J.Univ.Ruhuna 2017 5(1-2):14-25
Volume 5 Number 1-2, September 2017
ISSN2345-9387 DOI: http://doi.org/10.4038/jur.v5i1-2.7890

RESEARCH ARTICLE

Milk protein polymorphism of buffaloes in southern Sri Lanka

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Abstract: Water buffaloes contribute around 17% of the total milk production of the country. According to the department of census and statistics of Sri Lanka the buffalo population shows an increment during last decade and it has spread all over the country including southern province of Sri Lanka. However due to the present breed structure of the population a detailed study has to conduct to improve buffalo production through breeding. Selection of suitable animals plays an important role and genetic markers have a great potential. New sophisticated techniques which are based on DNA technology is more expensive to practice and more sophisticated facilities are essential. Therefore, conventional protein evaluating techniques based on polymorphism can be used as low cost methods. The study was conducted to identify the milk protein polymorphism of the buffalo milk and identify the relationships of the animals according to the milk protein polymorphism. Milk samples of four distinct groups of buffaloes (Surti cross bred, Local wild type, graded Murah and Local crossed) which had never meet one another were collected and used for protein separation by $(NH_4)_2SO_4$. The separated proteins were run in Polyacrylamide gel using Sodium Dodecyl Sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method and the polymorphism in the milk proteins were studied. Coomassi blue and silver staining methods were used for staining the gels. Milk proteins of the local wild type animals show a clear difference from other three groups of animals due to absence of a protein band of 87 kDa in 10 % SDS gel. There were differences in proteins between animal groups as well as within the same group. The most possible reason for that is the genetic diversity between the groups and within the group. For further studies on protein identification, sequencing of bands and studying the co- relation of genetic diversity and their morphological characters are essential.

Keywords: *milk protein polymorphism, buffaloes, Southern Province of Sri Lanka*

Introduction

The dairy industry is an important sector in Sri Lanka. Both neat Cattle and Buffaloes contribute to the Sri Lankan dairy sector. The buffalo population in Sri Lanka was 278,530 in 2008 and approximately 50,950 of them (18.29% of total population) were found from the Southern Province. The buffaloes are reared meagerly for milk production. The national buffalo milk production in the last year was 2,970,890 liters and it accounted for 17.13% of the total milk production of the country. (*Department of Census and Statistics ;2009*).

products capacities such as cheese, altering shelf life. The genetic diversity of the animals leads to the polymorphism of milk proteins among them. The Polymorphism of milk protein has been identified as an indicator of the genetic diversity of the mammalians because the composition of milk proteins is defined by the genetic properties of a particular animal.

The milk polymorphism is due to different forms of DNA sequences which define milk protein, especially Casein. We can use the milk protein polymorphism to identify the genetic similarities and differences among the animals. SDS Polyaccrylamide gel electrophoresis (SDS-PAGE)

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can be used to identify the protein composition of sample of serum or milk.

A few or no studies have been conducted to identify the milk protein polymorphism of the Buffaloes in Sri Lanka. There may be some relationship between the buffalo populations which are phenotypicaly different from each other. By identifying and comparing the milk protein polymorphism Relationships or similarities of the buffaloes can be identify according to milk protein genotypes.

Based on the results of the study and considering the phenotypic character and the milk protein composition we can select better animals for breeding programmes.

Materials and method

Selection of Animals

Buffaloes were selected for the research according the breed characters which can be clearly identified phenotypically. Colour of the animal, characteristic features of the horns and overall appearance of the animals were used as selection criteria.

Four groups of animals were selected from four separate populations and numbered them as follows.

B1R: Surti Cross bred animals (for "_Group 1" the replicate number, 1 to 6)

B2R: Local indigenous type animals (for "Group 2_" the replicate number, 1 to 6)

B3R: Graded murrah (for "Group 3" the replicate number, 1 to 6)

B4R: Local Cross bred animals(for "Group 4" the replicate number, 1 to 6)

Group 01:- Surti cross bred animals; Animals which show the characters of Surti Animals and clearly identified separately from other groups which were selected.The breed has got a fairly broad and long head with a convex shape at the top in between horns. Horns are sickle-shaped and flat which grow in a downward & backward direction and then upwards at the tip forming a hook. The skin color is black or brown, average milk production is 5-6*l/day*. Animals were selected from the faculty farm, Faculty of Agriculture, University of Ruhuna, Mapalana, Kamburupitiya

Group 02:- Local indigenous type animals; Animals have grayish to dirty whitish coloured coat and long hairs in the skin. Horns are long and not curved as surti animals, Average milk production is 3-4*l/day*; From Walsapugala, Sooriya wewa

Group 03:- Graded Murrah type animals; jet black coloured high yielding animals which show the typical Murah characters; comparatively small head and long face. Horns are short, tight, turning backward and upward and finally spirally curving inward. The animals have above 90% pure Murrah characters; Average milk production is about 6-8*l/day*; From Aliolu-ara, Sooriya wewa

Group 04:- Local cross bred animals; Animals which showing mixed up of characters of local animals and improved animals. Horne types are different and skin colour also showing a variance, Average milk production is 4-6*l/day*. From Udawalawa, Embilipitiya.

Six animals form each group were selected as replicates. Milk Production of the animal groups is different from each other.

Collecting of milk samples

Milk samples of 50ml from each animal were collected. The samples were taken in to clean; clear 50 ml plastic bottles and a tablet of *Sodium azide* was added into each bottle and inverted to dissolve the tablets in milk to prevent the spoilage of milk by micro organisms. The collected samples were frozen under deep freezing condition and were transported to the laboratory.

Separation of Fat

The milk samples were thawed in a water bath at the temperature of 37^{o}C, poured into 50ml centrifuge tubes and centrifuged at 4000rpm, 5^{o}C temperature for 20 minutes. A sample of 1.5ml supernatant was taken from that centrifuged and micro centrifuged under the conditions of 15000 rpm speed and 15^{o}C for 10minutues for further removal of fat if present.

Separation of Protein

A 0.325ml sample of fat free milk was taken into 1.5ml centrifugal vessel and 0.975ml of saturated ammonium sulphate was added. The tubes were vortexed well by using a tube mixture. All the tubes were kept for 1-1.5 hr in the refrigerator for the precipitation of protein. The tubes were re-vortexed and centrifuged at 15000 rpm and 15^{o}C for 10minutes. The protein was separated as a pellet and amoniumsulphate was removed. The tubes were re-centrifuged at 5000rpm speed and 15^{o}C for 3minutes. When all the amoniumsulphate was removed a 100µl of PBS was added in to each protein sample and pellets were dissolved in PBS. The tubes were kept overnight under the freezer conditions.

Estimation of Protein

The amount of protein in each sample was estimated using the Bicinchoninic Acid (BCA) method.

Preparation of apparatus

BIORAD Mini-PROTEAN®II Electrophoresis Cell was used to run the SDSPAGE. The apparatus was prepared according to the instruction manual of the BIORAD Mini-PROTEAN ®II Electrophoresis Cell.

Preparation of stock solutions

Following stock solutions were prepared according to the instruction manual of the BIORAD Mini-PROTEAN ®II Electrophoresis Cell.

- Acrylamide/bis (30% T, 2.67% C)
- 1.5 M Tris-HCl, pH 8.8
- 0.5 M Tris-HCl, pH 6.8
- 10% SDS
- Sample buffer (SDS reducing buffer)
- 5X electrode (Running) buffer, pH 8.3

Preparation of gels

Monomer solutions for the separating gel and staking gel were prepared according to the following recipe.

Table 1: Recipe for Separating and Stacking gels.

	Separating Gel (10%)	Stacking Gel (4%)
Deionized water	1.78 ml	3.05 ml
1.5M Tris – HCl pH 8.8	1.25 ml	--------
0.5M Tris – HCl pH 6.8	--------	1.25 ml
10% (w/v) sds stock	50 µl	50 µl
Acrylamide/bis (30% stock)	1.9 ml	0.665 ml
10% ammonium persulfate	25 µl	25 µl
TEMED	2.5 µl	5 µl
Total monomer	5 ml	5 ml

All the ingredients for the monomer solutions were mixed except APS and TEMED. APS and TEMED were added to the mixture for separating gel and it was poured into the glass plate. A few drops of deionized water were poured and the gel was allowed to polymerize.

After the polymerization, the water layer on the separating gel was removed. Ingredients of Monomer solution except APS and TEMED for the Stacking gel were mixed. ASP and TEMED were added to the Stacking gel monomer solution when the separating gel was polymerized and was poured on to the separating gel. The comb was inserted in to the stacking gel to mark the wells as the lower end of the comb reach at about 0.5 cm to the separating gel.

Preparation of samples for run

The milk from each sample was taken to get equal amount of total protein according to the results of the BCA protein assay and was added 6X sample buffer stock solution to make equal dilution. The mixture was vortexed well by using the tube mixter and was kept in 5^{o} C – 10^{o} C for 5 minutes.

Loading samples in to the wells

The comb was removed carefully to open the wells and the wells were washed by distilled water. The Upper Buffer Chamber was assembled as mentioned in the manual. The clamp assembly was placed in the lower buffer chamber. The electrode buffer (Running Buffer) was prepared by diluting 60 ml of 5X running buffer stock solution in 240 ml deionized water to make the total volume of 300 ml. The running buffer was poured in to the inner cooling core and rest of the buffer was poured in to the lower buffer chamber. A 20 µl of each prepared sample were loaded into the wells of the stacking gel using a fine pipette. With the milk samples, 5µl of a genetic marker and 20 µl of cattle serum sample were run in every gel.

Running the Gel

After the loading of every sample into the gel the lid of the apparatus was closed and the electrical leads were connected to the power supply unit. The power supply unit was programmed as

Step 1; 100V, 15 minutes
Step 2; 200V, 45 minutes

And the gel was run.

Removing the Gel

After the electrophoresis was completed the electrical leads were disconnected. The lid of the cell was removed and the buffer of the inner cooling core was removed carefully. The screws loosened and the glass plate sandwich was taken off. The upper glass plate was gently twisted and removed. The stacking gel was discarded and the separating gel was used for staining.

Staining the Gel

MALTISKAN EX PRIMARY EIA V. 2.3

ABSORBANCE MODE
CONTINUOUS MOVEMENT

Two different staining methods were used.

I) Coomassi Blue Staining
II) Silver Staining

Coomassi Blue Staining

The Coomassi Blue Staining solution was used for the Coomassi blue staining and the gel was let over night for staining. The Silver Staining was a procedure which was completed in many steps.

Silver Staining

The silver staining done according to the protocol of Blum *et al.* (1987)

De-staining of the Gel

The Coomassie blue stained gels were de-stained after staining overnight.

Pre-tests

Pre-tests were done to determine the best amount of the protein that gives the clear results for the staining methods. For that various known concentration of proteins were run in gels.

Comparing the gel pictures

The gel pictures were scanned and compared with each other to compare the proteins in the milk samples. The genetic marker was used for the identification of the specific molecular masses to get molecular mass estimation of milk proteins.

Results and discussion

Protein estimation

The result of the BCA assay was as follows. The well A_1 was considered as the blank well due to there was no any protein substances. There were only distilled water and the protein determination regent.

BCA assay results

FILTER 571
SINGAL WELL BLANK 0.181
BLANK WELL A1
MIXING TIME 10 SLOW

ABSORBANCES
20. JUN 2009 10:19:27

1	2	3	4	5	6	7	8	9	10	11	12
0.000	0.069	0.137	0.224	0.308	0.309	0.421	0.253	0.188	0.300	0.227	0.298
0.126	0.228	0.225	0.357	0.375	0.228	0.273	0.467	0.393	0.494	0.370	0.278
0.220	0.263	0.185	0.317	0.193	0.225	0.185	0.377	0.257	0.165	0.336	0.246
0.310	0.316	0.339	0.221	0.216	0.450	0.195	0.236	0.087	0.456	0.399	0.308
0.412	0.500	0.467	0.386	0.169	0.472	0.119	-0.138	-0.142	-0.139	-0.142	-0.142
-0.138	-0.139	-0.142	-0.138	-0.140	-0.142	-0.141	-0.139	-0.142	-0.139	-0.142	-0.137
-0.143	-0.141	-0.144	-0.143	-0.141	-0.143	-0.142	-0.142	-0.142	-0.144	-0.142	-0.142
-0.137	-0.141	-0.143	-0.141	-0.142	-0.142	-0.143	-0.142	-0.144	-0.142	-0.140	-0.141

Figure 1: BCA assay results

Above result sheet of the BCA assay gives the relative absorbance of each well reference to the blank well (A_1). According to the relative absorbance of A_1 to A_7 wells, an x y scatter plot was plotted and the trend line was obtained as the standard trend line for the relative absorbance as follows.

Standard curve

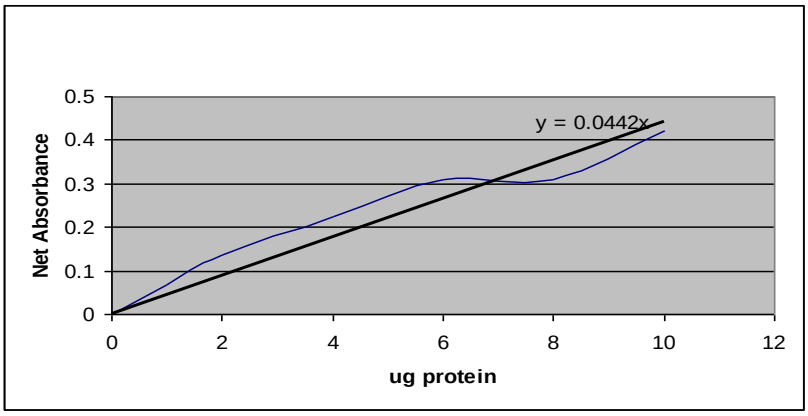

Figure 1: standard curve

Protein content in milk samples

According to the net absorbance of each sample the protein concentration was calculated by using the equation

$$\frac{(\mu g \text{ unknown protein per assay})\ (\text{dilution factor})}{(\mu l \text{ unknown used for assay})}$$

Table 2: calculation of protein levels of samples

Sample	Sample	Absorbance	X=Y/0.0442	DF	Volume	[P]=(X*DF)/V
01	B1R1	0.22	4.9773756	20	10	9.95475113
02	B1R2	0.263	5.9502262	20	10	11.9004525
03	B1R3	0.212	4.7963801	20	10	9.59276018
04	B1R4	0.227	5.1357466	20	10	10.2714932
05	B1R5	0.366	8.280543	20	10	16.561086
06	B1R6	0.25	5.6561086	20	10	11.3122172
07	B2R1	0.43	9.7285068	20	10	19.4570136
08	B2R2	0.432	9.7737557	20	10	19.5475113
09	B2R3	0.249	5.6334842	20	10	11.2669683
10	B2R4	0.224	5.0678733	20	10	10.1357466
11	B2R5	0.317	7.1719457	20	10	14.3438914
12	B2R6	0.225	5.0904977	20	10	10.1809955
13	B3R1	0.317	7.1719457	20	10	14.3438914
14	B3R2	0.336	7.60181	20	10	15.2036199
15	B3R3	0.278	6.2895928	20	10	12.5791855
16	B3R4	0.327	7.39819	20	10	14.7963801
17	B3R5	0.218	4.9321267	20	10	9.86425339
18	B3R6	0.45	10.180995	20	10	20.361991
19	B4R1	0.236	5.3393665	20	10	10.678733
20	B4R2	0.427	9.6606335	20	10	19.321267
21	B4R3	0.36	8.1447964	20	10	16.2895928
22	B4R4	0.483	10.927602	20	10	21.8552036
23	B4R5	0.386	8.7330317	20	10	17.4660633
24	B4R6	0.472	10.678733	20	10	21.3574661

(DF:-Dilution factor) [P] Protein concentrates (µg/µl)

According to the level of protein in the milk samples the required volumes of sample and 6X buffer (sample buffer) was calculated. The best combination was determined by the results of the preliminary trials.

Better combination which gives more clear results were as follows.

Table 3: better combination of Sample and 6X buffer

Sample	Sample	Sample Volume	6X buffer Volume
1	B1R1	1.80	23.20
2	B1R2	1.51	23.49
3	B1R3	1.87	23.13
4	B1R4	1.75	23.25
5	B1R5	1.08	23.92
6	B1R6	1.59	23.42
7	B2R1	0.92	24.08
8	B2R2	0.92	24.08
9	B2R3	1.59	23.41
10	B2R4	1.77	23.23
11	B2R5	1.25	23.75
12	B2R6	1.76	23.24

13	B3R1	1.25	23.75
14	B3R2	1.18	23.82
15	B3R3	1.43	23.57
16	B3R4	1.21	23.79
17	B3R5	1.82	23.18
18	B3R6	0.88	24.12
19	B4R1	1.68	23.32
20	B4R2	0.93	24.07
21	B4R3	1.10	23.90
22	B4R4	0.82	24.18
23	B4R5	1.03	23.97
24	B4R6	0.84	24.16

In the above combinations each sample was adjusted to obtain same amount of protein from each sample of 17.9 µg

Documentation of the Gels

Two staining methods were used for the staining the gels, but using the coomassi blue method it is difficult to identify a clear band pattern due to lack of sensitivity. Some distinct bands were observed in some animals.

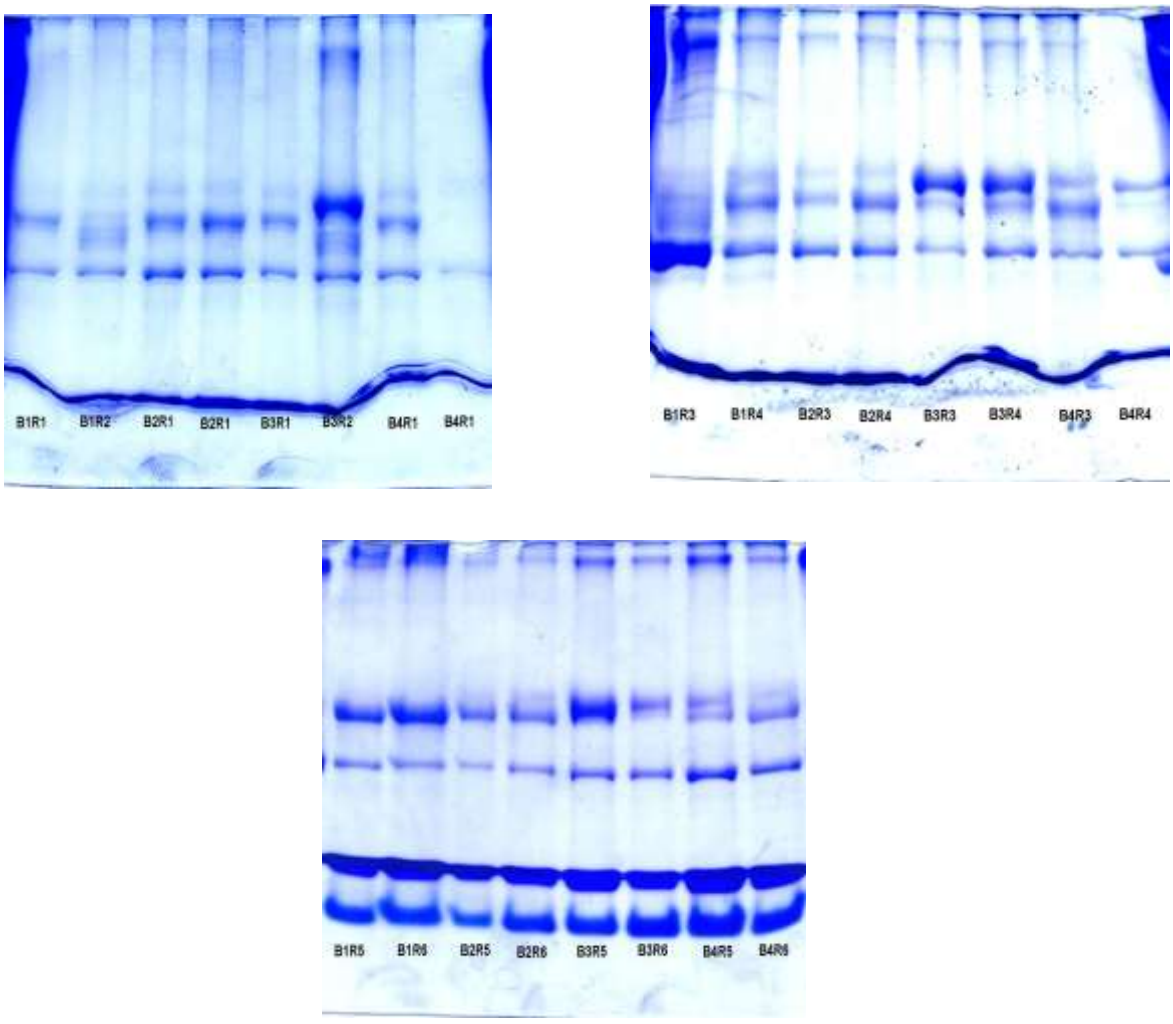

Figure 2: Coomassi Blue stained gels

The silver stained gel pictures are more descriptive than the coomassi blue stained gels due to the high sensitivity. So the silver staining method was used for compare gels for final results .

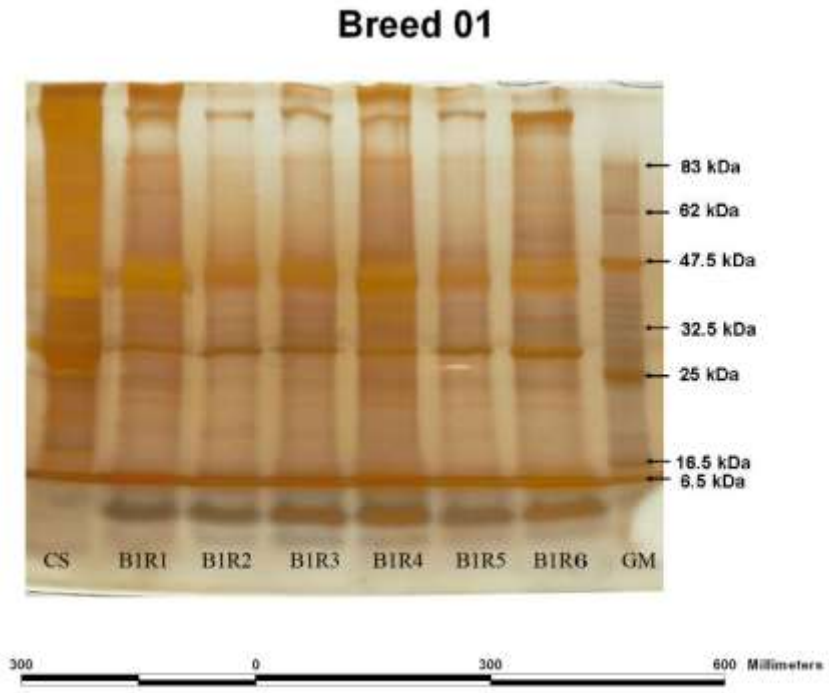

Figure 3: Silver Stained Gel picture Breed 01

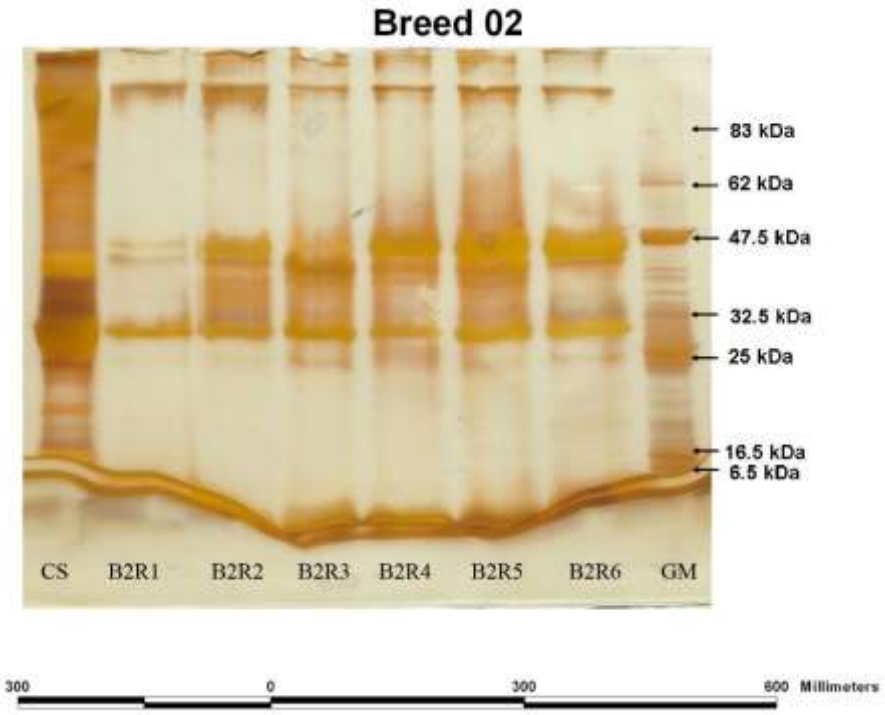

Figure 4: Silver Stained Gel Picture Breed 02

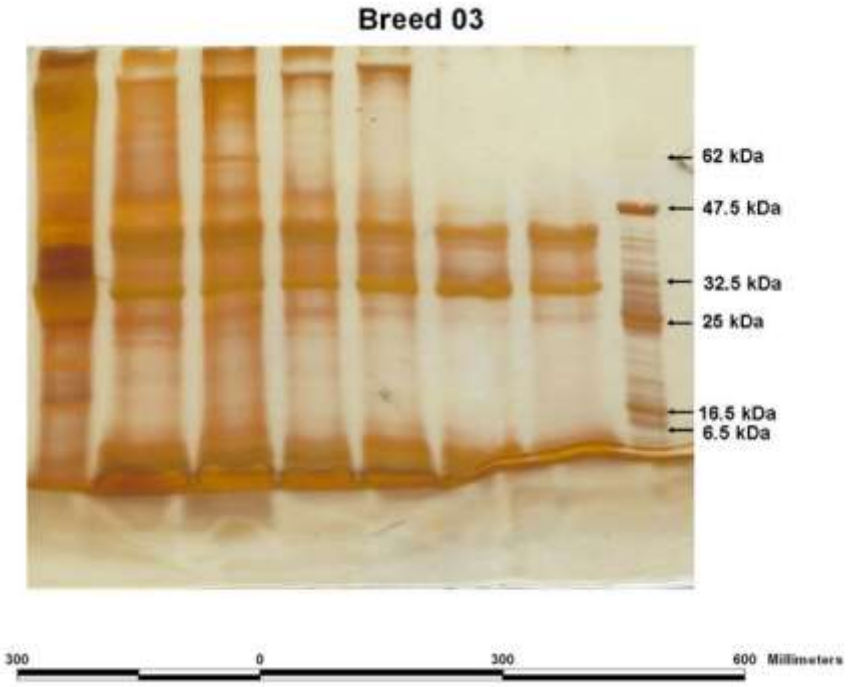

Figure 5: Silver Stained Gel picture Breed 03

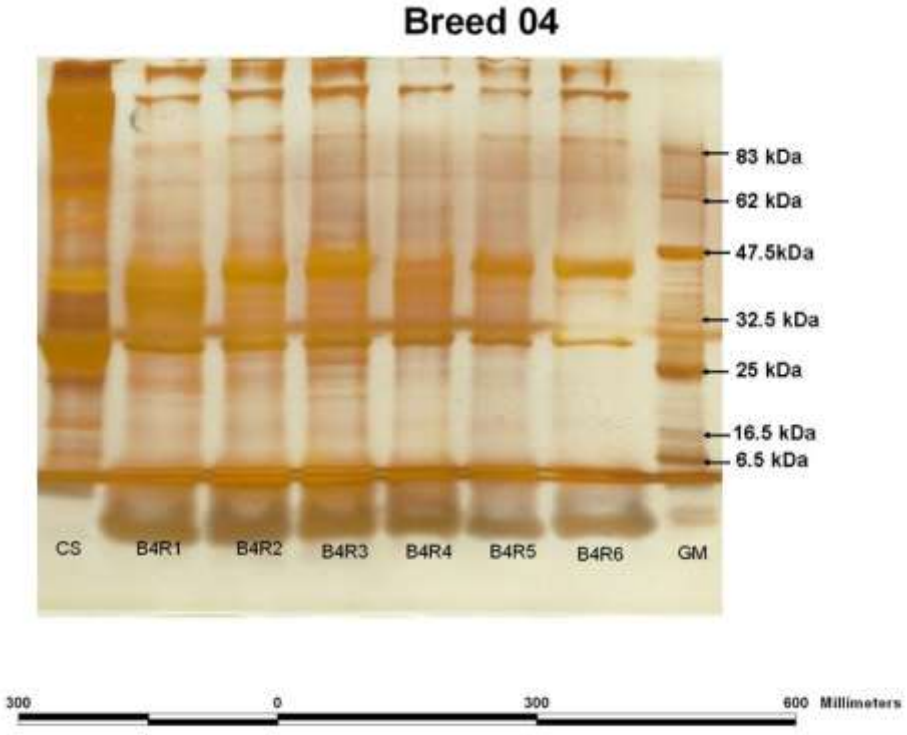

Figure 6: Silver Stained Gel picture Breed 04

According to the genetic marker which was run with the milk samples, the molecular masses at particular levels were identified and using graphs which were plotted by using the distance of the particular marker from the beginning of the separating gel and the log $_{(10)}$ value of the molecular mass, the unknown molecular masses were calculated. For example;

Table 4: Distance vs. Log (10) Molecular Mass

Distance	Molecular Mass(kDa)	Molecular Mass (log)
110.04	83000	4.919078092
160.62	62000	4.792391689
226.29	47500	4.67669361
302.6	32500	4.511883361
361.18	25000	4.397940009
434.82	16500	4.217483944
467.66	6500	3.812913357

(The distances were measured by using mm scale in a enlarged picture using the computer software "Arc View GIS 3.2a". The distance in the picture in the above page may not be equal to mm scale, but the ratios of the distances are equal to the original ratio).

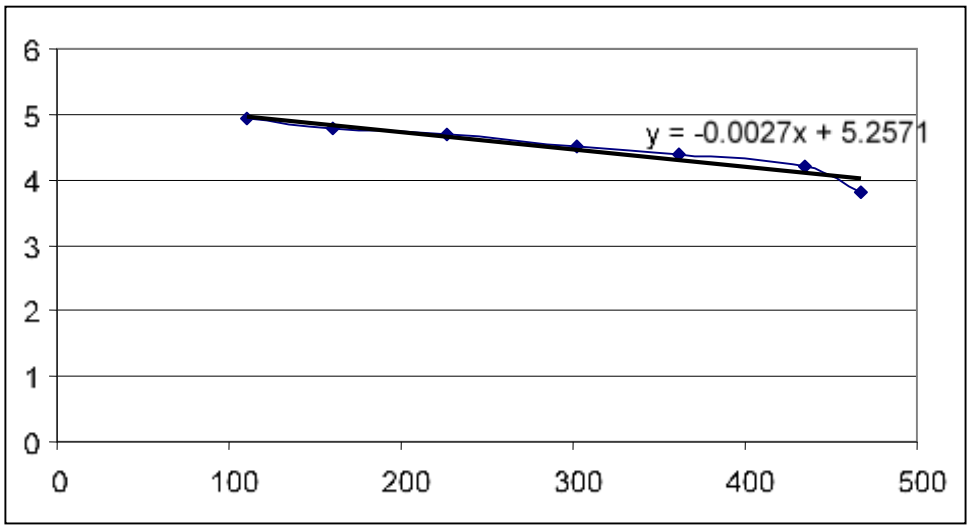

Figure 7 : Distance vs. Log $_{(10)}$ Molecular Mass

The molecular weights of the proteins which are showed by the bands were calculated and compared among the groups. According to the results of the calculations among groups and within group variations were identified.

Discussion

The variation of the milk protein can be identified by the study. The variation between the groups may due to the absence or variation in composition of the protein. In some groups there was a variation within the group. The variation may be due to the genetic variation within the animals of the same group.

In group, 02 the number of bands present was very low and some common bands for other three groups were not identified. That may due to the absence of such a protein in the animals of group 02 or may be due to the low concentration of the particular protein. By using a large sized gel we can improve the resolution of the gel. By improving the resolution we can clearly identify the band pattern.

The objective of the study to identify the correlation of the phenotypic characters and the milk protein polymorphism was unable to achieve due to the lack of time and sample size. To do such study, it needs more animal in the sample. By increasing the sample size, we can go to a cluster analysis and cluster the animals and identified the relationships among them according to the milk protein composition.

Many research works reported that Single Nucleotide Polymorphisms (SNP) potentially change the gene expression and may be linked with differences in milk yield and the quality of milk. Therefore, these SNP(s) in milk can be used as a genetic marker to identify a specific type of milk product or its origin; the animal type. According to the Wega *et al.* (1988) some of bovine milk proteins are lactation specific and used as lactation specific marker in modern industries.

In Sri Lanka there are different types of buffalo breeds. Among them Murrah and Surti are well-known, but local breeds too popular among Sri Lankan farmers. In this study inter as well as intra breed comparisons among protein bands in Acrylamide gels were made.

Variations in protein bands were observed within the breeds as well as among the breeds. According to the Ramesha *et al.* the quality and quantity of milk proteins differ between the species, breeds as well as individuals within the breed. The possible reason for these variations may be the absence of particular milk proteins because of genetic variation in tested animal breeds.

In Local Indigenous type animals (Group 02), the number of bands present was very low and some bands were not observed. The observed proteins of this breed showed in figure14. According to the figure 14 it is clearly observed that the composition of milk in the breed consist of proteins with molecular mass of 47.5kDa, 32.5kDa, 25kDa, 16.5kDa and 6.5kDa as the major proteins.

The other gel pictures clearly illustrated some higher protein bands which are absent among milk proteins of Local Indigenous type animals. A protein which is about the molecular mass of 87kDa was the significantly absent protein in this breed.

In other breeds some of the protein bands were not observed too. This may be due to the low concentration of protein and also absence of such protein. This is also possible to observe because there are lactation specific proteins in buffaloes. Not only can that, due to genetic variation of buffaloes similar results be observed within the breed. These protein variations may be potentially associated with differences in milk yield and quality. Especially the absent protein of local breed may be contributed to the high milk yield of the other breeds, which considered as milk breeds. In addition to that the absence of such protein of 87kDa may be a species characteristic. On the other hand the absence of the above mentioned protein indicates the genotype of local breed is significantly differing from other breeds. Ramesha *et al.* (2008) repotted that the quality and quantity of milk proteins differ between the species, breeds as well as individuals within the breeds. Therefore, the results of our study is further confirmed the milk protein polymorphism in buffaloes.

This milk protein polymorphism can be used as gene markers to identify the breed or the origin of a milk product of the present market.

Further studies such as sequencing, identification and gene expression of these proteins are essential to carry out.

Conclusions

Milk proteins of buffalo groups show some differences between the group and within the groups too. The differences of the molecular masses of the proteins show the difference of the proteins.

The milk samples of local wild type buffaloes show some prominent differences from other three groups. It shows low number of bands and the absence of the band which lay in about 87-90kDa is more clear difference from other groups.

The variation of milk proteins within the groups may due to many reasons. One possible reason may be genetic mutations.

The silver staining method is more appropriate method than the coomassi blue staining method to staining the gels.

The buffaloes can be genotyped by subjecting them for milk protein polymorphism and this can be used as a genetic marker to identify the origin or breed of the prevailing milk products.

References

1. Department of census and Statistics, Sri Lanka (2009); "National Livestock Statistics 2003 – 2009"

2. Blum, H., Beier, H., and Gross, H.J. (1987) *Electrophoresis* 8, 93-99.

3. Census of Agriculture, (2002), Agriculture and Environment Statistics Division, Department of Census and Statistics, Sri Lanka.

4. Ramesha1.K.P. Khosravinia.H., Gowda.S, RaoM.R.S., (2008), Alpha-Lactalbumin Gene Polymorphism: A preliminary study on two breeds of the river Buffalo (Bubalus bubalis), Asia Pacific Journal of Molecular Biology and BiotechnologyVol. 16 (2): 47-52