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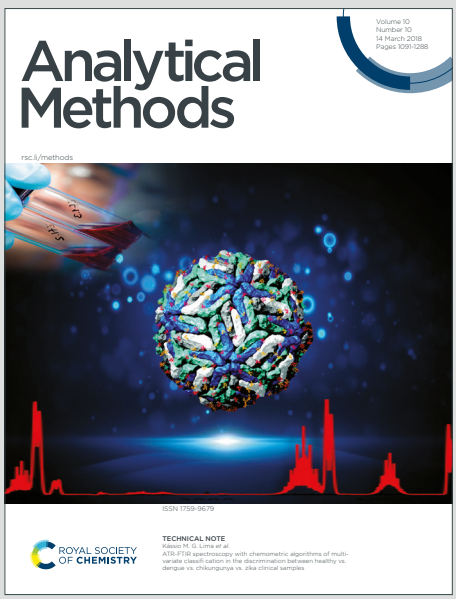

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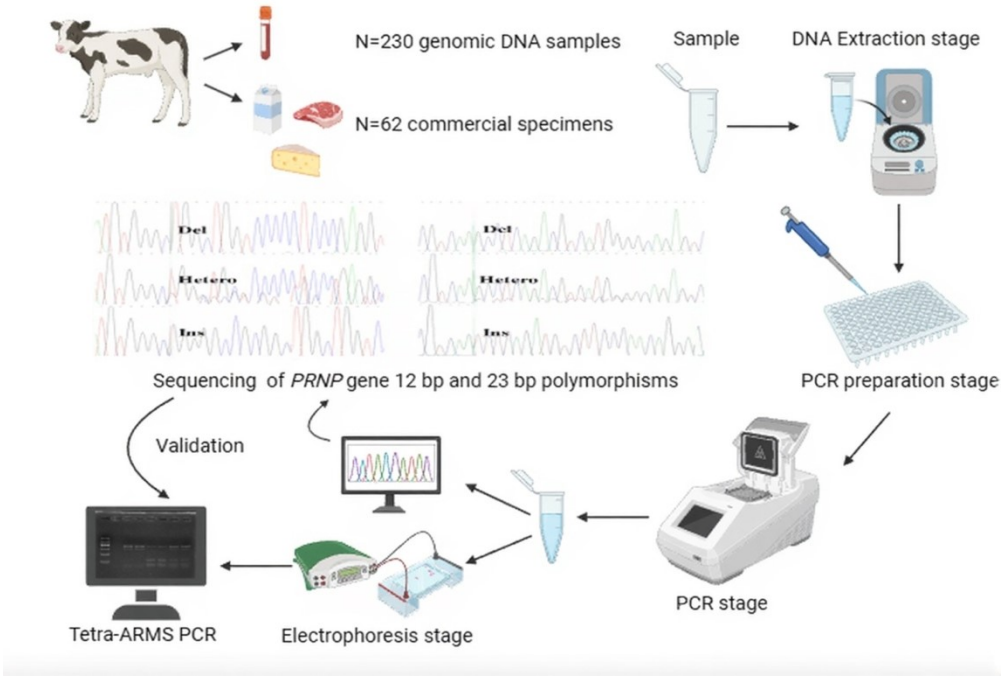

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ARTICLE

Development and Application of a Tetra-ARMS PCR Assay for Detecting Indel Polymorphisms in the Bovine *PRNP* Gene

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Insertion/deletion (indel) polymorphisms in the promoter region (23 bp) and intron 1 (12 bp) of the bovine *PRNP* gene influence gene expression and susceptibility to bovine spongiform encephalopathy (BSE). Conventional detection strategies dependent on DNA sequencing are cumbersome and costly. A tetra-primer amplification refractory mutation system PCR (Tetra-ARMS PCR) assay was developed which can be able to enable efficient and cost-effective genotyping of each indel locus. Optimized tetra-ARMS PCR primers and multiplex conditions allowed electrophoretic genotyping of the 23 bp indel via the size and presence of 422 bp, 259 bp, and 193 bp amplicons, whereas the 12 bp indel was genotyped based on the size and presence of 598 bp, 472 bp, and 153 bp fragments. Furthermore, the compatibility of these primer sets was preliminarily investigated, demonstrating the potential for co-amplification in a single-tube multiplex format. Validation against Sanger sequencing using 62 randomly selected cattle-derived retail samples demonstrated complete concordance. This straightforward, specific and cost-effective method requires only conventional PCR instrumentation, thereby establishing a robust and accessible platform for *PRNP*-assisted breeding and cattle product genotyping.

1 Introduction

Bovine spongiform encephalopathy (BSE), commonly known as 'mad cow disease', is a fatal neurodegenerative disorder resulting from conformational aberrations in the scrapie prion protein (PrP^{Sc})[1]. Initially identified in the United Kingdom in 1986, BSE later underwent rapid global dissemination, causing devastating livestock losses and precipitating an international public health crisis[2-4]. Although the global incidence of BSE has substantially declined following the implementation of control measures (e.g., prohibitions on ruminant-derived feed), elucidating its pathogenesis and establishing effective management strategies remain significant challenges[5-7]. The pathogenic isoform PrP^{Sc} exhibits pronounced resistance to conventional disinfection and thermal decontamination[8-10]. Furthermore, multiple transmission routes of BSE are documented, including milk and meat products[11, 12], while environmental prion contamination may constitute secondary vectors[13, 14]. Collectively, these effective factors impede eradication efforts for this disease[13, 15-17]. As the primary determinant of prion protein expression, polymorphisms in the bovine prion protein gene (*PRNP*) modulate its susceptibility to BSE and its heritability[18-22]. Following the bovine *PRNP* gene sequencing in 2001[6, 20, 23, 24], studies have demonstrated that 23 bp indel in the promoter region and 12 bp indel in the intronic region are significantly associated with BSE resistance,

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thereby informing DNA marker assisted selection of resistant individuals[7, 18, 22, 25-27]. Current strategies encompass *PRNP* gene editing to enhance resistance in cattle breeding[28]. Consequently, detection of *PRNP* polymorphisms in any locus for BSE risk assessment provides assisted molecular markers for breeding programs, enabling strategic selection of resistant populations and guiding containment policies—imperative for safeguarding livestock industries and public health.

Current DNA genotyping of the 23 bp and 12 bp indel polymorphisms in bovine *PRNP* gene primarily employs Sanger sequencing[22, 29-34], PCR-RFLP analysis[35], quantitative PCR (qPCR)[36], next-generation sequencing (NGS)[37], and microarray genotyping[38], et al. These methodologies are constrained by protracted processing times, elevated costs, and technical limitations, notably incomplete restriction enzyme digestion. Specifically, Sanger sequencing, despite providing precise sequence resolution, incurs high per-sample costs; PCR-RFLP is susceptible to interpretive errors owing to partial enzymatic cleavage, compromising its reliance on restriction site specificity; NGS, although permitting high-throughput multi-locus analysis, requires substantial instrumentation investment and sophisticated bioinformatics assay, thereby precluding routine diagnostics in resource-limited settings. Compared to the conventional detection methods mentioned above, amplification refractory mutation system PCR (ARMS-PCR), which exploits deliberate primer-template mismatches and constitutes a robust DNA analysis platform featuring operational simplicity, elimination of enzymatic digestion/fluorescent probes, cost-effectiveness, broad applicability, visual electrophoretic resolution, and inherent heterozygote discrimination[39-43]. Subsequent to its inception, the tetra-primer ARMS-PCR incorporated internal control primers to increase its accuracy, and has been widely applied to species identification and SNP genotyping across diverse biological systems[44-49], thus showing exceptional procedural efficiency and accessibility for routine genetic screening.

A Tetra-ARMS PCR-based DNA mismatch detection system was established in this study through optimized designed primers for the rapid and accurate genotyping of the 23 bp and 12 bp indel polymorphisms in the bovine *PRNP* gene. This study developed and validated two independent Tetra-ARMS PCR assays, and explored their potential for integration into a multiplex system. This assay enhances genotyping efficiency, broadens methodological capabilities for indel screening, and thereby provides technical support for DNA marker-assisted breeding programs and disease risk assessment in cattle populations.

2 Materials and Methods

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2.1 Sample Sources and DNA Extraction

A total of 230 genomic DNA samples were stored in the laboratory and were isolated from blood samples of Holstein cattle (n = 200), Yellow cattle (n = 10), and Wenzhou water buffalo (n = 20). Additionally, commercial specimens (fresh milk, beef, milk powder, cheese; n = 62) were purchased from retail markets, representing diverse production batches and dates. The blood samples of Holstein cattle and Wenzhou water buffalo were provided by the Key Laboratory of Dairy Cow Genetic Improvement and Milk Quality Research of Zhejiang Province, while those of Yellow cattle were from the Dairy Cattle Breeding Farm of Shandong Academy of Agricultural Sciences. Genomic DNA was extracted using a commercial Animal Tissue DNA Extraction Kit (Hangzhou Xinjing Biotechnology Co., China). All animal-related procedures were approved by the Animal Experiment Ethics Committee of China Jiliang University (Approval No. 2021-005).

Genomic DNA was extracted from commercial specimens using the TakaRa MiniBEST Universal Genomic DNA Extraction Kit (Takara Bio, China) according to the manufacturer's instructions. Approximately 25 mg aliquots underwent manufacturer-specified pretreatment, followed by genomic DNA isolation according to the instructions. Extracted DNA was quantified via NanoDrop 2000 spectrophotometry (Thermo Fisher Scientific) and assessed for integrity through 1.5% agarose gel electrophoresis with GelRed staining.

2.2 Sequencing Analysis of Two *PRNP* Polymorphic Loci

Sequencing and validation analysis of the 12 bp and 23 bp indel polymorphisms was performed using primer pairs reported by Imran et al[50], with the bovine *PRNP* gene sequence (GenBank accession No AJ298878.1) as the reference template. The polymorphic sequences were defined as 5'-GGGGGCCGCGGC-3' (12 bp) and 5'-TCTCAGATGTCTTCCCAACAGCA-3' (23 bp).

All primers were synthesized by Hangzhou Tsingke Biotechnology Co. (China), with sequences detailed in Table 1.

Table 1 Bovine *PRNP* Gene PCR Sequencing Primers

Polymorphic Locus	Primer	Primer Sequence (5'→3')
12bp indel	12indel F	GTGCTCGTTGGTTTTTGC
	12indel R	TCCTACACACCACCCACATA
23bp indel	23indel F	AGCCAGGTAAGAAGCTCATC
	23indel R	CATGAATTGTGTAGGCCAAA

PCR amplification targeting the 12 bp and 23 bp indel fragments in the bovine *PRNP* gene was performed separately using two independent primer sets under identical reaction conditions. The 20 μL reaction system followed the same PCR profile: 95°C for 5 min; 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s; 72°C for 10 min; and storage at 4°C. Amplification products were electrophoresed on 1.5% agarose gels, and fragments matching predicted sizes were purified and transferred to Hangzhou Tsingke Biotechnology (China Hangzhou) for bidirectional Sanger sequencing.

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2.3 Tetra-ARMS PCR Primer Design

Tetra-ARMS PCR primers targeting the 12 bp and 23 bp indel polymorphic loci were designed based on the bovine *PRNP* gene reference sequence (GenBank accession No AJ298878.1) and sequencing data described in section 2.2, following primer designed principles[39, 45, 47] with the expected amplicon sizes constrained to 150-600 bp for clear electrophoretic resolution. The 12 bp inner primer (12-IF2) has 5-nucleotide complementarity at the 3'-terminus to the insertion sequence; and the 23 bp inner primer (23-IFA4) has 22-nucleotide complementarity at the 3'-terminus to the insertion sequence. To enhance the PCR specificity, an additional T-G weak mismatch was introduced at nucleotide position 5 (from the 5'-terminus) in the upstream inner primer 23-IFA4 for the 23 bp polymorphic locus, and a T-G mismatch at nucleotide position 3 (from the 3'-terminus) in the downstream inner primer 23-AR2. The primer design strategy is illustrated in Figure 1, with sequences provided in Table 2. All primers were synthesized by Hangzhou Tsingke Biotechnology Co., Ltd. (China), and diluted to 10 μM, stored at 4°C for future use.

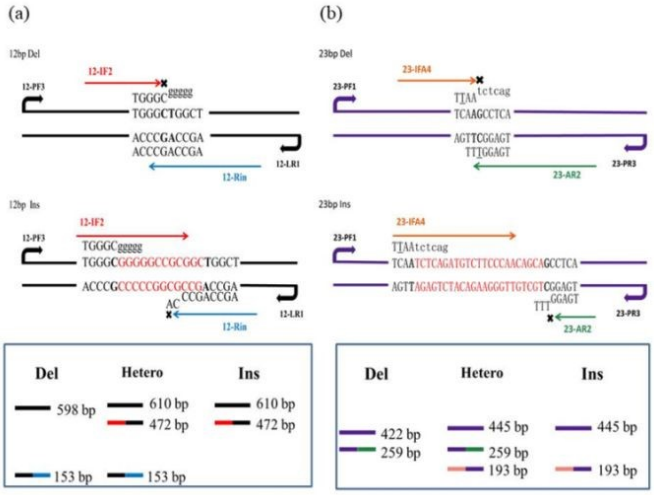

Fig. 1 Schematic of Tetra-ARMS PCR primer design and expected electrophoretic results. (a) 12 bp polymorphic locus; (b) 23 bp polymorphic locus. Colour-coded arrows denote primers: for the 12 bp locus, outer primers (black), insertion-specific inner primer (red), deletion-specific inner primer (blue); for the 23 bp locus, outer primers (purple), insertion-specific inner primer (orange-red), deletion-specific inner primer (green). Lowercase bases indicate complementarity to insertion sequences; underlined bases represent engineered mismatches. Genotype abbreviations: Del genotypes (homozygous deletion), Hetero genotypes (heterozygous insertion/deletion), Ins genotypes (homozygous insertion).

Table 2 Tetra-ARMS PCR Primer Sequences for Bovine *PRNP* indel Genotyping

Polymorphic Locus	Primer	Primer Sequence (5'→3')	Fragment length (bp)
12bp indel	12-PF3	ACCTTGACCGTGAGTAGGGCTGGG	
	12-IF2(I)	ACTCGGAATGTGGGCggggg	472(I)
	12-Rin(D)	GGGGGACCAGCCAGCCCA	153(D)
	12-LR1	CGCCAGGTGGCCCATGATT	598/610
23bp indel	23-PF1	GCAATGCAGCTTCCATGTTGTTTACTGA	
	23-IFA4(I)	ACGTTAAtctcagatgtcttcccaacagc	193(I)
	23-AR2(D)	ACTCTGCCCCATGACGTCTGAGGTTT	259(D)
	23-PR3	CGTGAGGGTTTGAGGGAACGAAATGAC	422/445

In the Tetra-ARMS PCR primer system: In primer nomenclature "I" denotes the upstream inner primer for the insertion sequence, and "D" denotes the downstream inner primer for the deletion sequence. Lowercase letters in "I" primers indicate bases complementary to the insertion sequence, while letters enclosed in boxes represent intentionally introduced mismatch bases.

2.4 Tetra-ARMS PCR Amplification

2.4.1 Basic PCR System and Optimization

Based on previously reported ARMS-PCR references system and conditions[39, 45, 47], the 20 μL PCR reaction system was prepared containing 2.0 μL 10× PCR buffer, 1.6 μL dNTP mixture (25 mM), 2.0 μL $MgCl_2$ (25 mM), 0.5 μL each of upstream/downstream outer primers (10 μM), 0.8 μL each of upstream/downstream inner primers (10 μM), 0.4 μL Taq DNA polymerase (5 U/μL; Thermo Fisher Scientific), 3.0 μL template DNA, and 8.4 μL ddH_2O.

The basic PCR program used was as follows: initial denaturation at 95°C for 8 min; 35 cycles of 94°C for 35 s, annealing at (57°C, 60°C, 63°C, 66°C, 69°C and 72°C) for 40 s, 72°C for 1 min; followed by a final extension at 72°C for 10 min, and finally the PCR products were maintained at 4°C in the end. Optimal reaction conditions were determined through systematic optimization of annealing temperature (57-72°C gradient) and cycle number (30, 32, 35, 38 cycles). Subsequent optimizations included Mg^{2+} concentration (1.5, 2.0, 2.5, 3.0 mM) and primer concentration ratios (outer:inner from 5:1 to 1:5). PCR products were resolved by 2.0% agarose gel electrophoresis, with optimal reaction conditions selected based on product specificity and amplification efficiency.

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2.4.2 Genotyping System for the 12 bp Polymorphic Locus

Based on optimization results, the Tetra-ARMS PCR system for the 12 bp polymorphic locus comprised a 20 μL reaction volume, which contained 2.0 μL 10× PCR buffer, 1.6 μL dNTP mixture (25 mM), 2.0 μL $MgCl_2$ (25 mM), 0.5 μL upstream outer primer 12-PF3 (10 μM), 0.8 μL upstream inner primer 12-IF2 (10 μM), 0.4 μL downstream inner primer 12-Rin (10 μM), 0.8 μL downstream outer primer 12-LR1 (10 μM), 0.4 μL Taq DNA polymerase (5 U/μL; Thermo Fisher Scientific), 3.0 μL template DNA, and 8.5 μL ddH_2O. Thermal cycling was as follows: initial denaturation at 95°C for 5 min; followed by 35 cycles at 94°C for 35 s, 69°C for 40 s, and 72°C for 1 min; the extension at 72°C for 10 min, and finally the PCR products were maintained at 4°C in the end.

2.4.3 Genotyping System for the 23 bp Polymorphic Locus

Based on optimization results, the Tetra-ARMS PCR system for the 23 bp polymorphic locus comprised a 20 μL reaction volume, which contained 2.0 μL 10× PCR buffer, 1.6 μL dNTP mixture (25 mM), 2.0 μL $MgCl_2$ (25 mM), 0.4 μL upstream outer primer 23-PF1 (10 μM), 0.4 μL upstream inner primer 23-IFA4 (10 μM), 0.3 μL downstream inner primer 23-AR2 (10 μM), 0.8 μL downstream outer primer 23-PR3 (10 μM), 0.4 μL Taq DNA polymerase (5 U/μL; Thermo Fisher Scientific), 3.0 μL template DNA, and 9.1 μL ddH_2O. Thermal cycling was identical to the 12 bp system.

2.4.4 Single-Tube Multiplex ARMS-PCR System

A single-tube multiplex PCR system was preliminarily established to explore the feasibility of simultaneous detection. This system combines the primer sets from the two independently optimized single-locus assays under their consistent PCR conditions. The final 20 μL reaction mixture contained:2.0 μL 10× PCR buffer, 1.6 μL dNTP mixture (25 mM), 2.0 μL of $MgCl_2$ (25 mM), 0.4 μL upstream outer primer 12-PF3 (10 μM), 2.2 μL upstream inner primer 12-IF2 (10 μM), 0.4 μL downstream inner primer 12-Rin (10 μM), 1.0 μL downstream outer primer 12-LR1 (10 μM), 0.4 μL upstream outer primer 23-PF1 (10 μM), 0.8 μL upstream inner primer 23-IFA4 (10 μM), 0.8 μL downstream inner primer 23-AR2 (10 μM), 0.8 μL downstream outer primer 23-PR3 (10 μM), 0.4 μL Taq DNA polymerase (5 U/μL; Thermo Fisher Scientific), 3.0 μL template DNA, and 4.2 μL ddH_2O. Thermal cycling was identical to the 12 bp system.

2.5 Limit of Detection (LOD) Assessment for Tetra-ARMS PCR

To further explore the detection limit of ARMS-PCR, the optimized detection system was used to assess it for different genotypes using continuous gradient dilution of DNA. Genomic DNA samples from individuals with three genotypes (Ins, Del, Hetero) for both the 12 bp and 23 bp loci were selected (two samples per genotype) as the template. DNA samples of identical genotypes were mixed and subjected to 10-fold serial dilution for detection limit assay.

2.6 Genotyping of Commercial Cattle-Derived Products

The *PRNP* genotypes of 62 commercial cattle-derived products (raw milk, milk powder, beef and cheese) were determined using an optimized single-locus Tetra-ARMS PCR system for both the 12 bp and 23 bp polymorphic sites. Concurrently, three samples per genotype were randomly selected for bidirectional sequencing verification according to the method in Section 2.2.

Based on ARMS-PCR electrophoretic profiles, allele and genotype frequencies for both loci in the bovine *PRNP* gene were calculated.

3 Results and Analysis

3.1 DNA Extraction and Quality

The detection for concentration and OD value of 62 extracted DNA samples revealed that the DNA concentration for meat samples was 46.2 ± 9.55 ng/μL, $OD_{260}/_{280}$ ratio was 1.93 ± 0.07; for raw milk samples was 2.69 ± 1.09 ng/μL and $OD_{260}/_{280}$ was 1.66 ± 0.21; for milk powder samples was 6.93 ± 2.65 ng/μL and $OD_{260}/_{280}$ was 1.74 ± 0.23. PCR products showed that all the DNA samples could amplify the specific *PRNP* gene containing the 12 bp and 23 bp polymorphic sites, demonstrating that the DNA concentrations and purity meet the PCR analysis requirements.

3.2 Sequencing-Based Genotyping for the two *PRNP* Polymorphic loci

Electrophoretic analysis of *PRNP* amplicons indicated the obtained fragment sizes was 119/131 bp for 12 bp locus and 245/268 bp for 23 bp locus and showed in Figure 2, consistent with expected sizes. However, the close proximity of fragment sizes impeded definitive genotyping by electrophoresis. Sequence alignment using EditSeq and MegAlign software confirmed the presence of three genotypes per locus,

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including homozygous deletion (Del genotypes), homozygous insertion (Ins genotypes), and insertion-deletion heterozygote (Hetero genotypes), as shown in Figure 3.

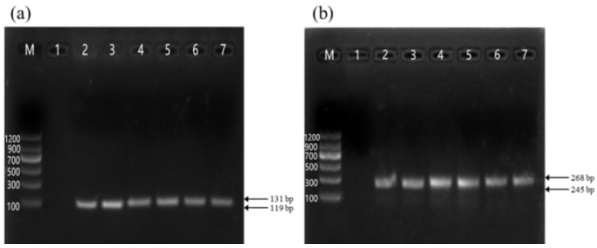

Fig. 2 Electrophoretic analysis of *PRNP* gene 12 bp and 23 bp polymorphic loci. Lane M represents DNA Marker C (100–1200 bp). (a) PCR products amplified with 12 indel FR primers; (b) PCR products amplified with 23 indel FR primers. Lane 1 represents negative control (NTC), Lanes 2–3 represent Del genotypes (–/–), Lanes 4–5 represent Hetero genotypes (+/–), Lanes 6–7 represent Ins genotypes (+/+)

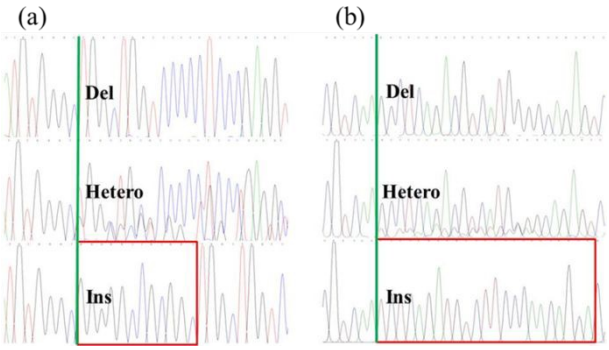

Fig. 3 Sanger Sequencing Chromatograms of *PRNP* gene 12 bp and 23 bp polymorphisms. (a) 12 bp indel polymorphism; (b) 23 bp indel polymorphism. Del genotypes (–/–), Hetero genotypes (+/–), Ins genotypes (+/+).

3.3 ARMS-PCR Genotyping Systems

3.3.1 Single-Locus Detection Systems

Using the known *PRNP* genotype individuals (Ins, Del, Hetero) as standard referenced DNA templates for both loci, tetra-primer ARMS-PCR amplifications were carried out and the results showed distinct electrophoretic profiles in Figure 4. For the 12 bp polymorphic locus, all the DNA samples from three genotypes produced positive control products, the individuals with Del genotypes obtained a control product of 598 bp, the individuals with Ins genotypes or Hetero genotypes obtained a control product of 610 bp. Correspondingly, the individuals with Ins genotypes obtained products of a control 610 bp fragment, and a specific 472 bp fragment. The individuals with Del genotypes obtained products of a control 598 bp and a specific 153 bp. The individuals with Hetero genotypes obtained products of a control 610 bp fragment, and also 472 bp and 153 bp specific fragments. For the 23 bp polymorphic locus, the positive control products were 422 bp and 445 bp, corresponding with Del genotypes and Ins genotypes or Hetero genotypes, respectively. Furthermore, the individuals with Ins genotypes obtained 445 bp and 193 bp fragments. The individuals with Hetero genotypes obtained 445 bp, 259 bp and 193 bp fragments. The individuals with Del genotypes only obtained 422 bp (control) and 259 bp PCR products. These fragment patterns clearly distinguished all genotypes at both loci, demonstrating the high efficiency and accuracy of the ARMS-PCR system.

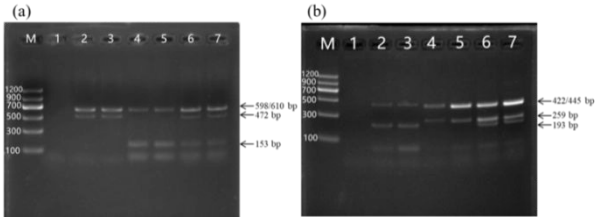

Fig. 4 ARMS-PCR Electrophoresis results for 12 bp (a) and 23 bp (b) Polymorphisms.Lane M represents DNA Marker C (100–1200 bp); Lane 1represents negative control (NTC); Lanes 2–3 represent Ins genotypes (+/+); Lanes 4–5 represent Del genotypes (–/–); Lanes 6–7 represent Hetero genotypes (+/–).

3.3.2 Preliminary Exploration of a Multiplex Detection System

Based on the optimized conditions for each single-locus assay, a preliminary multiplex PCR system containing both primer sets was configured to explore the feasibility of simultaneous detecting system. Following the conditions, this system simultaneously detected both 12 bp and 23 bp indel polymorphisms in the bovine *PRNP* gene. All genotypes were successfully verified using the DNA samples. The reactions yielded the expected positive control bands (598/610 bp for the 12 bp locus ; 422/445 bp for the 23 bp locus) and genotype-specific fragments. No non-specific amplification was observed. Electrophoretic results allowed clear genotype interpretation in Figure 5, with PCR product combinations detailed in Table 3. It should be noted that this multiplex system represents a preliminary proof of concept, established to validate its fundamental feasibility. Its stability, general applicability, and performance with larger sample sets require further systematic optimization and validation.

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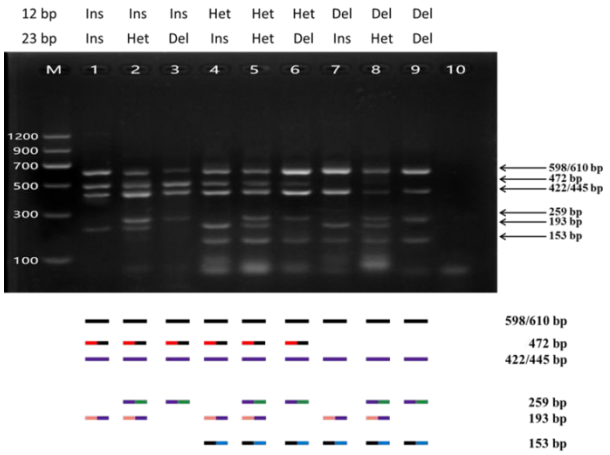

Fig. 5 Multiplex ARMS-PCR for simultaneous genotyping of 12 bp and 23 bp indel polymorphisms. Lane M represents DNA Marker C (100–1200 bp); Lanes 1–9 represent Genotyped samples (annotated above figure); Lane 10 represents negative control (NTC).

Table 3 Fragment Profile Matrix for Multiplex ARMS-PCR Genotyping

12 bp genotype	23 bp genotype	Fragment length (bp)	Total Bands
+ +	+ +	610、472、445、193	4
+ +	+ -	610、472、445、259、193	5
+ +	- -	610、472、422、259	4
+ -	+ +	610、472、445、193、153	5
+ -	+ -	610、472、445、259、193、153	6
+ -	- -	610、472、422、259、153	5
- -	+ +	598、445、193、153	4
- -	+ -	598、445、259、193、153	5
- -	- -	598、422、259、153	4

Symbols '+' and '-' represent insertion and deletion at the gene locus, respectively.

3.4 Limit of Detection for ARMS-PCR

Using the verified DNA templates of three genotypes at the 12 bp and 23 bp polymorphic loci as the test samples, the detection limits of the individual single-locus tetra-ARMS PCR assays were assessed using 10-fold serial dilutions. Following PCR amplification with the two separate tetra-primer systems, agarose gel electrophoresis results in Figure 6 revealed that locus-specific LOD values were as follows: For the 12 bp locus, the minimum detectable concentration of genomic DNA template with Del genotypes was 4.53 ng/μL (0.6795 ng/μL in PCR system), for Hetero genotypes was 5.39 ng/μL (0.8085 ng/μL in PCR system), for Ins genotypes was 5.01 ng/μL (0.7515 ng/μL in PCR system). For the 23 bp locus: the minimum concentration of DNA template with Del genotypes was 4.14 ng/μL (0.621 ng/μL in PCR system), for Hetero genotypes was 4.15 ng/μL (0.6225 ng/μL in PCR system), for Ins genotypes was 0.516 ng/μL (0.0774 ng/μL in PCR system). The overall detection limit ranged from 0.516 to 5.39 ng/μL (0.0774-0.8085 ng/μL in PCR system). To contextualize the relative sensitivity level of the developed assay, its detection limits were compared with those reported for other applications of the Tetra-ARMS PCR technique in the literature, as summarized in Table 4. The 23 bp Ins genotypes showed 10-fold higher sensitivity, potentially attributable to the lower GC content in insertion-specific primers enhancing amplification efficiency.

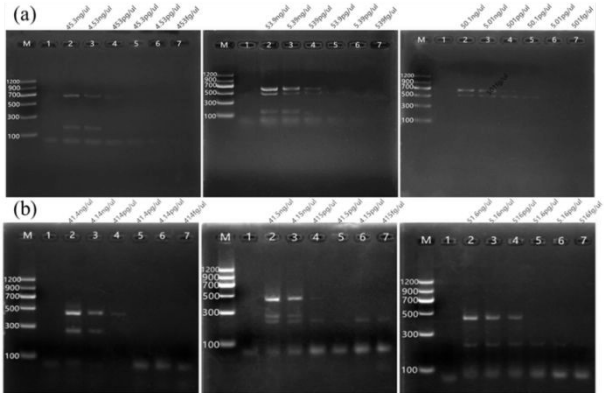

Fig. 6 LOD Analysis of Tetra-ARMS PCR. (a) 12 bp locus (left to right: Del genotypes–/–, Hetero genotypes +/–, Ins genotypes +/+); (b) 23 bp locus (left to right: Del genotypes–/–, Hetero genotypes+/–, Ins genotypes+/+). Lane M represents DNA Marker C (100–1200 bp); Lane 1represents No-template control; Lanes 2–6 represent 10 fold serial dilutions (10^0 to 10^{-4}).

Table 4 Comparison of the limit of detection (LOD) of Tetra-ARMS PCR methods across different studies

Method	Target Locus	LOD	Reference
Tetra-ARMS PCR	Bovine PRNP gene 12/23 bp indel	0.516-5.39 ng/μL	This work
Multi ARMS-PCR	Apolipoprotein E genotyping	10ng	Lian et al., 2016[51]
Tetra-ARMS PCR	SARS-CoV-2	78.91 copies/μL	Wang et al., 2022,[52]
TaqMan ARMS-PCR	mitochondrial DNA (mtDNA) 1555A>G	0.1 ng/μL	Tan et al., 2024[53]

3.5 Commercial product Genotyping

The two aforementioned primer sets were used to amplify the 62 commercially available bovine-derived products. The results

for the 12 bp locus were 18 Del, 13 Ins, and 31 Hetero; for the 23 bp locus, they were 12 Del, 9 Ins, and 41 Hetero.. Among these, the identification results of 24 samples are shown in Figure 7. The electrophoresis detection results were completely consistent with DNA sequencing, demonstrating that this study provides an accurate and intuitive detection method for genotyping the bovine *PRNP* gene. Based on electrophoretic genotyping results, allele and genotype frequencies were calculated for the 12 bp and 23 bp loci in the bovine *PRNP* gene. For the 12 bp locus, 21.9% of samples belonged to Ins genotype, 49.2% belonged to Hetero genotype and 28.9% belonged to Del genotype. For the 23 bp locus, 20.9% belonged to Ins genotype, 51.2% belonged to Hetero genotype, and 27.9% belonged to Del genotype. Polymorphism differences between loci are detailed in Table 4.

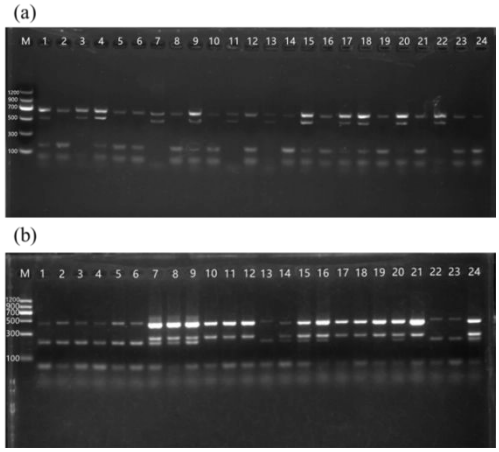

Fig. 7 Genotyping Results of Commercial Samples. (a) 12 bp indel polymorphism. Lane M represents DNA Marker C (100–1200 bp); Lanes 2,5,6,8,10,12,14,16,19,21,23,24 represent Del genotypes (–/–); Lanes 1,4,9,15,17,18 represent Hetero genotypes (+/–); Lanes 3,7,11,13,20,22 represent Ins genotypes (+/+). (b) 23-bp indel polymorphism. Lanes 10,11,12,17,18,19,21 represent Del genotypes (–/–); Lanes 7,8,9,14,15,16,20,24 represent Hetero genotypes (+/–); Lanes 1,2,3,4,5,6,13,22,23 represent Ins genotypes (+/+).

Table 5 Allele and genotype frequencies of insertion/deletion polymorphisms in the bovine *PRNP* gene

Locus	n	Allele frequency		Genotype frequency		
		Insertion Allele	Deletion Allele	+/+	+/-	-/-
12 bp+/-	62	0.460	0.540	0.219	0.492	0.289
23 bp+/-		0.465	0.535	0.209	0.512	0.279

4 Discussions

Since the completion of bovine *PRNP* gene sequencing in 2001, extensive research has characterized its genetic polymorphisms[23]. Among 60 polymorphic sites identified in the gene sequence, the 23 bp indel polymorphism in the promoter region showed significant association with BSE susceptibility, with deletion homozygotes and heterozygotes exhibiting higher disease risk[2]. Subsequent studies revealed the 12 bp indel polymorphism in the intron region similarly correlates with BSE resistance/susceptibility. Individuals carrying both 23 bp and 12 bp insertions demonstrate the strongest resistance to BSE, while double-deletion homozygotes show maximal susceptibility[54]. These polymorphic sites regulate prion protein (PrP) expression levels and conformational stability, directly impacting pathological accumulation and transmission efficiency of PrP^{Sc}[6, 21, 55, 56]. Molecular analyses indicated that 12 bp deletion abolishes transcription factor SP1 binding sites, reducing transcriptional efficiency, while 23 bp deletion interferes with 58 kDa repressor protein (RP58) binding, altering chromatin conformation. Both mechanisms converge on *PRNP* gene regulatory networks, ultimately affecting prion protein metabolic homeostasis[57-59]. Additionally, the *PRNP* gene contains other polymorphic sites such as E211K that influence prion protein structure[60, 61]. Nevertheless, the 12 bp and 23 bp polymorphic sites remained central to disease-resistant breeding applications and BSE safety assessments in cattle[28, 62-64].

The critical impact and significance of *PRNP* gene polymorphisms in animal breeding has driven extensive development of detection methodologies, including Sanger sequencing[22, 29-34], PCR-RFLP analysis[35], quantitative PCR (qPCR)[36], next-generation sequencing (NGS)[37], and microarray genotyping[38]. However, these platforms and developed methods are constrained by expensive instrumentation, high reagent costs, operational complexity, and prolonged processing times. In contrast, ARMS-PCR provides a simple, cost-effective solution for detecting of single nucleotide polymorphisms (SNPs) and small indels. Currently, this

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technology is used in medical diagnostics for precision genotyping of breast cancer susceptibility alleles[65], genetic risk assessment for mosquito-borne diseases[66], and polygenic analysis of colorectal cancer[67]. The applications in agricultural field include screening for bovine complex vertebral malformation (CVM) susceptibility[68] and authentication of Thai bird's nest products[69]. Within livestock genetics, rapid genotyping of leptin receptor mutations in rats[70] and ASB-3 gene polymorphisms affecting bovine growth traits[71]. Extensive applications demonstrated that optimized ARMS-PCR with refined primer design and reaction conditions delivered visual electrophoretic results within 2-3 hours. With minimal costs and scalability for large-scale screening, this method provides an efficient and economical solution for genetic analysis.

This study established two streamlined, independent genotyping assays for bovine *PRNP* 12 bp and 23 bp indel polymorphisms based on Tetra-ARMS PCR technology. The developed single-locus assays can efficiently identify *PRNP* 12 bp and 23 bp indel genotypes of cattle and cattle-derived products with simplicity, low cost, and high efficiency. ARMS-PCR amplifies target sequences only with perfect 3'-end complementarity to template DNA, blocking amplification otherwise. Incorporating outer primers as positive controls creates a tetra-primer system[39, 45]. This design enhances accuracy by reducing false negatives and ambiguous results in practical applications.

This study designed an optimized Tetra-ARMS PCR system targeting for 12 bp and 23 bp indel polymorphisms in the bovine *PRNP* gene, which provided a better method for *PRNP* genotyping technology. It is worth mentioning that, the Tetra ARMS PCR primers designed in this study are different from other SNP genotyping primers, all the primers were designed only for inserting genotype sequences with multiple bases complementary to the insertion sequence at the 3' end of the inner primer which had higher accuracy and sensitivity than other common Tetra ARMS PCR single base mismatches. Critically, inner primer sequences fully featured extended 3′-terminal complementarity to insertion sequences, specifically, 5 nucleotides in 12-IF2 and 22 nucleotides in 23-IFA4 were complete pairing the 12 bp and 23 bp insertion sequences, which ensure the specificity of PCR amplification. This design achieved detection limits as low as 0.516 ng/μL genomic DNA, is comparable to that of other established Tetra-ARMS PCR applications, includes the assay reported for apolipoprotein E genotyping[51]. Moreover, the specificity was enhanced through introduced a T-G mismatch at position 5 (from 5′-terminus) in primer 23-IFA4 and position 3 (from 3′-terminus) in primer 23-AR2. These strategically positioned weak suppress non-specific amplification while improving allelic discrimination[39, 72], aligning with established principles for enhancing ARMS-PCR accuracy.

The concentration ratio of primers in the Tetra ARMS PCR reaction system is also a key factor affecting specificity and sensitivity. Due to the presence of multiple mismatch sites in the inner specific primer, the amplification efficiency of the outer primers is higher than that of the inner primer, as they are completely matched. Therefore, adjusting primer concentration ratios were adopted to counterbalance amplification efficiency disparities between fully matched outer primers and mismatch-containing inner primers[73]. Specifically, by increasing inner primer concentrations, this study resolved amplification imbalances.

Furthermore, the annealing temperature is another critical factor influencing PCR amplification, and its effect in the Tetra-ARMS PCR system indicates that as the annealing temperature gradually increases, the specificity of the two sets of primers will also be correspondingly enhanced. This study has demonstrated this phenomenon that annealing temperature optimization addressed the problem of low PCR efficiency due to high GC content (73%) in the 12 bp insertion sequence (GGGGGCCGCGGC). At 69°C, exceeding conventional PCR Tm values, the specific target amplification was achieved while eliminating non-specific products.

The principal value of the assay developed herein is its potential to significantly reduce the economic and temporal barriers associated with screening for these important PRNP indels. Compared to the gold-standard Sanger sequencing, which is accurate but costly and slower for batch processing,

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our method requires only a conventional PCR thermocycler and standard gel electrophoresis apparatus, instruments accessible in most molecular biology laboratories. Reagent costs are minimal, and the workflow from DNA to genotype can be completed within approximately 3 hours, enabling high-throughput analysis. This makes it a viable and attractive option for large-scale screening within breeding programs aimed at increasing BSE resistance in cattle populations. By facilitating the cost-effective identification of resistant (Ins/Ins) individuals, the method directly supports DNA marker-assisted selection. Additionally, its successful application to commercial products (milk, beef, cheese) demonstrates utility in the genetic traceability and risk assessment of animal-derived foods, contributing to safety monitoring in the food chain.

Nevertheless, this study has certain limitations. First, the developed multiplex detection system requires further systematic optimization to improve its stability and reproducibility across different laboratory conditions. Second, although validated using commercial processed products (e.g., milk powder, cheese), the performance of the method on highly degraded or low-quality DNA[74], or with extreme sample matrices containing complex inhibitory substances[75], remains to be specifically evaluated in future work. Finally, as a technique based on end-specific amplification, its accuracy depends on precise primer design and stringent PCR condition control, which requires careful attention to experimental technique.

Additionally, the ARMS-PCR framework demonstrates potential prospects for integration with advanced platforms. Combination it with lateral flow chromatography enabled SARS-CoV-2 detection limit to 1.90 copies/μL[61], coupled with digital PCR achieved detection limit to 0.308 copies/μL for epidermal growth factor receptor mutations[76]. Such integrations highlight its promise for future ultra-sensitive diagnostics.

In summary, the Tetra-ARMS PCR method established in this study offers significant advantages of low cost and high efficiency, over existing techniques for analysing 12 bp and 23 bp indel polymorphisms in bovine *PRNP* gene. It provides a viable option for large-scale screening of disease-resistant animal individuals. Furthermore, disease resistance breeding represents an important safeguard for animal husbandry safety and public health, holding significant scientific value and societal value.

5 Conclusions

This study successfully developed and validated two independent tetra-ARMS PCR assays for the efficient genotyping of 12 bp and 23 bp indel polymorphisms in the bovine *PRNP* gene. Furthermore, a preliminary multiplex configuration was demonstrated, outlining a clear pathway for future development toward a single-tube, high-throughput screening tool. The established single-locus methods offer a cost-effective, high-throughput screening, addressing limitations of existing methods. This approach significantly advances disease-resistant cattle DNA marker assisted breeding and safety assessment of animal-derived food products.

Author contributions

Ye Xu: conceptualization, methodology, investigation, writing – original draft. Siyu Yang: conceptualization, methodology. Siling Ding and Ting Xu: investigation. Jian Ge: investigation, formal analysis. Weiming Xiao: resources. Like Zhu: funding acquisition, project administration. Feng Guan: supervision, writing – review & editing.

Conflicts of interest

There are no conflicts to declare.

Data availability

The raw sequence data reported in this paper have been deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2025) in National Ge-nomics Data Centre (Nucleic Acids Res 2025), China National Centre for Bioinformation / Beijing Institute of Genomics, Chinese Academy of Sciences (GSA: accession number CRA030580) that are publicly accessible at https://ngdc.cncb.ac.cn/gsa. All data generated or analysed in this study are available.

Acknowledgements

Not applicable.

Ethical approval

The blood samples used for genomic DNA isolation were originally collected as part of routine herd health monitoring programs by the collaborating farms and research institutes. All animal-related procedures in this study were conducted in strict compliance with the national regulations and guidelines of the People's Republic of China, including the "Regulations for the Administration of Affairs Concerning Experimental Animals" and the "Guideline on the Humane Treatment of Laboratory Animals". The protocol for the retrospective genetic analysis of these pre-existing DNA samples was reviewed and formally approved by the Animal Experiment Ethics Committee of China Jiliang University (Approval No. 2021-005). This research did not involve human participants.

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