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RESEARCH ARTICLE

Frequencies of the *PRNP* Gene Polymorphisms in Binglangjiang Buffalo (*Bubalus bubalis*) for Comparing Potential Susceptibility to BSE

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ABSTRACT

The bovine spongiform encephalopathy (BSE), is a neurodegenerative disorder initiated by miss folded prion protein affecting in cattle. It is associated with a 23 bp indel polymorphism in the putative promoter and a 12 bp indel in intron 1 of the *PRNP* gene. There is no prior record of investigating for these indels polymorphism from Binglangjiang (BLJ) buffalo. The results of collected 100 samples from BLJ buffalo demonstrated that high insertion of genotype frequencies in 23++ (0.98) and 12++ (0.93) with the allelic frequencies also high in 23 bp (0.980) and 12 bp (0.965) while deletion is considerably low or absent in the allelic as well as in genotype frequencies. Haplotypes data showed different indel polymorphism with absent deletion (0) in both regions of this *PRNP* gene. A comparative analysis of BLJ buffalo with healthy and BSE affected German and Swiss cattle breeds from European countries was done with the results signifying a difference ($P<0.001$) in healthy and affected cattle. Significant outcomes were observed after comparison with previous studies on BLJ buffalo. Result demonstrated no genetic vulnerability to in BJL buffalo. Thus, BLJ buffalo can therefore prove to be the most likely model for genetic, selection, breeding and production. To best of our knowledge, this was first study describing indels polymorphism 23 bp & 12 bp in BLJ buffalo (river buffalo) of China.

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INTRODUCTION

Bovine spongiform encephalopathy (BSE) is a neurodegenerative disorder and poses life-threat to the health of cattle. Prion diseases are most devastating among sheep and goat as scarapie, in humans as Creutzfeldt Jacob disease, are among the fatal degeneration of brain and known to be caused by miss-folded prion protein. The clinical features include aggressive behavior and ataxia, while pathological features of BSE prion are miss folding prion protein plus aggregation, followed by brain's spongiform degeneration (Imran *et al*., 2012). This protein is associated with indels polymorphism in the promoter and intron 1 regions of *PRNP* gene (Oztabak *et al*., 2009). Among these, two indels polymorphisms which are characterized by a 23 bp indel in putative promoter and a 12 bp indel in intron 1, octapeptide repeats in coding sequence and polymer-phism in amino acids (Zhao *et al*., 2015). Earlier reports have shown the deletion of alleles either at single or both indels sites resulted in these two sites, which are further related to susceptibility of classical BSE (Sander *et al*., 2004; Juling *et al*., 2006; Haase *et al*., 2007).

The river buffalo and swamp buffalo are two different species of domestic water buffalos. Interestingly, river buffalo have a different chromosome complement of 2n=50 while the swamp buffalos have 2n=48 (Oztabak *et al*., 2009). The Binglangjiang (BLJ) buffalo is the first native river-type in Yunnan Province, China. The BLJ has been developed as a new breed of river type milking buffalo in China. BLJ buffalo has good quality milk with rich in protein and milk fat and

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lactation 8-15% higher than other river type buffaloes Murrah and Nili-Ravi. The genetic background of BJL buffalo shows significant genetic variability and diversity and most of them contain diploid chromosome number of 50 (2n=50). They have typical body shape, but is comparatively small in size compare with others river buffalo breeds (Zhang *et al*., 2011).

Bovine *PRNP* gene related to expression levels of prion is responsible for indels polymorphism when assays with reporter genes and epidemiological surveys have shown (Sander *et al*., 2005). It was interesting to know about genetic differences in *PRNP* polymorphisms associated with susceptibility of BSE between buffalo and cattle. In present scenario, results of previous findings have displayed that Anatolian, Chinese, Pakistani and Thai buffalo species compared to cattle showed dramatically differences in the two *PRNP* indels polymorphisms (Oztabak *et al*., 2009; Imran *et al*., 2012; Uchida *et al*., 2014; Zhao *et al*., 2015). Although the genetic resources of buffalo in China is enormous, but still little has been reported on genetic vulnerability of the Chinese buffalo to BSE (Zhao *et al*., 2015).

The objective of this study was to identify the allelic, genotypic and haplotypic frequencies of *PRNP* indels polymorphism in BLJ buffalo from Yunnan Province of China and compare with reported healthy or affected-BSE cattle for evaluating their potential resistance to BSE.

MATERIALS AND METHODS

Animals and sample collection: In total, we collected 100 Binglangjiang buffalo (BLJ buffalo) samples from Tengchong county located in the upper reaches of Binglangjiang River in Yunnan Province, China. About 5 ml of blood sample from each animal was carefully withdrawn from the jugular vein in presence of a trained veterinarian. Blood samples were collected and then stored at −20°C.

Polymerase chain reaction (PCR) and sequencing: Total genomic DNA was extracted following Sambrook *et al*. (1989). The *PRNP* region was amplified using previously described primers following Nakamistu *et al*. (2006) - F 5'-CTTCTCTCTCGCAGAAGCAG-3' and R 5'CCCTTGTTCTTCTGAGCTCC3' for the 12-bp indel and (Brunelle *et al*., 2008) - forward primer 5'-AAGGCACTTCAATCAGTACAC-3' and reverse primer 5'-AAGAGTTGGACAGGCACAATG-3' for the 23-bp indel, respectively. The PCR was carried out in a reaction volume of 25 µl, containing 2.0 µl DNA (approximately 50 ng), 2.0 µl 250 µmol/l dNTPs, 2.5 µl buffer ($MgCl_2$ in the buffer provided by the manufacture already), 2.0 µl 10 µmol/l forward primer, 2.0 µl 10 µmol/l reverse primer and 0.2 µl 10× Taq DNA polymerase (5 U/µl, Beijing TransGen Biotechnology Co., Ltd, China). PCR cycling conditions were as follows: 5 min at 94°C, 35 cycles of amplification (45 s at 94°C, 45 s at 54°C, 60 s at 72°C), and finally 6 min at 72°C. All PCR products were electrophoresed on 2% agarose gel for 45 mins at 30 volts. Electrophoresis of the PCR product conducted in either a 191 bp (+) or 168 bp (-) for indel 23 polymorphism and either in a 215 bp (+) or a 203 bp (-) for indel 12 polymorphism. PCR products were selected randomly and bidirectional sequencing was performed on ABI 3730xl Genetic Analyzer (Applied Biosystems, USA) at Sango Biotechnology Company (Shanghai, China) to verify their genotype.

Data analysis: Genotype and allele frequencies of the BLJ buffalo *PRNP* promoter and intron 1 region variants were examined manually and the deviation of Hardy-Weinberg (H-W) equilibrium was calculated using the chi-squared test (X^2) (Oztabak *et al*., 2009; Xi *et al*., 2011). The haplotype frequencies were analyzed from the genotypic data by using the program Haploview 4.0 (Barrett *et al*., 2005) and diversity indices were calculated using method by (Nei and Li, 1979).

RESULTS

Allelic genotypic frequencies: Insertion in both alleles 23bp in *PRNP* promoter and 12bp in intron 1 region was high in frequency (0.980 and 0.965 respectively) resulting extremely low frequencies of deletion in both alleles (0.020 in 23bp deletion and 0.035 in 12bp deletion) as shown in Table 1. The allelic and genotypic frequencies for two indels polymorphisms in BLJ buffalo were calculated and compared with BSE-affected and Healthy cattle (Table 1). The allelic and genotypic frequency of 23 bp and 12 bp indels polymorphism was significantly higher (P<0.001) from affected as well as BSE affected cattle of both breeds (Table 1).

Table 1: Distribution and comparison of allele and genotype frequencies of 23bp and 12bp-indels of BLJ Buffalo with BSE-affected and healthy cattle

(a) 23bp-indel

Breed	Health Status	n	Allele Frequency		p-value		Genotypic frequency			p-value	
			D23	I23	BSE	Healthy	D23/D23	D23/I23	I23/I23	BSE	Healthy
BLJ Buffalo		100	0.02	0.98			0.02	0	0.98		
Germen cattle[1]	BSE	43	0.73	0.27	< 0.00001	–	0.51	0.44	0.05	< 0.00001	–
	Healthy	48	0.57	0.43	–	< 0.00001	0.35	0.44	0.21	–	< 0.00001
Germen & swiss[2]	BSE	670	0.68	0.32	< 0.00001	–	0.45	0.47	0.09	< 0.00001	–
	Healthy	574	0.61	0.39	–	< 0.00001	0.37	0.47	0.16	–	< 0.00001

(b) 12bp-indel

Breed (n)	Health Status	n	Allele Frequency		p-value		Genotypic frequency			p-value	
			D12	I12	BSE	Healthy	D12/D12	D12/I12	I12/I12	BSE	Healthy
BLJ Buffalo		100	0.035	0.965			0	0.07	0.93		
Germen cattle[1]	BSE	43	0.67	0.33	< 0.00001	–	0.44	0.47	0.09	< 0.00001	–
	Healthy	48	0.51	0.49	–	< 0.00001	0.23	0.56	0.21	–	< 0.00001
Germen & swiss[2]	BSE	670	0.58	0.42	< 0.00001	–	0.34	0.49	0.17	< 0.00001	–
	Healthy	574	0.54	0.46	–	< 0.00001	0.31	0.46	0.23	–	< 0.00001

[1]- Sander *et al.* (2004); [2]Haase *et al.* (2007). All values found to be significant on comparison of BLJ Buffalo with German BSE affected, German Healthy, German and Swiss BSE affected and German and Swiss Healthy cattle.

Haplotypic frequencies: The four different haplotypes (23+/12+, 23+/12-, 23-/12+ and 23-/12-) were measured in the promoter and intron 1 region of the *PRNP* from BLJ buffalo (Table 2). The most numerous haplotype obtained was 23+/12+ with a frequency 0.945 which was higher than 23-/12+ (frequency=0.020), 23+/12- (frequency= 0.035) and 23-/12- (frequency=0). The haplotypes frequencies for two indels polymorphism in BLJ buffalo were calculated and compared with affected and healthy German and Swiss cattle. The haplotypes of 23 bp and 12 bp indels polymorphism was also significantly higher (P<0.001) from BSE affected as well as healthy cattle of both breeds (Table 2).

DISCUSSION

The existence of correlation between BSE and the two indel polymorphism is well established. Results indicated that BLJ buffalo had extremely high insertion of 23bp (0.98) and 12bp (0.965) allelic frequencies. A comparative analysis of the results with cattle and buffalo was done and is mentioned in Table 3. In-variant frequency of 12 bp polymorphism vary between 0.104/0.11 Guangxi buffalo breed and Hereford cattle breed (Zhu *et al*., 2011; Zhao *et al*., 2015) and 0.99/1.0 Guangnan, Dehong, Chongqing and Guizhou buffalo breed (Zhao *et al*., 2015). The 12 bp indels polymorphism in BLJ buffalo is similar to others breed of buffalo such as Anatolian Water Buffalo, Azikheli, Chinese swamp buffalos (Chongqing and Guizhou, Dehong, Fuhan and Hainan, Guangnan and Guandong), Kundhi, Murrah, Nili and Ravi buffalos (Oztabak *et al*., 2009; Imran *et al*., 2012; Zhao *et al*., 2015; Yaman *et al*., 2016) except Guangxi breed of Chinese swamp buffalo the 12-bp indel is vary from others buffalo (Zhao *et al*., 2015) (Table 3). In accordance of the previous reports, present study showed in variant of 23 bp polymorphism (frequency 0.980) close to be others buffalo except Guangxi and Guangdong buffalos (Zhao *et al*., 2015). Previous studies revealed that indels polymorphism of 23 bp in the promoter and 12 bp in intron 1 region with BSE in cattle (Sander *et al*., 2004; Haase *et al*., 2007). However, (Zhu *et al*., 2011) presented a contradictory observation of the association of a 23 bp deletion in promoter and a 12bp deletion in intron 1 region in Hereford cattle. Results from our experiment correlate with the findings of previous studies on a variety of cattle and buffaloes (Table 3).

Results from current and previous studies were analyzed for frequency distribution of 23 bp and 12 bp indels polymorphism of buffalo, healthy and BSE cattle. Data for the comparative analyses and frequency distribution on buffalo, BSE cattle and healthy cattle was acquired from almost published documents (Sander *et al*., 2004; Seabury *et al*., 2004; Juling *et al*., 2006; Nakamitsu *et al*., 2006; Brunelle *et al*., 2007, 2008; Czarnik *et al*., 2007, 2011; Haase *et al*., 2007; Kerber *et al*., 2008; Muramatsu *et al*., 2008; Kim *et al*., 2009; Msalya *et al*., 2009; Oztabak *et al*., 2009; Murdoch *et al*., 2010; Shimogiri *et al*., 2010; Zhao *et al*., 2010; Qin *et al*., 2011; Zhu *et al*., 2011; Gurgul *et al*., 2012; Imran *et al*., 2012; Uchida *et al*., 2014; Zhao *et al*., 2015). The allelic, genotypic and haplotypic frequencies of *PRNP* indels polymorphism in buffalo, taking into account the Anatolian buffalo (Oztabak *et al*., 2009), Pakistani buffalo (Imran *et al*., 2012), Vietnam, Indonesian and Thai buffalo (Uchida *et al*., 2014), Chinese swamp buffalo (Zhao *et al*., 2015) and BLJ buffalo (in presentstudy, river type), were dramatically vary from that of BSE and healthy cattle (P<0.001; Fig. 1A–E). Results indicate a significantly higher (P<0.001) allele frequency (D23 and

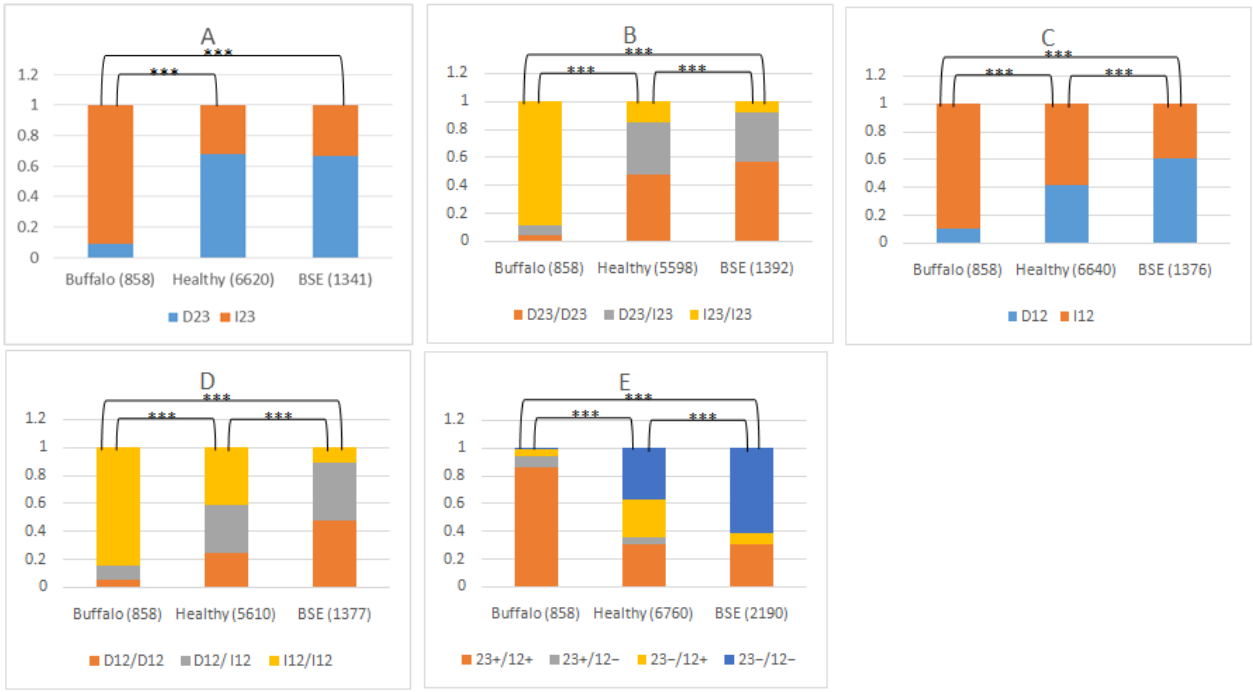

Fig. 1: Comparison of the frequency distributions of the Chinese BLJ with different healthy and BSE cattle. *** denotes highly significant differences (P<0.001) in frequency distribution. A and B show the allele and genotype distributions in the 23-bp indel polymorphism, while C and D indicate the allele and genotype frequencies in the 12-bp indel polymorphism respectively. E corresponds to the haplotype distributions assembled by two polymorphism.

Table 2: Comparison of haplotype frequencies for the 23-bp and 12-bp indel polymorphisms between BLJ Buffalo with UK Holstein BSE-affected and German Holstein healthy cattle

Breed	n	Haplotype frequency				P-value	
		23+/12+	23+/12−	23−/12+	23−/12−	BSE+	BSE-
UK Holstein BSE affected	706	0.242	0	0.041	0.717	< 0.00001	–
Germen Holstein Healthy	615	0.391	0.005	0.91	0.513	–	< 0.00001
BLJ Buffalo	100	0.945	0.035	0.02	0		

All values found to be significant on comparison of BLJ Buffalo with German and Swiss BSE affected and German and Swiss BSE Healthy cattle (Juling *et al* 2006).

Table 3: Comparison of allele frequencies of 12bp-indel and 23bp-indel polymorphism among different breeds of cattle and buffalo

Country	Species	n	Allele frequencies of 12 bp and 23 indel polymorphisms				References
			12 bp		23 bp		
			+	-	+	-	
Cattle breed (*Bos taurus)*							
Germany	German Cattle	48	0.49	0.51	0.43	0.57	Sander *et al.* 2004
Germany	German Holstein	127	0.57	0.43	0.38	0.62	Juling *et al.* 2006
	German Brown	43	0.86	0.14	0.65	0.35	Juling *et al.* 2006
	Fleckvieh	106	0.38	0.62	0.32	0.68	Juling *et al.* 2006
Japan	Japanese Holstein	278	0.26	0.74	0.21	0.79	Nakamitsu *et al.*2006
	Japanese Brown	186	0.43	0.57	0.41	0.59	Nakamitsu *et al.*2006
Poland	Polish Holstein	234	0.46	0.54	0.37	0.63	Czarnik *et al.* 2007
Brazil	Abderdeen Angus	99	0.44	0.56	0.27	0.73	Kerber *et al.* 2008
	Charolais	82	0.42	0.58	0.32	0.68	Kerber *et al.* 2008
	Franqueiro	73	0.67	0.33	0.36	0.64	Kerber *et al.* 2008
Vietnam	Vietnamese Cattle	206	0.52	0.43	0.15	0.85	Muramutsu *et al.* 2008
South Korea	Korean Cattle	437	0.44	0.56	0.44	0.56	Kim *et al* 2009
Vietnam	Vietnam Native	100	0.93	0.07	0.34	0.66	Shimogiri *et al.* 2010
Laos	Laos Native	72	0.92	0.47	0.37	0.63	Shimogiri *et al.* 2010
Myanmar	Myanmar Native	110	0.81	0.29	0.20	0.80	Shimogiri *et al.* 2010
Mongolia	Mongolia Native	44	0.49	0.51	0.30	0.70	Shimogiri *et al.* 2010
Bangladesh	Bangladesh Native	30	0.75	0.25	0.17	0.83	Shimogiri *et al.* 2010
China	Chinese Native	349	0.842	0.158	0.404	0.596	Zhao *et al.* 2010
	Hereford	33	0.11	0.88	0.017	0.98	Zhu *et al.* 2011
	Simmental	30	0.19	0.79	0.160	0.830	Zhu *et al.* 2011
	Black Angus	30	0.37	0.63	0.210	0.780	Zhu *et al.* 2011
	Mongolian	31	0.58	0.42	0.480	0.510	Zhu *et al.* 2011
Poland	Polish Holstein	837	0.473	0.527	0.378	0.622	Czarnik *et al.* 2011
	Polish Holstein	651	0.475	0.525	0.381	0.619	Gurgul *et al.* 2011
Pakistan	Cholistani	43	0.884	0.116	0.105	0.895	Imran *et al.* 2012
	Dhanni	11	0.818	0.182	0.091	0.909	Imran *et al.* 2012
	Lohani	28	0.911	0.089	0.125	0.875	Imran *et al.* 2012
	Sahiwal	83	0.958	0.042	0.217	0.783	Imran *et al.* 2012
	Therparker	29	0.983	0.017	0.207	0.793	Imran *et al.* 2012
	Red Sindhi	26	01	0	0.25	0.75	Imran *et al.* 2012
	Achai	16	0.969	0.031	0.219	0.781	Imran *et al.* 2012
Vietnam	Vietnamese Cattle	99	0.95	0.05	0.33	0.67	Uchida *et al.* 2014
Indonesia	Indonesian Cattle	121	0.79	0.21	0.49	0.51	Uchida *et al.* 2014
Thailand	Thai Cattle	68	0.88	0.13	0.53	0.47	Uchida *et al.* 2014
Buffalo Breed (*Bubalis bubalis)*							
Turkey	Anatolian Water Buffalo	106	0.86	0.14	0.92	0.08	Oztabak *et al.* 2009
Pakistan	Nilli	66	0.86	0.14	0.94	0.06	Imran *et al.* 2012
	Ravi	39	0.83	0.17	0.97	0.03	Imran *et al.* 2012
	Azikhali	20	0.95	0.05	1	0	Imran *et al.* 2012
	Kundhi	34	0.88	0.12	0.97	0.03	Imran *et al.* 2012
	Nili Ravi	122	0.87	0.13	0.94	0.06	Imran *et al.* 2012
Indonesia	Indonesian	14	1	0	1	0	Uchida *et al.* 2014
Thailand	Thai	45	0.84	0.16	0.53	0.47	Uchida *et al.* 2014
China	Dehong	88	0.99	0.01	0.91	0.08	Zhao *et al.* 2015
	Guangnan	59	1.00	0	0.94	0.06	Zhao *et al.* 2015
	Guangxi	24	0.104	0.896	0.104	0.896	Zhao *et al.* 2015
	Guangdong	73	0.952	0.048	0.027	0.973	Zhao *et al.* 2015
	Chongqing and Guizhou	43	1	0	1	0	Zhao *et al.* 2015
	Fuan and Hainan	25	0.940	0.060	1	0	Zhao *et al.* 2015
Turkey	Anatolian Buffalo	89	1	0	1	0	Yaman *et al.* 2016
	Murrah Buffalo	20	1	0	1	0	Yaman *et al.* 2016
	Murrah×Anatolian	86	1	0	1	0	Yaman *et al.* 2016
China	BLJ Bufallo	100	0.965	0.035	0.980	0.020	In present study

D12) in BSE cattle (Fig. 1-A&C). There were significant contrasts observed in genotypic frequencies of D23 and D12 while comparing buffalo, healthy and affected cattle (Fig. 1-B&D). Furthermore, the buffalo haplotypic frequencies were also significant from healthy and affected cattle (Fig. 1-E). For instance, the insertion of 23bp and D12bp allelic frequencies were higher in buffalo (0.92 and 0.903 respectively) and in contrast to healthy cattle (0.32 and 0.591) or in affected cattle (0.332 and 0.393). Resultantly, the affected cattle displayed drastically increased haplotypic frequency of D23-D12 compare to healthy cattle (0.613 vs. 0.372; P<0.001),

whereas the low in buffalo compared to the cattle (0.012; P<0.001). The findings of this experiment comply with the results of Zhao *et al*. (2015).

BSE vulnerability has been reported to be majorly linked with deletion of 12-bp (Juling *et al*., 2006; Msalya *et al*., 2011) D23-D12 haplotypes. Low 12-bp deletion leads to a less susceptible animal. In the study of Zhao *et al*. (2010), as the appearance was low in Chinese cattle resulting is less susceptibility to BSE. Similar results were observed in present study where haplotype D12-D23 was 0 and showed least tendency towards susceptibility. Besides, allele frequency 12-bp D was 0.035 which also complies with the previous reports. Our finding indicates a low expression of D12 and D12-23 in BLJ buffalo resulting in rare BSE attacks.

Comparison of our results with previous studies indicated that D12 of the present study was significantly lower in both the disease and the healthy cattle. Additionally, D23/12 was not detected in the BLJ buffalo compared with previous result for healthy and affected cattle. These results agree with our observation that the BLJ buffalo is not susceptible to BSE. Based on our findings and our investigations, we hypothesized that increase in insertion compared to deletion results in less expression of the *PRNP* genes which leads less susceptibility to BSE. Through our study, we established the fact that BLJ buffalo is not vulnerable to BSE and are therefore recommended for breeding and production purposes. These results will prove valuable for the future breeding programs in order to establish a resistance against BSE.

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Authors contribution: SM, GL, WD, DX designed and conceived the study; SM, HX, MY, YY performed experiments and collected data; LW, XL analyzed the data; and SM, GL, WD, DX wrote the manuscript.

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