATGGAGCGCGTGGTGGAGCAGATGTGCATCACCCAGTACCAGAAAGAGT 670
gi|119489983 Sus        GATAGAGCGCGTGGTGGAACAGATGTGCATCACCCAGTACCAGAAAGAGT 676
gi|119489801 Bos        GATGGAGCGAGTGGTGGAGCAAATGTGCATTACCCAGTACCAGAGAGAAT 697
gi|54125480 Bos         GATGGAGCGAGTGGTGGAGCAAATGTGCATTACCCAGTACCAGAGAGAAT 697
gi|54125508 Bubalus     GATGGAGCGAGTGGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 697
gi|54125464 Syncerus    GATGGAGCGAGTGGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 697
gi|119514499 Capra      AATGGAGCGAGTGGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|119655282 Ammotragus AATGGAGCGAGTGGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|89160951 Ovis        AATGGAGCGAGTGGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|73697718 Rangifer    GATGGAGCGAGTTGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|50442265 Rangifer    GATGGAGCGAGTTGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|50442321 Capreolus   GATGGAGCGAGTTGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|50442307 Alces       GATGGAGCGAGTTGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|158714095 Cervus     GATGGAGCGAGTTGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|50442285 Cervus      GATGGAGCGAGTTGTGGAGCAAATGTGCATCACCCAGTACCAGAGAGAAT 673
gi|308194928 Homo       GATGGAGCGCGTGGTTGAGCAGATGTGTATCACCCAGTACGAGAGGGAAT 664
                        **.*****.** ** **.**.***** ** ********* ***..**.*
gi|27733849 Equus       ACGAGGCTTTTCAACAAAGAGGGGCGAGCGTGGTCCTCTTCTCCTCCCCG 720
gi|119514511 Equus      ACGAGGCTTTTCAACAAAGAGGGGCGAGCGTGGTCCTCTTCTCCTCCCCG 720
gi|119489983 Sus        ACGAGGCGTACGCCCAAAGAGGGGCCAGTGTGATCCTCTTCTCCTCCCCT 726
gi|119489801 Bos        CCCAGGCTTATTACCAACGAGGGGCAAGTGTGATCCTCTTCTCTTCCCCT 747
gi|54125480 Bos         CCCAGGCTTATTACCAACGAGGGGCAAGTGTGATCCTCTTCTCTTCCCCT 747
gi|54125508 Bubalus     CCCAGGCTTATTACCAACGAGGGGCAAGTGTGATCCTCTTCTCTTCCCCT 747
gi|54125464 Syncerus    CCCAGGCTTATTACCAACGAGGGGCAAGTGTGATCCTCTTCTCTTCCCCT 747
gi|119514499 Capra      CCCAGGCTTATTACCAAAGGGGGGCAAGTGTGATCCTCTTTTCTCCCCCT 723
gi|119655282 Ammotragus CCCAGGCTTATTACCAAAGGGGGGCAAGTGTGATCCTCTTTTCTTCCCCT 723
gi|89160951 Ovis        CCCAGGCTTATTACCAAAGGGGGGCAAGTGTGATCCTCTTTTCTTCCCCT 723
gi|73697718 Rangifer    CCCAGGCTTATTACCAAAGAGGGGCAAGTGTGATCCTCTTCTCCTCCCCT 723
gi|50442265 Rangifer    CCCAGGCTTATTACCAAAGAGGGGCAAGTGTGATCCTCTTCTCCTCCCCT 723
gi|50442321 Capreolus   CCCAGGCTTATTACCAAAGAGGGGCAAGTGTGATCCTCTTCTCCTCCCCT 723
gi|50442307 Alces       CCCAGGCTTATTACCAAAGAGGGGCAAGTGTGATCCTCTTCTCCTCCCCT 723
gi|158714095 Cervus     CCGAGGCTTATTACCAAAGAGGGGCAAGTGTGATCCTCTTCTCCTCCCCT 723
gi|50442285 Cervus      CCGAGGCTTATTACCAAAGAGGGGCAAGTGTGATCCTCTTCTCCTCCCCT 723
gi|308194928 Homo       CTCAGGCCTATTACCAGAGAGGATCGAGCATGGTCCTCTTCTCCTCTCCA 714
                        . **** *:..**..*.**. * ** .**.******* ** * **
gi|27733849 Equus       CCTGTGGTCCTCCTCATCTCTT---------------------------- 742
gi|119514511 Equus      CCTGTGGTCCTCCTCATCTCTTTCCTCATTTTCCTCATAGTGGGCTGA 768
gi|119489983 Sus        CCTGTGATCCTCCTCATCTCTTTCCTCCTTTTCCTCATAGTGGGCTGA 774
gi|119489801 Bos        CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 795
gi|54125480 Bos         CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 795
gi|54125508 Bubalus     CCTGTGATCCTCCTCATCTCTTTGCTCATTTTTCTCATAGTAGGATAG 795
gi|54125464 Syncerus    CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 795
gi|119514499 Capra      CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|119655282 Ammotragus CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|89160951 Ovis        CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|73697718 Rangifer    CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|50442265 Rangifer    CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|50442321 Capreolus   CCTGTGATCCTCCTCATATCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|50442307 Alces       CCTGTGATCCTCCTAATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|158714095 Cervus     CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|50442285 Cervus      CCTGTGATCCTCCTCATCTCTTTCCTCATTTTTCTCATAGTAGGATAG 771
gi|308194928 Homo       CCTGTGATCCTCCTGATCTCTTTCCTCATCTTCCTGATAGTGGGATGA 762
                        ******.******* **.****
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Notes:
** indicates identical nucleotide sequences;*
–, shift of the nucleotide sequences for a more efficient alignment;
. or :, nucleotide substitution.

Fig. 2. *Ending.* Alignment of the PRNP gene nucleotide sequences.

As follows from Fig. 2, there are point differences at positions 5, 7–12, 16, 21–22, 24, 30, 33, 47, 50, 61, 63, 66, 94, 108, 110, 114, 126, 135, 138, 153, 156, 160, 162, 171, 174, 190, 193, 196, 201–223 (numbered as in *Bos taurus* protein) between *Bos taurus*, *Bos javancus, Bubalus bubalis*, and *Syncerus caffer caffer*; positions 225, 231, 237, 240, 246, 255, 261, 264, 270, 273, 283, 294, 296–298, 318, 320, 324, 339, 348, 366, 375, 378, 381, 399, 408, 411, 414, 438, 444, 447,453, 456, 459, 462, 496, 528, 532, 540, 543, 554–555, 564, 570, 576, 582, 589, 600, 606, 630, 636, 642, 648, 660, 663, 676, 686, 698–699, 705, 708–709, 721, 723, 726, 738, 741–742, 744, 747, 762, 769–795 are different only in *Equus caballus*.

To better visualize the level of evolutionary relatedness of the prion protein sequences, a phylogenetic tree presented in Fig. 3 was built in the ClustalW software (see Fig. 2 for designations).

Also, gene sequences of pathogenic and normal prion protein from *Ovis aries* was performed (Fig. 4). It demonstrated that the nucleotide sequences of PrP^{c} and PrP^{sc} are identical.

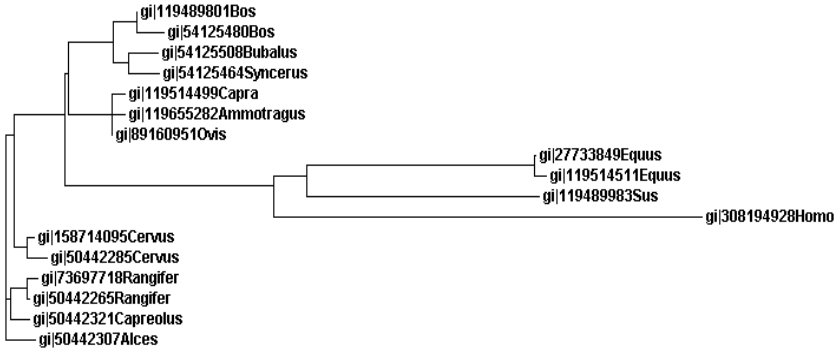

Fig. 3. Phylogenetic tree of the PRNP protein gene sequences.

```
   gi|341942290PrPsc
GGGTCAAGGTGGTAGCCACAGTCAGTGGAACAAGCCCAGTAAGCCAAAAACCAACATGAA 60
   gi|47028553PrP    -
GGTCAAGGTGGTAGCCACAGTCAGTGGAACAAGCCCAGTAAGCCAAAAACCAACATGAA 59
            ************************************************************
   gi|341942290PrPsc
GCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGGTAGGGGGCCTTGGTGGCTACATGCT 120
   gi|47028553PrP
GCATGTGGCAGGAGCTGCTGCAGCTGGAGCAGTGGTAGGGGGCCTTGGTGGCTACATGCT 119
            ************************************************************
   gi|341942290PrPsc
GGGAAGTGCCATGAGCAGGCCTCTTATACATTTTGGCAATGACTATGAGGACCGTTACTA 180
   gi|47028553PrP
GGGAAGTGCCATGAGCAGGCCTCTTATACATTTTGGCAATGACTATGAGGACCGTTACTA 179
            ************************************************************
   gi|341942290PrPsc
TCGTGAAAACATGTACCGTTACCCCAACCAAGTGTACTACAGACCAGTGGATCAGTATAG 240
   gi|47028553PrP
TCGTGAAAACATGTACCGTTACCCCAACCAAGTGTACTACAGACCAGTGGATCAGTATAG 239
            ************************************************************
   gi|341942290PrPsc
AACCAGAACAACTTTGTGCATGACTGTGTCAACACCACAGTCAAGCAACACACAGTCAC 300
   gi|47028553PrP
TAACCAGAACAACTTTGTGCATGACTGTGTCAACATCACAGTCAAGCTACACACAGTCAC 299
            ********************************** ***********:************
   gi|341942290PrPsc
CACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGATAATGGAGCGAGTGGT 360
   gi|47028553PrP
CACCACCACCAAGGGGGAGAACTTCACCGAAACTGACATCAAGATAATGGAGCGAGTGGT 359
            ************************************************************
   gi|341942290PrPsc GGAGCAAATGTGCATCACCCAGTACCAGAGAGAATCCCAGGCTT 404
   gi|47028553PrP    GGAGCAAATGTGCATCACCCAGTACCAGAGAGAATCCCAGGCT- 402
            ****************************************
```

Fig. 4. Alignment of normal (PrP^{c}) and pathogenic (PrP^{sc}) forms of PRNP prion protein from *Ovis aries*.

The phylogenetic analysis confirmed that prion protein sequences are rather conserved and differ only by conformation and relative stability to proteolysis it associates with. This does not allow choosing a DNA target among the prion sequences for further analysis with PCR. Therefore, here we chose a variety of the PCR method, i.e. the real-time immuno-PCR, where DNA molecule is used as a marker, to detect infectious prion proteins. Immuno-PCR allows to detect pathogenic prion protein using specific antibodies labeled with double-strand DNA. Immuno-PCR combines the universality of the enzyme-linked immunosorbent assays with sensitivity of PCR. The method allows for protein detection at the level of several hundred molecules.

To choose an appropriate antibody reacting with pathogenic prion proteins, we analyzed commercial antibodies. Because of the high inter-species homology noted for the PrP protein, antibodies against peptide conjugates are the most feasible.

Therefore, we chose a mouse monoclonal antibody 15B3 (Prionics) obtained using 3 different sequences (epitopes) of human PrP: 15b3-1 includes amino acid residues 142–148 GSDYEDR(YY); 15b3-2, residues 162–170 YYRPVDQYS; and 15b3-3, residues 214–226 CITQYQRESQAYY (Fig. 5).

```
   gi|56180813Sus
MVKSHIGGWILVLFVAAWSDIGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGG- 59
   gi|119514512Equus MVKSHVGGWILVLFVATWSDVGLCKKRPKPGG-
WNTGGSRYPGQGSPGGNRYPPQGGGG- 58
   gi|6110615Ovis
MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGG- 59
   gi|1149617Capra
MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGG- 59
   gi|34334038Bos
MVKSHIGSWILVLFVAMWSDVGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYPPQGGGGR 60
   gi|89160954Homo  --MANLGCWMLVLFVATWSDLGLCKKRPKPGG-
WNTGGSRYPGQGSPGGNRYPPQGGGG- 56
                 :::* *:****** ***:********** *************************

   gi|56180813Sus  -------
WGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTN 112
   gi|119514512Equus -------WGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-
SHGQWNKPSKPKTN 110
   gi|6110615Ovis  -------WGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-
SHSQWNKPSKPKTN 111
   gi|1149617Capra  -------WGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-
SHSQWNKPSKPKTN 111
   gi|34334038Bos  GQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGG-
THGQWNKPSKPKTN 119
   gi|89160954Homo  -------WGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGG-
WGQGGGTHSQWNKPSKPKTN 108
                 ******************************** ***** :*.**********

   gi|56180813Sus
MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQ 172
   gi|119514512Equus
MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVNE 170
   gi|6110615Ovis
MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGNDYEDRYYRENMYRYPNQVYYRPVDQ 171
   gi|1149617Capra
MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLMHFGNDYEDRYYRENMYRYPNQVYYRPVDQ 171
   gi|34334038Bos
MKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYYRENMHRYPNQVYYRPVDQ 179
   gi|89160954Homo
MKHMAGAAAAGAVVGGLGGYMLGSAMSRPIIHFGSDYEDRYYRENMHRYPNQVYYRPMDE 168
             ***:************************..***.**********:**********:::

   gi|56180813Sus
YSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERVVEQMCITQYQKEYEAYAQR 232
   gi|119514512Equus
YSNQNNFVHDCVNITVKQHTVTTTTKGENFTETDVKIMERVVEQMCITQYQKEYEAFQQR 230
```

Fig. 5. *Beginning.* Amino acid sequence of the PRNP prion protein.

```
    gi|6110615Ovis
YSNQKNFVHDCVNITVKQHTVTTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQR 231
    gi|1149617Capra
YSNQNNFVHDCVNITVKQHTVTTTTKGENFTETDIKIMERVVEQMCITQYQRESQAYYQR 231
    gi|34334038Bos
YSNQNNFVHDCVNITVKEHTVTTTTKGENFTETDIKMMERVVEQMCITQYQRESQAYYQR 239
    gi|89160954Homo
HSNQNNFVHDCVNITIKQHTVTTTTKGENFTETDVKMMERVVEQMCITQYERESQAYYQR 228
             :***:.*********:*:*****************:*::***********::* :*: **

    gi|56180813Sus    GASVILFSSPPVILLISFLLFLIVG 257
    gi|119514512Equus GASVVLFSSPPVVLLISFLIFLIVG 255
    gi|6110615Ovis    GASVILFSSPPVILLISFLIFLIVG 256
    gi|1149617Capra   GASVILFSPPPVILLISFLIFLIVG 256
    gi|34334038Bos    GASVILFSSPPVILLISFLIFLIVG 264
    gi|89160954Homo   GSSMVLFSSPPVILLISFLIFLIVG 253
             *:*::***.***:******:*****
```

Fig. 5. *Ending.* Amino acid sequence of the PRNP prion protein.

15B3-1 and 15B3-2 bind beta-sheets that accumulate in PrP^{sc}, and 15B3-3 recognizes amino acid residues near the C-terminus.

15B3 is an antibody specifically recognizing an aberrantly folded PrP^{sc} protein, and not the normal PrP molecules (PrP^{c}).

The Prionics company proved experimentally that 15B3 reacts with pathogenic PrP^{sc} prions of man, cattle, sheep, deer, mouse, and hamster, but does not react with the normal prions. Therefore, 15B3 can be used as a detecting antibody for further analysis.

The procedure goes as follows. Antigen (prion protein) is introduced into a 96-well polysterene plate, 100 μL per well, and the plate is incubated at 37°C for 60 min. To choose the optimal conditions for antigen adsorption on the plastics, the antigen was incubated at concentrations of 0.5, 1, 2.5, 5, 10, 25, and 50 μg/mL for 30, 60, 90, 120, 150, or 180 min. Unbound material is removed from the wells with a simple shaking followed by three washes (washing buffer: 50 mM Tris, 150 mM NaCl, and 0.5 mL/L Tween 20). Non-specific binding sites are blocked by a 30-min incubation with PBS supplemented with bovine serum albumin, 100 μL per well. After removal of the blocking solution (by washing), biotinylated monoclonal antibody to pathogenic prion protein 15B3 is added, 100 μL per well, to determine the adsorbed material, and the plate is incubated for 2 h at 18°C. Unbound antibodies are removed by a triple washing of the wells with PBS containing 1 mL/L Tween and triple washing with PBS containing 15 g/L bovine serum albumin. To prepare DNA reporter agent, streptavidin–biotin complex was chosen as a binding unit between the antibody and the DNA reporter.

A molecule of streptavidin comprises four identical subunits and is capable of binding four biotin molecules, which allowed using it as a binding unit between two biotin-containing compounds. In this case, DNA tail is also biotinylated, and streptavidin functions as a bridge binding the two molecules containing biotin residues.

Preparation of the conjugates of antibodies and DNA with biotin is accompanied by minimal changes in their immunological activity.

Recombinant streptavidin is pre-incubated for 45 min at 4°C with biotinylated DNA reporter in the molar ratio of 1 : 2. Then, the streptavidin–DNA complex is added to the wells and the plate is incubated for 30 min at room temperature. The wells are washed 5 times with PBS and 10 times, with distilled water, and then subjected to PCR.

By this stage of the study, we have already aligned the PRNP gene nucleotide sequences and have built the phylogenetic tree. Alignment of gene sequences of pathogenic and normal prion proteins from *Ovis aries* has demonstrated that the nucleotide sequences of PrP^{c} and PrP^{sc} are identical.

To choose a high-performance DNA target, we performed the analysis of GenBank, Sol Genomic Network, and EMBL-EBI databases, which proved that the prion protein gene sequences are rather conserved; therefore, it is not possible to choose a DNA target among the prion sequences for further analysis with PCR. Therefore, we have chosen the real-time immuno-PCR method for detection of infectious prion proteins, where DNA molecule is used as a marker.

Mouse monoclonal antibody 15B3 obtained using three different sequences (epitopes) of the human PrP peptide (15b3-1 includes amino acid residues 142–148 GSDYEDR(YY); 15b3-2, residues 162–170 YYRPVDQYS and 15b3-3, residues 214–226 CITQYQRESQAYY) was chosen for the work.

It has been shown experimentally that 15B3 reacts with pathogenic PrP^{sc} prions of man, cattle, sheep, deer, mouse, and hamster, but does not react with the normal prions. Therefore, 15B3 can be used as a detecting antibody for further analysis.

The primer design as such is preceded by the construction of a detailed model of the target gene or another nucleotide sequence to be amplified.

To perform the immuno-PCR analysis, a DNA (or a DNA tail) template was needed.

To decrease the risk of false response due to exogenous contamination of DNA in the assay, we designed a DNA tail which does not exist in nature. A synthetic random 194 bp long sequence (fragment length in the range of 150–300 bp is considered optimal) was prepared (see Fig. 6) [11, 12].

AGGAGGTGGCCACGACTGCGAAGGAGGTGGCGTAGGATAGAGT-
CAGTCCTTGGCCTCCTTGGCCCAGTTAAGAAGTTGCAGCCACA-
CACGCTGTTGTTGGGTTCGGGGCGGAGTTGCAGCCATCTACACAAACGA-
TACCCTCGTGCAGCTGGAGAAGCAGCACGGCCTATTACCTGGAGGAGGATCGAAACTGA

Fig. 6. DNA template sequence.

The created sequence was analyzed in GenBank using the BLAST software to confirm that there are no homologs of the sequence.

One of the key factors in the reaction are the primers, synthetic oligonucleotides 20–30 nucleotide long. Primers are complementary to DNA chains in regions at the boarder of a chosen DNA fragment and are oriented with their 3'-ends facing each other and along the chosen DNA sequence to be amplified. The length of the amplified fragment is determined by the distance between the primers.

In the PCR amplification, two oligonucleotide primers are used. Primers are chosen so that the synthesis by polymerase would proceed only between them, doubling the number of copies of this DNA region. As a result, the amount of a specific fragment grows exponentially.

Primer construction, probably, is the most critical parameter for a successful PCR analysis. Primer sequence determines a whole number of parameters, such as the position and length of the product, its melting temperature, and yield of the product. Poorly constructed primer may lead to small amount of the product, its absence due to non-specific amplification and/or dimer formation by a primer, which may become a competitive process inhibiting the product formation [11].

Taking into account the above-mentioned issues, the following two 20-nucleotide long primers were selected for the synthesized DNA tail:

>>>>>> left primer – starting from 41 bp-AGTCAGTCCTTGGCCTCCTT;

<<<<<< right primer – starting from 193 bp-CAGTTTCGATCCTCCTCCAG.

Using the Primer3 software, melting temperature (t_m) and other parameters of the primers were chosen (Table 7).

The melting temperature of the left primer t_m = 59.8°C and the right primer t_m = 60.25°C.

Annealing temperature is set 4–5°C below the melting temperature.

Therefore, the optimal annealing temperature in the amplification program will be t_a = 56°C for the left primer and t_a = 55.8°C, for the right one.

Table 7. Parameters of the primers

Primer type	Starting position, bp	CG, %	Length, bp	t_m,°C	Sequence
Left primer	41	55	20	60.25	AGTCAGTCCTTGGCCTCCTT
Right primer	193	55	20	59.80	CAGTTTCGATCCTCCTCCAG

Table 8. Final characteristics for primer construction

Main requirements to the primers	Values	Comparison of the characteristics of chosen primers with the requirements
Primer length	from 15 to 30 nucleotides	20 bp, fits
GC content	from 45% to 55%	55%, fits
Melting temperature (t_m)	from +55°C to +75°C	t_m = 60.25°C and t_m =59.80°C fits
Annealing temperature (t_a)	4–5 degrees below the melting temperature	t_a =56°C и t_a =55.80°C, fits
Secondary structure of the primer	Primer should not fold into a secondary structure with melting temperature equal to or above the t_m of the primer	Fits (verified using the *Mfold* 3.2 software package)
Secondary structure of the target site	Target site should not fold into a secondary structure with melting temperature equal to or above the t_m of the primer	Fits (verified using the *Mfold* 3.2 software package)
Homo- and heterodimerization of the primers	Excluded, especially at the 3'-end	Fits (verified using the Hybrid software package)
Primer specificity	The degree of complementarity to the target site is close to 100%; less than 70% homology with other nucleotide sequences	Fits (verified using the BLAST software)

Therefore, at this stage of the study, random synthetic target DNA sequence (DNA tail) 194 bp long has been created. Analysis of the GenBank using the BLAST software demonstrated that the created sequence has no homologs among the sequences of the database.

Two 20-bp primers were synthesized for the DNA tail. Using the Primer3 software, primer parameters were chosen.

Studies of the specificity of the developed PCR system were performed by the example of meat chop containing the mixture of muscle tissues of beef and pork and supplemented with 1.0, 2.0, 5.0, 10.0, and 15.0 pork meat infected with a pathogenic prion protein. Each stage of DNA isolation was accompanied by the addition of an internal standard. The presence of the pathogenic prion protein in pork tissues was confirmed using the commercial TeSeE^{TM} ELISA test-system.

The analysis demonstrated high specificity of the developed PCR system: no non-specific response to 100-% fish flour or chicken chop, as well as their mixtures, was registered. The results are presented in Fig. 7.

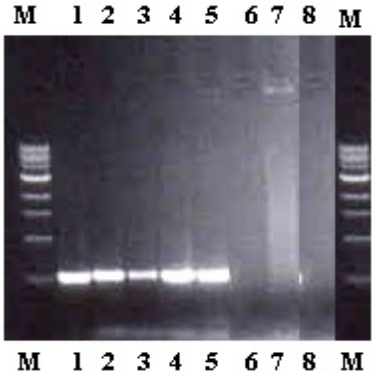

Fig. 7. Evaluation of the specificity and sensitivity of the test-system: M, marker; 1, 1.0% infected pork meat in the meat chop; 2, 2.0% infected pork meat in the meat chop; 3, 5.0% infected pork meat in the meat chop; 4, 10.0% infected pork meat in the meat chop; 5, 15.0% infected pork meat in the meat chop; 6, 100% meat chop; 7, fish flour; 8, chicken chop.

Besides, the specificity of the developed test-system was studied based on the comparative analysis of the results on determination of the pathogenic prion protein obtained using the proposed PCR test-system and a commercially available ELISA assay TeSeE^{TM}. Over 200 samples of clinical material were tested in parallel. Test results of the commercially available TeSeE^{TM} assay and the proposed test-system matched in 198 out of 200 cases. Sensitivity of the reference (commercially available) method was 96.5%. In five of the positive samples not detected by the TeSeE^{TM} ELISA assay, initial DNA target concentration did not exceed 100 copies/mL. Therefore, the higher stability of detection of low DNA concentrations in the PCR method, if compared with other methods, is confirmed by the results of clinical samples study (Table 9).

Table 9. Comparison of the results of pork testing using the PCR test-system and the TeSeE^{TM} ELISA assay

	Number of analyzed samples					
	PCR test-system			TeSeE^{TM} ELISA assay		
	«+»	«–»	«inh»	«+»	«–»	«inh»
«+» n = 60	58	1	1	57	2	1
«–» n = 140	1	139	0	2	134	4
Relative specificity	96,5			96,5		

Notes: «+», positive samples; «–», negative samples; «inh», inhibited samples.

Comparison of the results obtained with the proposed PCR test-system and the reference method evidence real-time high specificity of the developed PCR method.

Therefore, high specificity of the developed test-system and oligonucleotide primers was confirmed by three ways: 1) using the Primer3 software; 2) by electrophoretic separation of the meat chop samples with different percent content of pork tissues infected with a pathogenic prion protein; and 3) by comparative analysis of the results of pathogenic prion protein determination using the proposed PCR test-system and a commercial ELISA assay TeSeE^{TM}.

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