Sickle Cell Trait, Malaria and Sensorineural Hearing Loss—A Case-Control Study from São Tomé and Principe

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Abstract

Background: Hearing loss is a problem with higher incidence in South Asia, Asia Pacific and sub-Saharan Africa. In these countries there is also associated history of anemia and malaria.

Objective: This study aims to identify a putative role of Beta globin mutation - sickle cell trait and HL in São Tomé and Principe population.

Methods: A retrospective case-control study of a convenience sample was collected during Otolaryngologist Humanitarian Missions in São Tomé and Principe. Control group includes individuals with normal hearing in both ears, and the case group has participants presenting bilateral or unilateral HL. It was evaluated the potential risk factors and sickle cell trait with HL, as well self-report of malaria infection, consanguinity, familial history of HL. The HbS gene point mutation (Glu6Val) was determined by PCR-RFLP.

Results: Our results showed a statistical significance between HL - oral language and self-report of HL. Taken altogether, our data did not reveal association between sickle cell trait and HL. However, a statistical association between HL and self-report of malaria was found.

Conclusion: No association between sickle cell trait and the high prevalence of HL was found. Self-report of Malaria was found as a risk factor for the development of HL in São Tomé and Principe population. The multifactorial profile of HL shall not exclude the relevance of other etiologic factors than Malaria to justify the high prevalence of HL in São Tomé and Principe and further investigation must be applied.

Keywords Hearing loss; Sensorineural hearing loss; São tomé and principe; Sickle cell trait; Sickle cell disease; Malaria; Hemoglobinopathies

Introduction

More than 360 million people in the World have disabling hearing loss (HL). According to new World Health Organization (WHO) Global Estimates on prevalence of the HL [1,2], there is a higher incidence in South Asia, Asia Pacific and sub-Saharan Africa [1-3]. Sub-Saharan Africa holds 10% of the world’s population and two thirds of the world’s least developed nations.

More than 1.2 million of the children living in sub-Saharan Africa aged 5 to 14 years old have moderate to severe bilateral HL [3]. The consequences of hearing problems are well known. HL in children can result in developmental delays and lead to significant inability to engage in oral/aural communication.

Amenia has been proposed as an etiologic factor in sensorineural deafness for many years, but there is little supporting evidence [4]. In these African countries there is a high prevalence of anemia and this could be associated with infections, hemoglobinopathies or stunting [5].

Several gene mutations and polymorphisms in the human hosts confer survival advantage and have increased in frequency through natural selection over generations. These include hemoglobinopathies like Sickle Cell Disease (SCD), thalassemias and glucose-6-phosphate dehydrogenase (G6PD) deficiency. Sickle Cell Disease (SCD) refers to a group of symptomatic disorders associated with a specific mutation on the Beta globin (HBB) gene [6]. Sickle hemoglobin (Hbs), a structural variant of normal adult hemoglobin, results in the substitution of glutamic acid with valine in the sixth position of β-chain of the hemoglobin (β6Glu-Val) [7]. HBS is the most common pathological hemoglobin variant worldwide [7].
Several studies have suggested that HbS has an effect of protection against Plasmodium falciparum, the etiologic agent of malaria [6], showing that heterozygous people carrying the sickle-cell trait (HbAS) are protected against severe malaria (prevalence of HbAS, in some populations, is above 90%) [6,8].

This mechanism act as natural selection and is co-responsible for the high prevalence of HbS in malaria endemic regions as a result of natural selection over generations [9].

The red cells of individuals with the mutant homozygous gene (HbSS) become sickle shaped in low oxygen tension pressure with reduced oxygen-carrying capacity. The basal turn of the cochlea is particularly sensitive to anoxia, due to the high oxygen consumption rate of the stria vascularis and poor capacity for anaerobic metabolism. In this cases, SNHL start with the loss on the higher frequencies, then the lower frequencies, resulting from the damage at the apical region (low frequencies) and finally loss of the function throughout the entire cochlea [8].

SNHL is one of the several complications of this disease and has been found to occur in 8% of SCD children in Nigeria, 12% in USA, 22% in Jamaica, 36.5% in Kenya and 60% in Ghana [3,8].

In some studies, HbAS in malaria exposure could be also a cause of SNHL [10].

São Tomé and Príncipe it’s an archipelago in western equatorial Africa, near Gabon, Equatorial Guinea, Camaroon and Nigeria, with Portuguese as official language [11]. São Tomé and Príncipe was discovered by Portuguese explorers in 1470. The resident population is about 187,000 inhabitants who have a low average age distribution (17-18 years) with low socioeconomic conditions and poor sanitary conditions, as well as a public health infection problem–malaria [12–14].

Since 2011 an otolaryngologist group formed by 2 doctors, 2 nurses and 1 audiologist began humanitarian missions in São Tomé and Príncipe (Project “Health for all”–Instituto Marqués de Valle Flor (IMVF)). Apparently according their clinical registries, this was the first hearing evaluation performed in these islands. Upon the first mission, a high prevalence of SNHL in the population was identified and it was observed upon subsequent missions.

As far as we know this is the first study developed in São Tomé and Príncipe population to evaluate the causes inherent to the high incidence of HL in this population. Such way, this study proposes to answer the question: will it be possible to establish an association between the sickle cell trait (HbAS) and the high incidence of SNHL in São Tomé and Príncipe?

Materials and Methods

Subjects

We present a case-control study, with a convenience sample where the control group include individuals with normal hearing in both ears, and patients with unilateral or bilateral HL compose the case group. The project was submitted and approved by the Medical Ethics Committee of São Tomé and Príncipe and Ethics Research Committee NMS|FCM-UNL.

A total of 316 individuals (136 HL patients and 180 controls with bilateral normal hearing), ranging from 2 to 35 years old, agreed to participate in this study. The limitation to 35 years was chosen to avoid action of other risk factors of HL, like acoustic trauma, age and the effect of other diseases. We organized into two age groups based on WHO: 1) below 14 years old (2–14 years) as children group and 2) above 15 years old (15–35 years) as adults group. The patients were recruited during consultation provided by the humanitarian missions in São Tomé and Príncipe over a period from February 2012 to May 2014. The controls (normal hearing bilateral) were recruited at consultation at health services, primary schools from São Tomé and local hotel.

All patients signed an informed consent and answered a clinical questionnaire recording risk factors and clinical history. An otolaryngologist observed all. The risk factors included were family history of HL, consanguinity, self-report of malaria infection, pre-natal and perinatal history and history of infections.

All 316 individuals were evaluated regarding their hearing status with Pure Tone Audiogram (PTA) or Auditory Brainstem Response (ABR) depending on collaboration to participate on the audiometric exams.

Audiometric exams were carried out by an audiologist without an audiometric cabin, with earphones-TDH39, in a closed room, with a level of noise measured by iPhone using SchableDoesIT Gbr, Munich, Germany (version 1.0.0), considered acceptable (ANSI S3.1-1999) (R2013). The equipment used was the Madsen Midimate 622 and Vivosonic Integrity V500 audiometer (auditory brainstem response), calibrated according to calibration ISO389 1975/Oslo Recommendation. The Integrity™ V500 system used to collect auditory brainstem is a modular equipment comprised by 4 main components: the computer, the VivoLink (SN: V10026), the Amplitrode (SN: AJ0270) and the earphones. The earphones used were the ER-3A (ER-3A Left SN:63762 e ER-3A Right SN: 63763) and were calibrated according to ANSI S3.6-1996 and the stimulus used was the CLICK, calibrated in dB equivalent to the sound pressure level (dBpeSPL) according to the procedure IEC 60645-3 for the calibration of short duration stimulus.

Electrophysiological thresholds were translated into the audiometric thresholds for frequencies 2000 Hz and 4000 Hz. No correction factor was applied as is advocated in studies conducted by Jerger and Mauldin [15]; Gorga et al. [16]; van der Drift et al. [17]; Gorga et al. [18] in which establish a strong correlation between the electrophysiological thresholds with the “click” and the audiometric thresholds at 2000 and 4000 Hz.

The hearing loss of each patient was determined based on the better ear and following the WHO classification [19].

Patients with less than 2 years and more than 35 years of age, conductive hearing loss, syndromic hearing loss, obvious environmental causes such as meningitis, cerebral malaria, intrauterine or neonatal complications, ototoxicity, severe head trauma or developmental delay were excluded.

DNA extraction

Blood samples of all patients and controls were collected into Guthrie cards. Genomic DNA was obtained from each sample using a commercially available kit (QIAamp® DNA micro kit, Qiagen) according to the manufacturer's instructions. All DNA samples were stored at −20°C.
SNP screening

The HbS gene point mutation (Glu6Val) was determined by PCR-RFLP. The primers and PCR conditions for the mutation site of this gene is shown in Table 1. For all of them the nucleotide mutated resulted in either gain or loss of restriction site, which therefore allowed the wild-type (HbAA) and variant alleles to be discriminated by RFLP after appropriate restriction enzyme digestion.

PCR was carried out with 50 ng of DNA in 50 ml reaction volume, containing 1.3x ImmoBuffer, 1.5 mM MgCl₂, 0.6 mM dNTP, 1.0 mM of each primer, and 0.75 U of Immolase (Bioline). The amplification started with an initial denaturation step at 95°C for 7 min, cycling parameters were 35 cycles of 95°C for 30 s, specific annealing temperature (60°C) for 30 s, 72°C for 30 s and a final extension at 72°C for 10 min. After amplification, 10 ml of each PCR products were digested with appropriate restriction enzymes (DdeI) for 3 h at 37°C followed by an inactivation step at 65°C for 20 min and electrophoresed in 2% agarose gel with ethidium bromide (0.5 mg/ml) for visualization under ultraviolet light. The expected products for each genotype of the tested gene are shown in Table 1. All the genotype determinations were carried out twice in independent experiments and inconclusive samples were reanalyzed.

Table 1: PCR-RFLP conditions for identification of HbAS (rs334) polymorphism.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>PCR fragment</th>
<th>Patterns after restriction enzyme digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbAS codon 6</td>
<td>Rv 5'- AGG GTG GGA AAA TAG ACC AA -3'</td>
<td>395 bp</td>
<td>HbAA: 202, 180, 13 bp</td>
</tr>
<tr>
<td></td>
<td>FW 5'- CGG CTG TCA TCA CTT AGA CCT -3'</td>
<td></td>
<td>HbAS: 382, 202,180,13 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HbSS: 382, 13 bp</td>
</tr>
</tbody>
</table>

Table 1: PCR-RFLP conditions for identification of HbAS (rs334) polymorphism.

Statistical Analysis

The Hardy-Weinberg equilibrium of HbS was assessed by Qui-square test, calculated by HW calculator™ - Michael H Court (2005-2008).

Description of the sample was made with descriptive statistics, considering frequency analysis, means and standard deviation (SD).

To study the association between HL and each of the following parameters as district origin, oral language, perception of HL, sex and history of malaria infection, have been used the Qui-squared test.

The association between HbS genotype and the degree of HL of each ear and was analysed with Qui-square test by Monte Carlo Simulation.

To identify risk factors of HL we adopted a Binary Logistic Regression, where HL is a response variable and independent variables were HbS, age groups and self-report of Malaria infection.

All analyses were performed using the IBM, SPSS 20 version.

Results

We evaluated 316 subjects (Table 2) of whom 146 (45.6%) were men and 172 (54.4%) were women, with an age mean of 17.4 ± 9.74 years and a median of 15 years.

<table>
<thead>
<tr>
<th>Sex</th>
<th>0.256</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
<tr>
<td>Female</td>
<td></td>
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</table>

<table>
<thead>
<tr>
<th>Oral Language</th>
<th>0.0001</th>
</tr>
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<tbody>
<tr>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Indefined</td>
<td></td>
</tr>
</tbody>
</table>

| Family History | Hearing Loss | 0.278 |
|----------------|--------------|
| No             |       |
| Yes            |       |
| Missing        |       |

<table>
<thead>
<tr>
<th>Consanguinity</th>
<th>0.443</th>
</tr>
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<tr>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Missing</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Malaria</th>
<th>0.07</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td></td>
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<tr>
<td>Yes</td>
<td></td>
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<tr>
<td>Missing</td>
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</tbody>
</table>

According to our results, HL is not associated with the district of origin (p=0.058) of each individual.

Among the 316 subjects, we found 180 (57.0%) individuals with bilateral normal hearing, 44 (13.9%) unilateral HL (UHL) and 92 (29.1%) with bilateral HL (Figure 1). The individuals with UHL and bilateral HL were included in the case group.

Table 2: General characteristics for convenience sample of São Tomé and Príncipe population.

<table>
<thead>
<tr>
<th></th>
<th>Case</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbAA</td>
<td>112</td>
<td>142</td>
</tr>
<tr>
<td>HbAS</td>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td>HbSS</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 2: The Hardy-Weinberg Principle for HbS genotype was confirmed (p>0.05).

The Hardy-Weinberg Principle for HbS genotype was confirmed based on the distribution of HbS genotype in control and case group (Figure 2).
When we calculate the media of the tone threshold, we found the left ear (40.02 dB) slight the same than the right (39.85 dB). The audiometric curve in the global sample has a horizontal shape, around mild HL.

The self-reported HL was confirmed by the audiometric evaluation, showing a positive association (p<0.0001) between both parameters. The tendency for self-reported HL was confirmed by the exams (ϕ=0.553; p<0.0001).

Considering oral language for communication, this was absent in 33 (24.3%) of the individuals from case group and in 4 (2.2%) of the control groups these showing a negative association (ϕ=-0.379; p<0.0001) between oral language and HL (p<0.0001). There was a tendency to have oral language in the control group.

A familial history of HL was found to be more prevalent in the control group than in the case group, respectively with 34 controls (18.9%) and 20 patients (14.7%). In the case group, 16 (80%) had bilateral HL and 4 (20%) unilateral HL.

From our sample 3 individuals (1.7%) in the control group and 4 patients (2.9%) in case group confirmed the existence of consanguinity in their families. Thus, no association between HL and consanguinity was performed.

In São Tomé and Principe we did not found any registry about history of malaria infection. At same time, we found that malaria infection is a public health problem and all individuals enrolled recognize malaria infection and can answer about their clinical history regarding this endemic infection. Self-report of malaria infection was more prevalent in the case group with 91 patients reporting a history of malaria (66.9%), being reported by 103 individuals (57.2%) of the control group.

The genotype distribution in our population was shown in Table 2. Concerning our results the HbAS showed no significant predisposition to HL (Table 3).

By applying the model of binary logistic regression the self-report of malaria was identified as a risk factor for HL (Table 3). The history of malaria infection almost doubled the risk of HL (OR=1.840; CI 95% [1.097-3.085]). We also found that, even not statistically significant, the oldest age group presented a decreased risk of HL in 36.7%.

For sickle cell trait we did not verify a significant association in contrast to what we are expecting, since has been reported the relevance of these factors in HL [18,22].

The horizontal audiologic curve in tonal audiogram was not specific or characteristic to a specific risk factor, meaning that innumerable causes may be responsible for SNHL in São Tomé and Principe and not one in particular.

Analysing the risk factors, the history family of HL and consanguinity were excluded from the analysis because they have a small number of cases. They haven't significant association in contrast to what we are expecting, since has been reported the relevance of these factors in HL [21,22].

The individuals included in this study rarely reported to have consanguineous parents, even knowing that São Tomé and Principe are small islands, with high probability of consanguinity.

São Tomé and Principe is a population of Sub-Saharan Africa in which several haemoglobinopathies have been identified, including HbSS as the most prevalent [7,23]. It would be expected to obtain an increased prevalence of HbS homozygous (HbSS) and increased HbAS [4,8,10,24] in the São Tomé and Principe population. However in our

| Table 3: Binary logistic regression between HbS and HL without and with risk factors (age group and history of malaria infection), HL: Normal hearing–0–Reference Category is a response variable; independent variables: HbS (0–HbAA wild type, 1- HbAS trait, 2–HbSS homozygotic), age groups (0–[2-14] years, 1–[15-35] years) and history of Malaria infection (0–no, 1–yes). |
|---|---|---|---|---|
| Case | No | Malaria infection | HbS |
| No | Yes |
| HbS | HbAA | HbAS | HbSS |
| Reference | Reference | Reference |
| [2-14] | 69 (45.7%) | 82 (54.3%) | 0.362 | 0.07 |
| [15-35] | 98 (59.4%) | 98 (59.4%) | 0.812 | 0.633 | [0.386-1.038] |
| Malaria infection | 0.071 | Reference | Reference |
| No | 41 (30.1%) | 72 (40%) | 0.573 |
| Yes | 91 (66.9%) | 103 (57.2%) | 1.552 | 1.840 | [0.964-2.497] |
| HbS | 0.744 | Reference |
| (HbAA) | 112 (62.3%) | 142 (78.9%) |
| (HbAS) | 22 (16.2%) | 35 (19.4%) | 0.449 | 0.842 | [0.463-1.530] |
| (HbSS) | 2 (1.5%) | 3 (1.7%) | 0.855 | 0.821 | [0.133-5.063] |

**Discussion**

The results of this study revealed a prevalence of bilateral SNHL in 92 (29.1%) individuals and unilateral SNHL in 44 (13.9%) of subjects from our convenience sample. The unilateral and bilateral SNHL, accounting for 43% of the total sample, were included in the case study group since the unilateral SNHL also contribute to worse language acquisition and decreased school performance [20].

The horizontal audiologic curve in tonal audiogram was not specific or characteristic to a specific risk factor, meaning that innumerable causes may be responsible for SNHL in São Tomé and Principe and not one in particular.

Analysing the risk factors, the history family of HL and consanguinity were excluded from the analysis because they have a small number of cases. They haven't significant association in contrast to what we are expecting, since has been reported the relevance of these factors in HL [21,22].

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results, HbSS genotype were found only in 16% and 18% for HbAS of the sample.

The HbAS has been reported in many studies as a factor of protection from malaria, reducing its severity [6]. The mechanism that contribute for this protection is the accelerated sickling of parasite-infected HbAS erythrocytes, low growth rates and parasite invasion in HbAS erythrocytes (in low oxygen conditions) and increased phagocytosis of infected HbAS erythrocytes [6]. Recently, had been show that intra-erythrocytic parasite growth is greatly inhibited by HbS polymerization when oxygen levels drop below 5% and, higher parasite-infected sickle erythrocyte phagocytosis by host immune cells has been observed when compared to infected normal erythrocytes [6]. Moreover, HbS erythrocytes infected by P. falciparum lower the surface expression of PEMP-1, which results in a reduction of cytoadherence and thus protection against severe malaria [6]. This haemoglobin alteration otherwise may lead to thromboembolic events which sought being the source of SNHL [10].

The study model adopted did not confirm this assumption that the HbAS can promote SNHL. In our study we found a significant association between SNHL and a self-report of malaria. Although the patient provides the clinical malaria episode information, we considered their report valid. We must also consider that in the São Tomé Príncipe population exists a strong public awareness of Malaria infection as public health problem and standard procedures and guidelines for infection evaluation are followed, including the collection of thick blood film Plasmodium sp. in the presence of fever and illness.

Our results are supported by others, suggesting also that mild clinical malaria may also be associated with SNHL [25,26].

The malaria infection may trigger the onset of SNHL by pathophysiological processes, thromboembolism and release of inflammatory mediators [26,27]. On the other hand, malaria treatment adopted may be ototoxic. Some studies report reversibility of ototoxicity of some antimalarial [28–32]. In the specific case of children it does not apply [33] especially when is not performed a drug weight adjustment, and children ingest high doses of antimalarial therapy which may induce irreversible ototoxicity [32,33]. Eventually the higher association of the youngest patients of the case group with HL could be supported by the report of children malaria infection in this country during last 14 years [34,35].

The epidemiological profile of Malaria in São Tomé and Príncipe reveals a significant decrease of malaria admissions and deaths over 2006-2007 [34]. Since then, the children were most affected, representing a high proportion relatively to all patients. In 2003 they started some measures to control the disease which include indoor residual spraying (IRS). In 2004 initiated the intermittent preventive treatment (IPT) with and sulfadoxine and pyrimethamine and changed the antimalarial policy with association of artesunate (AS) and amodiaquine (AQ), as first line treatment, in 2005 implemented the use of insecticide-treated nets (ITN) [34].

Our results support the hypothesis that the sickle cell trait (HbAS) acts as a protective role against malaria and SNHL in São Tomé and Príncipe.

Moreover, although São Tomé and Príncipe region is also affected by other HBB mutations, their prevalence is not relevant [23], justifying no screening for them in our study. There are others haemoglobin diseases, like deficiency of glucose-6-phosphate dehydrogenase, which ototoxicity is well known when combined with primaquine antimalarial therapy [36,37].

Conclusion

No association between sickle cell trait (HbAS) and the high prevalence of HL was found. However, our study suggests that in this sample, HbAS is preventing HL because is protecting against malaria. Malaria was found as a risk factor for the development of HL in São Tomé and Príncipe population. The multifactorial profile of HL and the horizontal audiologic curve, highly suggests the relevance of other etiologic factors than Malaria to justify the high prevalence of HL in São Tomé and Príncipe and further investigation must be applied.

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Competing Interests

There are no conflicts of interest.

Ethics Approval and Consent to Participate

The project was submitted and approved by the Medical Ethics Committee of São Tomé and Príncipe and Ethics Research Committee NMS|FCM-UNL (nº02/2014/CEFCM). The Ethics Research Committee is aligned with the Declaration of Helsinki for the Protection of Human Subjects. A full consenting process was applied in respect of all participants. Consent to use the survey data was also obtained.

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