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Polymorphic Variation of Hemoglobins in Buffaloes (*Bubalus bubalis*)

Variação Polimórfica de Hemoglobinas em Búfalos (*Bubalus bubalis*)

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SUMMARY

In the present report the biochemical polymorphism of hemoglobins in 96 Indian buffaloes from different breed groups of Murrah, Jafarabadi and Mediterranean races reared in the experimental farm Lageado - UNESP - Botucatu, State of São Paulo was studied. Hemoglobins variants were identified through agarstarch gel electrophoresis, in discontinuous buffer system, pH 8.6. Animals showing one fast band (A_1) were considered AA (4.4%); buffaloes with two bands (A_1 and A_2) were considered AB (31.87%) when A_2 was weak and BB (31.87%), when A_2 was strong. A third band was detected, named N. Phenotypes with three bands with the respective frequencies: ABN (3.3% and BBN (30.77%). Densitometric analyses and the lack of AAN phenotype drove us to conclude that, in some way, the mutant β chains synthesis that originated N band was related probably to α^{II} allele. Probably, the high frequency of N band was due to the intensive use of few breeders.

UNITERMS: Buffaloes; Electrophoresis; Hemoglobin; Genetics

INTRODUCTION

The first genetical protein variation was detected by Pauling *et al.*[19] (1949), when they demonstrated that people with sickle cell anemia had a different type of hemoglobin form. Giri; Pilai[8] (1956) were the first to demonstrate the two hemoglobin bands phenotype in buffaloes (*Bubalus bubalis*). Loypetjra[11] (1962) and Sen *et al.*[21] (1966) confirmed such pattern and found some animals with a weak third band. Naik; Sukumaran[18] (1967) noticed a pattern of two bands when they studied 250 buffaloes; only three animals showed just the fast band. The hemoglobin proportion in the two bands pattern was 60% - 70% for the fast band and 30% - 40% for the slower one. They did not observe any elctrophoretical or alkaline denaturation differences between fetal and adult hemoglobins. This was confirmed by Ranjekar; Barnabas[20] (1969). Patterns showing two bands were perceived in European buffaloes by Braend; Gasparsky[6] (1967) and by Makaveev[14] (1968). A pattern of three bands was observed in four of 507 buffaloes studied by Khanna; Braend[10] (1968). Abe *et al.*[1] (1969) and Khanna[9] (1973) observed some animals with a pattern of two bands with the slowest one showing a lower proportion than the noticed by other authors. Khanna[9] (1973) studied the biochemical polymorphism of hemoglobins in seven buffalo breeds: Bhadawari (91 buffaloes), Marathawada (34), Murrah (1255) from 10 differents herds, Nagpuri (75), Nili (115), Ponderpuri (37), Surti (139). From the total number of animals under study, 1730 showed the pattern of two bands, including calves younger than one month. The fast band, strongly dyed, was named A_1, while the slowest one was named A_2. Their electrophoretic mobilities were similar to HBC and HBA bovine hemoglobins, respectively. The components were quantified and A_1 was about 68% - 75% while A_2 was about 25% - 32%. In 11 Murrah buffaloes it was observed that component A_2 was much weaker than A_1 (15% and 85% respectively). The presence of a third band, slower than A_1 and A_2, named N, considerably weak, was another phenotype found. A_2 component in this phenotype seemed to be more stained than in phenotypes with two bands. Structural differences in adult buffaloes hemoglobins were studied by Balani; Barnabas[2] (1965). The two hemoglobins components were named by HB_1 and HB_2, which were different in α chains. These components were named $\alpha_2^I \beta_2$ and $\alpha_2^{II} \beta_2$. They were different in two pairs of peptides, one of them involving thriptofane, acording to Balani *et al.*[3] (1968). Two fetal hemoglobins and few content of adult hemoglobin were detected in newborn buffalo. Structural differences between fetal hemoglobins were located in α chains, seeming to be type $\alpha_2^I \gamma_2$ and $\alpha_2^{II} \gamma_2$. Ranjekar; Barnabas[20] (1969) observed that during post-natal development γ chain was changed by

β chain, keeping constant α^{I} and α^{II} chains concentration. Masina *et al.*[16] (1971) observed in buffaloes three hemoglobins electrophoretic phenotypes named as BB, AB and AA. BB animals showed two bands with the slowest one in fewer proportion. In animals AB, the slowest components was weaker than in BB animals. In AA phenotype only the fastest component was observed. Masina *et al.*[17] (1977) prescribed some hypothesis to explain genetical mechanisms involved in buffaloes hemoglobin synthesis. Luccia *et al.*[13] (1989) employed alkaline starch electrophoresis and classified buffaloes hemoglobin samples in AA, AB and BB. By means of electrophoretic and chromatographic techniques, they observed that α^{I} and α^{II} chains differed in two aminoacid residues, identified as lysine and cysteine. Luccia *et al.*[12] (1991) employing BB buffaloes hemoglobin samples, analyzed by means of isoelectric focusin and by ultranarrow immobilized pH gradient, found three types which were called BB, BsBs and Bbs. Analyses of constituent globin chains showed a polymorphism in β chains level. Luccia *et al.*[12] (1991) used electrophoretic and HPLC techniques to study hemoglobin polymorphism in italian river buffaloes. They were able to identify six different phenotypes, characterized by two of four different components. The AA phenotype was associated to two α chains (I-α-1 and II-α3) that gave two hemoglobins with similar mobilities (Hb1 and Hb3). Two α chains were associated with the BB phenotype (I-α-2 and II-α-4), that gave hemoglobins with different mobilities (Hb2 and Hb4). Double hemoglobins were associated with the AB phenotype, that suggested allelic polymorphism at the two alpha loci. Ferranti *et al.*[7] (1992) studying the fast band of AA phenotype in buffaloes observed that it was formed by two components (Hb1 and Hb3) whose differences were in the α chains (α1 and α3). Such differences were due to the action of a neutral aminoacid substitution. In the present report, the polymorphism of hemoglobins of Indian buffaloes reared in the State of São Paulo, Brazil, from different genetic composition is described.

MATERIAL AND METHODS

Blood samples (10 ml) were obtained by vacutainer from 96 outbred buffaloes (28 male and 68 female), of different genetical composition among breeds Murrah, Jafarabadi and Mediterranean, reared in the Lageado Experimental Farm, UNESP, Botucatu, São Paulo, Brazil. Samples were collected in Alserviers (0.54 M sodium citrate, 1.44 M sodium chloride, 0.228 M dextrose, 40.0 mg rivanol, pH 6.2) and kept under refrigeration (4°C) until use. Age varied from 4 to 216 months. Three times washed blood cells were hemolysed by adding distiled water and chloroformiun equal amounts (v:v:v), obtain hemoglobin solution, as stated by Bortolozzi *et al.*[5] (1990). Electrophoresis was carried out employing alkaline discontinuous buffer system (gel buffer: TRIS 0.42 M, EDTA 0.01 M, boric acid 0.06 M, pH 8.6; diluted at 10%; electrode buffer: boric acid 0.30 M, sodium hydroxide 0.06 M, pH 8.6, diluted at 33%) and agarstarch gel (100 mg% and 380 mg%, respectively), as recommended by Martins[15] (1982). Gel was prepared on a Bunsen flame. After gel melting, 3 ml was poured on a microscope slide glass; hemoglobin was applied at 2 cm from anode. To set seven glasses, 150 V with 25 mA was applied. Electrophoresis was completed after 90 minutes. Glasses were then stained in Amido Schwartz 10 B (1% in destaining solution) for three minutes and washed in destaining solvent (methanol 800 ml, acetic acid 200 ml, water 1000 ml) until complete band resolution. After drying, the glasses that exhibited two or more bands were analyzed in a densitometer (Zeiss MD 100) and an index was calculated (fast band - A_1/slow band - A_2). Using hypothesis stated by Masina *et al.*[17] (1977). X^2 with Yates correction was employed to verify equilibrium conditions. Densitometric data were submitted to Descriptive Statistics and to Variance Analyses according to Berquó *et al.*[4] (1980).

RESULTS

In Fig. 1 are describe the hemoglobin elctrophoretic patterns obtained in the present work. These patterns were called as stated by Masina *et al.*[16] (1971) and Khanna[9] (1973). Animals that showed only the fast band (A_1) were considered AA. Buffaloes with two bands, but the slow one (A_2) weak, were considered AB. Animals that exhibited two bands, but the A_2 strong, were considered BB. In many buffaloes the occurrence of a third band (N), slower and weaker than the second one, was observed. These animals were considered ABN and BBN. Relative frequencies are described in Tab. 1. In Tab. 2 densitometric measurements show that there is a clear difference between phenotypes found in this work. Relative frequencies of alleles A and B are described in Tab. 3. The herd under study are in Hardy - Weinberg equilibrium for this characteristic. Densitometric indices analyses are presented in Tab. 4.

TABLE 1
Relative frequencies of buffaloes hemoglobin phenotypes obtained by electrophoresis in agar-starch gel, alkaline discontinuos buffer system

Phenotypes	n	Relative Frequencies
AA	4	0.0440
AB	29	0.3187
BB	29	0.3187
ABN	3	0.0330
BBN	28	0.3077

TABLE 2

Descriptive Statistics: Central tendency and dispersion measurements relative to densitometric analyses of buffaloes hemoglobins electrophoretic patterns

Band	Phenotypes	n	Mean (%)	s	s^2
A_1	AB	29	77.15	4.83	23.29
	BB	29	60.25	6.50	42.29
	BBN	28	49.49	6.59	43.47
A_2	AB	29	22.73	4.74	22.49
	BB	29	39.79	6.40	40.99
	BBN	28	35.20	4.86	23.60
A_1 / A_2	AB	29	3.73	1.68	2.82
	BB	29	1.60	0.47	0.22
	BBN	28	1.45	0.34	0.11

TABLE 3

Observed and expected distribution of buffaloes hemoglobin phenotypes, detected in alkaline electrophoresis and respective relative frequencies.

	Phenotypes				Allelic Frequencies	
	AA	AB	BB	Total	HB^A	HB^B
Observed	4	34	53	91	0.23	0.77
Expected	4.81	32.23	53.96	91		

Statistic $X^2 = 0.073$ f. D. = 2 $p < 0.05$

TABLE 4

Variance analyses relative to densitometric measurements of buffaloes hemoglobin variants detected by alkaline electrophoresis.

Variable	F	P	Comment
A_1	153.18	0.001	AB > BB > BBN
A_2	77.59	0.001	AB < (BB = BBN)
A_1 / A_2	43.26	0.001	AB > (BB = BBN)

DISCUSSION AND CONCLUSIONS

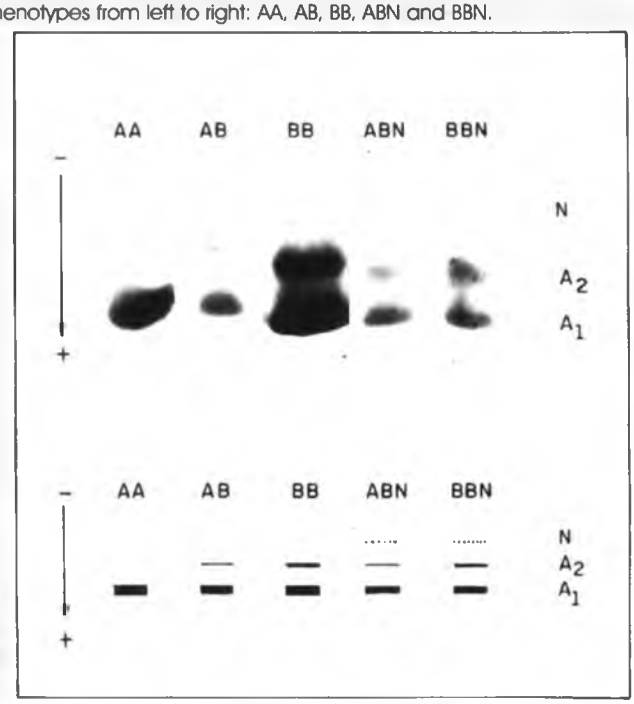
Animals with the phenotypes AA, AB, BB, ABN and BBN were found, as can be seen in Fig. 1. Relatively to the one band pattern, data presented here agree with Naik; Sukumaran[18] (1967) and Masina *et al.*[16,17] (1971 and 1977), that found few animals showing this phenotype. Buffaloes hemoglobin pattern showing two bands is in agreement with available reports in literature. Naik; Sukumaran[18] (1967) measured hemoglobin bands obtaining 60% - 70% to A_1 and 30% - 40% to A_2, which agrees in part with the present work. In Tab. 2 it can be seen that data obtained by cited authors can confuse BB buffaloes with AB when A_1 band is considered. When A_1 is analysed, animals showing AB phenotype reveal lesser hemoglobin quantity than the studied by Naik; Sukumaran[18] (1967).

Khanna[9] (1973) observed that some animals showed A_1 stronger than the others (85%), exceeding values here presented. Average values found by the author were 71% to A_1 and 29% to A_2, that are quite similiar to ours. These animals constitute an intermediary group between buffaloes that showed AB and BB in the present report. Such differences may be attributed to techniques employed on the band hemoglobins quantity measurements. Relating to the third band (N), available data are in agreement with Loypetjra[11] (1962), Sen *et al.*[21] (1966), Naik; Sukumaran[18] (1967), Khanna; Braend[10] (1968) and Khanna[9] (1973). This later author explains the third band appearance by a β chain aminoacid substitution that could cause an electrophoretic mobility retard. Four hemoglobins molecules are expected: $\alpha^{A1}_2\beta^A_2$, $\alpha^{A2}_2\beta^A_2$, and the mutant chains $\alpha^{A1}_2\beta^N_2$ ans $\alpha^{A2}_2\beta^N_2$, causing overlapping. The third band was not reported by Luccia *et al.*[13] (1989), Luccia *et al.*[12] (1991)

and Ferranti *et al.*[7] (1992) that studied Italian buffaloes. Probably this band is a breed effect because the herd in study is performed by Murrah and Jafarabadi which have an Indian origin, plus Mediterranean race. Considering that no animals AAN were found neither in the herd under study nor in the literature, this fact suggests the possibility that in any way β^N mutant chains synthesis are related to the allele B, α^{II}_B or α^{II} as stated by Masina *et al.*[17] (1977), or more recently, to α^{I}_2 and α^{II}_4, according to Luccia *et al.*[12] (1991). Through Variance analyses (Tab. 4) it is observed that there are statistical differences relatively to A_1 densitometric measurements.

FIGURE 1

Electrophoretic patterns of buffaloes hemoglobins in agar-starch gel, alkaline discontinuous system buffer, pH 8.6, showing the following phenotypes from left to right: AA, AB, BB, ABN and BBN.



Buffaloes AB showed highest values. BB animals showed higher values than BBN. Considering A_1/A_2 index, it may be seen in Tab. 4 that differences between BB and BBN do not occur. Masina *et al.*[17] (1977) suppose the α^I gene existence, which is responsible to 68% of α chain synthesis, and α^{II}, that is represented by two genes $\alpha_A{}^{II}$ and $\alpha_{B1}{}^{II}$. Similar chains to α^I are produced by $\alpha_A{}^{II}$ allele, which forms the fast band (A_1). In such way, AB animals should present the $\alpha^I\ \alpha^{II}/\alpha_B{}^{II}$ genotype, whereas BB buffaloes should have the $\alpha_B{}^{II}/\alpha_B{}^{II}$ genotype. The fast band densitometric differences obtained, when AB and BB phenotypes were considered, may be explained regarding to the α_I and α_{II} genes that could lead to a higher quantity A_1 formation than the α^{II} by itself. Relating to slow band densitometry (A_2) it is observed that, in AB buffaloes B^{II} is responsible for 16% of α chain production and in animals BB for 50%, which could explain the differences observed. The same explanation is valid when the second hypothesis is considered, where the α^{II} allele may be monomorphic. AA buffaloes would be homozygous to α^I; in AB animals, α^I would be duplicated and in heterozygosis with α^{II}. This condition would lead to the 84% of α production of α^I, whereas in BB, because both alleles are in duplication, 66% are produced. Densitometric differences between BB and BBN may not be considered as a biological event, but only a statistical artifact, because biochemical and genetical bases to the production of N band are just related to $\alpha_B{}^{II}$ or α^{II} alleles, what does not make them directly responsible for B mutant chain, as stated by Khanna[9] (1973). Such conditions could explain the fact that BB and BBN buffaloes do not differ in densitometric index; differences show in AB animals could be explained by chain polymorphism. Relating to A and B alleles, obtained data permitted to conclude that the herd under study was in Hardy-Weimberg equilibrium, which is in agreement with Masina *et al.*[17] (1977). The fact that the population keeps the equilibrium reveals that there probably are no relationships between genetical biochemical hemoglobins polymorphism and characteristics that drove animals selection in that herd. No fetal hemoglobins were detected because buffaloes older than four months were examined in the present work. The high frequency of N band is probably due to intensive use of few breeders in the flock.

RESUMO

No presente trabalho foi estudado o polimorfismo bioquímico das hemoglobinas de 96 búfalos indianos de diferentes cruzamentos envolvendo as raças Murrah, Jafarabadi e Mediterrâneo, criados na Fazenda Experimental Lageado - UNESP - Botucatu, Estado de São Paulo. As variantes de hemoglobinas foram identificadas através de eletroforese em gel de Agar-Amido em sistema de tampão descontínuo, pH 8,6. Animais portando uma banda rápida (A_1) foram considerados AA (4,4%); búfalos com duas bandas (A_1 e A_2) foram considerados AB (31,87%) quando A_2 era mais fraca e BB (31,87%) quando A_2 era mais forte. Uma terceira banda foi encontrada, chamada N. Fenótipos com três bandas foram encontrados com as respectivas freqüências: ABN (3,3%) e BBN (30,77%). Análises densitométricas e a falta de animais AAN levaram à conclusão que, de alguma forma, a síntese da cadeia β mutante que origina a banda N esteja relacionada provavelmente ao alelo α^{II}. Provavelmente, a alta freqüência de animais com banda N seja devida ao uso intenso de poucos reprodutores.

UNITERMOS: Búfalos; Eletroforese; Hemoglobina; Genética

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