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Bovine hemoglobin polymorphism: a novel alpha-globin variant identified in the Agerolese breed from southern Italy

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ABSTRACT: This study describes the results of molecular screening in the Agerolese cattle breed from southern Italy, which constitutes an economical source and contributes to the regional biodiversity. Integrated electrophoretic, chromatographic, mass spectrometric, and DNA sequencing procedures were used to qualitatively and quantitatively evaluate globin chain polymorphism in a population of animals from different farms. In addition to α-, β^A-, and β^B-globin-bearing animal phenotypes, our data demonstrate the occurrence of individuals characterized by the presence of a novel α-globin variant, whose primary structure differs from the normal counterpart for the p.Ala27Thr substitution. The SNP *c.79G>A* located at the exon 1 of the *HBA2* gene is responsible for this amino acid change and it represents a third example of α-globin gene polymorphism observed in *Bos taurus*. This phenomenon was ascertained only in two Mediterranean breeds from southern Italy and highlights the uncommon occurrence of α-globin variants in this geographical region, similarly to what has already been described in goat and human.

Keywords: *Bos taurus*; native cattle breed; amino acid substitution; *HBA2* gene; SNP

INTRODUCTION

Agerolese is a local cattle breed occurring in southern Italy (Monti Lattari and Sorrento peninsula), which is well adapted to a mountainous environment, fed on products of pruning and undergrowth (Peretti et al. 2013). It originated from Podolian cows crossed with Swiss Brown, Dutch Friesian, and Jersey bulls, and was selected over the centuries as a good milking animal, yielding 17–18 l of milk/day. Its milk is used to produce the Provolone del Monaco, a cheese recognized by the Protected Designation of Origin (Reg. CE n. 121/2010) and Slow Food Foundation for Biodiversity.

Characterization of native animal breeds through the screening of genetic polymorphisms is important for breeding protection and safeguard of the genetic heritage. This is particularly relevant for animal breeds at risk of extinction and/or subjected to frequent replacement cross-breeding. Studying genetic variants is also important because of the impact that single amino acid substitutions can have on protein function. A prototype example is hemoglobin, where different globin genes contribute to a protein phenotype in which amino acid replacements can affect development of a pathological state or, more generally, organism adaptation to environmental conditions, which are peculiar for the breed and/or related to the uniqueness of its products (Haenlein et al. 1987; Storz and Moriyama 2008; Pieragostini et al. 2010; Weber and Campbell 2011). In bovine, hemoglobin polymorphism was originally described for alleles *Hb A* and *Hb B* (Bangham and Blumberg 1958). A

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number of other hemoglobin variants were reported later on (Efremov and Braend 1965; Braend et al. 1966; Braend 1971). Most of these hemoglobin variants originated from polymorphisms in the subunit beta chain (beta-globin; HBB) (Namikawa et al. 1983; Pieragostini et al. 2010, Tanaka et al. 2011), among which one characterizes the Podolian cattle from southern Italy (Pieragostini et al. 2000). On the other hand, bovine α-globin gene cluster consists of two genes, *HBA1* and *HBA2*, which are expressed at different levels. Only two natural allelic variants of these genes have been reported so far and both have been described in the Podolian cattle (Scaloni et al. 1998; Pieragostini et al. 2010).

The aim of the present study was to screen the genetic polymorphisms of α- and β-globins of the local Agerolese cattle breed by an integrated protein approach and to characterize the newly found variants at the amino acid and DNA sequence levels.

MATERIAL AND METHODS

Blood sampling and proteins/nucleic acids preparation. Blood samples from 68 Agerolese (about a sixth of the whole Agerolese population), 2 Friesian (control), and 2 Bruna Alpina (control) breed individuals were collected in vacutainer tubes (BD, Franklin Lakes, USA) supplemented with K_2EDTA (1 mg/ml) as anticoagulant. They were taken from different farms and treated according to standard hemolysis/globin precipitation procedures (Scaloni et al. 1998; Pieragostini et al. 2000). DNA was isolated from the corresponding leucocytes according to Montgomery and Sise (1990). DNA concentration and $OD_{260/280}$ ratio were measured with a Nanodrop ND-2000C instrument (Thermo Fisher Scientific Inc., Waltham, USA).

Electrophoretic and mass spectrometric analysis. Blood lysates were analyzed by polyacrylamide isoelectrofocusing (PAGIF) over a 6.6–7.6 pH gradient (Scaloni et al. 1998). They were also analyzed by acid-urea triton X-100 polyacrylamide gel electrophoresis (AUT-PAGE), as previously reported (Pieragostini et al. 2005). Blood lysates were also diluted (1 : 100) in 10% acetonitrile (ACN), 0.1% trifuoroacetic acid (TFA), and analyzed by matrix assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) using a UltrafleXtreme™ instrument (Bruker Daltonics, Bremen, Germany). Samples were mixed (1 : 1) with sinapinic acid as matrix (10 mg/ml in 40% ACN, 0.1% TFA) before analysis. A standard protein mixture was used for calibration of mass values.

Structural characterization of the globin chains. Globins were resolved by reversed-phase high pressure liquid chromatography (RP-HPLC) over a C_4 column (4.6 × 250 mm, 5 μm) (Phenomenex, Torrance, USA) (Scaloni et al. 1998). A step gradient of solvent B (40% ACN, 0.1% TFA) in solvent A (20% ACN in 0.25% TFA) was used: from 50 to 54% B over 16 min, from 54 to 60% B over 4 min, and from 60 to 70% B over 33 min, at a flow rate of 1 ml/min. Peaks were detected at 220 nm. Purified globin chains were then hydrolyzed with trypsin (1/50 w/w) in 50mM NH_4HCO_3, pH 8.0, containing 10mM dithiotreitol for 3 h, at 37°C.

Globin chain tryptic digests were directly analyzed by MALDI-TOF-MS as reported above, using α-cyano-4-hydroxycinnamic (10 mg/ml in 50% ACN, 0.1% TFA), or by liquid chromatography-electrospray ionization-linear ion trap-tandem mass spectrometry (nLC-ESI-LIT-MS/MS) with a LTQ XL mass spectrometer (Thermo Fisher Scientific Inc.), equipped with a Proxeon nanospray source connected to an EASY-nanoLC (Proxeon–Thermo Fisher Scientific Inc.). Peptides were resolved over a C_{18} column (100 × 0.075 mm, 3 μm) (Thermo Fisher Scientific Inc.) using a linear gradient from 5 to 60% of solvent B (ACN, 0.1% formic acid) in solvent A (0.1% formic acid) over 65 min, at flow rate of 300 nl/min. MS and MS/MS data were searched with MASCOT software (Version 2.3, 2010) against a home-made database of all bovine globin sequences.

PCR amplification and bioinformatics. DNA fragments corresponding to *HBA* (mixed amplicons from *HBA1* and *HBA2* genes) and *HBB* genes were amplified by iCycler (BioRad Laboratories, Hercules, USA) using the primers reported in Supplementary Table S1 (see the eletronic version). A typical PCR reaction mix (25 μl) comprised: 50 ng of genomic DNA, 1X PCR Buffer (Promega, Madison, USA), 2.5mM $MgCl_2$, 5 pmol of each primer, dNTPs each at 200μM, 1 U of *Taq* DNA Polymerase (Promega). PCR was performed under the following thermal conditions: 95°C for 4 min, 35 cycles at 95°C for 30 s, annealing temperature according to the amplicon for 30 s, 72°C for 30 s, and the final extension at 72°C for 5 min. Product specificity was confirmed by ethidium-bromide-stained

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1.5% agarose gel electrophoresis. PCR products from 10 informative samples already screened by MALDI-TOF-MS were sequenced on both strands in outsourcing at MWG (www.eurofinsgenomics.eu/sequencing). Homology searches and multiple alignments were obtained with DNAsis-MAX (Hitachi, San Francisco, USA).

HBA1 and HBA2 gene discrimination and allele detection by Hin6I PCR-RFLP. Using the bovine EMBL ID: AC_000182.1 as reference and exploiting the differences of the gene promoter sequences, specific primers were designed in order to discriminate *HBA1* and *HBA2* genes (Table S1 – for the supplementary material see the eletronic version). Product specificity was confirmed by 2.5% agarose gel electrophoresis stained with ethidium-bromide.

A PCR-RFLP method was developed in order to clarify which of these two genes is the carrier of the SNP *c.79G>A* responsible for the p.Ala27Thr variant. Digestion of 15 ml of each amplicon was accomplished with 10 U of *Hin*6I endonuclease (5'...G↓CGC...3') (Thermo Fisher Scientific Inc.) overnight at 37°C. The digestion products were analyzed directly by electrophoresis in 2.5% agarose gel in 0.5X TBE buffer and stained with ethidium-bromide.

RESULTS AND DISCUSSION

Integrated proteomic approach based on PAGIF, AUT-PAGE, RP-HPLC, and MALDI-TOF-MS allowed the identification of 4 phenotypic groups among the 68 individuals that were subjected to screening for genetic polymorphisms (Figure 1, Table 1). The most abundant one (group a) (about 76% of total animals) showed the simultaneous occurrence of the normal α-globin chains (GenBank ID: CAB56827.1 and CAB56828.1 for the proteins encoded by *HBA1* and *HBA2* genes, respectively) and β^A-globin chain (GenBank protein ID: BAJ05100.1 encoded by allele *A* of *HBB* gene). The remaining groups presented these globins in their eventual association with allelic/variant forms. The presence of the variant B of the β-globin (β^B) (GenBank protein ID: BAJ05099.1), already reported in other cattle breeds (Braend 1971; Scaloni et al. 1998; Pieragostini et al. 2010), was assessed in 13 Agerolese individuals (groups b and c) (about 19% of total animals). β^B-globin

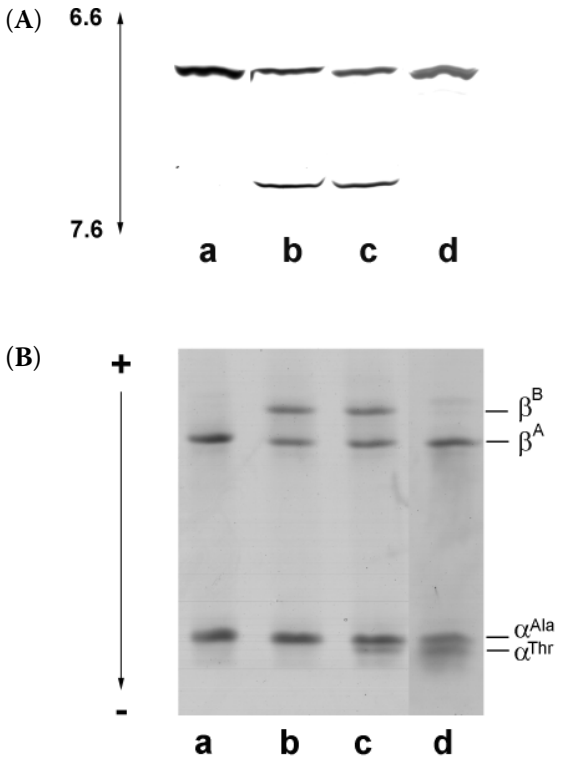

Figure 1. Hemoglobin profiling of samples from the Agerolese breed. Comparative analysis was performed by PAGIF (**A**) and AUT-PAGE (**B**). Four phenotypic groups were recognized, namely a, b, c, and d. Tentative assignment to specific α- and β-globin chains was performed according to the observed electrophoretic mobility and the measured molecular mass (Table 1). The novel α-globin variant is indicated as α^{Thr}

Table 1. MALDI-TOF-MS analysis of globin chains from the four phenotypic groups observed in the Agerolese cattle breed. Molecular mass (MH^+) average values are reported

Group	Number of animals (%)	α^{Ala}	α^{Thr}	β^A	β^B
a	52 (76.5)	15 053.7	–	15 955.2	–
b	12 (17.6)	15 053.7	–	15 955.9	15 979.4
c	1 (1.5)	15 053.5	15 083.4	15 954.8	15 979.4
d	3 (4.4)	15 053.4	15 083.7	15 955.6	–
Theoretical MH^+		15 054.2	15 084.2	15 955.4	15 980.3

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Table 2. Genotype distribution and allele frequencies detected for α- and β-globin variants in the Agerolese cattle breed

α-Globin (HBA2)						β-Globin (HBB)					
genotype				allele frequency		genotype				allele frequency	
$\alpha^{Ala}\alpha^{Ala}$	$\alpha^{Ala}\alpha^{Thr}$	$\alpha^{Thr}\alpha^{Thr}$	total	α^{Ala}	α^{Thr}	β^{AA}	β^{AB}	β^{BB}	total	β^{A}	β^{B}
64	4	–	68	0.970	0.030	55	13	–	68	0.904	0.096

α^{Ala} = normal (wild type) variant p.Ala27 (c.79G) in HBA2, α^{Thr} = mutation in HBA2 – p.Thr27 (c.79A), β^{A} = allele *A*, β^{B} = allele *B*

(p.Gly15Ser; p.Lys18His; p.Lys119Asn) occurred in heterozygosity with the β^{A} counterpart (Table 2). On the other hand, a novel α-globin variant was evidenced in 4 animals from different farms (about 6% of total individuals), which presented it together with α-, β^{A}-, and β^{B}-globin chains (group c), or with α- and β^{A}-chains (group d), respectively. This new variant was highlighted by an evident band in AUT-PAGE (Figure 1) and a unique mass shift of +30 Da (with respect to the normal counterpart) during MALDI-TOF-MS analysis of blood lysates (Table 1). PAGIF analyses were unsuccessful in clearly resolving the novel α-globin variant from the normal counterpart or the hemoglobin tetramers (Figure 1A), as well as RP-HPLC analyses (data not shown). AUT-PAGE analysis of all individuals bearing this new species revealed that the normal and the variant α-globin occurred with a 70 : 30 quantitative ratio.

MALDI-TOF peptide mass mapping experiments on isolated globin chains confirmed the nature of the α-, β^{A}-, and β^{B}-globins observed in groups a–d, and assigned the mass shift observed in the intact, novel α-globin Agerolese variant to a specific peptide region. In particular, MALDI-TOF-MS analysis of this partially purified variant showed the presence in the spectrum of two signal pairs, both spaced by a Δm = +30 Da, the first at *m/z* 1529.75 and 1559.76, the second at *m/z* 2043.03 and 2073.03. They were assigned to peptides 18-32 and 13-32 in the normal and variant α-globins, respectively; in fact, signals at *m/z* 1529.75 and 2043.03 were ascribed to the co-occurrence of the normal counterpart within the purified α-globin

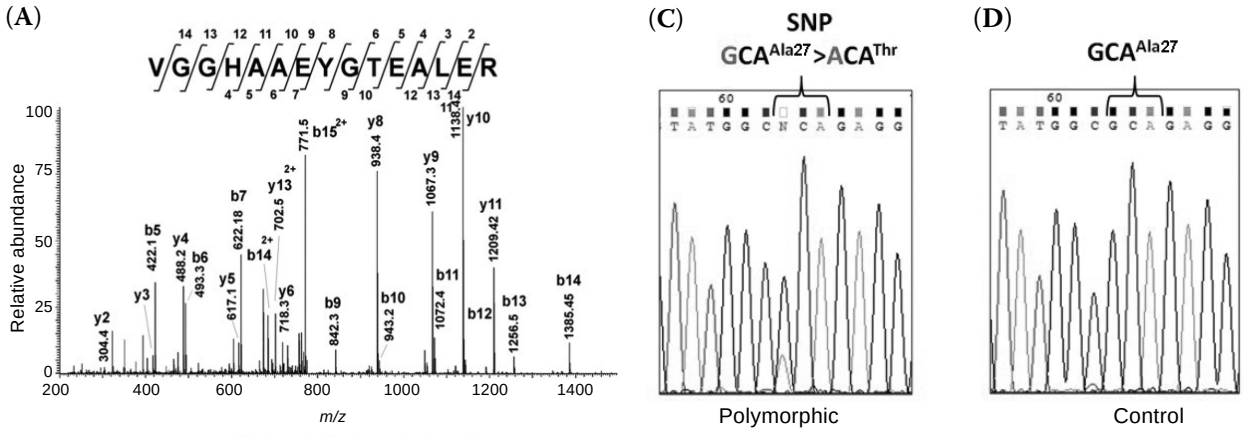

Figure 2. Assignment of the mutated amino acid and base in the variant Agerolese α-globin and the corresponding *HBA* gene. (**A**) Tandem mass spectrometric analysis of the peptide 18-32 bearing the p.Ala27Thr substitution. (**B**) Tandem mass spectrometric analysis of the peptide 18-32 in normal α-globin. (**C**) DNA sequencing chromatogram showing the nucleotide transition in the codon responsible for the p.Ala27Thr substitution. (**D**) DNA sequencing chromatogram corresponding to the same codon mentioned above but from an animal carrier for the wild type *HBA* gene

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Agerolese variant. nLC-ESI-LIT-MS/MS analysis of the mutated peptide 18-32 definitively assigned the observed mass shift to the amino acid substitution p.Ala27Thr, as demonstrated by the fragment signals y_6 and y_5 at *m/z* 718.3 and 617.1, respectively, and b_{10} and b_9 at *m/z* 943.2 and 842.3, respectively (Figure 2A). Ascertained p.Ala27Thr replacement also accounted for the mass value measured for the whole α-globin variant (15083.7 Da), which was here designated as α^{Thr} (according to standard nomenclature), excluding the occurrence of additional substitutions at other sites. Finally, nLC-ESI-LIT-MS/MS analysis of the corresponding tryptic digests definitively proved the nature of the other α- and β-globin chains detected in this study.

To confirm mass spectrometric data, phenotype variability was also investigated at genetic level within the α- and β-globin genes (*HBA1*, *HBA2*, *HBB A*, and *HBB B*, respectively). Three DNA fragments were amplified and sequenced. The comparison of the obtained sequences showed a total of 4 polymorphic sites (Figure 2C,D). The first SNP (*c.79G>A*, taking *HBA1* sequence EMBL AJ242797 as reference) was located at the nucleotide 79 of exon 1 of the *HBA* genes (either *HBA1*, or *HBA2*, or both); it is responsible for the amino acid substitution p.Ala27Thr mentioned above (Figure 2C). However, the result of sequencing did not clarify the specific location of this SNP on *HBA1* or *HBA2* gene. Therefore, a specific amplification protocol followed by a RFLP digestion was developed to overcome this problem. *HBA1* and *HBA2* genes were discriminated by the size of the promoter/exon 1 amplification of the same DNA sample. The first gene (*HBA1*), in fact, produced an amplicon of 334 bp, whereas *HBA2* was characterized by a fragment 283 bp long (Figure 3). Both amplicons were then digested by *Hin*6I, which removes a restriction site in presence of adenine and allows the identification of both *c.79G>A* alleles. In fact, one band of 164 bp is undigested for the *AA* carriers, whereas it is further restricted into two fragments of 133 bp and 31 bp for the *GG* carriers. Smaller restriction fragments are also generated for both amplicons but these are not important for the discrimination of the genotypes.

All the informative samples carriers of the p.Ala27Thr variant resulted in homozygous *GG* at the *HBA1* gene and heterozygous *AG* at *HBA2* gene (Figure 3). This result clearly indicates that the molecular event responsible for the p.Ala27Thr globin variant was realized at the *HBA2* gene.

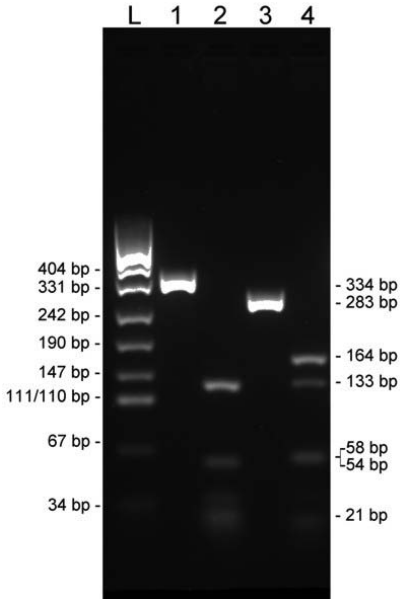

Figure 3. *HBA1* and *HBA2* gene discrimination pattern and PCR-RFLP digestion profile for the *c.79G>A* allele detection by *Hin*6I (5'...G↓CGC...3'). Line 1 = *HBA1* undigested amplicon (334 bp), line 2 = *HBA1 GG* homozygous sample, digested, line 3 = *HBA2* unrestricted DNA fragment (283 bp), line 4 = *HBA2 AG* heterozygous sample, digested, line L = pUC19 DNA/*Msp*I (*Hpa*II) Marker, 23 (Thermo Fisher Scientific Inc., Waltham, USA)

The SNP *c.79G>A* represents a novel variant for the *HBA2* gene never characterized so far, whereas the remaining 3 non-synonymous SNPs (*c.43G>A*, *c.52A>C*, and *c.357G>C*, numbering relative to the EMBL ID: X00376) observed in the *HBB* gene are responsible for amino acid replacements (p.Gly15Ser, p.Lys18His, and p.Lys119Asn, respectively; Figure S1 – for the supplementary material see the eletronic version) already reported in literature (Bangham and Blumberg 1958; Schroeder et al. 1967; Braend 1971; Pieragostini et al. 2010).

By comparing the amino acid sequence of the α-globins found in Agerolese cattle with that of other Bovidae (data not shown), we may note that the differences in the primary structure of these globins are mainly located at six well-defined positions: 20, 24, 72, 90, 116, and 132. The p.Ala27Thr at the α-globin reported here is the first example of a bovine α-globin variant where the amino acid substitution is located at position 27. A visual inspection of the 3D structure of bovine hemoglobin (PDB code 2QSS) revealed that the side chain of the amino acid at position 27 is located at the pro-

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tein surface pointing out toward the solvent, in a region that is far from the heme groups, and is not involved in intra- and/or inter-dimer contacts, or in binding to allosteric effectors (data not shown). On this basis, no functional consequences of the p.Ala27Thr substitution can be predicted according to known site mutation determinants (Storz and Moriyama 2008; Weber and Campbell 2011). The same amino acid replacement at the same position has already been detected in α-chain globin variants from goat breeds present in southern Italy (Puglia and Sardegna) (Pieragostini et al. 2005; Pirastru et al. 2009) and from human families in Caserta (Campania) (Lacerra et al. 2004). Either in Agerolese bovine, Apulian/Sardinian goat, and human Casertian α-globin variants, this substitution involves a CpG dinucleotide that is considered as a mutation hotspot (Perutz 1990). In all these mammalian variants, this silent mutation needed dedicated analytical procedures for its detection, since it was not visible by conventional electrophoretic methods.

Regarding quantitative polymorphism, a reasonable estimate of the level of expression from the different α-globin haplotypes can be deduced by considering the ratio (70 : 30) of the major α-globin with the novel variant here identified, as evaluated by direct quantification of the corresponding chains in AUT-PAGE. These data are suggestive of a heterozygous condition where the wild type variant ($\alpha^{Ala}\alpha^{Ala}$) is in combination with the novel variant ($\alpha^{Ala}\alpha^{Thr}$). Such a result is also confirmed by the molecular genetic data (PCR-RFLP), where 3 out of 4 *HBA* alleles (in genotypes *HBA1 GG* and *HBA2 AG*) were carriers of guanine representing a potential 75% of the protein expression vs only one allele (in genotype *HBA2 AG*) was a carrier of adenine representing the remaining 25%. Being tightly linked to a well-defined quantitative polymorphism, the Agerolese breed represents the third example of α-globin gene polymorphism observed in *Bos taurus* where the *HBA2* is the carrier of the DNA mutation (*c.79G>A*) encoding for the new rare α^{Thr} variant form in position 27. Additional studies are now in progress to assay the occurrence of this novel α-globin variant in a broader Agerolese population and in other, more diffused, bovine breeds.

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