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Polymorphism In The PrPC Prion Protein Gene In Pigs

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POLYMORPHISM IN THE PrP^C PRION PROTEIN GENE IN PIGS

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ABSTRACT

Protein coding regions of the PrP^C gene in 51 pigs belonging to 3 breeds namely Hampshire, Yorkshire, Blue Butt Cross (blue spotted cross from a Hampshire X Yorkshire), and a wild pig were studied. These breeds are used nationwide for commercial purposes. The *PrP^C* gene of the pigs that code for prion proteins were sequenced and studied. Deletions of the octapeptide (WGQPHGGG) were observed in 13 samples that belongs to the crossbreed Dark Cross sow 71 by Blue Butt Boar (Gummy bear). This hydrophobic repeat has been used in experiments as a model to investigate neurodegeneration in prion diseases. Also, it was used as vaccine where the octapeptide sequence was administered in a liposomal bilayer and when administered to an animal, the vaccine elicited a local or systemic, immunogen-specific immune response against amyloid proteins, peptides or fragments. In previous studies, prion proteins commonly have five or six repeats, while in this study, four octarepeats were obtained in all samples except the few that contained only three octarepeats. The presence of the octapeptide is said to be correlated with increased susceptibility of prion disease. If this is the case, these individuals may be less susceptible to prion disease as compared to other breeds. Still, research needs to be done to confirm this. Those sequences were then compared with the PrP^C genes of other mammals. Based on these sequences, a phylogenetic tree was constructed. Also, the gender of the pigs does not seem to play role in whether the octapeptide deletion occurs.

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PREFACE

This thesis follows the style of American Society for Microbiology.

INTRODUCTION

Prion diseases

Prion diseases are neurodegenerative disorders caused by the pathogenic isoform of a normal constituent of cell membrane called prion protein (Johnson 2005). These diseases are collectively called transmissible spongiform encephalopathies (TSEs) and are named differently depending on the species they infect, and includes Kuru and Creutzfeldt-Jakob disease (CJD) in humans, bovine spongiform encephalopathy (BSE) in cows, scrapie in sheep, and chronic wasting disease (CWD) in deer and elk (Choi et al. 2012).

Prion disease leads to impairment of brain function, decline in intellectual function, and abnormal movements (National Institutes of Health 2014). About 10 -15% of the cases of human prion diseases are the result of mutations in the PrP^{C} gene, while the remaining 85-90% are classified as either sporadic or acquired.

Sporadic prion disease does not have a genetic predisposition or an identified mutation in the PrP^{C} gene and occurs when the normal prion protein (PrP^{C}) is transformed to an abnormal sporadic form of prion protein (PrP^{SC}) (Figure 1). The mechanism for this transformation is yet unknown, but prion diseases are transmitted by consumption of infected feed or by contact with infected animals (Johnson 2005). The sporadic form of human prion disease includes sporadic CJD (sCJD), sporadic fatal insomnia (sFI), and variably protease-sensitive prionopathy (VPSPr). On the other hand, acquired prion disease is caused by the exposure to PrP^{SC} from an external source, as in vCJD, BSE, and Kuru. In some cases, prion disease is caused through accidental exposure to PrP^{SC} during

a medical procedure. This method of transmission is classified as iatrogenic (National Institutes of Health 2014).

History of prion research

The historical background of prion disease dates back to 1700, when a disease 'scrapie' was first reported in sheep (Hunter 1993) but it did not receive public attention until the 1980s. Scrapie once was thought to be caused by sexual overactivity. It was then considered to be a viral disease. Broad scale research and production of several hypotheses occurred rapidly after reports that the scrapie from sheep was transmitted to mice (Morris and Gajdusek 1963) and from goats to mice (Chandler 1961, Chandler 1963). Hunter (1993) showed that scrapie passed through an ultrafilter of 30 nm and was highly hydrophobic. He concluded its replication did not depend on intrinsic nucleic acid. Kuru, a type of TSE, was first identified in humans as a strange encephalitis-like disease in the Fore tribe of Papua New Guinea. A young virologist, D. Carlton Gajdusek and his team were working to isolate a virus but all attempts were in vain (Gajdusek and Gibbs 1964). They concluded cannibalism, a culture followed in Papua New Guinea, to be the vehicle of kuru transmission, but the causative organism or substance was still unknown (Liberski et al. 2012). Later, William Hadlow recognized a micrograph of kuru pathology to be reminiscent of scrapie, and kuru was finally identified as a type of TSE (Farquhar and Gajdusek 1981). Other TSE related diseases have their own history of identification and prevalence.

Stanley B. Prusiner (1982), forwarded the idea that prion disease is caused by protein and nothing else. Unlike other diseases, including viral diseases, prion disease does not follow the basic principles of the central dogma of molecular biology, which holds that diseases that are transmissible contain genetic material like DNA or RNA. Hence, he gave the term 'prion' to the protein, which was to be pronounced as 'pree-on' and derived from two words 'protenacious' and 'infection' (Prusiner 1982). Prion protein then changes its structure to an abnormal form, causing disease. He, along with other researchers, then concluded that abnormal prion protein converts normal prion protein to abnormal form and this process occurs rapidly (Prusiner 1995)

BSE was first discovered in Great Britain in 1986. The neuropathology of BSE was similar to that of scrapie in sheep, and so, the disease was recognized as a spongiform encephalopathy. Afterwards several cases of BSE were reported that resulted in epidemics among cows. Epidemiological studies revealed that meat-and-bone-meal (MBM) might be used as a vehicle to transfer the disease. Hence in June 1988, the U.K. government banned the use of ruminant-derived MBM to be fed to ruminants, but it was still allowed to be feed to pigs and poultry, as these animals were not thought to be susceptible to BSE. Several other cases occurred even after the ban, which gave rise to a new hypothesis that pigs and poultry might act as carriers and cross-contaminate the infected feeds. After the first case of vCJD was reported in 1996, feeding mammalian protein to any cattle was banned. After this ban in 1996, the BSE cases declined from 44,000 in 1992 to 57 after 1996 (Smith and Bradley 2003). vCJD, which was thought to be acquired in people by consuming BSE contaminated beef, had some similarities with the previously known

human prion disease CJD (Ingram 2013). Also, during the same decade when BSE outbreak in cattle and the vCJD outbreak in people, Feline Spongiform Encephalopathy (FSE) was confirmed in domestic cats in Great Britain as the result of eating pet food made with BSE. By the end of 1999, the number of cases of FSE recorded reached 87 (World Health Organization, 1999).

Prion protein overall structure:

The widespread distribution of prion protein among mammals prompted biologists to determine its structure. Three-dimensional structure and functional properties of the proteins were then compared with nucleotide and amino acid sequences (Calzolai et al. 2005). Due to the low yield of the purified prion protein in previous work, it was difficult obtain structural results using NMR spectroscopy or X-ray crystallography. So, recombinant PrP^{C} was developed to study the protein structure, which suggested that recombinant PrP^{SC} contains three alpha helices and one small anti-parallel beta sheet (Acevedo-Morantes and Wille 2014).

Based on NMR measurements, conformation of recombinant human PrP^{C} was determined (Hosszu et al. 1999) as shown in Figure 2. It consists of a signal peptide, and with the addition of glycophosphatidylinisitol (GPI) anchor functions as a membrane attachment signal. PrP^{C} is further made up of a flexible N- terminal domain and a largely helical C- terminal domain. The N-terminal domain is involved in the regulation of copper and zinc whereas the C-terminal domain is involved in the conversion of alpha helix to beta sheets during the development of prion disease. Both of these domains are independent of one another and do not interact (Stockel et al. 1998). The C-terminus

forms a globular domain, comprising three alpha helices and a short double stranded beta sheet, stabilized by a disulfide bond. The domain contains two N-linked glycosylation sites (Rheede et al. 2003). Similarly, conformations of PrP^{C} of hamster (James et al. 1997), bovine (Garcia et al. 2000), mouse (Riek et al. 1996, 1997), and others were also determined using NMR. Globular folds of all these mammals were highly similar (Rheede et al. 2003). This globular domain is highly conserved among various species, as the nucleotide sequences of different species encoding this globular domain are almost identical. (Dominikus et al. 2005). A study based on NMR structures has shown the pig prion protein has a single charge-effective amino acid substitution in the position 223 relative to cow prion protein. In addition, amino acid replacement from Asn-173 in the cow prion protein to Ser-173 in pigs is found to stabilize the 100-173 loop in the protein due to its neutral charge. It is hypothesized that this extra stability may explain why naturally occurring prion diseases have not been reported in pigs. The study thus has presumed that this substitution might affect the protein structure (Prusiner 1998). Change in the conformation of normal PrP^{C} to abnormal PrP^{SC} is carried out by the increase in beta-sheet content from 3% to around 40%, whereas alpha-helix content is decreased from 40% to 30% (Cohen and Prusiner 1998).

Prion protein octarepeat region

Physiological function of prion protein explains how membrane-bound prion protein binds to copper in the N- terminal domain (Whittal et al. 2000). The octarepeat region in the N- terminal domain has been observed in residues 60-91 in PrP^{C} of humans and other mammals, including pig, syrian hamster, and others. The prion protein in these

animals consists of four or more repeats of the residue sequence PHGGGWGQ. Those humans who contain extra octarepeats in their prion protein are prone to Creutzfeldt-Jakob disease (Goldfarb et al. 1991). Similarly, in an experiment done in mice, removal of the octarepeat domain helped in the slowing the progression of disease (Flechsig et al. 2000). Also, when copper was added to wild prion protein, protease resistance was favored, which is a characteristic feature of the pathogenic form (Quaglio et al. 2001). The cellular membrane prion protein (PrP^{C}) is GPI-anchored, which exists on the membrane surface (Vey et al. 1996), where exchange of divalent copper occurs (Peña et al. 1999, Waggoner et al. 2000). The above mentioned octarepeat peptide domain binds four Cu^{2+} ions with significant cooperativity as compared to other divalent metal ions (Whittal et al. 2000). Circular dichroism (CD) studies have indicated that coordination in PHGGGWGQ and HGGG peptides develops from the imidazole ring of histidine amino acid and three amide nitrogen within HGGG unit (Bonomo et al. 2000). These studies suggested fundamental ideas on the functions of PrP^{C} as a copper sensor as well as transporter, and also the role of octarepeat in transmembrane signaling leading to the copper-mediated conversion of PrP^{C} to PrP^{SC} (Burns et al. 2002).

The octapeptide repeat (OR) region is a highly conserved part of PrP^{C} in mammals (Harris et al. 1991). The 92-111 region is denoted as a non-OR region which is adjacent to the hydrophobic residues (112-127), and contains two additional His residues and which are also hypothesized to play a part in the prion conversion. A high degree of conservation is observed in the residues 90 to 127 among different mammals. Because the

OR region does not seem to be play a role in the conversion of prion to the abnormal form, the non-OR copper binding site is being studied currently.

The non- OR copper binding site is found to be adjacent to the palindromic amino acid motif sequence AGAAAAGA. This palindromic motif is involved in causing the structural changes during the initial prion converting phase. A recent study has also shown that the interaction among copper and peptide, including both palindromic motif and non-OR residues induce beta-sheet formation, which results in the aggregation of the peptide segment (Giachin et al. 2015).

Species barriers

Though PrP^C sequence is highly conserved among mammals, prions are not observed to transfer easily from one species to another (Collinge 2012). Slight polymorphisms in the amino acids might play a major role in the transmission efficiency (Beringue et al. 2008). For example, in the case of sheep, homozygosity for the $V_{136}R_{154}Q_{171}$ (VRQ) allele has shown high susceptibility to classical scrapie whereas homozygosity in allele $A_{136}R_{154}Q_{171}$ (ARQ) exhibits major resistance (Belt et al. 1995, Goldmann et al. 2016). In the same way, human susceptibility to TSE is influenced by the amino acid polymorphism at residue 129 (Collinge 2001, Wadsworth and Collinge 2007). Transmission of the TSE agent within the species was noted first in sheep scrapie in the 1930's (Belt et al. 1995). Cross species transmission of prion is less common than intraspecies transmission. The emergence of new vCJD strain in humans, after the BSE outbreak, has demonstrated the ability of prion disease to be transferred from one species

to the other. Conformational flexibility of the prion protein, along with the ability to infect foreign hosts, seems to play an important role in strain variation, but this is a complex process, which is difficult to comprehend (Beringue et al. 2008).

Previous studies explained that the difference in the primary structure of protein between host and donor species is the major point where the species barrier resides. In an experiment by Scott et al. (1989), hamster PrP^C was expressed in transgenic mice, which developed susceptibility to hamster scrapie (Kimberlin and Walker 1978) which resulted in the apparent lack of species barrier. Here, the PrP^C gene of host and infecting species were identical. In this way, mice transgenic lines were developed for sheep (Crozet et al. 2001, Vilotte et al. 2001, and Westaway et al. 1994), bovine (Beringue et al. 2006, Buschmann and Groschup 2005), human (Asante et al. 2002), and other mammals. Also, transgenic lines have been made by using gene replacement method. In this work, a series of inbred lines of transgenic mice were produced and PrP^C genes were expressed in each, which allowed the study of the direct influence of the host PrP^C gene in TSEs. Hence, these experiments gave the conclusion that identity in the PrP^C sequence among transgenic host and donor leads to the increased susceptibility to TSE when compared to the wild type mice (Cancellotti et al. 2007).

Hence, interspecies transmission of prions results in varying outcomes based on the type of species. For example, some species can show resistance to infection, while some might possess asymptomatic disease which might act like a carrier and can pass disease to a different species more easily. Also, certain species can show permanent or

reversible change of strain properties, and some might show faithful propagation (Beringue et al. 2008).

Past studies with the recombinant prion protein PrP^C have explained prion assembly as a nucleation-dependent polymerization process, which also gives different insight into the species barrier concept. In these studies, fibrils were made of bacterially-expressed PrP^C in such a way that those were able to polymerize into amyloid fibrils either spontaneously or by adding preformed fibrils(Surewicz et al. 2006, Noinville et al. 2008). In the study done by Surewicz et al. (2006), recombinant PrP^C fibrils were generated using mouse, human or hamster PrP^C. They were morphologically different to one another. Also, experiments were carried out to examine the cross-seeding capacities of the fibrils which concluded that mouse monomers could be converted by hamster fibril seeds, whereas the opposite could not happen. Once formed, the mouse fibril generated from the hamster can then polymerize the hamster PrP^C monomers into fibrils. These newly formed hamster PrP^C fibrils adopted different secondary structure as compared to that of mouse fibrils generated by mouse PrP^C monomers. This study has also explained that point mutations can influence the seeding barrier by changing the conformation of amyloid fibrils with mutant fibrils and adopting PrP^C of a different species. For example, in case of human variant PrP^C, mutation at position 138 and 139 where isoleucine is replaced by methionine, the seeding capacity is affected, which is similar to that seen in the hamster protein. It resulted in the hamster- like fibril conformation (Jones and Surewicz 2005, Vanik et al. 2004).

Risk of prion zoonoses

Transmission of prion disease from cattle to human is a major concern to public health. There is evidence of clinical disease in the transgenic mouse expressing human PrP^C protein which were exposed to BSE prions (Beringue et al. 2012), whereas prion infection in spleen was observed in high proportion and infection in brain was seen in less than 10% of mice kept for longer incubation periods. Prions might not be harmful by the subclinical infection caused from foreign prions, but when they adapt to new host species, high lethality was obtained in the individuals of that species (Hill et al. 2000). This is important in terms of public health because humans colonized with silent vCJD prions can transfer prions to healthy individuals via blood transfusion, tissue donation, and other contamination during surgery (Weissmann 2002). In 1996, vCJD was found in a human caused by exposure to BSE strain (Collinge 2001). But the rate of vCJD transmission was very low which might be the result of the species barrier. Research has shown that during BSE outbreak, rate of vCJD was 1 in 4000 of UK population. (Spongiform Encephalopathy Advisory Committee position statement, 2008).

Prions in pigs

Naturally occurring prion diseases have not yet been identified in pigs (*Sus scrofa*). As pigs were clearly exposed to the ruminant derived meal during the British BSE epidemic, studies were conducted to examine if BSE was transmitted to them. But BSE was not found to be transmitted from feeding infected brain materials to pigs, while BSE was observed in animals when brain homogenate from BSE infected cows was inoculated to healthy pigs through intracerebral, intravenous, and intraperitoneal routes (Gibbs et al.

1979). Study of prion proteins in pigs is important because they are widely used in human daily life. More than one billion pigs are consumed by humans every year. Although natural TSEs have not yet been identified in pigs, there is a possibility these prion-disease-resistant pigs can be carriers of replicating prions (Hammarstrom and Nystrom 2015). Since pigs are also considered to be excellent organ donors for humans, because humans and pigs share similar physiology, there is another possibility that prion disease could be transmitted to humans during xenotransplantation (Meng et al. 2005).

Purpose of my research

Based on species barrier, transmission of PrP^{SC}, and to develop therapeutic interventions, the basic goal of all prion related research is to have knowledge and understanding of the structure and function of prion protein regions that are involved in the generation and aggregation of PrP^{SC}. In this research, my objective was to sequence the prion encoding gene extracted from pig tissues (Figure 3) and look for polymorphisms in the sequences. The aim of this study was to see if the polymorphisms in the sequences have contributed to changes in copper binding domain of the prion protein along with studying if these have links to species diversity. Subsequently, the sequences were compared with other prion disease susceptible and non-susceptible mammals. Because pigs live in close proximity to humans and though there are no naturally reported pig prion disease, research on pig prion protein should be given prior importance and should be continued.

MATERIALS AND METHODS

Sample collection:

A total of 51 samples were sampled, 50 from domestic piglets collected from Fort Hays State University farm belonging to 3 breeds, namely Hampshire, Yorkshire, Blue Butt Cross (blue spotted cross from a Hampshire X Yorkshire), and 1 from a wild pig. Table 1 shows a list of 50 samples taken from the FHSU farm. These breeds are used nationwide for commercial purposes. Out of 50 domestic pigs, 23 were female and 27 were male. The pigs' tails had been docked and placed in 70% ethanol and stored in -20°C freezer until DNA extraction.

Genomic DNA isolation:

Total genomic DNA was isolated from each sample using the extraction kit from Qiagen (Hilden, Germany) as per the instruction from manufacturer and eluted in 50 µl of nuclease-free water. Before starting extracting procedure, samples were washed with double-distilled water. The extracted DNA was visualized in 1% agarose gel to verify the DNA was non-degraded. Furthermore, the quality and quantity of DNA was measured using a spectrophotometer (Nanodrop Technologies, Wilmington, DE).

Polymerase Chain Reaction:

Polymerase Chain Reaction (PCR) was used to detect the presence of PrP^C genes in samples of isolated genomic DNA. Primer sequences from Martin et al. (1995), obtained from Sigma, were used in PCR reactions.

The forward primer was Por PrP fwd 5'CATTTGATGCTGACACCCTCTTTA3' and reverse primer was Por Prp rev 5'ATGAGACACCACCACTACAGGGCT3'. DNA was amplified using Phusion Polymerase kit obtained from Biolabs Inc. (Ipswich, MA). Annealing temperature was identified as 60 ^oC which did not match with the one used by Martin et al. (1995).

Amplification of selected regions was carried out in 50 µL of reaction mixture. PCR components were combined in 0.5 mL micro centrifuge tube as follows: 10 µL of 5X Phusion High Fidelity Buffer, 2.5 µL of 10 µM PrP forward primer, 2.5 µL of 10 µM reverse primer, 1 µL of 10 mM dNTPs, 0.5 µL Phusion polymerase, 2 µL of template DNA, and 31.5 µL of nuclease-free water. The optimized PCR conditions were 98 ^oC for 30 seconds, followed by 30 cycles of 98 ^oC for 10 seconds, 60 ^oC for 30 seconds, and 72 ^oC for 30 seconds. The final elongation was occurred at72 ^oC for 10 minutes.

Agarose gel electrophoresis, PCR purification, and sequencing

The qualitative assessment and quantitative estimation of amplicons generated by PCR was verified by gel electrophoresis using Tris-acetate-EDTA in 1% agarose gels (Agarose low EEO, Thermo Fisher Scientific, Waltham, MA) and stained with Sybersafe obtained from Invitrogen (Carlsbad, CA). The one Kb DNA ladder, obtained from Promega, (Madison, WI), was used in the gel and the loading dye used was from

GelPilot. Correct amplicons were purified using QIAquick PCR purification kit (Qiagen, Hilden, Germany) following manufacturer's instructions and eluted in nuclease-free water. Each sample having a concentration of around 30 ng/L was then sequenced at the Sequencing and Genotyping Facility of Kansas State University (Manhattan, KS) using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA) and Genewiz (South Plainfield, NJ).

Sequence alignment and data analysis:

From the nucleotide sequences, protein accession, nucleotide accession, gene annotation, and full length protein sequences were obtained using National Center for Biotechnological Information (NCBI). Based on the sequence of pig prion protein obtained from NCBI, sequences of all 51pig prion proteins were compared and polymorphisms were identified. Nucleotide sequences were aligned in Clustal Omega (Version 1.2.1, Thompson et al. 1994) and BioEdit Sequence Alignment Editor (Version 7.2.5, Hall 1999).

Phylogenetic tree construction

Prion sequences of other mammals were retrieved from NCBI GenBank; *Cricetulus griseus* (Chinese hamster), *Canis familiaris* (dog), *Bos taurus* (cow), *Felis catus* (cat), *Ovis aries* (sheep), *Homo sapiens* (humans), and Cervus *Nippon* (sika deer) and analyzed with the sequences obtained from pig samples. A phylogenetic tree based

on Maximum Likelihood method was constructed using mega software (Version 7. 0. 18, Kumar et al. 2016).

RESULTS

PrP^C Gene Detection by PCR

The PCR assay demonstrated the presence of the PrP^C gene in all 51 samples, including domestic and wild pigs (Figure 4). Based on the sequences obtained from the DNA Sequencing and Genotyping Facility at Kansas State University and Genewiz, the nucleotide sequences of length 774 base pairs were obtained and those sequences were translated into amino acid sequences of length 257. A few multiple peaks of nucleotides were obtained in samples 23-1, 23-4, 24-1, and 24-7.Sequence was not obtained for sample 23-5.

Polymorphisms in the PrP^C gene

Four octapeptide repeats (PHGGGWGQ) were observed in all the samples based on the aligned translated protein sequences of PrP^C gene. But in samples, 9-1, 9-2, 9-4, 9-5, 9-6, 9-7, 9-8, 9-9, 9-11, 23-6, 23-7, 23-8, and 23-10, eight amino acids WGQPHGGG or one complete octapeptide repeat domain were deleted (Figure 5 and 6). These samples with missing amino acids belong to the same pig cross, namely 'Dark Cross sow 71 by Blue Butt Boar (Gummy bear)'. The octapeptide repeat was conserved in all other samples from domestic pigs as well as the wild pig. The octapeptide repeat region was followed by 21 non-octapeptide repeats, after which an alanine rich palindromic amino acid motif sequence AGAAAAGA was observed in all the samples, including other mammals. Hydrophobic amino acids were found in a row, starting immediately following

the palindromic sequence AGAAAGA. Polymorphism was not observed to be related with the gender of the pigs. Both male and female pigs had presence and absence of the octapeptide deletion.

Phylogenetic tree

A molecular phylogenetic tree was obtained using Maximum Likelihood method with Mega 7 software. All gaps and missing data were eliminated and an unrooted analysis was performed. The branch length signifies the rate of nucleotide substitutions. In the tree obtained, branch lengths of most of the pig samples were equal due to almost identical sequences. Branch lengths of rest of the pig samples and other mammals are shown in Figure 7. Rest of the mammals appeared in a different clade.

DISCUSSION

The deletion of one octapeptide as observed in the crossbreed samples 9-1, 9-2, 9-4, 9-5, 9-6, 9-7, 9-8, 9-9, 9-11, 23-6, 23-7, 23-8, and 23-10 can be the result of mutated PrP^C. Such mutation might result in the mutation of the PrP^C protein (Acevedo-Morantes and Wille 2014). Experiments conducted previously have shown that the octapeptide repeat mutation might result in genetic prion disease in humans. Insertion of additional octapeptide repeats makes an individual more prone towards the prion disease and thus the degree of prion aggregation was found to be proportional to the number of octapeptide repeats present. The increased octapeptide repeats resulting in increased aggregation can be the result of inheritance of mutated octapeptide repeat sequence (Yu 2007). The wild pig sample used in this study did not show any deletion of octapeptides, which might suggest that wild pigs have the same prion susceptibility to prion disease as that of domestic pigs.

The octapeptide repeat motif PHGGGWGQ (Figure 5 and 6), observed in the N-terminal domain of the PrP^C protein has a high affinity for copper ions (Cu^{2+}) and other divalent cations such as zinc in specific pH conditions. Copper binding plays an important role in converting alpha helix to a beta sheet structure. The above mentioned 13 samples with one octapeptide deletion in each, contain only three octapeptide repeats which might have reduced the copper binding domain from two to one, since two octapeptide repeats bind to one Cu2+ ion (Stockel et al. 1998). This might contribute to fewer possibility of converting an alpha helix to a beta sheet. This can be one of the

reasons behind why the crossbreeds Dark Cross sow 71 by Blue Butt Boar (Gummy Bear) might be less susceptible to prion disease than the rest of the pig breeds. Research has shown that one Cu^{2+} ion binds to two octapeptides and the imidazole side chain of the histidine present in the octapeptides acts as a chelating ligand. NMR spectroscopy has demonstrated that in the absence of copper ions, octapeptide repeats fail to achieve secondary or tertiary structure. (Stockel et al. 1998). Recent research has suggested that zinc binds to octapeptide and the C-terminal domain via electrostatic interaction which contributes in the tertiary structure contact. This contact then further results to the familial prion disease (Spevacek 2013). In the mice without prion protein, or with infectious prion protein, copper and iron metabolism is abnormal (Brown 2003).

AGAAAAGA, is the hydrophobic core of the protein and is the N-terminal palindrome of PrP^{C}. It was present in all 51 samples sequenced and is shown in Figure 5 and 6. This is a highly conserved sequence motif found in most of the mammals studied so far (Acevedo-Morantes and Wille 2014). Norstrom and Mastrianni (2005) suggested that this AGAAAAGA palindrome is required for obtaining the PrP^{SC} conformation. Mutants without the palindromic sequence were unable to convert PrP^{C} to PrP^{SC}. Also, the palindromic motif was found to facilitate the association of PrP^{C} with PrP^{SC},which results in the aggregation of abnormal protein (Norstrom and Mastrianni 2005).

The deleted sequences WGQPHGGG as seen in pigs of crossbreed Dark Cross sow 71 by Blue Butt Boar (Gummy bear) was noted to be used as vaccine against prion disease. In the past, Nicolau and Kapetanovic (2004) had worked on the vaccine against prion diseases. They used different segments of prion protein as a vaccine, including the

deleted sequences WGQPHGGG, where it is administered in a liposomal bilayer. When administered to an animal, the vaccine elicits a local or systemic, immunogen-specific immune response against amyloid proteins, peptides or fragments, and prevents, stops or hinders amyloid deposition caused by prions. Traditional vaccines, made from either purified antigens or the attenuated form of pathogen, which illicit the immune responses in the individual did not work for prion disease. This might be because it is molecular structure of the abnormal prion protein not the nucleic acid sequences, that is involved in causing prion disease (Nicolau and Kapetanovic 2004).

The phylogenetic tree (Figure 7) shows the evolutionary history of the prion proteins. Branch lengths of most of the pig samples was equal due to almost identical sequences and the remaining samples, which were seen in a different clade, are those having octapeptide deletion . The sequences of mammals subjected for phylogeny analyses were chosen in such a way that both prion susceptible mammals like cow, sheep, cat, human, and prion non-susceptible mammals like dog and pig are included for study. Taking into consideration that both pigs and dogs do not easily get prion disease, their phylogeny was expected to be closely related, but result was not obtained as expected.

CONCLUSION

In the samples 9-1, 9-2, 9-4, 9-5, 9-6, 9-7, 9-8, 9-9, 9-11, 23-6, 23-7, 23-8, and 23-10, eight amino acids WGQPHGGG were deleted. All these samples with deletions are from the same breed (Dark cross sow 71 by Blue butt boar). From the observed results, and based on the research done by Stockel et al. (1998), we can hypothesize that the Dark Cross sow 71 by Blue Butt Boar (Gummy Bear) crosses might be less susceptible to prion disease as these crosses have one less octapeptide repeat as compared to rest of the domestic breeds and wild pig. Because two octapeptide repeats bind to one Cu^{2+} ion, there will be one less octapeptide repeat in these breeds that can bind Cu^{2+} ion, which might contribute to lower possibility of conversion of alpha helix to beta sheets. The result might also indicate that wild pig's prion susceptibility is similar to that of the domestic pigs having four octapeptide repeats and wild pigs might have been separated from the herd of domestic pigs in the course of time. This study might be valuable if this possibility is investigated in future animal studies. Differences in the susceptibility might be observed when the prion disease infected brain material is injected in the brain of these pigs. Those pigs with one less octapeptide repeat might show disease at a lower rate as compared to the others with four octapeptide repeats. Also, the gender of the pigs does not seem to play role in disease susceptibility.

LITERATURE CITED

Acevedo-Morantes CY, Wille H. The Structure of Human Prions: From Biology to Structural Models- Considerations and Pitfalls. Viruses 2014; 6: 3875-3892.

Asante EA, Linehan JM, Desbruslais M, Joiner S, Gowland I, Wood AL. BSE prions propagate as either variant CJD-like or sporadic CJD-like prion strains in transgenic mice expressing human prion protein. The EMBO Journal 2002; 21: 6358-6366.

Belt PB, Muileman IH, Schreuder BE, Bosde Ruijter J, Gielkens AL, Smits MA. Identification of five allelic variants of the sheep PrP gene and their association with natural scrapie. Journal of General Virology 1995; 76:509-517.

Beringue V, Bencsik A, Le Dur A, Reine F, Lai TL, Chenais N. Isolation from cattle of a prion strain distinct from thet causing bovine spongiform encephalopathy. PLOS Pathogens 2006; 2: e112.

Beringue V, Vilotte JL, Laude H. Prion agent diversity and species barrier. Veterinary research 2008; 29 (4): 47.

Beringue V, Herzog L, Jaumain E, Reine F, Sibille P, Le Dur A, Vilotte JL, Laude H. Facilitated cross-species transmission of prions in extraneural tissue. Science 2012; 335 (6067): 472-475.

Bonomo RP, Impellizzeri G, Pappalarado G, Rizzarelli E, Tabbi G. Copper(II) Binding Modes in the Prion Octapeptide PHGGGWGQ: A Spectroscopic and Voltammetric Study. Chem-Eur J 2000; 6: 4195-4202.

Brown DR. Prion protein expression modulates neuronal copper content. Journal of Neurochemistry 2003; 87: 377–385.

Burns SC, Aronoff-Spencer E, Dunham CM, Lario P, Avdievich NI, Antholine WE, Olmstead MM, Vrielink A, Gerfen GJ, Peisach J, Scott WG, Millhauser GL. Molecular Features of the Copper Binding Sites in the Octarepeat Domain of the Prion Protein. Biochemistry 2002; 41:3991-3992.

Buschmann A, Groschup MH, Highly bovine spongiform encephalopathy-sensitive transgenic mice confirm the essential restriction of infectivity to the nervous system in clinically diseased cattle. Journal of Infectious Disease 2005; 192: 934-942.

Calzolai L, Lysek DA, Perez DR, Guntert P, Wuthrich K. Prion protein NMR structures of chickens, turtles, and frogs. Proceedings of the National Academy of Sciences USA 2005; 102: 651-655.

Cancellotti E, Barron RM, Bishop MT, Hart P, Wiseman F, Manson JC. The role of host PrP in transmissible spongiform encephalopathies. Biochimica et Biophysica Acta 2007; 1772: 673-680.

Chandler RL. Encephalopathy in mice produced by inoculation with scrapie brain material. Lancet 1961; 1: 1378-1379.

Chandler RL. Transmissible encephalopathy in mice inoculated with scrapie brain. Journal of Gentic Microbiology 1963; 31: XIV.

Choi S, Woo HJ, Lee J. Sequence variations of the bovine protein gene (PNRP) in native Korean Hanwoo cattle. Journal of Veterinary Science 2012; 13: 127-137.

Cohen FE and Prusiner SB. Pathologic conformations of prion proteins. Annual Review Biochemistry 1998; 67: 793-819.

Collinge J. Prion diseases of humans and animals: their causes and molecular basis. Annual review Neuroscience 2001; 24: 519-550.

Collinge J. The Risk of Prion Zoonoses. Science 2012; 335: 411-413.

Crozet C, Flamant F, Bencsik A, Aubert D, Samarut J, Baron T. Efficient transmission of two different sheep scrapie isolates in transgenic mice expressing the ovine PrP gene. Journal of Virology 2001; 75: 5328-5334.

Dominikus AL, Schorn C, Nivon LG, Esteve-Moya V, Christen B, Colzalai L, Schroetter Cv, Fiorito F, Herrmann T, Guntert P, Wuthrich K. Prion protein NMR structures of cats, dogs, pigs, and sheep. Proceedings of the National Academy of Sciences USA 2005; 102 (3): 640-645

Farquhar J, Gajdusek DC eds. Kuru. Early letters and field-notes from the collection of D. Carleton Gajdusek. Raven Press, New York 1981.

Flechsig E, Shmerling D, Hegyi I, Raeber AJ, Fischer M, Cozzio A, von Mering C, Aguzzi A, Weissmann C. Neuron. Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. Neuron 2000; 27: 399-408.

Gajdusek DC, Gibbs CJ. Attempts to demonstrate a transmissible agent in kuru, amyotrophic lateral sclerosis, and other sub-acute and chronic nervous system degenerations of man. Nature 1964; 204: 257-259.

Garcia LF, Zahn R, Riek R, Wuthrich. NMR structure of the bovine prion protein. Proceedings of the National Academy of Sciences USA 2000; 97: 8334-8339.

Giachin G, Mai PT, Tran TH, Salzano G, Benetti F, Migliorati, Arcovito A, Longa SD, Mancini G, D'Angelo P, Legname G. The non-octarepeat copper binding site of the prion protein is a key regulator of prion conversion. Scientific reports 2015; 5: 15253.

Gibbs CJ Jr, Gajdusek DC, Amyx H. Strain variation in the viruses of Creutzfeldt-Jacob disease and kuru. Slow transmissible diseases of the nervous system, Academic Press, New York 1979: 2: 87-110.

Goldfarb LG, Brown P, McCombie WR, Goldgber D, Swergold GD, Wills PR, Cervenakova L, Baron H, Gibbs CJ Jr, Gajdusek, DC. Transmissible familial Creutzfeldt-Jakob disease associated with five, seven, and eight extra octapeptide coding repeats in the PRNP gene. Proc. Natl. Acad 1991; 88: 10926-10930.

Goldmann W, Marier E, Stewart P, Konold T, Street S, Langeveld J, Windl O, Ortiz-Pelaez A. Prion protein genotype survey confirms low frequency of scrapie-resistant K222 allele in British goat herds. BMJ Journals 2016; 103521.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symposium Series 1999; 41: 95-98.

Hammarstrom P, Nystrom S. Porcine prion protein amyloid. Taylor and Francis Group, LLC 2015.

Harris DA, Falls DL, Johnson FA, Fischbach GD. A Prion-Like Protein from Chicken Brain Copurifies with an Acetylcholine Receptor-Inducing Activity. Proceedings of the National Academy of Sciences of the United States of America 1991; 88: 7664-7668.

Hill AF, Joiner S, Linehan J, Desbruslais M, Lantos PL, Collinge J. Species-barrier-independent prion replication in apparently resistant species. Proceedings of the National Academy of Sciences of the United States of America 2000; 97 (18): 10248–10253.

Hosszu LL, Baxter NJ, Jackson GS, Power A, Clarke AR, Waltho JP, Craven CJ, Collinge J. Structural mobility of the human prion protein probed by backbone hydrogen exchange. Nature Structural and Molecular Biology 1999; 6: 740-743.

Hunter GD. Scrapie and mad cow disease. The smallest and most lethal thing. Vantage Press, New York 1993.

Ingram J. Fatal Flaws. Yale University Press 2013; 109-163.

James TL, Liu H, Ulyanov NB, et al. Solution structure of a 142-residue recombinant prion protein corresponding to the infectious fragment of the scrapie isoform. Proceedings of the National Academy of Sciences USA 1997; 94: 10086-10091.

Johnson RT. Prion diseases. THE LANCET Neurology 2005; 4 (10): 635-642.

Jones EM, Surewicz WK. Fibril conformation as the basis of species- and strain-dependent seeding specificity of mammalian prion amyloids. Cell 2005; 121: 63-72.

Kimberlin RH, Walker CA. Evidence that the transmission of one source of scrapie agent to hamsters involves separation of agent strains from a mixture. Journal of Genetics Virology 1978; 39: 487-496.

Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Molecular Biology Evolution 2016; 33 (7): 1870-1874.

Liberski PP, Sikorska B, Lindenbaum S, Goldfarb LG, McLean C, Hainfellner JA, Brown P. Kuru: genes, cannibals and neuropathology. Journal of Neuropathol ex Neurol 2012; 71: 92-103.

Martin T, Hughes S, Hughes K, Dawson M. Direct Sequencing of PCR amplified pig PrP genes. Central Veterinary Laboratory 1995.

Meng LP, Zhao DM, Liu HX, Yang JM, Ning ZY. Single nucleotide polymorphisms of the prion protein gene (PRNP) in Chinese pig breeds. Xenotransplantation 2005; 12: 324-326.

Morris J, Gajdusek DC. Encephalopathy in mice following inoculation of scrapie sheep brain. Nature 1963; 197: 1084-1086.

National Institutes of Health. Prion disease 2014. http://ghr.nlm.nih.gov/condition/prion-disease. Accessed 11 November 2015.

Nicolau YC, Kapetanovic E. Vaccination against prion diseases. United States Patent Application Publication 2004. https://docs.google.com/viewer?url=patentimages.storage.googleapis.com/pdfs/US20040208919.pdf

Noinville S, Chich JF, Rezaei H, Misfolding of PrP protein: linking biophysical and biological approaches. Veterinary Research 2008; 39: 48.

Norstrom EM, Mastrianni JA. The AGAAAAGA Palindrome in PrP Is Required to Generate a Productive PrP^{SC}-PrP^{C} Complex That Leads to Prion Propagation. Journal of Biological Chemistry 2005; 280: 27236-27243.

Peñã MM, Lee J, Thiele DJ. A delicate balance: homeostatic control of copper uptake and distribution. Journal of Nutrition 1999; 129: 1251-1260.

Prusiner SB. Novel Proteinaceous Infectious Particles Cause Scrapie. Science 1982; 216: 136-144.

Prusiner SB. The Prion Diseases. Scientific American 1995; 272: 48-57.

Prusiner SB. Prion. Proceedings of the National Academy of Sciences USA 1998; 95: 13363-13383.

Quaglio E, Chiesa R, Harris DA. Copper Converts the Cellular Prion Protein into a Protease-resistant Species That Is Distinct from the Scrapie Isoform. Journal of Biological Chemistry 2001; 276: 11432-11438.

Rheede T, M. W Smolenaars Marcel, Madsen O, W. de jong W. Molecular Evolution of the Mammalian Prion Protein. Molecular Biology and Evolution 2003; 20: 111-121.

Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K. NMR structure of the mouse prion protein domain PrP (121-321). Nature 1996; 382: 180-182.

Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K. NMR characterization of full-length recombinant murine prion protein mPrP (23-231). Nature 1997; 413: 282-288.

Scott M, Foster D, Mirendra C, Serban D, Coufal F, Walchli M, et al.. Transgenic mice expressing hamster prion protein produce species-specific scrapie infectivity and amyloid plaques. Cell 1989; 59: 847-857.

Spongiform Encephalopathy Advisory Committee position statement: prevalence of subclinical variant Creutzfeldt-Jakob disease (2008) http://webarchive.nationalarchives.gov.uk/20110316163005/http:/www.seac.gov.uk/statements/state-cjd-infections.pdf.

Smith PG, Bradley R. Bovine spongiform encephalopathy (BSE) and its epidemiology. British Medical Bulletin 2003; 66: 185-198.

Spevack AR, Evans EGB, Miller JL, Meyer HC, Pelton JG, Millhauser GL. Zinc Drives a Tertiary Fold in the Prion Protein with Familial Disease Mutation Sites at the Interface. Structure 2013; 21: 236-246.

Stockel J, Safar J, Wallace AC, Cohen FE, Prusiner SB. Prion protein selectively binds copper(II) ions. Biochemistry 1998; 37: 7185-7193.

Surewicz WK, Jones EM, Apetri AC. The emerging principles of mammalian prion propagation and transmissibility barriers: insight from studies in vitro. Accounts of Chemical Research 2006; 39: 654-662.

Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 1994; 22: 4673-4680.

Vanik DL, Surewicz KA, Surewicz WK. Molecular basis of barriers for interspecies transmissibility of mammalian prions. Molecular Cell 2004; 14: 139-145.

Vey M, Pilkuhn S, Wille H, Nixon R, DeArmond SJ, Smart EJ, Anderson RG, Tarabolous A, Prusiner SB. Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proceedings of the National Academy Sciences 1996; 93: 14945-14949.

Vilotte JL, Soulier S, Essalimani R, Stinnakre MG, Vaiman D, Lepourry L, et al.. Markedly increased susceptibility to natural sheep scrapie of transgenic mice expressing ovine PrP. Journal of Virology 2001; 75: 5977-5984.

Wadsworth JD, Collinge J. Update on human prion disease, Biochimica et Biophysica Acta 2007; 1772: 598-609.

Waggoner DJ, Drisaldi B, Bartnikas TB, Casareno RL, Prohaska JR, Gitlin JD, Harris DA. Brain Copper Content and Cuproenzyme Activity Do Not Vary with Prion Protein Expression Level. Journal of Biological Chemistry 2000; 275: 7455-7458.

Weissmann C, Enari M, Klohn PC, Rossi D, Flechsig E. Transmission of prions. Proceedings of the National Academy Sciences 2002; 99 (4): 16378-16383.

Westaway D, DeAromond SJ, Cayetano-Canlas J, Groth D, Foster D, Yand SL, et al.. Degeneration of skeletal muscle, peripheral nerves, and the central nervous system in transgenic mice overexpressing wild- type prion proteins. Cell 1994; 76: 117-129.

Whittal RM, Ball HL, Cohen FE, Burlingame FE, Prusiner SB, Baldwin MA. Protein Sci. 2000; 9: 332-343.

World Health Organization Consultation on Public Health and Animal Transmissible Spongiform Encephalopathies: Epidemiology, Risk and Research Requirements. Geneva, Switzerland 1999.

Yu S, Yin S, Li C, Wong P, Chang B, et al. Aggregation of prion protein with insertion mutations is proportional to the number of inserts. Jounal of Biochemistry 2007; 403: 343–35.

Sample No.	Individual Crosses
9-1 to 9-11	Dark Cross sow 71 by Blue Butt Boar (Gummy bear)
10-1 to 10-7	Pure York sow by Blue Butt Boar (Gummy bear)
19-1 to 19-7	Pure Duroc sow by Berkshire Boar
22-1 to 22-5	Pure York sow by Blue Butt Boar (Gummy bear)
23-1 to 23-10	Dark Cross sow 71 by Blue Butt Boar (Gummy bear)
24-1 to 24-10	Dark Cross (Half Hampshire by Half Duroc) bred by Blue Butt Boar (Gummy bear)

Table 1. Overview of DNA samples used in the study of polymorphism in the PrP^c prion protein gene in pigs.

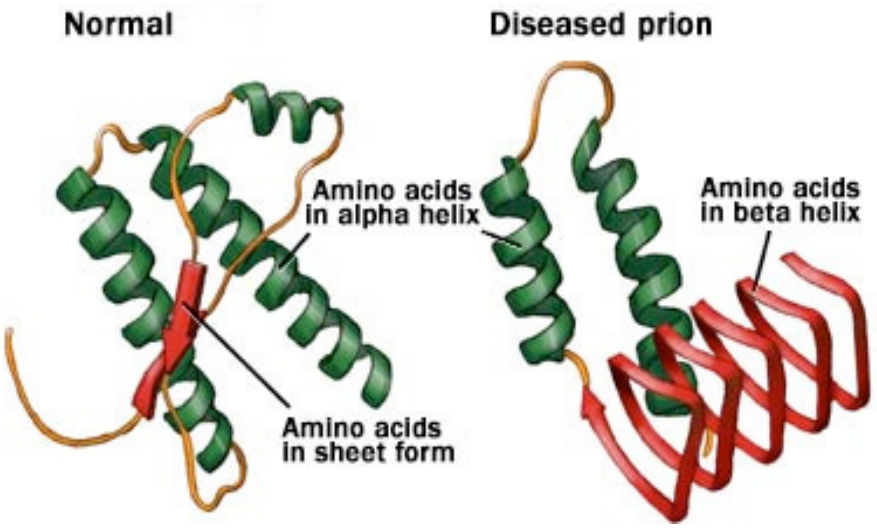

Figure 1. Normal and abnormal form of prion protein. Normal prion protein has amino acids in alpha helix with less than 5% of beta sheets. Abnormal prion protein is a misfolded protein, where majority of alpha helices are converted into beta sheets resulting in more than 50% of beta sheets. (Source: Mayo Foundation of Medical Education and Research). Accessed on: 3/16/2017

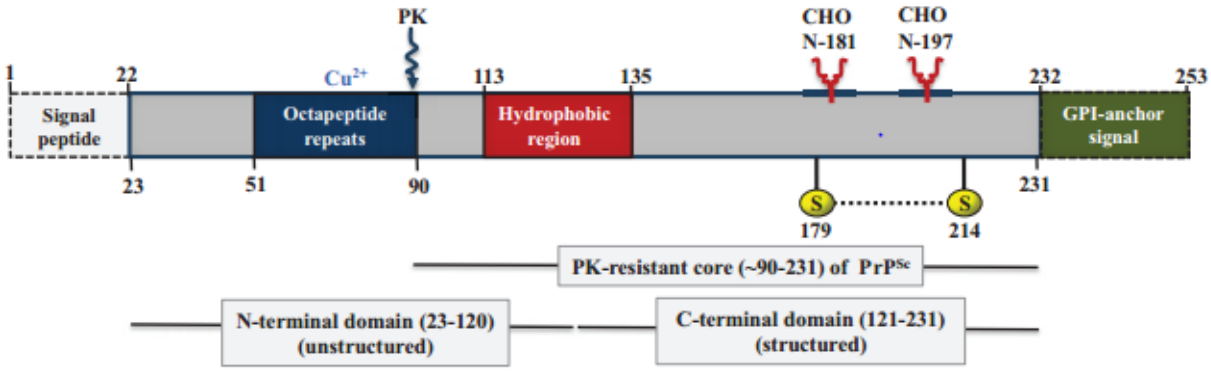

Figure 2. Organization of human PrP^{C}. The PrP^{C}, having 253 amino acid residues, contains one signal peptide (1-22), four octapeptide repeats (113-135), one hydrophobic region (113-135), two N-linked glycosylation sites (181 and 197 residues), and one glycophosphatidylinositol-anchor signal (232-254). Mutated prion protein contains up to nine additional octapeptide repeats or deletion of one octapeptide. The palindromic amino acid motif AGAAAAGA lies in the hydrophobic region. (Source: Acevedo-Morantes and Wille 2014)

Figure 3. Piglets of approximately 10 days old at Fort Hays State University farm. 23 female and 27 male pigs were studied.Tails of the piglets are docked a few days after their birth so they do not hurt each other.

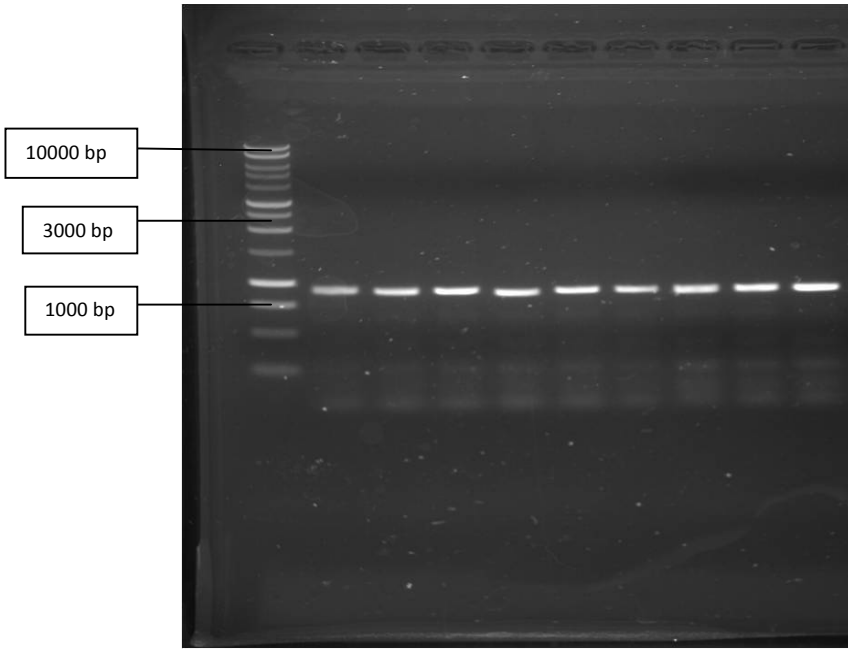

Figure 4. Electrophoresis gel picture of amplified PCR fragment of PrP^C gene obtained from the pigs at Fort Hays State University farm. Lane 1: 1 Kb ladder (Promega). Lane 2-10: PCR amplicons of sample 9-1 to 9-9.

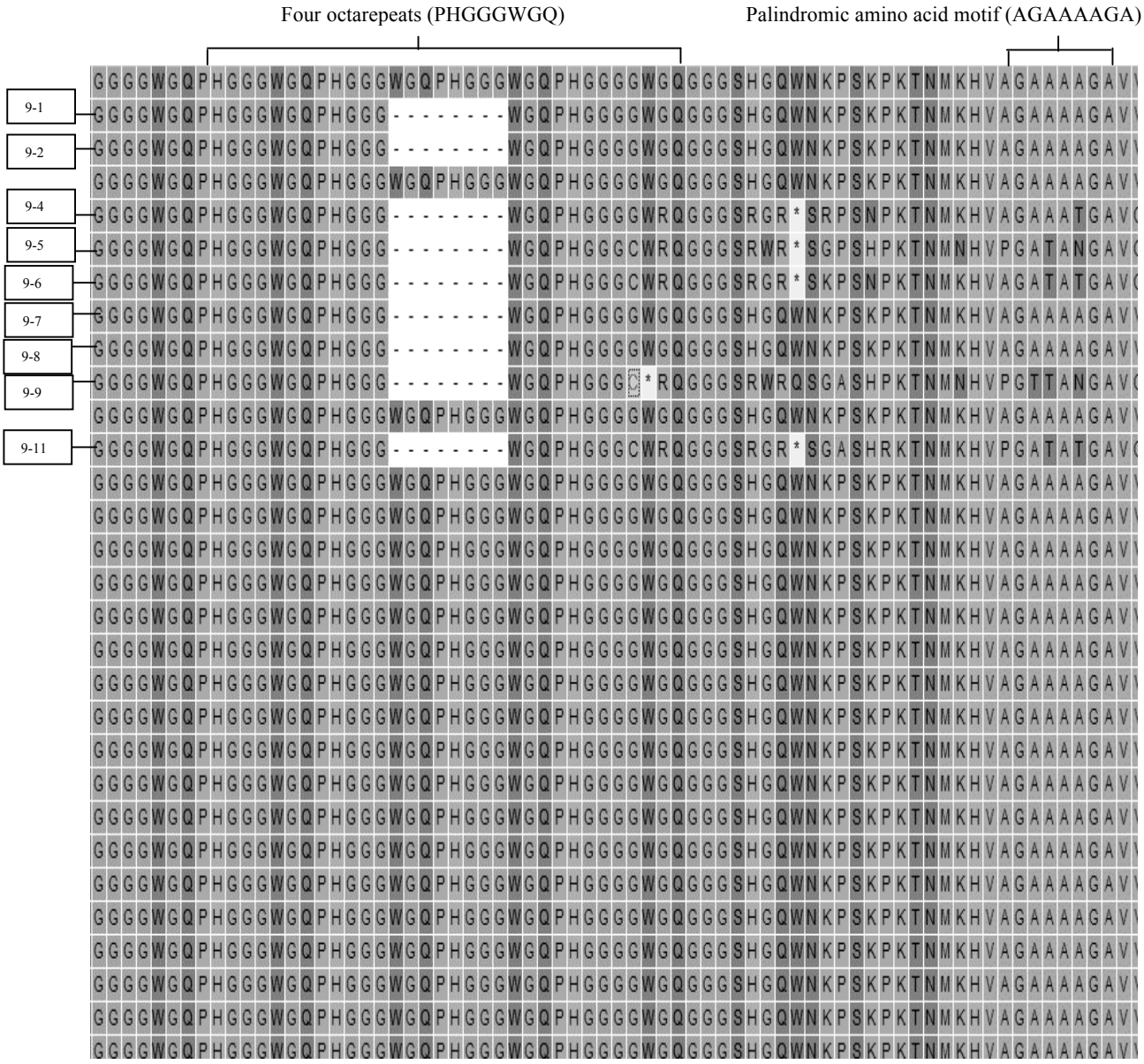

Figure 5: Deletion of octapeptide WGQPHGGG observed during multiple alignment of PrP^{C} prion protein in pig samples 9-1, 9-2, 9-4, 9-5, 9-6, 9-7, 9-8, 9-9, and 9-11. All these samples with deletions are from the same breed (Dark cross sow 71 by Blue butt boar) 'Dashes' represent Deleted amino acid, 'Asterisks' represent gaps. A- Alanine, G- Glycine, V- Valine, K- Lysine, W- Tryptophan, Q- Glutamine, P- Proline, H- Histidine, S- Serine, T- Threonine, and N- Asparagine.

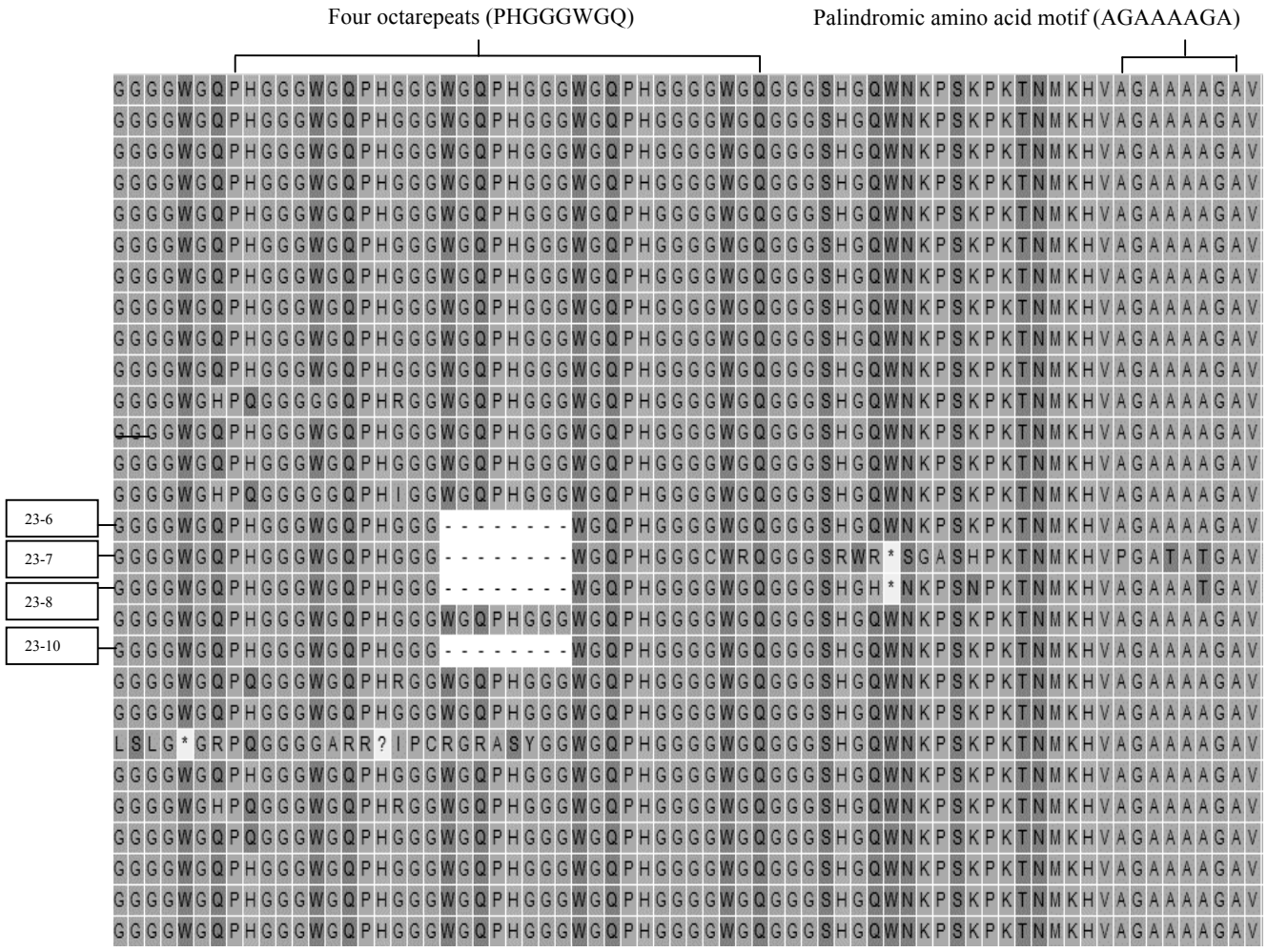

Figure 6: Deletion of octapeptide WGQPHGGG observed during multiple alignment of PrP^{C} prion protein in pig samples 23-6, 23-7, 23-8, and 23-10. All these samples with deletions are from the same breed (Dark cross sow 71 by Blue butt boar). 'Dashes'represent deleted amino acid, 'Asterisks' represent gaps. A- Alanine, G- Glycine, V- Valine, K- Lysine, W- Tryptophan, Q- Glutamine, P- Proline, H- Histidine, S- Serine, T- Threonine, and N- Asparagine.

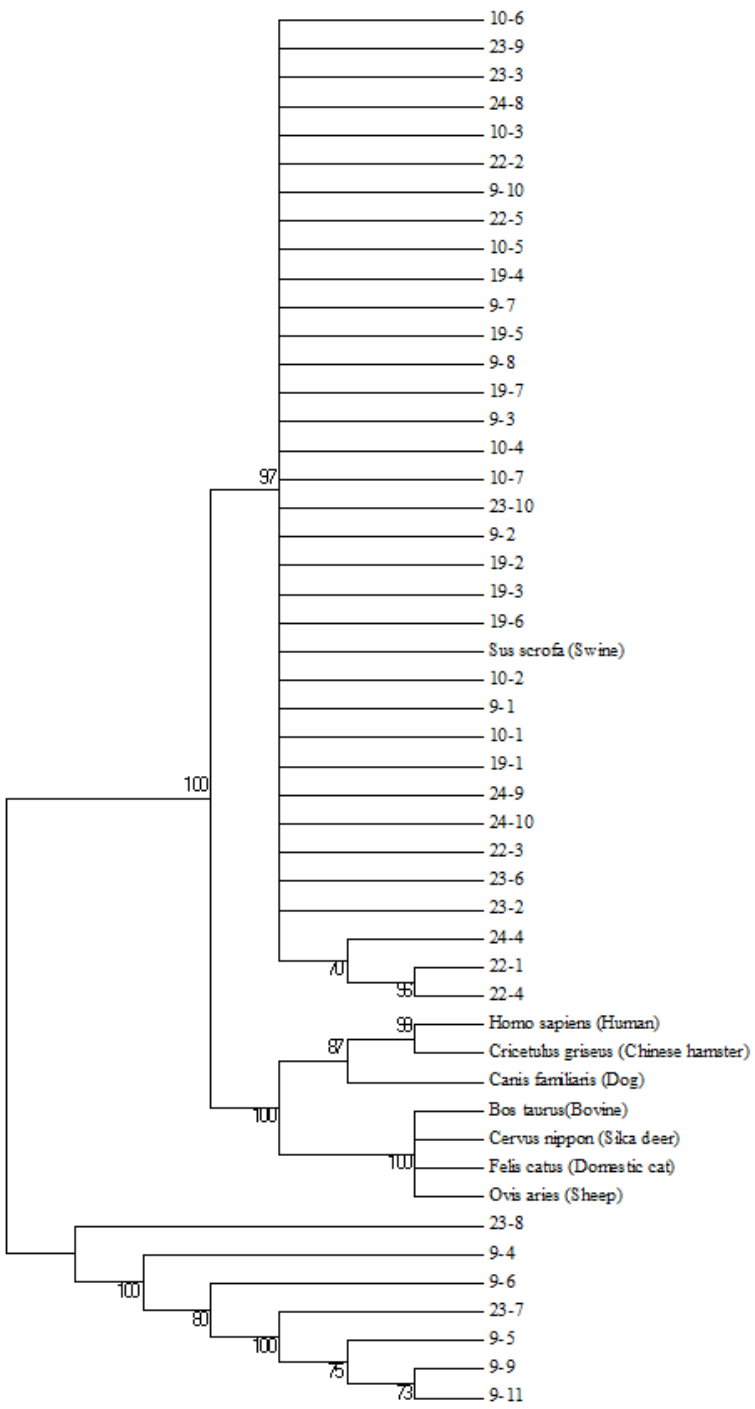

Figure 7. Phylogenetic tree derived from prion nucleotide sequences of pigs and few other mammals.

Appendix 1: Amino acid sequences

1. *Sus scrofa* (GenBank)
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
   QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
   MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
   RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLISFLLFPPFXS

2. 9-1
   MVXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
   HGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSR
   PLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERVV
   XTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

3. 9-2
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
   HGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSR
   PLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERVV
   XTDVHHPVPERVRGVRPKXGPV*SSXSSPPVILLXLFPPFXS

4. 9-3
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
   QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
   MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
   RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

5. 9-4
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
   HGGGWGQPHGGGWGQPHGGGGWRQGGGSRGR*SRPSNPKTNMKHVAGAAATGAVGGGLGGYMLGSAMSKP
   LIHFGSDYEDRFYRENMYRYPNQMYYRPLDQFSNQNIFVHDCLNISVNHCTMTTTTKGENFTNTDVKNIDRVEXT
   DVHHPVPTKVQGLLP-KXGPM*SSXSSAPVILFXLSPPVXS

6. 9-5
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
   HGGGWGQPHGGGWGQPHGGGCWRQGGGSRWR*SGPSHPKTNMNHVPGATANGAVGGGLGGDMLGGAMSNP
   LVRFVSDYQDRFYRENKYRYPHQIFYRPLDQFYYQPIFVHDCLNISVHHCTMTTTTKGENSTTTDGEIINHVEXTD
   EHHPVPTKVQGLLP-KXGPRRSSXSWPPVILFXLSPPDXS

7. 9-6
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
   HGGGWGQPHGGGWGQPHGGGCWRQGGGSRGR*SKPSNPKTNMKHVAGATATGAVGGGLGGYMLGSAMSNP
   LIHFVSDYEDRFYRENMYRYPNQMYYRPLDQFNYQTIFVHDCLNISVHHCTVTTTTKGENFTNTDVKIIDRAEXT
   DVHHPVPTKVRGVLP-KXRPMRSSXSSPPVILLXLSPPFXS

8. 9-7
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
   HGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSR
   PLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERVV
   XTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

9. 9-8
   MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
   HGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSR
   PLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERVV
   XTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

10. 9-9
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
HGGGWGQPHGGGWGQPHGGGC*RQGGGSRWRQSGASHPKTNMNHVPGTTANGAVGGGLGGYMLGGPMSNP
LVRFVSNYEDRFYRENKYRYPNQNFYRPPNQFNYQPIFVHDCLNISVHDCTMTTTTKGENSTNTDVKNINHTEXT
DDHHPVPTKVQGLLP-KXSPRRSSXSWATVILLXLSPRDXS

11. 9-10
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

12. 9-11
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
HGGGWGQPHGGGWGQPHGGGCWRQGGGSRGR*SGASHRKTNMKHVPGATATGAVGGGLGGDMLGGAMSNP
LVRFVSDYQDRFYRENKYRYPNQNFYRPLNQFNYQPIFVHDCLNIFVHHCTMTTTTKGENSTNTDGKNIDHAEXT
DDHHPVATKVQGLLPKXGPMRSSXSWAPVILFXLSPPDXS

13. 10-1
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

14. 10-2
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

15. 10-3
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

16. 10-4
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

17. 10-5
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

18. 10-6
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

19. 10-7
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

20. 19-1
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

21. 19-2
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRPKXGPV*SSXSSPPVILLXLFPPFXS

22. 19-3
MXXKSXXGGWILVLXCGRME*HRXSAXSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

23. 19-4
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

24. 19-5
MXXKSXIGGWILVLXCGRME*HRXSARSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

25. 19-6
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
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MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRPXKXGPV*SSXSSPPVILLXLFPPFXS

26. 19-7
MXXKSXIGGWILVLXCGRME*HRXSARSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

27. 22-1
MXXEXPIGGWVLVLXCGSME*HMXSSKSDQXLAEEGTLXXGAYTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRPXKXGPV*SSXSSPPVILLXLFPPFXS

28. 22-2
MXXKXXXGGWILVLXCGRME*HRXSARSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

29. 22-3
MXXKIXIGGWILVLXCGRME*HRXSAKSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

30. 22-4
MXXENPIGGWILVLXCGSME*HMVSSQNDQXLAGEGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRXXKXGPV*SSXSSPPVILLXLFPPFXS

31. 22-5
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

32. 23-1
V*XKLHILCWILVLGRFRNXQWXVASYKKXPSLAW**TLXXGAYPXGRVDMEXNPYPPQGGGGWGHPQGGGG
GQPHRGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVGTDVHHPVPERVRGVRP-KEGPV*SSXSSPPVILLXLFPSFSS

33. 23-2
MXXKIXIGGWILVLXCGSME*HRXSAKSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

34. 23-3
XXXKTXXGGWILVLXCGRME*HRXSARSDXSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPEXVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

35. 23-4
VLXNLQVLLXXAXLGRYRNXQWXVRSYERXQXLAGW*SLXXGAWTLGRVVMEXNPFPPKGGGGWGHPQGGG
GGQPHIGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGS
AMSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMI
ERVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

36. 23-6
MXXESXIGGWILVLXCGRME*HRXSAKSDQXLAEEGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
HGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSR
PLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERVV
XTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

37. 23-7
MXXKSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
HGGGWGQPHGGGWGQPHGGGCWRQGGGSRWR*SGASHPKTNMKHVPGATATGAVGGGLGGYMLGGAMSNP
LVRFVSDYEDRFYREYKYRYSNQNFYRPLNQFYYQPIYVHDCLNIFVHHCTMTTTTKGENFTTTDGEIIDRAEXT
DEHHRVATKVQGVLP-KXGPMRSSXSSPPVILFXLSPRFXS

38. 23-8
MXKVSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQP
HGGGWGQPHGGGWGQPHGGGGWGQGGGSHGH*NKPSNPKTNMKHVAGAAATGAVGGGLGGYMLGSAMSK
PLIHFGSDYEDRFYRENMYRYPNQMYYRPLDQFSNQNIFVHDCVNISVKHCTVTTTTKGENFTNTDVKMIDRVV
XTDVHHPVPKRVQGVLP-KXSPM*SSXSSPPVILLILFPPFXS

39. 23-9
MXXKSXIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

40. 23-10
MXXKVSHIGGWILVLXCGRME*HRXSARSDQSLAEDGTLXXGADTQGRVVLEXNRYPPQGGG~~~~~~~~GWGQ
PHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMS
RPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERV
VXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

41. 24-1
V*XKLEILYWXMEASDLWGSXQ*HITSYKKMQGLAGCGTLXXGAYTLGRVVLGXNPFPPQGGGGWGQPQGGG
WGQPHRGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLG
SAMSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVK
MIERVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

42. 24-2
XKXXQNPILGWVLVXXCGSXE*XXAS*KNEQXLAGEGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

43. 24-3
VXXTLHIRGPWTVVGGYHNPQLXVVDXGRX*YL*RWCSAXXGPWXXDWVLAKTRKGXPPMLSLG*GRPQGGG
GARRXIPCRGRASYGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSFSSPPVILLXLFPPFPS

44. 24-4
MXRNPIFGWILXIXWGNXQ*XRXSAKKEQGLAGGGTLXXGAYTQGRVVLEXNRFPPQGGGGWGQPHGGGWGQ
PHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMS
RPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIERV
VXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

45. 24-5
VXXTLPIRGWILVLGGFSNRXWXLVS*KKXQXLAW**SLXXGAYPXGWVVMEXNRYPPKGGGGWGHPQGGGW
GQPHRGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

46. 24-6

XTXRNPMFGMXSLXIXCGRXQ*HRXSAKNDQGLAGGGTLXXGAYTQGRVVLEXNRYPPQGGGGWGQPQGGG
WGQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLG
SAMSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVK
MIERVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

47. 24-7

XPXXQILILGEXVLVLXFGNXE*XVVSYKNEQXLAGEGTLXXGAYTLGRVVMEXNRYPPQGGGGWGQPHGGG
WGQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLG
SAMSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVK
MIERVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

48. 24-8

MXXKSXIGGWILVLXCGRME*HRXSARSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

49. 24-9

MXXKIXIGGWILVLXCGRME*HRXSARSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGWG
QPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

50. 24-10

MXXKXXIGGWILVLXCGRME*HRXSARSDQXLAEDGTLXXGADTQGRVVLEXNRYPPQGGGGWGQPHGGGW
GQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQWNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSA
MSRPLIHFGSDYEDRYYRENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDVKMIE
RVVXTDVHHPVPERVRGVRP-KXGPV*SSXSSPPVILLXLFPPFXS

Appendix 2. Pig prion protein information

Nucleotide accession: L07623.1
Protein accession: AAA92862.1
Gene annotation: Sus scrofa prion protein (PrP) gene, complete cds
Coding sequence: 1-774 base pairs
Amino acid feature: Putative glycosylation sites, base pairs 185 and 201
Protein translation: MVKSHIGGWILVLFVAAWSDIGLCKKRPKPGGGWNTGGSRYPGQGSPGGNRYP PQGGGGWGQPHGGGWGQPHGGGWGQPHGGGWGQPHGGGGWGQGGGSHGQ WNKPSKPKTNMKHVAGAAAAGAVVGGLGGYMLGSAMSRPLIHFGSDYEDRYY RENMYRYPNQVYYRPVDQYSNQNSFVHDCVNITVKQHTVTTTTKGENFTETDV KMIERVVEQMCITQYQKEYEAYAQRGASVILFSSPPVILLISFLLFLIVG