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Short Communication

PRNP gene variation in Pakistani cattle and buffaloes

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ABSTRACT

Bovine spongiform encephalopathy (BSE) is a neurodegenerative prion protein misfolding disorder of cattle. BSE is of two types, classical BSE and atypical BSE which in turn is of two types, H-type BSE and L-type BSE. Both H-type BSE and L-type BSE are primarily sporadic prion disorders. However, one case of H-type BSE has recently been associated with E211K polymorphism in the prion protein gene (*PRNP*). Two polymorphisms in the bovine *PRNP* are also associated with susceptibility to classical BSE: a 23 bp insertion/deletion (indel) in the *PRNP* promoter region and a 12 bp indel in the first intron. No information regarding BSE susceptibility in Pakistani cattle is available. The present study aimed at achieving this information. A total of 236 cattle from 7 breeds and 281 buffaloes from 5 breeds were screened for E211K polymorphism and 23 bp and 12 bp indels employing triplex PCR. The E211K polymorphism was not detected in any of the animals studied. The 23 bp insertion allele was underrepresented in studied cattle breeds while the 12 bp insertion allele was overrepresented. Both 23 bp and 12 bp insertion alleles were overrepresented in studied buffalo breeds. Almost 90% of alleles were insertion alleles across all studied buffalo breeds. The average frequency of 23 bp and 12 bp insertion alleles across all studied cattle breeds was found to be 0.1822 and 0.9407, respectively. There were significant differences between Pakistani and worldwide cattle in terms of allele, genotype and haplotype frequencies of 23 bp and 12 bp indels. The higher observed frequency of 12 bp insertion allele suggests that Pakistani cattle are relatively more resistant to classical BSE than European cattle. However, the key risk factor for classical BSE is the dietary exposure of cattle to contaminated feedstuffs.

1. Introduction

Bovine spongiform encephalopathy (BSE) is a neurodegenerative disorder affecting cattle. The clinical features of BSE include ataxia and aggressive behavior while pathological features of BSE include prion protein misfolding and aggregation followed by spongiform degeneration of the brain (Novakofski et al., 2005). BSE cases have occurred worldwide in nearly 0.2 million Holstein–Freisian *Bos taurus* cattle, 1 *Bos indicus* animal, and 1 *Bos taurus*×*Bos indicus* cow (Ducrot et al., 2008; Nicholson et al., 2008; Novakofski et al., 2005; Richt and Hall, 2008; Seuberlich et al., 2006). BSE first appeared in mid 1980s in UK and soon evolved to epidemic proportions with 1000 cases occurring per week in 1992 (Collinge and Clarke, 2007). The dietary exposure to BSE has been evidenced to cause a variant form of Cruetzfeldt–Jacob disease (vCJD) in humans (Bruce et al., 1997), feline spongiform encephalopathy (FSE) in domestic and wild captive cats and exotic ungulate spongiform encephalopathy (EUE) in bovids (Sigurdson and Miller, 2003).

BSE is one of 7 types of animal prion disease and 9 types of human prion disease (Parchi et al., 2011; Sigurdson and Miller, 2003). The central molecular event responsible for the pathogenesis of prion diseases is the conversion of host-encoded normal cellular prion protein (PrP^{C}) into a pathogenic misfolded scrapie prion protein (PrP^{Sc}). Both

Abbreviations: PRNP, Prion protein gene; PrP^{C}, Cellular isoform of prion protein; PrP^{Sc}, Scrapie isoform of prion protein; CJD, Cruetzfeldt–Jacob disease; vCJD, variant CJD; TSE(s), Transmissible spongiform encephalopathy(ies); BSE, Bovine spongiform encephalopathy; FSE, Feline spongiform encephalopathy; EUE, Ungulate spongiform encephalopathy; Indel, Insertion/Deletion; MBM, Meat and bone meal; RP58, Repressor protein 58; SP1, Specificity protein 1; I, Shannon's information index; Fis, Fit and Fst, Measures of heterozygosity loss; Nm, Gene flow; HWE, Hardy–Weinberg equilibrium; LD, Linkage disequilibrium; A, Alanine; E, Glutamic acid; H, Histidine; K, Lysine; R, Arginine; Q, Glutamine; V, Valine; PCR, Polymerase Chain Reaction; ARMS, Amplification Refractory Mutation System.

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PrP^C and PrP^{Sc} have similar primary structure but they vary in their state of conformation. PrP^C is predominantly α-helical while PrP^{Sc} is rich in β-pleated sheets. The spontaneously formed or acquired PrP^{Sc} conformers bind PrP^C conformers and catalyze the process of PrP^C to PrP^{Sc} conversion and aggregation (Collinge and Clarke, 2007; Prusiner, 1998).

Prion diseases are unique human and animal disease conditions as they may occur sporadically, be inherited or be acquired. BSE is of two types, classical BSE and atypical BSE. Atypical BSE is also of two types, H-type BSE and L-type BSE. Both H-type BSE and L-type BSE are primarily sporadic prion disorders while classical BSE is associated with dietary exposure of cattle to infectious PrP^{Sc} aggregates contained in meat and bone meal (MBM) originating from sporadic BSE cases, sheep with scrapie or CJD patients (Capobianco et al., 2007; Colchester and Colchester, 2005; Hill et al., 1998; Nicholson et al., 2008; Richt and Hall, 2008). A ban on the use of MBM in the cattle feed ultimately resulted in a progressive decline of the BSE epidemic (Ducrot et al., 2008).

Similar to human prion disorders, several *PRNP* variations have been found to be associated with susceptibility to animal prion disorders (Goldmann, 2008; Parchi et al., 2011; Sigurdson and Miller, 2003; Wadsworth and Collinge, 2011). All H-type BSE cases have occurred sporadically except one case that was recently found to be associated with E211K polymorphism in the bovine *PRNP* (Nicholson et al., 2008; Richt and Hall, 2008). The E211K polymorphism is analogous to E200K mutation in the human prion protein, which is the major cause of familial or genetic human prion disease worldwide (Capellari et al., 2011; Heaton et al., 2008; Kovács et al., 2005; Nicholson et al., 2008; Richt and Hall, 2008). Two non-coding polymorphisms in the bovine *PRNP* are also associated with susceptibility to classical BSE: a 23 bp indel in the *PRNP* promoter region and a 12 bp indel in the first intron (Sander et al., 2004). The 12 bp indel is the major component of resistance to classical BSE in UK Holsteins cattle (Juling et al., 2006). However, these indel polymorphisms are not associated with susceptibility to atypical BSE (Brunelle et al., 2007).

The 23 bp and 12 bp insertion alleles contain transcription binding sites for Repressor Protein 58 (RP58) and Specificity Protein 1 (SP1), respectively. Both RP58 and SP1 transcription factors interact with their corresponding binding sites and regulate the expression of messenger RNA (mRNA) from *PRNP* (Kashkevich et al., 2007; Msalya et al., 2009; Nakamura et al., 2007; Sander et al., 2005; Xue et al., 2008). The presence of deletion alleles either at single or both of 23 bp and 12 bp indel sites are associated with increased susceptibility to classical BSE (Juling et al., 2006; Sander et al., 2004). A recent study evidenced that the mRNA levels were greater in the medulla oblongata of Japanese Black cattle harboring del/del genotype at the 23 bp indel site (Msalya et al., 2011). It has been reported that the reduced PrP^C expression prolongs the disease progression and that mice lacking the PrP^C expression are completely resistant to the disease development (Bu¨eler et al., 1993).

A brief report to the European Union regarding the BSE and/or scrapie free status of Pakistan was drawn up by the Government of Pakistan, Ministry of Food, Agriculture & Livestock at the end of 1998 (http://ec.europa.eu/food/fs/sc/ssc/out199_en). According to this report, Pakistan has passive BSE surveillance system and is free of BSE and scrapie. No information regarding BSE susceptibility in Pakistani cattle is available. The present study aimed at achieving this information. A total of 236 unrelated Pakistani cattle representing 7 breeds were screened for the presence of E211K polymorphism and 23 bp and 12 bp indels. These polymorphisms were also studied in 281 buffaloes from 5 breeds to examine why BSE has not occurred in buffaloes which are closely related to cattle.

2. Materials and methods

2.1. Blood sampling of Pakistani cattle and buffaloes

The study was approved by Ethical Review Committee of University of Health Sciences Lahore, Pakistan. Five milliliter blood samples were collected into K_2-EDTA vacutainers from the jugular vein of cattle and buffaloes. Only unrelated animals were sampled from their breeding home tracts as well as Government livestock farms. The number of animals sampled per breed is given in Table 1. DNA was isolated from whole blood samples using standard organic extraction procedure.

2.1.1. PCR amplification of 12 bp and 23 bp indels loci and E211K polymorphism in the bovine PRNP

Primers were designed using online Primer3 software (frodo.wi.mit.edu/primer3/) and the bovine *PRNP* genomic sequence (GenBank ™ accession number AJ298878). Melting temperature (Tm) of primers was calculated using online UCSC In-Silico PCR server (www.genome.ucsc.edu/). Non-specificity of these primers against human *PRNP* sequence was verified by the UCSC BLAT analysis (www.genome.ucsc.edu/). Gradient PCRs were performed at annealing temperatures (A^T) in the range of 51–65 °C (51, 52.1, 53.8, 56.3, 59.9, 62.5, 64.1 and 65 °C) to find optimum A^T for each primer pair. Optimum A^T used for *PRNP* amplifications with all primer pairs 1–8 was 60 °C (Supplementary file 1).

The 12 bp and 23 bp indels and E211K polymorphism were multiplexed in a single PCR reaction. Details on the development of multiplex PCR are provided in Supplementary file 1. All PCR amplifications were carried out in 25 μl reactions consisting of 50 ng DNA, 1× Taq buffer [75 mM Tris–HCl (pH 8.8 at 25 °C), 20 mM $(NH_4)_2SO_4$ and 0.01% (v/v) Tween 20], 2.5 mM $MgCl_2$, 200 μM of each dNTP, 10 pmol of each primer and 1U of *Taq* DNA polymerase (Fermentas Inc., USA). Temperature profile consisted of an initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 45 s, followed by a 10 min final extension step at 72 °C. The thermocycler used was iCycler PCR machine (Bio-Rad, Hercules, CA, USA). PCR products were resolved in 2–3% agarose gels containing ethidium bromide (EtBr), visualized under UV and photographed.

2.1.2. Statistical procedures

Significance of pair-wise differences for allelic frequencies was tested by two-tailed Fisher exact test and χ^2 test with Yate's correction and for genotypic frequencies by χ^2 test only. These tests were performed using online GraphPad Prism statistical software package (www.graphpad.com/prim). Pair-wise comparisons of allele, genotype and haplotype frequencies of 12 bp and 23 bp indels were made between all possible breed pairs of Pakistani cattle and buffaloes. The cumulative allele, genotype and haplotype frequencies of 12 bp and 23 bp indels were also compared between Pakistani cattle and worldwide cattle as well as between Pakistani buffalo and Anatolian water buffalo. Pair-wise differences were considered significant at P-value ≤ 0.05.

Allelic and genotypic variation of indels in all cattle and buffalo breeds was analyzed using Popgene 1.32 (32-bit) software package (Yeh et al., 2000). The calculated parameters included genotypic frequency, allelic frequency, observed and expected homozygosity and heterozygosity, homogeneity of alleles distribution, two-locus linkage disequlibrium (LD), Hardy–Weinberg equilibrium (HWE), neutrality of loci, Shannon's and fixation indices, genetic distance, *F*-statistics and gene flow (Nm).

HWE tests were performed to assess whether 12 bp and 23 bp indels loci in Pakistani cattle and buffaloes had been under selection against prion diseases. Neutrality of 12 bp and 23 bp indels loci was tested to estimate whether these loci are also associated with any phenotypic trait in Pakistani cattle or buffaloes other than BSE. All other parameters were calculated to testify differences in allele, genotype and haplotype frequencies of 12 bp and 23 bp indels.

Table 1
PRNP alleles, genotypes and haplotypes distribution in various breeds of Pakistani cattle and buffalo.

		Alleles				Genotypes						Haplotypes			
	No. of animals	23 bp idel		12 bp indel		23 bp indel			12 bp indel			23 bp indel + 12 bp indel			
Breed		+	−	+	−	++	+−	−−	++	+−	−−	23+/12+	23-/12-	23-/12+	23+/12-
Cattle															
Cholistani	43	9	$77^{1,2}$	76	10^{1}	2	5	36^{1}	35	6	2	9	10	67	$0^{1,3}$
Dhanni	11	2	20^{2}	18	4^{1}	0	2	9^{1}	7	4	0^{1}	2	4	16	$0^{1,3}$
Lohani	28	7	49^{2}	51	5	1	5	22^{1}	24	3	1	7	5	44	$0^{2,3}$
Sahiwal	83	36	$130^{1,2}$	159	$7^{1,2}$	10	16	57^{1}	76	7	$0^{1,2,3}$	36	7	123	$0^{1,3}$
Therparker	29	12	46^{2}	57	$1^{1,2}$	1	10	18^{1}	28	1	0^{1}	12	1	45	$0^{1,3}$
Red Sindhi	26	13	39^{2}	52	$0^{1,2}$	3	7	16^{1}	26	0	$0^{1,2}$	13	0	39	$0^{1,\ 2,3}$
Achai	16	7	25^{2}	31	1	1	5	10^{1}	15	1	0	7	1	24	0^{3}
Buffalo															
Nili	66	124	8^{2}	113	19^{2}	59	6	1^{1}	52	9	5^{2}	108	3	5	16^{3}
Ravi	39	76	2^{2}	65	13^{2}	37	2	0^{1}	30	5	4^{2}	64	1	1	12^{3}
Azikheli	20	40	0^{2}	38	2	20	0	0^{1}	18	2	0	38	0	0	2^{3}
Kundhi	34	66	2^{2}	60	8^{2}	32	2	0^{1}	28	4	2	58	0	2	8^{3}
Nili Ravi	122	229	15^{2}	213	31^{2}	110	9	3^{1}	99	15	8^{3}	206	8	7	23^{3}

Given are observed numbers of alleles, genotypes and haplotypes. Identical digits above numbers of alleles, genotypes and haplotypes indicate significant χ^2 differences between different breed pairs. Some identical digits have been underlined so that breed pairing of numbers of alleles, genotypes and haplotypes could be established.

3. Results

Genotyping revealed the absence of the E211K polymorphism from all animals studied. Both 23 bp and 12 bp indels sites were found to be polymorphic in both cattle and buffaloes. The number of alleles, genotypes and haplotypes of 23 bp and 12 bp indels observed for Pakistani cattle and buffalo breeds is presented in Table 1. The frequency of 23 bp and 12 bp insertion alleles in cattle was found to be the lowest in Dhanni and the highest in Red Sindhi cattle breed. The frequency of these alleles across all buffalo breeds was greater than 90% and 80%, respectively. The distribution of alleles, genotypes and haplotypes of 23 bp and 12 bp indels varied at $P \leq 0.05$ between some of cattle breed pairs but not between any of buffalo breed pairs. These variations were more significant for the 12 bp than the 23 bp indel site as confirmed by *P*-values of homogeneity tests which were performed for the pooled data of cattle breeds. The *P*-values of χ^2/G2 likelihood tests for the homogeneity of alleles at 12 bp and 23 bp indels sites were 0.006/0.004 and 0.152/0.123, respectively. The pooling of data on buffalo breeds resulted in these values as 0.473/0.398 for the 12 bp and as 0.307/0.143 for the 23 bp indel site.

Genotypic data for following breed × locus pairs deviated from HWE: Cholistani (12+/−\1n2y and 23+/−\1n2n), Lohani (12+/−\1n2y), Sahiwal (23+/−\1n2n), Nili (12+/−\1n2n), Ravi (12+/−\1n2n), Kundhi (12+/−\1n2n), Nili Ravi (12+/−\1n2n and 23+/−\1n2n) and the pooled data for cattle breeds and for buffalo breeds (12+/−\1n2n and 23+/−\1n2n). The sign of \1n2n or \1n2y wherever used means the relevant breed × locus pair does not obey/obeys HWE assumptions (n/y) by χ^2 test (1) or by G2 likelihood test (2). For example, Cholistani × 12 bp indel locus pair does not obey HWE assumptions by χ^2 test ($p = 0.024$) but obeys by G2 likelihood test ($P = 0.060$) (12+/−\1n2y).

Consistent with the excess of homozygous genotypic data for both 12 bp and 23 bp indels, the values of Shannon's information index (I) were lower having a range of 0.0937 ± 0.2296 in Red Sindhi to 0.1298 ± 0.2081 in Dhanni with a mean of 0.1166 ± 0.1972 across all cattle breeds. The range of (I) values in buffalo was 0.0331 ± 0.0810 in Azikheli to 0.1068 ± 0.1753 in Nili with a mean of 0.0965 ± 0.1615 across all buffalo breeds. Observed homozygosity was greater than expected homozygosity and the converse was true for observed and expected heterozygosities for both indels loci in cattle as well as in buffalo, with an exception of Dhanni, Therparker and Azikheli breeds. These breeds revealed a values pattern for these parameters opposite to other breeds. The heterozygote deficiency as indicated by Wright's fixation index (Fis) was more prominent in cattle at the 23 bp (mean Fis = 0.2891) than the 12 bp indel locus (mean Fis = 0.1647). This pattern reversed in buffalo with mean Fis of 0.2608 for the 23 bp and 0.4490 for the 12 bp indel locus. The mean Fis values became 0.1507 in cattle and 0.3538 in buffalo with a combined analysis of the data set for both indels and were supported by the values of Fit, Fst, gene flow (Nm) and genetic distance. The respective values for Fit, Fst, Nm and genetic distance were 0.1789, 0.0333, 7.2654 and 0.0000–0.0105 for cattle and 0.3634, 0.0149, 16.5784 and 0.0000–0.0020 for buffalo. No significant results were obtained for Ewens–Waterson neutrality test for any of studied bovid breeds. Significant and strong linkage disequilibria existed in Nili Ravi between all possible allele pairs of the two indels loci (12+/23+, 12+/23−, 12−/23+ and 12−/23−). The 12+ and 23− allele pairs in all cattle breeds and 12+ and 23+ allele pairs in all buffalo breeds were also in linkage disequilibrium, although the results were not statistically significant at a *P*-value of 0.05.

χ^2 testing revealed significant differences when allele, genotype and haplotype frequencies of 23 bp and 12 bp indels were compared

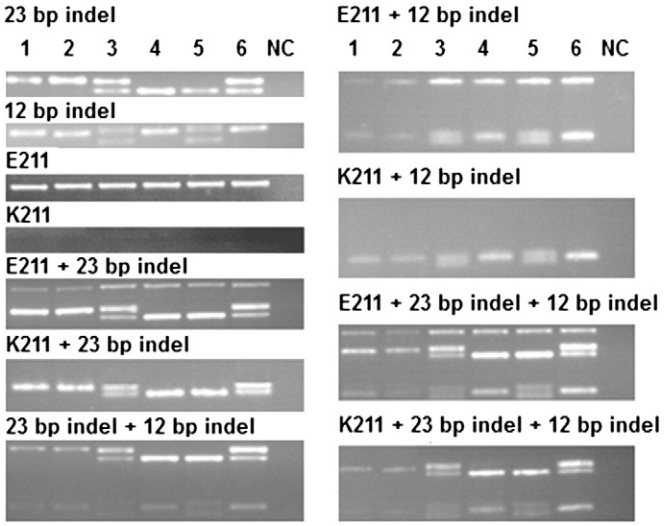

Fig. 1. Mono-, di- and triplex PCR amplifications of studied *PRNP* alleles. The fragment sizes are given in Supplementary file 1 (see Materials and methods). NC = negative control, lanes 1–6 = DNA samples from buffalo (lane 1) and cattle (lanes 2–6).

between Pakistani and worldwide bovid populations (cattle and buffalo) (Supplementary files 2, 3 and 4). Significant differences between BSE cattle groups, except German Holstein BSE cattle, and Pakistani cattle were also found. No significant differences in allele, genotype and haplotype frequencies of 23 bp and 12 bp indels were found between Pakistani and Anatolian water buffalo.

4. Discussion

Our genetic data provide support to the inference that Pakistani cattle are more resistant to classical BSE as compared to European cattle. Sequence alignments of *PRNP* regions harboring 23 bp and 12 bp indels loci revealed that the 12 bp insertion was more conserved across bovids and cervids than the 23 bp insertion (Fig. 2). Although the 12 bp insertion is the major genetic factor conferring resistance to classical BSE in UK Holsteins cattle (Juling et al., 2006) and is more conserved across bovids and cervids, it does not mean that the 12 bp indel locus is functionally more relevant to BSE susceptibility than the 23 bp indel locus. No case of prion disease has been reported in buffaloes even in those European countries where millions of buffaloes are present and many BSE cases have been found. One reason for the absence of prion disease in buffalo might be the higher frequency of 23 bp and 12 bp insertion alleles in this species (Oztabak et al., 2009).

The frequencies of 12 bp insertion (+) allele and 12+/+ genotype were significantly higher in Pakistani cattle (94.07% and 89.41%, respectively) as compared to worldwide cattle (Supplementary file 2). Previous association studies have demonstrated that the 23 bp and 12 bp deletion (−) alleles, 23−/− and 12−/− genotypes and 12−/23− haplotype are associated with increased susceptibility of European cattle to classical BSE (Haase et al., 2007; Juling et al., 2006; Sander et al., 2004). Conversely, the presence of insertion alleles at the 23 bp and 12 bp indels loci is associated with resistance to classical BSE. The basic component of BSE resistance was considered to be the 12 bp indel locus as both 12+/23− and 12+/23+ haplotypes were equally associated with BSE resistance (Juling et al., 2006; Kashkevich et al., 2007). Studies on *PRNP* regulation have also provided a proof that the intron 1 in which the 12 bp indel locus is located, has a key role in controlling *PRNP* expression levels (Bellingham et al., 2009; Elmonir et al., 2010; Haigh et al., 2007; Kashkevich et al., 2007; Msalya et al., 2009; Xue et al., 2008).

On the contrary, the 23 bp insertion allele contains binding site for RP58 transcription factor which interacts with SP1 transcription factor having binding site within the 12 bp insertion allele and represses the SP1 binding and the *PRNP* expression (Aoki et al., 1998; Becker et al., 1997; Lee et al., 2002; Sander et al., 2005). Decreasing the level of prion protein increases the incubation period of BSE (Msalya et al., 2011). This provides a plausible explanation for the association of 12+/23+ haplotype with increased resistance to

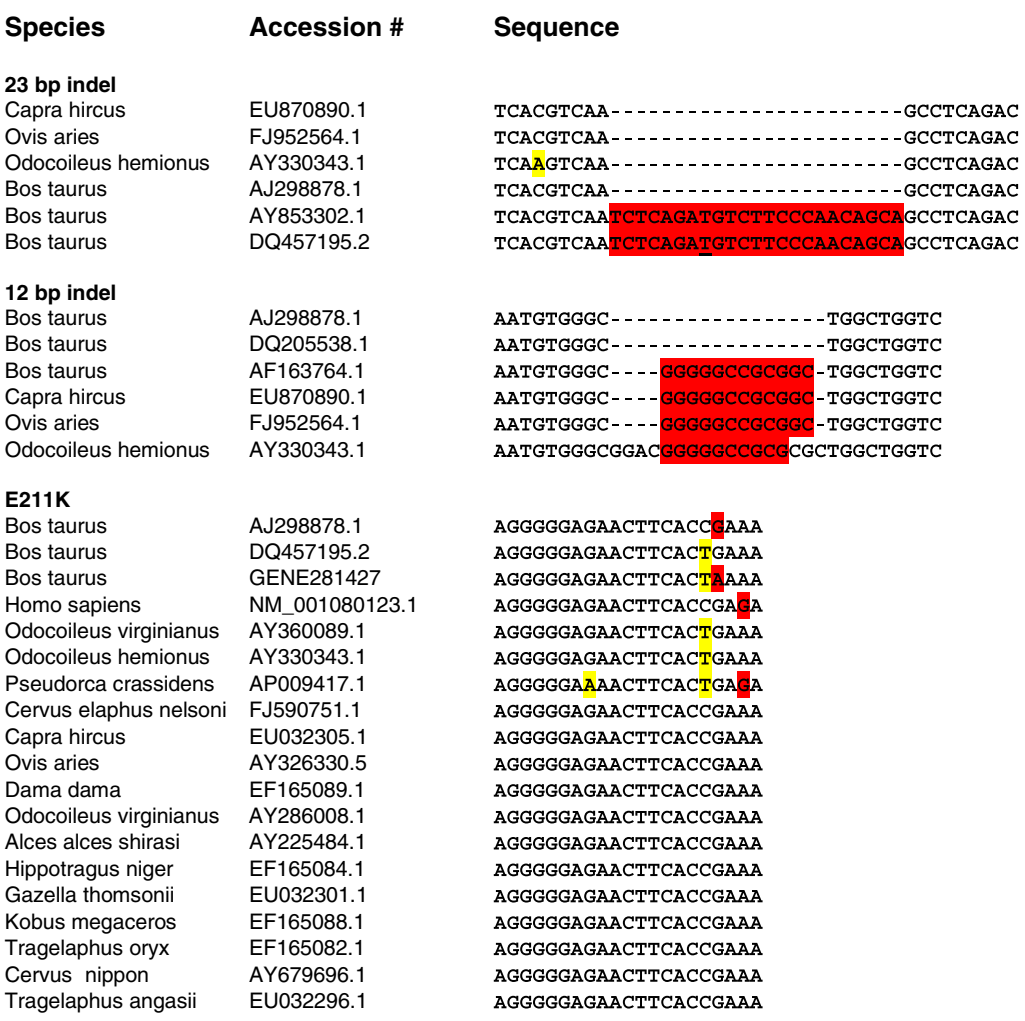

Fig. 2. Sequence alignments of studied *PRNP* alleles. Sequence alignments were performed using NCBI BLAST (www.ncbi.nlm.nih.gov/blast/). In red color is illustrated within alignment the position of 12 bp and 23 bp indels and E211K (GAA→AAA) polymorphism.

classical BSE (Juling et al., 2006; Msalya et al., 2009; Sander et al., 2005). The SP1 transcription factor was recently reported to be a copper-sensing transcription activator which enhanced the *PRNP* expression in response to elevated copper level (Bellingham et al., 2009). The *PRNP* expression will decrease when the interaction of RP58 and SP1 transcription factors will occur. The reason for association of the 12+/23− haplotype with resistance to classical BSE in UK Holsteins cattle remains to be answered (Juling et al., 2006; Kashkevich et al., 2007). BSE resistant 12 bp and 23 bp insertion alleles have also been found in BSE cattle, suggesting that these alleles are associated with relative resistance to the disease rather than absolute protection. There should be other genetic variations within or nearby the 23 bp and 12 bp indels loci controlling the BSE susceptibility (Haase et al., 2007; Juling et al., 2006; Nakamura et al., 2007).

In contrast, the 12 bp and 23 bp indel polymorphisms are not associated with L-type or H-type atypical BSE. The atypical BSE cases have occurred in almost 30 cattle worldwide. The atypical BSE cases were 4 years older (10 years old) than the classical BSE cases (6 years old) (Brunelle et al., 2007; Clawson et al., 2008; Heaton et al., 2008; Nicholson et al., 2008; Richt and Hall, 2008). Although genetic factors are also important in the context of disease susceptibility, the environmental exposure of cattle to contaminated feedstuffs is the major factor contributing to BSE incidence (Juling et al., 2006). The 23 bp and 12 bp insertion alleles may artificially be selected to produce BSE resistant cattle, preferably in countries where the practice of feeding ruminants-derived MBM to cattle has been common for enhancing the milk and meat production (Goldmann, 2008; Juling et al., 2006). It was the ban on the use of MBM by which the BSE epidemic was controlled. So, the continuity of this ban is necessary to avoid the BSE epidemics to occur in future. In order to fulfill the market demands for milk and meat, the illegal use of MBM may, however, be kept on even without screening for the presence of prions. The following facts suggest that Pakistani cattle are highly unlikely for acquiring BSE infection: 1) no BSE case has been observed hitherto in cattle from Pakistan or neighboring countries (Shankar and Satishchandra, 2005), 2) Pakistani cattle are not fed ruminant-derived MBM (http://ec.europa.eu/food/fs/sc/ssc/out199_en), and 3) the 12 bp insertion allele is predominantly present in *PRNP* of Pakistani cattle (Supplementary files 2, 3 and 4).

The BSE susceptibility depends on breed type; most of BSE cases have occurred in Holstein–Friesians than in other cattle breeds (Ducrot et al., 2008; Novakofski et al., 2005). As *Bos taurus* and *Bos indicus* are two subspecies of cattle with independent origins (Loftus et al., 1994), they may exhibit differential susceptibility to BSE. Although origins of classical BSE are unknown, some observations have convinced a group of investigators to suggest that the source of BSE infection may have originated from the Indian sub-continent (Baron and Biacabe, 2006; Colchester and Colchester, 2005, 2006; Nicholson et al., 2008; Pennington, 2006; Richt and Hall, 2008). These observations demonstrate the occurrence of spongiform encephalopathy (SE) in a 19 years old miniature zebu in Basel, Switzerland (Seuberlich et al., 2006), the association of BSE in a *Bos taurus*×*Bos indicus* cow with a *PRNP* polymorphism E211K (Nicholson et al., 2008; Richt and Hall, 2008) and a marginal correlation between periods of the BSE epidemic in UK and excessive import of the mammalian bones and soft tissues from the Indian sub-continent to UK (Colchester and Colchester, 2005). Such a possibility may have some major social, ethical, policy, and trade-related concerns for at least Pakistan, India and Bangladesh (Chatterjee and Van Marck, 2006; Shankar and Satishchandra, 2005).

Although BSE origins may have been linked to the dietary exposure of cattle to contaminated MBM derived from sheep or goats with scrapie, cattle with atypical BSE and humans with Cruetzfeldt–Jacob disease (CJD) (Colchester and Colchester, 2005; Hill et al., 1998; Nicholson et al., 2008; Richt and Hall, 2008), the contaminated MBM derived from cattle with atypical BSE may be the likely candidate for being causative of the BSE epidemic, given that prion infection occurs more faithfully within animals of the same species (Capobianco et al., 2007; Nicholson et al., 2008; Richt and Hall, 2008). Atypical L-type BSE may be more infectious to humans than classical BSE (Kong et al., 2008), but no one from naturally occurring human prion cases has been associated with atypical BSE. Most of atypical BSE cases were found in subclinical stages after active BSE surveillance was carried out in Europe (Ducrot et al., 2008). This indicates that the infectivity titer in subclinical atypical BSE cases may, perhaps, have never reached to levels required to cause sustainable human infection.

Atypical BSE cases are thought to have existed in cattle even long ago before the BSE epidemic first started to occur in 1986 (Ducrot et al., 2008). Some visually normal cattle propagating atypical BSE infection may also exist in any of cattle-rearing countries. In the context of BSE origins, the Indian sub-continent is therefore more concerned about surveillance of atypical BSE. No BSE case was observed from the Indian-subcontinent in bovines (cattle and buffalo) after screening the animals from slaughter houses, dead animals or those ailing with neurological disorders (Shankar and Satishchandra, 2005). To elucidate whether the only polymorphism (E211K) associated with atypical H-type BSE is present in Pakistani cattle or buffaloes, genotyping of this polymorphism was performed for these bovid species (Fig. 1). The K211 allele was absent from peripheral DNA of the studied animals and the results of 12 bp and 23 bp indel polymorphisms suggested that Pakistani cattle are more resistant to classical BSE as compared to European cattle. However, genetically resistant cattle can also acquire prion infection. Therefore, the most critical step for controlling the BSE occurrence in a locality should be the prohibition of import of contaminated ruminants-derived animal diet from BSE-endemic countries. The import of mammalians-derived MBM from any region of the world can possibly be associated with the BSE epidemic. Since no clustering of animal or human prion or prion-like disorders have been reported from the Indian subcontinent (Pakistan, India and Bangladesh) throughout the human history, currently, BSE is not a matter of concern for this region. However, rare sporadic CJD cases have been reported from India (Shankar and Satishchandra, 2005) and may also occur in Pakistan (Imran et al., 2012).

4.1. Conclusion

The higher observed frequency of 12 bp insertion allele suggests that Pakistani cattle are relatively more resistant to classical BSE than European cattle. However, the key risk factor for classical BSE is the dietary exposure of susceptible cattle to contaminated feedstuffs.

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Competing interests

The authors declare that they have no competing interests.

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