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Evidence of over-dominance for sickle cell trait in a population sample from Buenaventura, Colombia
Diana Carolina Ortega, Cristian Fong, Heiber Cárdenas and Guillermo Barreto
Evidence of over-dominance for sickle cell trait in a population sample from Buenaventura, Colombia

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Sickle cell disease is a hereditary disease caused by a hemoglobin variant, known as hemoglobin S. This hemoglobin variant has a high frequency in areas where malaria is prevalent, because heterozygous genotype has a protective action against malaria infection. We evaluated evidence of over-dominance or deviation from Hardy-Weinberg equilibrium for the HbS gene in Buenaventura, an afro-descendent population with actual medium endemicity of malaria, located in the Colombian Pacific Coast. 820 healthy individuals were analyzed (from eight months to 80 years old) from Buenaventura, Colombia, and the β-globin locus was genotyped by PCR-RFLP. It was analyzed to establish deviation from Hardy – Weinberg and selection coefficient. Hemoglobin S frequency was 3.1% and the population was found in Hardy-Weinberg equilibrium. However, the selection coefficient for allele S decreased its value of 1 (under 12 years old) to 0.625 (13 to 17 years old), similar to the selection coefficient of allele A (s=0.61). Although the total population found in H-W equilibrium, there was a decrease in the coefficient of selection on hemoglobin S in the range of 13 to 17 years old, which is within the age range of the greatest malaria infection in this population. This may be an indication of a selection to the S over-dominance hemoglobin possibly, at least in this age group of the population.

**Keys words**: Hemoglobin S, Buenaventura, malaria resistant, over-dominance, fitness, selection coefficient.

INTRODUCTION

Sickle cell anemia is an autosomal recessive disease, caused by a mutation on beta-globin gene (HBB). This mutation is caused by a transversion (A→T) in the twentieth nucleotide, resulting in the change of glutamic acid to valine in the six codon in the globin (Ingram, 1956). This mutated hemoglobin is also known as S hemoglobin (HbS) and its presence characterizes sickle cell disease (Pauling et al., 1949). When this mutation is in homozygous state (Hb SS), it is called denominated Sickled Cell Anemia (SCA) and when it is in heterozygous state (Hb AS), it is known as sickle cell trait. The individuals with sickle cell anemia present the most severe clinical cases such as: vaso-occlusive crisis, aplastic crisis, hyper hemolytic crisis, splenic sequestration, bacterial infections (in the first months of life), among others. Individuals with sickle cell trait are usually asymptomatic, because of the reasonable amounts of HbA (Ashley-Koch et al., 2000).

The presence of HbS is a well-known example of over-dominance or heterozygous advantage, because AS
genotype provides protection against malaria. However, natural selection can alter that balance. When the selection favors heterozygote (over-dominance), the gene frequency tends towards an intermediate equilibrium value in which both alleles remain in the population.

Even without mutation, in the cases of over-dominance, the alleles must affect two fitness components in opposite directions. In sickle cell anemia, the homozygote’s fitness reduces through a component serious anemia, while the AA homozygote’s fitness reduces due to its susceptibility to malaria and its complications (Falconer and Mackay, 1996).

Haemoglobin disorders contribute to equivalent 3.4% of mortality in children below five years worldwide or 6.4% in Africa (Modell and Darlison, 2008). In 1994, it was reported that life expectancy of those affected by sickle cell disease is not longer than 50 years and 1% children die before they reach the age of 3 (Platt et al., 1994). Nevertheless, this gene is present in Africa and its descendants in America with much higher frequencies than it would be expected due to the compensation between mutation and selection against homozygotes (Haldane, 1949).

Increased homozygous frequencies converge with increased malaria incidence, according to Haldane in 1949; later, Allison (1954) suggested that the presence of these frequencies as well as the maintenance of the sickle-cell polymorphism might have been caused by heterozygous advantage (AS). This is because they have great resistance to Plasmodium infection, which is the causative agent of malaria and this is known as “the hypothesis of Haldane” (Haldane, 1949; Allison, 1954; Piel et al., 2010).

Recently Piel et al. (2010) have contributed to the first geographic and quantitative confirmation of the hypothesis of malaria on a global scale. While comparing the frequencies of HbS with worldwide frequencies of malaria endemicity, they found that these relationships are considerably a match in the African continent. However, this relationship is still not clear in America and Asia (Piel et al., 2010). Recently, a study estimated that the global number of neonates affected by HbS in 2010 included 5,476,000 AS and 312,000 SS (Piel et al., 2013). The disease occurs in 1 in every 500 African-American births and 1 in every 1000 to 1400 Hispanic-American births (WHO, 2015).

The maximum predicted frequency in South America to HbS was about 5% (Piel et al., 2013). In Brazil, a HbS frequency of 0.9 to 14% has been reported (Cardoso et al., 2012; De Mello Auricchio et al., 2007; Bandeira et al., 1999), and in Venezuela, up to 7% HbS has been reported (Salazar-Lugo, 2004).

In Colombia, there have been records of hemoglobinopathies frequencies between 2.4 and 14% in different regions (Silva et al., 1998; Bernal et al., 1995; Espinel and Valenzuela, 1991). In the city of Buenaventura, Moyano and Méndez kept records of AS heterozygous prevalence of 7% (Moyano and Méndez, 2005). In 2010, hemoglobinopathies frequencies for a total of 399 infants was revealed, of which 5.8% were heterozygous AC followed by 4.8% heterozygous AS. No presence of homozygous SS was recorded (Bernal et al., 2010).

In the Colombian Pacific Coast, sickle cell disease is an issue of public health. Whether the HbS frequencies are influenced by environmental factors is still unknown. One of those factors can be malaria.

In this study, a healthy population sample was taken from Buenaventura in the Colombian Pacific coast in order to evaluate whether HbS might be favored by natural selection in this population.

MATERIALS AND METHODS

Sample taking in the studied population

Blood samples (4 ml) were collected through venipuncture in ethylenediaminetetraacetic acid (EDTA) tubes. A random stratified sampling of 820 people without visible clinical condition participated in the study; they consisted of both genders and with a age range of 8 months to 80 years old (mean = 35.04 years). Samples corresponding to the 12 zones of the City of Buenaventura (Figure 1), an afro-descendent population with actual medium endemicity of malaria, in Colombian Pacific Coast, were included. Visitors or residents who could not prove their original birth in the city were included in this study. Likewise, participants without any kinship relationship with themselves in the same generation (was sampled one only sibling, cousin, grandchild or a family uncle) and members of up to 2 generations per family were allowed.

All participants authorized their entrance to the study by signing an informed consent. This project has the approval of the ethics committee of the Universidad del Valle (Act No. 02-013, 288-012 code).

Molecular diagnosis

DNA was extracted through the method of “salting out” (Miller et al., 1988). DNA amplification was carried out according to the methodology provided by Swee Lay Thein, King’s College Hospital in London. The amplification mixture (25 µl of volume): consisted of 50 ng DNA, 1X Taq Buffer, 1.5 mM MgCl₂, 0.1 mM of the mixture of the four deoxynucleotides triphosphate (Fermentas, Vilnius, Lithuania), 1.8 uM primer (forward 5’-GGG GGC CTG AAA ATA GTG A-3’ and reverse 5’-GGA AGG AAG ATC AAC AAA GAA GGT C-3’) and 0.5 units of Taq DNA polymerase (Bioline, London, UK). The amplification program began with a warm up of 5 min at 94°C for denaturation, 30 cycles continuing as follows: denaturation for 30 s at 94°C, followed by annealing at 62°C for 1 min; finally, an extension at 72°C for 3 min.

The amplified fragment was 571 bp. This was digested with 2 units of restriction enzyme Ddel (Promega, Madison, WI, USA) following the manufacturer’s directions for use. The digestion was conducted for 5 h at 37°C. Subsequently, the resulting DNA fragments were separated by gel electrophoresis on 8% polyacrylamide and were examined by staining with silver nitrate. Homozygous individuals (SS) affected showed a single band of 308 bp; the heterozygous (AS) three bands, 308, 201 and 107 bp, and homozygous individuals (AA) showed 2 bands of 201 bp and 107 bp.
Figure 1. Map of the urban zones of the city of Buenaventura. It shows the Department of Valle del Cauca from the city of Buenaventura, Colombia, and South America country.

Data analysis

The calculation of allele and genotype frequencies, the comparison of the respective genotype frequencies observed and expected ($\chi^2$ test) and the genetic structures among communities (through AMOVA) were conducted with the statistical software GenALEX version 6 (Peakall and Smouse, 2006). The age groups management of data was carried out by the exact test of population differentiation of Raymond and Rousset (Raymond and Rousset, 1995) in the statistical package Arlequin 3.1 (Excoffier et al, 2005). Selection coefficients and fitness for each case were calculated using the formulas proposed by Falconer and Mackay (1996).

RESULTS

A total of 820 individuals were analyzed; there were 214 under-age (18 years old minors) and 606 adults. In the whole sample, 770 (94%) had AA (healthy) genotype, 49 (5.9%) had AS (sickle cell trait) and only one (0.1%) individual had SS (sickle cell anemia), with HbS allele having 3.1% frequency. The needed time to get the observed frequency was calculated from a hypothetical initial frequency of 0.25 using the equation $t = (1/q_0 - 1/q_t)$; thus the population would need 28.25 generations to reach the observed frequency (Table 1).

The total population was found in Hardy-Weinberg equilibrium, $\chi^2 = 0.656$ with $p = 0.7978$ (Table 1); no significant differences were observed when the population of neonates was compared, as reported by Bernal et al. (2010), with the general population in this research ($p = 0.51070$). In this study, no significant differences were found when both populations were compared; minors and neonates ($p = 0.37830$). Results are not shown.

Before we did the respective analysis of evidence of selection, we divided the groups of children and adults to know if they were homozygote and could be worked as such. The group of children was divided into three subgroups: first, 8 months to 5 years; second, 6 years to 12 years; and third, 13 to 17 years (to embrace the growth phases of childhood and adolescence). Among them, statistically significant differences were found in the
allele frequencies ($p = 0.00355$) and therefore were evaluated as separate groups (Table 2). In the group of adults, no differences between age ranges were found ($p = 0.34115$) and they were evaluated as a single group.

Every under-age group was compared with an adult group except the one with children of 13 and 17 years old group. This was compared with the 8 months to 5 years old group to evaluate biological fitness, selection coefficient and $q$ value of equilibrium for balanced selection (Table 2). An increase in efficiency for the heterozygous and $q$ increase in equilibrium were observed ($q_0^* = 0.031$), starting with a value of $q_{eq}^* = 0.0642$ for case 1 and ending with a value of 0.49 for case 3. It was also seen how the selection coefficient for allele S decreased its value of 1 (cases 1 and 2) to 0.625 (case 3) similar to the selection coefficient of allele A ($s_i = 0.61$). To validate these operations, we added, for every case, 1 to the absolute genotype frequencies of SS for the minors (Table 2).

The matching between the frequency of genotype AS for the average age of each age range was conducted (Figure 2). The graph shows a heterozygous genotype increase at the average age of 15 years, having its peak at the age of 22 (frequency = 0.0918). From there, there is a decrease in the frequency of the genotype and then a rise from 46 years old (frequency = 0.07) to a frequency of 0.051 at an age of approximately 70 years (Figure 2).

Finally, the genetic structure was observed between the zones ($p = 0.001$) studied by analysis of molecular variance (AMOVA), where the variation between zones was 3%; while the variation within zones was 97%. The $\Phi$PT value was 0.030 and $N_m = 8.104$ (Table 3).

**DISCUSSION**

The population studied was found in Hardy-Weinberg equilibrium, like that of Bernal's study of neonates in 2010. It was done in three different health institutions of the City of Buenaventura between 2007 and 2008 (Bernal et al., 2010). Unlike them, in this study a recessive homozygous individual was found for HbS (genotype SS) gene.

As the different individuals were evaluated in age range, a possible different selection effect was observed among them. In comparing the group of children between 13 and 17 years old with those between 8 month and 5 years old, a decrease of the coefficient of selection against gene HbS and an increase of $q$ value in equilibrium were observed (Table 2). Although in the adult groups age range there were not found significant differences in HbS frequencies, an increase in heterozygous genotype was seen in particular ages: 18-26 years old and 46-60 years old (Table 3).

The observed decrease in heterozygous individuals and homozygous AA and SS during early ages could be because these are affected by children's illnesses. Buenaventura has a substantial rate of child mortality. According to 2004 data, death was observed in 36/1000

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**Table 1.** Genotype and allelic frequencies and HW equilibrium for the population of 820 individuals in Buenaventura.

<table>
<thead>
<tr>
<th>Frequency</th>
<th>Genotype</th>
<th>HbS gene frequency</th>
<th>H-W equilibrium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>AS</td>
<td>SS</td>
</tr>
<tr>
<td>8 months to 5 years old</td>
<td>0.9444</td>
<td>0.0556</td>
<td>0</td>
</tr>
<tr>
<td>Minors 6 to 12 years old</td>
<td>0.9796</td>
<td>0.0204</td>
<td>0</td>
</tr>
<tr>
<td>13 to 17 years old</td>
<td>0.8548</td>
<td>0.1290</td>
<td>0.0161</td>
</tr>
<tr>
<td>Adults</td>
<td>0.9406</td>
<td>0.0594</td>
<td>0.0000</td>
</tr>
<tr>
<td>Total</td>
<td>0.9390</td>
<td>0.0598</td>
<td>0.0012</td>
</tr>
</tbody>
</table>

$N_g$ generations $= (1/q^* t) - (1/q^0 t) = 28.2580$

$q^* = 0.031; q^0 = 0.025$.

**Table 2.** Calculated coefficients selection and fitness for three age subgroups studied.

<table>
<thead>
<tr>
<th>Case</th>
<th>1) 8 months old to 5 years old vs. adults</th>
<th>2) 6 to 12 years old vs. adults</th>
<th>8 months old to 5 years old vs. 13 to 17 years old</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td>AA</td>
<td>AS</td>
<td>SS</td>
</tr>
<tr>
<td>Fitness relative</td>
<td>1.0144</td>
<td>1.0891</td>
<td>0.0000</td>
</tr>
<tr>
<td>Fitness relative to SA</td>
<td>0.9314</td>
<td>1.0000</td>
<td>0.0000</td>
</tr>
<tr>
<td>Coefficient selection</td>
<td>$s_1 = 0.0686$</td>
<td>$s_2 = 1.0000$</td>
<td></td>
</tr>
<tr>
<td>$q$ value of equilibrium</td>
<td>$0.0642$</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
children under 5 years old and in less than 1 year old (31.4/1000). The leading causes of deaths in infants are: respiratory disorders (28.14%), congenital malformations (10.18%), other conditions originating from the perinatal period (9.37%), and acute respiratory infections (22%) (Diagnóstico socio-económico de Buenaventura, 2015). Therefore, under these circumstances, healthy heterozygous and homozygous children are equally susceptible; except when cultural customs of preventing mosquito like using of mosquito nets for little children are used to reduce the incidence of malaria (Méndez and Carrasquilla, 1995). This shows the HbS allele can reach its highest value of equilibrium \((q = 0.494)\) in 13 to 17 years old group and its lowest value in 8 months to five years old \((q=0.064)\) group. This HbS allele equilibrium value during adolescence can be related with an acquired immunity when individuals begin to manifest AS. In malaria zones, there are 9 years old AS children with a minor risk of developing symptomatic malaria than 2 years old children. This suggests an acquired defense mechanism (Williams et al., 2005).

Moreover, an increase in frequency of heterozygote children is observed. They reach a frequency between 7.3 to 9.0% (Figure 1). These peaks suit the age range where malaria is lethal in Buenaventura, which is between 15 to 60 years old (Méndez and Carrasquilla, 1995). The frequency of heterozygous AS decreases in the range of 30 to about 40 years old. This may be due to the acquired resistance to malaria in people after repeated episodes of the infection. This immunity depends on the genotype acquired not present in the beta-globin. Therefore, individuals between 30 and 40 years old of AA genotype have equal probability of survival than heterozygous AS (Gong et al., 2012), implying a higher proportion of AA in this age range. This phenomenon would also explain why there was no variation in the selection of coefficient in adults.

In the infant population of Buenaventura, especially those older than 6 years, there is evidence of selection effect (Table 2). From 6 to 12 years old (case 2) group, the effect on the A allele is remarkable, since there is a significant increase in the selection of this coefficient with respect to that in 8 months to 5 years old group (case 1). This also is reflected in the q equilibrium, again according to case 1. Likewise, in 13-17 years old (case 3) group, there is an evidence of selection action but this time on the

Table 3. AMOVA and genetic structure found for the 12 zones of Buenaventura city.

<table>
<thead>
<tr>
<th>Level</th>
<th>df</th>
<th>Sum of squares</th>
<th>Mean squares</th>
<th>Variance</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among zones</td>
<td>11</td>
<td>1.988</td>
<td>0.181</td>
<td>0.002</td>
<td>3</td>
</tr>
<tr>
<td>Within zones</td>
<td>808</td>
<td>47.840</td>
<td>0.059</td>
<td>0.059</td>
<td>97</td>
</tr>
<tr>
<td>Total</td>
<td>819</td>
<td>49.828</td>
<td>0.061</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>

\(F_{PT} = 0.030\) \(P = 0.001\) \(Nm = 8.104\)

Figure 2. Graphic of the relationship between the average age \((X)\) and genotype SA frequency \((Y)\). Polynomial trend line type of order 6 show relationship between these two variables.
S allele, where there is a decrease in the allele coefficient of selection. The selection acts in a less severe way on this range of age allele (Table 2).

Although effects of natural selection were found in the children population, the total sample was found in Hardy-Weinberg equilibrium. These results can be an indication of the presence of a founder effect and/or bottleneck in the population. Buenaventura town, during late nineteenth and early twentieth centuries, suffered several catastrophic events: yellow fever and smallpox wiped out nearly a fifth of the population, a series of fires destroyed the few existing buildings, and in 1931 another fire damaged important areas of the city (Pérez, 2007). These events made the population to go through several bottlenecks, where drift prevails as evolutionary force. Under these conditions, selection effects will be superimposed on the drift, in addition, because the drift’s effects were permanent in the population after the bottlenecks (Luzzato, 2012).

The action of selection in favor of HbS is not observed in the whole population. In fact, the action of selection was reflected in only one segment of the population and not in all of it. Moreover, the frequency of HbS decreased faster in the population than expected: if the selection was only taken into account, the time required to achieve the current rate would be 262 generations (706 years) (Table 1). This time period is greater than the period of time since the creation of the first settlements in Buenaventura (~470 years), which leaves genetic drift as a most likely mechanism to explain the current frequency of HbS in Buenaventura.

Indeed, Buenaventura recently lived or might still be living a small phenomenon founder effect. The genetic structure is observed among the zones of the city (Table 3). This is a clear indication of the recent formation of these: until just 60 years ago, Buenaventura was concentrated on the Cascajal Island and the "mainland region" was gradually colonized.

These new subpopulations could take subsamples of the genetic diversity of the population "mother" (the town located on the Cascajal Island), a phenomenon that would add to the diversity which people bring from different regions of Colombia’s Pacific Coast; those who have come and are still coming to Buenaventura and those who are located in this island.

There are clear indications that there are dynamics of change in the frequency of HbS in the town of Buenaventura depending on the frequency of malaria infection. This could indicate this module disease and can be a factor of pressure on the HbS in Buenaventura. However, a confirmation data of a case of over-dominance could be tested if there are significant differences in density of the pathogen between heterozygote (AS) and AA people. A quantitative analysis of Plasmodium falciparum using molecular method like q-PCR or reverse-transcriptase PCR rounds up our results. Now, knowing the condition of over-dominance for gene HbS in Buenaventura, the risk for the occurrence of new cases of sickle anemia in the population (as well as the most susceptible age groups) is clearer, as long as the pressure of selection is maintained for heterozygous individuals.

Finally, although AS individuals are usually asymptomatic, it is important to give them genetic counseling and to educate them about their status in order not to give birth to homozygous children and to avoid psychosocial issues such as stigmatization and misunderstanding because of their status as carriers.

Conflicts of interest

The authors have not declared any conflict of interest.

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