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Study of β-Lactoglobulin Milk Protein Variants in Buffalo

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Abstract.- Polymorphism exists in β-LG milk encoding gene in dairy animals which results in different protein isoforms being expressed in the milk. Overall quality of raw milk, total milk protein and fat content, depends on which β-LG isoforms are being expressed. Present study was planned to identify Beta-Lactoglobulin (β –LG) protein isoforms in *Bubalus bubalis* (Riverine buffalo) Nili Ravi breed milk and to explore their association with major milk constituents. β-Lactoglobulin protein variants were typed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Total milk protein and whey contents were estimated using Bradford 1976 method while total milk fat by Gerber method. Three β-LG protein isoforms AA, BB and AB were identified with genotype frequencies; 0.49, 0.10 and 0.40 respectively. Allele A, with higher frequency of 0.69, was found more prevalent in studied animals as compared to B allele. The AA and AB isoforms were associated with high total milk protein contents (4.36%) while AB isoform had high whey protein (1.0%) and total milk fat (6.06%) contents. Overall we report three β-LG protein isoforms (AA, AB and BB) in Nili Ravi buffalo milk which show significant association with major milk constituents.

Key words: Protein isoforms, β-lactoglobulin, dairy animals.

INTRODUCTION

Protein polymorphism is nature's unique mechanism that leads to more than one isoforms of a single protein. Aschaffenburg and Drewry (1955) reported polymorphism in β-lactoglobulin (β-LG) milk protein. The identification of genetic isoforms of β-LG at protein level was based on differential migration patterns of A and B allele specific polypeptide chain.

Due to polymorphism in major milk protein fractions; caseins and whey, different isoforms are expressed in the milk of dairy animals belonging to the same breed (Amigo *et al.*, 2000). Existence of different protein variants has also been found to affect both quality and quantity of raw milk mainly total milk production, protein and fat contents (Daneila *et al*. 2008; Pena *et al*., 2000). β-Lactoglobulin is the major whey protein in cattle and most ruminants but is absent in the milk of rodents and humans (Amigo *et al.*, 2000). The bovine globular protein is 18.4 kDa in molecular weight and 162 amino acid residues long, mainly present in dimeric form under physiological conditions (Kontopidi *et al*., 2004). Studies have shown that frequency of the occurrence of β-LG polymorphic forms differ not only within a particular animal breed but also across a breed (Heidari *et al.*, 2009) with varying milk composition. ß-LG protein isoforms have shown direct association with milk production, processing and dairy products manufacture (Karimi *et al*. 2009). Thus ß-LG protein isoforms based genetic typing of major dairy animals has been adopted in many western countries (Stasio *et al*., 2000; Gouda *et al*., 2011; Karimi *et al*., 2009) to control quality of dairy food industries products.

β-Lactoglobulin protein polymorphism and its association with milk production traits has been extensively established in cattle both at protein and DNA level (Karimi *et al*., 2009). A total of 15 bovine β-LG protein alleles have been identified with variants A and B being the most prevalent. In contrast to extensive work in cattle, data regarding β-LG polymorphism in buffalos is scarce (Meignanalakshmi and Mahalinga, 2009; Gouda, *et al*., 2011). Buffalos are raised in many regions of the world especially Asian countries including Pakistan (Baber *et al.*, 2009). Nili-Ravi buffalo of

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Pakistan is claimed amongst the richest and finest dairy buffalos in the world contributing 67% of the total milk production of country (Bashir *et al*., 2007). For the improvement of milk production traits, conventional breeding strategies like superior breeds selection and their onward cross breeding have been employed in Nili-Ravi. However, genetic typing of animals based on milk protein isoforms and their association with milk composition has not been explored yet. Keeping in view of foregoing, the present study was planned to detect β-LG protein isoforms from Nili Ravi buffalo milk and association milk constituents.

MATERIALS AND METHODS

Nili-Ravi buffalos (n=200) were sampled from livestock research station maintained at National Agriculture Research Center (NARC), Islamabad, and private dairy farms at Gujrat and Peshawar, Pakistan. Animals were selected based on being in mid-lactation phase and with overall good health. The records maintained at selected farms were accessed for data collection. Approximately 50ml early morning fresh milk samples were collected in sterilized bottles and stored at -20^{o}C till further processing and analysis.

For whey protein extraction, 10mL skimmed milk sample was adjusted to pH 4.6 with 1N HCl to precipitate caseins. Whey supernatant was separated by centrifugation at 3500rpm for 10 minutes. Whey isolates were run on 15% SDS-PAGE along with pre-stained molecular weight marker (Thermo Scientific, USA). Purified bovine (AB) β-LG protein (Sigma-Aldrich, USA) was used as standard. SDS-PAGE gels were run in a Mini protein gel system (Bio-Rad, CONSORT E815 Electrophoretic Power Supply), coommassie blue stained and photographed (under white light) using Gel Documentation system. Results were interpreted by counting protein bands in whey samples in comparison with A and B isoforms of bovine β-LG protein standard.

Total milk protein and whey protein contents were quantified by using Coomassie® Brilliant Blue G 250 dye (Bradford, 1976), Casein protein was determined by subtracting whey protein contents from that of total protein contents for each sample. Total fat contents of milk were estimated by Gerber method (Kleyn *et al.,* 2001).Whole milk (10.94 ml) was mixed with Sulphuric acid and Iso-amyl alcohol in Gerber tube and centrifuged to separate fats from proteins. Fat layer, collected in the upper calibrated part of tube, is then measured in percentage.

Statistical analysis

The allele and genotype frequencies were calculated based on Hardy Weinberg equation. Chi-Square (χ^2) test was applied at 5% significance level to determine deviation of selected population from Hardy Weinberg Principle. Association of identified β-LG protein isoforms with total protein, whey protein and fat contents was carried out by ANOVA followed by Least Significant Difference Test (LSD) using SPSS (Special Package for Social Sciences, Chicago, IL) V.16 software.

RESULTS AND DISCUSSION

Our analysis revealed three distinct β-LG protein isoforms; AA, AB and BB in Nili-Ravi milk samples with high abundance of A allele as compared to B. Using SDS-PAGE analysis three distinct β-LG isoforms AA, AB and BB were identified in all Nili-Ravi buffalo milk samples being typed (Fig. 1). As already established in cattle (Ng-Kwai-Hang, 1998), homozygous AA and BB genotypes of β-LG were characterized by one major band on gel while heterozygous AB genotypes produced two overlapping bands (Fig. 1). Of 200 milk samples β-LG genotypes distributions were as follows; 99 homozygous AA, 81 heterozygous AB and 20 homozygous BB. Our results confirm existence of A and B being the major β-LG protein isoforms in Nili-Ravi buffalo with AA genotype the pre-dominant form in our sampled population. The β-LG genotype frequencies were in the order; 0.495 AA, 0.405 AB and 0.10 BB, respectively. While the allele frequency for β-LG A variant was 0.69 as compared to 0.31 for variant B indicating an abundance of β-LG A variant/allele in our sample population of Nili-Ravi buffalo (Table I). While studies on Iranian and native Egyptian buffalos report absence of AA genotypes while β-LG AB, BB genotypes with B allele as predominant β-LG isoforms (Gouda *et al*., 2011; Karimi *et al.,* 2009).

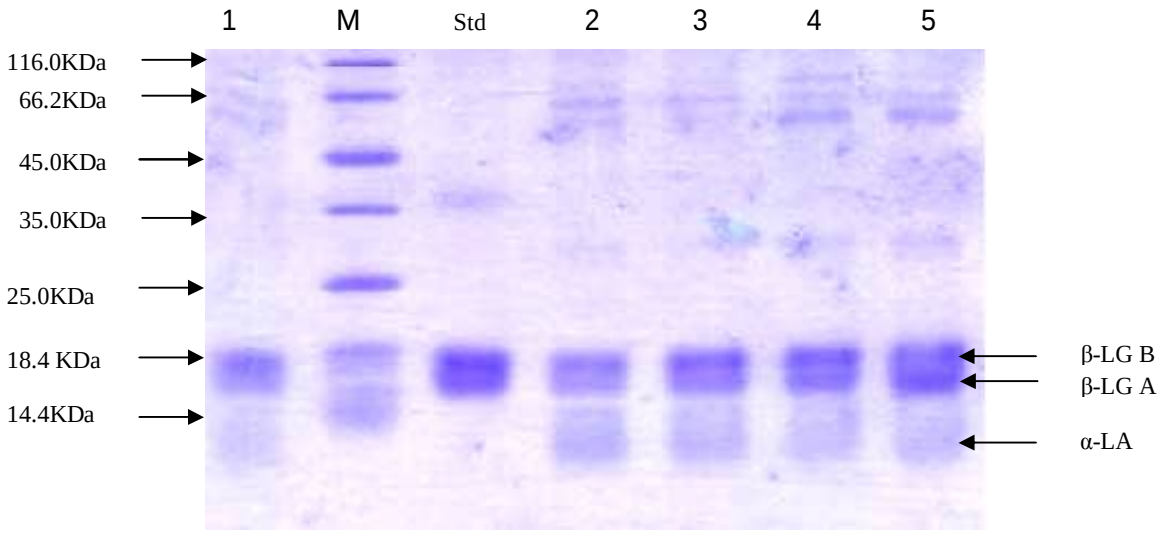

Fig. 1. SDS PAGE analysis of beta lactoglobulin isoforms in gel picture. Std, Standard bovine β-LG AB (18 KDa monomers); M, Molecular weight markers. Lane No. 1, 2, 3, 4, 5 β-LG AB isoforms.

Table I.- Allelic and genotype frequencies of buffalo β-lactoglobulin protein.

Breed	No.	χ^2 test (ρ value)	Allelic frequencies		Genotype frequencies		
			A	B	AA	AB	BB
Nili Ravi	200	0.56^{ns}	0.69	0.31	0.495	0.405	0.10

n.s, not significant.

Native Egyptian and Iranian buffalo breeds are distinct from Nili-Ravi and therefore β-LG BB isoform might have shown predominance in these animals as compared to β-LG AA in Nili-Ravi. Studies conducted on β-LG polymorphism at DNA level have also identified two genetic isoforms (Lama and Zago, 1996; Patel *et al*., 2007). In contrast, polymorphism was reported to be absent in Murrah buffalo at both DNA and protein levels for β-LG by Meignanalakshmi and Mahalinga (2009). So findings of present study in Nili-Ravi would sure add to the already existing data on β-LG milk protein polymorphism. Our study population of Nili Ravi buffaloes also followed Hardy-Weinberg equilibrium which might suggest lack of genetic diversity perhaps due to controlled breeding.

Major milk constituents *i.e.* total protein, whey protein, casein content and total fat of collected milk samples depicted in Table II. The total milk protein contents were significantly high ($P<0.05$) in AA and AB genotype milk samples (4.36% β-LG AA and 4.26% β-LG AB) as compared to BB (3.37% β-LG BB). In contrast, no significant differences were found in whey protein contents of AA and BB genotypes but heterozygous AB genotype was significantly ($P<0.05$) high than homozygous genotypes (Table III). However, casein protein contents of heterozygous AB was significantly lower than ($P<0.05$) homozygous β-LG genotypes. The milk fat contents were found significantly ($P<0.05$) high in heterozygous AB (6.06%) as compared to homozygous BB (5.50 %) and AA (5.87%) forms (Table III). Our findings are in contrast to Egyptian buffalo where high total protein and casein protein contents were found associated with β-LG BB (Gouda *et al*., 2011).

Table II.- Descriptive statistics of important milk constituents (n=200).

Milk constituents (%)	Mean±SD	Range
Total milk protein	4.22±0.93	2.00-6.70
Total milk fat	5.91±1.50	2.50-10.00
Whey protein	0.96±0.20	0.40-1.60
Casein protein	3.26±0.94	1.05-6.00

Table III.- β-lactoglobulin protein isoforms based distribution of milk constituents.

Milk constituents (%)	β-LG genotypes (Means ± SD)			*p*-Value
	AA	AB	BB	
Total milk protein	4.36[a] ±0.80	4.27[a] ±1.00	3.37[b] ±0.83	<0.0001
Total milk fat	5.87[n.s] ±1.49	6.06[n.s] ±1.59	5.52[n.s] ±1.05	0.315
Whey protein	0.93[a] ±0.20	1.00[b] ±0.19	0.93[a] ±0.25	0.037
Casein protein	3.43[a] ±0.84	3.01[b] ±1.00	3.49[a] ±0.93	<0.0001

Means within a column with different superscript differ significantly (P<0.05).
n.s, Non Significant results.

In conclusion, the frequency of allele A is high in sampled Nili-Ravi buffalo population as compared to allele B. Milk samples from animals with AA and AB genotypes have high total protein and casein contents as compared to BB. Therefore, it could be suggested that AA genotype being in abundance and of high protein contents while AB seem good β-LG based markers for improving milk production traits.

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