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Research Article

PRELIMINARY STUDIES ON FTA CARD-BASED DNA EXTRACTION USING NOVEL BUFFERS FOR A1/A2 GENOTYPING FROM DRIED BLOOD AND MILK SPOTS

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ABSTRACT
Genetic research and cattle development are supported by the effective extraction of high-quality genomic DNA, and the present study demonstrates dried milk spots (DMS) and dried blood spots (DBS) on filter paper as a straightforward but effective field-based substitute for traditional liquid samples. This investigation assessed the capacity of two novel purification buffers coated on Whatman No. 3 filter paper to separate DNA from milk and blood samples from Nattukuttai cattle. For PCR applications, both buffers produced DNA with sufficient concentration and purity (27–161ng/µl for DBS; 17–130ng/µl for DMS), while Buffer II performed marginally better. After β-casein exon 7 PCR amplification verified DNA integrity, restriction digestion was used to distinguish between A1/A2 variants. Genotyping revealed a predominance of the A2 allele, with 90% of cattle exhibiting the A2A2 genotype, which is consistent with the fact that indigenous breeds in India tend to carry the A2 allele rather than the more frequent A1 form found in taurine cattle. These findings show that buffer-treated filter paper provides an economically feasible, transportable, and trustworthy way to retain DNA and perform molecular analysis in non-refrigerated settings. This technology has practical uses in breeding programs, veterinary diagnostics, and sustainable dairy production.

Keywords: *Dried blood cards, FTA technology, DNA extraction, FTA Buffers, β-casein Genotyping, A1/A2 genotyping, Nattukuttai cattle*

INTRODUCTION
Genetic research has focused on identifying various types of genetic variations like Single Nucleotide Polymorphism analysis and the investigation of disease connection, for such research high-quality genomic DNA must be extracted from blood (*Queiros et al.,* 2021). Fresh blood samples require special handling like refrigeration increasing the cost and also logically difficult for large scale studies. Dried blood spots (DBS) are a practical alternative to liquid blood, allowing for easy, cost-effective transportation and preservation without the need for refrigeration, especially when collected on chemically processed filter papers like Whatman or FTA cards (*Denis et al.,* 2020). These cards disrupt cells and stabilize nucleic acids when they come into contact, rendering them perfect for large-scale and field-based genetic research. Despite the advantages, traditional FTA purification can be lengthy and time-consuming (*Goldberg et al.,* 2022). As a result, Novel buffers that make DNA extraction easier, reduce contamination and enhance PCR results (*Morin et al., 2023*), especially for veterinary applications have been developed (*Meyer et al.,* 2023). Qualitative filter papers, abundant in alpha cellulose, enhance DNA binding effectiveness (*Singh et al.,* 2023).

In the present study, the efficacy of the formulated novel buffers on dried cattle blood spots (DBS) and dried cattle milk spots (DMS) was evaluated by performing A1/A2 genotyping using well known primers

by Polymerase Chain Reaction (PCR), to confirm the quality of the extracted DNA. The role of A1 beta casein as an undesirable variant has led to the need for genotyping cows for A1/A2 allele of beta casein making selections based on their polymorphism. Given the significance of indigenous cattle to the local population, the goal of the current study apart from checking the efficacy of the novel buffers was also to identify the frequency of beta casein variants in the A1/A2 allele of this significant breed. This discovery advances our knowledge of genetic variance in native cow breeds and may have ramifications for breeding and dairy production initiatives.

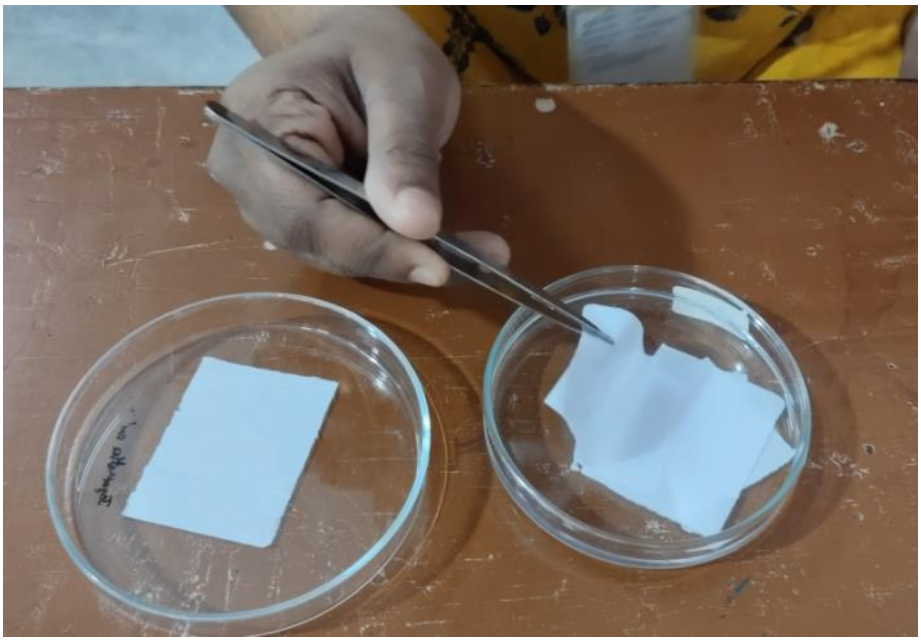

Figure 1: Whatmann-3 filter paper treated with DNA Binding Buffers I & II.

The A1 and A2 variants differ at the 67th amino acid position of the beta casein gene: A2 (CCT, proline) and A1 (CAT, histidine). This polymorphism results in significant conformational alterations in the secondary structure of expressed β-casein protein (*Eliott* 1999*, McLachlan* 2001), leading to the production of various bioactive peptides when specific variants are digested. Gastrointestinal proteolytic breakdown of A1 β-casein results in the release of a 7 amino acid bioactive peptide known as beta-casomorphins-7 (BCM 7) in the small intestine, whereas proline at the 67 position in A2 milk obstructs this cleavage, leading to the formation of a 9 amino acid peptide called beta-casomorphins-9 (BCM9) (*Hartwig et al.,* 1997*, Elliot et al.,* 1999). BCM7 attaches to the mu-opioid receptors found in the central nervous system and gastrointestinal tract, disrupting various pathways. BCM-7 is regarded as a risk factor for type I diabetes (DM-I), coronary heart disease (CHD), artherosclerosis, and sudden infant death syndrome (*Langersen and Elliot* 2003*, Truswell* 2005*; Kamiński et al.,* 2006*; Caroli et al.,* 2009*; Pal et al.,* 2015*; Sodhi et al.,* 2012).Genetic examination focused on A1/A2 β-casein variants in indigenous cattle revealed a dominance of the A2 variant, associated with health advantages by inhibiting the release of the bioactive peptide BCM-7. Unlike the taurine cattle that are A1-dominant (*EFSA,* 2009*; Kaminski et al.,* 2007), native Indian breeds are more likely to have A2 variations (*Mishra et al.,* 2009). These findings validate the use of A2 genotyping and Whatman cards in targeted breeding and on-site diagnostics to produce safer and healthier milk for human consumption (*Verma et al.,* 2022).

MATERIALS AND METHODS

SAMPLE COLLECTION AND TREATMENT

Blood samples (5 ml each) were obtained from 10 healthy Nattukuttai cattle via jugular vein puncture using 14G needles and stored in sodium heparin vacutainers at 4°C. Milk samples (15ml) were collected from 10 adult cows (2-3 years old) and stored at -20°C until processing. Whatman filter paper 3 (WHA1003185) 2x2 inches square has been treated with Novel buffers and air dried for 24 hours at room temperature. The composition of the buffer was as follows; Buffer I containing 20 mM Tris-HCl (pH 8.0), 50 mM EDTA, 30 mM NaCl, and 2% SDS; and Buffer II containing 50 mM EDTA, 30 mM NaCl, and 20 mM $MgCl_2$. Both buffers were adjusted to pH 7.4-7.6 and stored at 4°C. Milk and Blood samples (250 µl) was added to novel buffer -treated cards and air-dried. DBS and DMS stored in separate sterile zip lock covers until further analysis.

DNA EXTRACTION AND QUALITY ASSESMENT

DNA was extracted from DBS and DMS by punching 4 mm diameter circles into 1.5ml centrifuge tubes. 1ml of 20 mM Sodium hydroxide was added and centrifuged at 10,000 rpm for 3 minutes, the supernatant was discarded. The procedure was repeated thrice, followed by incubating the discs with100µl of Nuclease-free water at 70°C for 10 minutes. Centrifuging at 10,000 rpm for 10 minutes, the supernatant was collected and stored at -20°C. For reference, a standard kit extraction was carried out using a commercial DNA extraction kit according to the manufacturer's instructions. The concentration and purity of DNA were evaluated usingUV-Visspectrophotometry (Denov DS–11FX+) at wavelengths of 260/280nm with a NanoDrop One spectrophotometer (Thermo Fisher Scientific, United States).

PCR AMPLIFICATION AND STATISTICAL ANALYSIS

Detection of A1/A2 β-casein variants was carried out using allele-specific PCR with forward primers (A1: 5'-CTTCCCTGGGCCCATCCA-3'; A2: 5'-CTTCCCTGGGCCCATCCC-3') and a shared reverse primer (5'-AGACTGGAGCAGAGGCAGAG-3'). PCR reactions (total volume of 10µl) included 5µl of master mix, 2µl of template DNA, 1µl of each primer, and 1µl of nuclease-free water. A single amino acid alteration, from CCT (proline) to CAT (histidine) at the 67th position of the 209 amino acid chain, distinguishes the A1and A2 beta casein isoforms. One of the forward primers picks histidine because it contains CCT in its primer sequence. Proline is picked up by the CAT in the primer sequence of the other forward primer. The forward primers can distinguish between A1 and A2 in this manner.

Thermal cycling was performed in C1000 Touch system (Bio-Rad) comprised an initial denaturation step at 94°C for 5 minutes, followed by 5 cycles at 94°C/64°C/72°C (30 seconds each), then 30 cycles at 94°C/62°C/72°C (30 seconds each), and concluded with a final extension at 72°C for 5 minutes. PCR products (244bp) were examined on 2.5% agarose gel stained with ethidium bromide using ChemiDoc MP imaging system (Bio-Rad). Statistical analysis was conducted employing one-way ANOVA with significance set at $p<0.05$ utilizing MS Excel.

RESULTS AND DISCUSSION

DNA EXTRACTION EFFICIENCY AND BUFFER PERFORMANCE

The current study effectively showed the viability of using newly developed in-house binding buffers (Buffer I and Buffer II) with Whatman 3 filter cards for extracting DNA from dried blood spots (DBS) and dried milk spots (DMS) from indigenous Nattukuttai cattle. These indigenous cattle, predominantly found in the north-eastern agro-climatic zone of Tamil Nadu, including districts of Kancheepuram, Villupuram, and Tiruvallur, play a significant role in the regional economy and are characterized by their compact, robust physique with brown or grey coloration *(Vinothkumar,* 2014).

Buffer I produced DNA concentrations between 27.3 and 161.5 ng/µl from blood samples, with a mean concentration of 93.44 ng/µl. Buffer II exhibited similar effectiveness with concentrations between 27.3 and 129.4ng/µl (mean: 71.79ng/µl). The inclusion of $MgCl_2$ enhances DNA binding efficiency by acting as a cofactor for DNA-processing enzymes like polymerases, ligases, and restriction enzymes, ensuring proper catalytic activity and neutralizing DNA's negative charge to stabilize interactions with proteins and primers (Sambrook& Russell, 2001). Statistical analysis revealed no noteworthy difference between the two buffers ($p > 0.05$), highlighting equivalent DNA binding efficiency.

DNA QUALITY ASSESMENT AND BUFFER COMPOSITION EFFECTS

DNA purity assessment, determined by A_{260}/A_{280} ratios, showed unique traits across the two buffer systems. Buffer I regularly showed lower purity ratios (average: 1.635 for blood, 0.99 for milk), whereas Buffer II displayed better performance with higher ratios (average: 4.78 for blood, 1.02 for milk). The increased purity seen with Buffer II is due to its more alkaline characteristics from the addition of $MgCl_2$ and NaCl, in contrast to the mildly acidic Buffer I that include EDTA and Tris-HCl. The pH-related changes in A_{260}/A_{280} ratios correspond with the results from *Wilfinger et al.,* (1997), who found that acidic solutions generally underestimate the 260/280 ratio by 0.2–0.3, whereas basic solutions overestimate the ratio by 0.2–0.3. Even though the purity ratios varied from the established pure DNA standard of 1.8-2.0 set by *Wilfinger et al.,* (1997), the extracted DNA was still appropriate for PCR applications. This result aligns with *Mihuaiu et al.,* (2009), who showed that DNA at concentrations of 50 ng/µl and A_{260}/A_{280} ratios ranging from 1 to 2 are suitable for PCR applications. Additionally, a comparable reference for our buffer optimization efforts was provided by Usman *et al*., (2002), who reported employing modified nucleospin blood kit procedures to isolate DNA from milk and achieve standard 260/280 nm absorbance ratios of 1.8.

Table.1: The given table summarizes the DNA concentration of Dried Blood Spots (DBS) and of Dried Milk Spots (DMS) treated with Buffer I and Buffer II.

SAMPLE TYPE	SAMPLE ID	PARAMETER	BUFFER I	BUFFER II
DBS	Nk 1 – Nk 10	DNA Concentration (ng/µl)	93.4 ± 2.0	71.8 ± 2.3
DMS	N 1 - N 10	DNA Purity (260/280)	27.8 ± 3.3	46.7 ± 2.8
DBS	Nk 1 – Nk 10	DNA Concentration (ng/µl)	1.6 ± 0.3	1.7 ± 0.2
DMS	N 1 - N 10	DNA Purity (260/280)	0.99 ± 0.1	1.02 ± 0.1

Table 2: This table summarizes the key descriptive statistics of DNA concentration and Purity for the two groups (Buffer 1 and Buffer 2)

SAMPLE TYPE	BUFFER	CONCENTRATION mean ± SD (ng/µl)	RANGE	Absorbance (260/280) mean ± SD	RANGE
DBS	Buffer I	93.39 ± 28.08	69.5–161.5	1.61 ± 0.93	0.7–3.1
DBS	Buffer II	72.57 ± 30.04	27.3–129.4	2.18 ± 1.26	0.8–4.2
DMS	Buffer I	34.37 ± 14.11	17.4–56.2	0.97 ± 0.25	0.7–1.3
DMS	Buffer II	55.69 ± 31.81	24.6–130.2	1.02 ± 0.14	0.8–1.2

MOLECULAR VALIDATION AND GENOTYPING SUCCESS

Validation using PCR focused on the A1/A2 variants of the β-casein gene confirmed the effectiveness of DNA extracted with both buffer systems. The 251bp amplicon of exon 7 was effectively amplified from DBS and DMS samples, subsequently subjected to TaqI restriction enzyme digestion for genotype differentiation. The restriction analysis showed distinct fragment patterns: 251bp (A2A2 genotype), 213bp and 38bp (A1A1 genotype), and 251bp, 213bp, and 38bp (A1A2 genotype). By proving that the extracted DNA maintained enough integrity and quality for intricate downstream molecular processes like PCR amplification and restriction enzyme digestion, this molecular validation conclusively established the feasibility of the novel buffer-treated filter paper system for genetic analysis applications.

GENETIC ANALYSIS AND BREED EVALUATION

The genotyping examination of Nattukuttai cattle samples identified a major prevalence of the A2A2 genotype, as 90% of blood samples (9/10) and 90% of milk samples (9/10) displayed this advantageous variant. The other samples exhibited an A1A2 heterozygous genotype in blood and an A1A1 homozygous genotype in milk, leading to genotypic ratios of A2A2:A1A2:A1A1 = 9:1:0 and 9:0:1 for blood and milk samples, respectively. From a nutritional and commercial standpoint, the high incidence of the A2A2 genotype is especially noteworthy because A2 β-casein is linked to better digestibility and fewer inflammatory reactions when consumed by humans. These native cattle exhibit better heat tolerance than crossbred cattle and are preferred by small-scale farmers because they are easier to manage and have less tedious costs compared to Jersey crossbreds *(Vivekanandan and Alagumalai, 2013; Vinothkumar, 2014).*

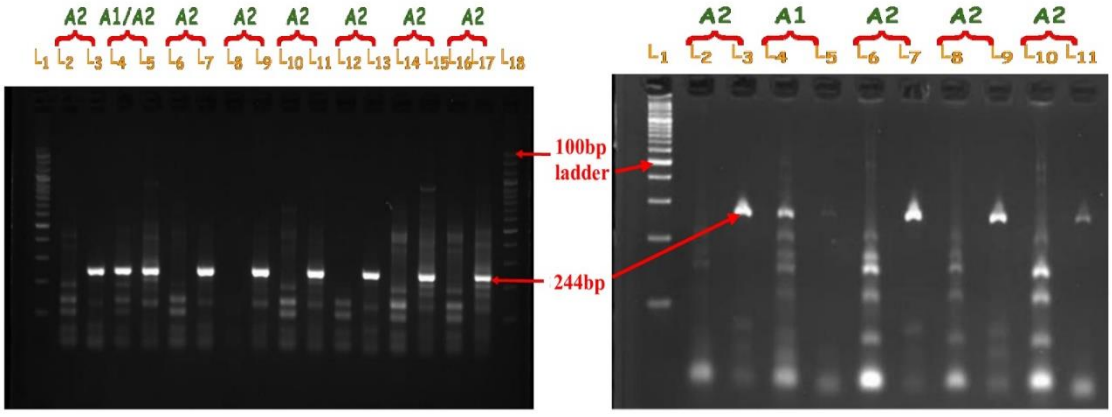

Figure 2: Gel image of the DNA extracted from Dried Blood Spots (DBS) and Dried Milk Spots (DMS)

Table 3. Genotypic Frequency Analysis of β-casein A1/A2 Variants in Blood and Milk Samples

S.NO	GENOTYPE	COUNT	FREQUENCY
BLOOD SAMPLES (DBS)	**A2/A2**	**9**	**0.9***
	A1/A2	1	0.1
	A1/A1	0	0
MILK SAMPLES (DMS)	**A2/A2**	**9**	**0.9***
	A1/A2	0	0
	A1/A1	1	0.1

For Blood samples, the ratio is expressed as A2/A2: A1/A2: A1/A1 = 9:1:0, For Milk samples. the ratio is expressed as A2/A2: A1/A2: A1/A1 = 9:0:1. These results underscore the conservation significance of the native Nattukuttai breed and offer molecular proof of their potential in sustainable dairy production systems, highlighting the necessity of preserving indigenous genetic resources as promoted by Food and agricultural organization (FAO) efforts for livestock biodiversity conservation.

CONCLUSION

This study developed two novel purification buffers for DNA isolation from dried blood spots (DBS) and dried milk spots (DMS). The buffer-treated filter papers, provide a cost-effective, non-refrigerated alternative to manual protocol and matching commercial kit performance. In PCR validation tests, Buffer II demonstrated a marginally higher DNA yield and purity in comparison to Buffer I. Analysis of the A1/A2 β-casein gene in Indigenous cattle verified the preponderance of the A2 allele and the related health advantages. This approach enables field-adaptable genetic screening for sustainable dairy production by offering the first comprehensive comparison to commercial kits. To increase PCR amplification success rates in veterinary molecular diagnostics, more buffer refining is required.

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