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G6PD Variants, Malaria and Sensorineural Hearing Loss in São Tomé and Príncipe: A Case-Control Study

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ABSTRACT

Background: *São Tomé and Príncipe (STP) is a least developed country (LDC) on Sub-Saharan Africa, in which was detected a high prevalence of sensorineural Hearing Loss (SNHL). HL is a common condition with both genetic and environmental causes, and it greatly impacts on global health. STP population has leading with additional health problems over the years, such as anaemia and malaria infection. The present study aims to identify the correlation between the most prevalent G6PD variants and the high prevalence of HL in STP population.* ***Methods:*** *A sample of 316 individuals collected during Humanitarian Missions in STP, was retrospectively studied in a case-control approach to evaluate the role of G6PD gene variants in individual susceptibility to HL and it correlation with other potential risk factors.* ***Results:*** *The results obtained showed an increased risk for those cases that have reported malaria infection (OR 1.867, CI 95% [1.107-3.48]) in global population. The same effect of increased risk was found after stratification for male gender (OR 3.721 CI 95% [1.631-8.489]).* ***Conclusions:*** *Our results did not allow us to correlate any specific variant of G6PD gene with HL. However, emphasize the hypothetical correlation between malaria infection and the increased risk for HL.*

Keywords: Anaemia, *G6PD* deficiency, hearing loss, malaria, São Tomé and Príncipe, Sub-Saharan Africa

INTRODUCTION

The hearing loss (HL) is a condition that could be related to genetic and environmental factors. The high prevalence of such condition in under developing countries, such as African countries has been described [1].

São Tomé and Príncipe (STP) is a least developed country (LDC) on Sub-Saharan Africa, which has been receiving humanitarian help from Portugal through a program called "Health for all-specialties". In this program, a team of otolaryngologists, nurses, audiologists, and speech therapist collaborate in STP.

During the humanitarian action, the clinical team was faced with a high prevalence of HL, mainly sensorineural hearing loss (SNHL) in STP population [2-4]. Anaemia and malaria infection were two additional health problems detected in this population4.

Anaemia can be caused by several factors as such hemoglobinopathies or enzymopathies which can act by themselves as a protector effect to malaria infection [5-8]. Previous case control studies reported to the hypothesis of the existence of enzymopathies which act as selectively advantageous in malarial endemic areas [7,9].

Glucose-6-phosphate dehydrogenase (*G6PD*) deficiency is one of the most common genetic enzymopathy in humans. This pathology is an X-linked disorder with recessive genetic trait.

G6PD is a cytoplasmic enzyme that catalyzes the first step in the hexose monophosphate pathway leading to synthesis of pentose phosphate. It also catalyzes conversion of nicotinamide adenine dinucleotide phosphate (NADP) to its reduced form (NADPH), thus protecting erythrocytes from oxidative damage. *G6PD* activity has been shown to be reduced in infected erythrocytes when compared to uninfected ones [9]. Clinical symptoms include acute or chronic haemolytic anaemia, neonatal jaundice, or hyperbilirubinemia, but also can remain asymptomatic and is rarely mortal [5].

The reduced capacity of cell defence against oxidative damage induced by the *G6PD* deficiency, has been correlated to a high resistance against malaria infection by *Plasmodium falciparum*, especially in populations living in malaria endemic regions, as Sub-Saharan Africans [5,10].

More than 400 different variants of *G6PD* gene have been described to date [11], from which at least 186 have been characterized as mutations [12]. The most common variants described are 376A (*G6PD* type B, normal phenotype), 376G (*G6PD* type A+, moderately deficient phenotype), both variants generated by *G6PD* (Asn126Asp) polymorphism and characterized by c.376A>G; and 202A (*G6PD* type A-, severely deficient phenotype) generated by *G6PD* (Val68Met) and characterized by c.202G>A [5,7,9]. However, the type A- is heterogeneous since it can result from a combination of the Asn126Asp replacement with any of three additional mutations, the most common of which is Val68Met [6,10].

The present exploratory study aims to identify the correlation between the most prevalent *G6PD* variants and the high prevalence of HL in STP population.

MATERIAL AND METHODS

Study subjects

A convenience sample of 316 individuals (136 HL patients and 180 controls), was collected during the humanitarian missions from February 2012 to May 2014. The individuals who agreed to participate were attended in medical consultation and were included individuals from 2 to 35 years old.

All enrolled population answered a clinical questionnaire identifying risk factors, clinical history, and clinical observation. The risk factors included were: family history of HL, consanguinity, self-report of malaria infection, pre-natal and perinatal history, and history of infections. In Table 1 can be find the general characteristics of the global population under study.

Table 1 Global distribution and characterization of the individuals enrolled in this study (n=316)

Variables	Control n (%)	Cases n (%)	P Value*
Age range			
[2-14]	82 (45.6)	69 (50.7)	0.361
[15-35]	98 (54.4)	67 (49.3)	
Sex			
Male	87 (48.3)	57 (41.9)	0.256
Female	93 (51.7)	79 (58.1)	
Oral Language			
Yes	170 (94.4)	85 (62.5)	**<0.0001**
No	4 (2.2)	33 (24.3)	
Missing	6 (3.3)	18 (13.2)	
Familial History HL			
Yes	34 (18.9)	20 (14.7)	0.278
No	140 (77.8)	115 (84.6)	
Missing	6 (3.3)	1 (0.7)	
Consanguinity			
Yes	3 (1.7)	4 (2.9)	0.443
No	171 (95)	127 (93.4)	
Missing	6 (3.3)	5 (3.7)	

Self-report of malaria			
Yes	103 (57.2)	91 (66.9)	0.070
No	72 (40)	41 (30.1)	
Missing	5 (2.8)	4 (2.9)	
***G6PD* (202G>A)**			
GG	137 (76.1)	99 (72.8)	0.857
GA	28 (15.6)	24 (17.6)	
AA	15 (8.3)	11 (8.1)	
Missing	---	2 (1.5)	
***G6PD* (376A>G)**			
AA	95 (52.8)	69 (50.7)	0.952
AG	42 (23.3)	32 (23.5)	
GG	43 (23.9)	34 (25)	
Missing	---	1 (0.7)	

To all was evaluated hearing status with pure tone audiogram (PTA) or auditory brainstem response (ABR) depending collaboration. There was collected a sample of blood to Guthrie paper after an informed consent and Medical Ethics Committee approval, to diagnosis *G6PD* deficiency, demonstrating specific mutations by DNA studies.

The audiometric exams were carried out by an audiologist. The equipment used was the Madsen Midimate 622 and Vivosonic Integrity V500 audiometer (auditory brainstem response). The audiometric exams were carried out without an audiometric cabin, with earphones-TDH39, in a closed room, with a level of noise measured by iPhone de SchabelDoesIT GbR, Munich, Germany (version 1.0.0), considered acceptable, based on ANSI S3.1-1999 (R2013). The audiometric equipment was calibrated according to calibration ISO389 1975/Oslo Recommendation. The IntegrityTM V500 system used to collect auditory brainstem is a modular equipment comprised by 4 main components: the computer, the VivoLink (SN: VL0026), 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) are 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 to the calibration of short duration stimulus.

Hearing loss is classified in mild (26 dB to 40 dB), moderate (41 dB to 60 dB), severe (61 dB to 80 dB) and profound (>80 dB) according pure tone averages in 500; 1000; 2000 and 4000 Hz. Based on the better ear, each patient was classified, according to the WHO classification.

The exclusion factors used were: individuals with less than 2 years and more than 35 years of age, conductive hearing loss, and history of cranial trauma, intra-uterine, neonatal complications, and obvious mental retardation.

This is a case-control study, where control group include patients with both ears normal, and case group have patients with one or both ears with HL, being a homogeneous group for gender and age.

The project was submitted and approved by the Medical Ethics Committee of STP and Ethics Research Committee NMS|FCM-UNL. 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.

DNA Extraction

A drop of peripheral blood samples of all enrolled population was collected into Guthrie paper by qualified assistants. Genomic DNA was obtained from each blood 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 until analysis.

SNP Selection

We selected for this study two polymorphisms in *G6PD* gene between the most prevalent single nucleotide polymorphisms (SNPs) described in this gene (rs1050828 and rs1050829) in Sub-Saharan Africa [10,12].

The SNPs selected were genotyped using Real-time polymerase chain reaction (RT-PCR 7300 Applied Biosystem), through TaqMan® SNP genotyping assays (Life Technology), according to manufacturer instructions. Real-time PCR genotype duplicate validations were carried in 20% of randomly selected samples in independent experiments and all the inconclusive samples were reanalyzed.

Statistical Analysis

All analyses were performed using the statistical package for the social sciences for Mac 20.0 version (SPSS).

The Hardy Weinberg Principle was tested with Qui-square test for one sample.

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

To study the association between Oral Language and HL, self-report of HL and HL, Sex and HL, self-report of malaria and District we used Qui-square test. The Hardy-Weinberg Equilibrium was carried out using exact probability tests available in SNPStat software [13].

To identify risk factors of HL, we adopted a binary logistic regression, where HL is a response variable and independent variables were age, *G6PD* variants and self-report of Malaria infection. In Table 2 could be find the genotype combination for both SNPs under study. The *G6PD* deficiency is an X-linked recessive disorder occurring much often in males, so deficient variants are expressed more commonly in males than in females. (Tables 2 and 3).

Table 2 Genotype distribution of both variants of *G6PD*: rs1050828 (202G>A) and rs1050829 (376A>G) by gender in global population of São Tomé and Principe

G6PD rs1050828 (202G>A)		
	Female, n (%)	Male, n (%)
G6PD WT (GG)	112 (65.1)	124 (86.1)
G6PD Heterozygotic (GA)	52 (30.2)	--
G6PD Homo/Hemizygotic (AA)	7 (4.1)	19 (13.2)
Missing	1 (0.6)	1 (0.7)
G6PD rs1050829 (376A>G)		
G6PD WT (AA)	72 (41.9)	92 (63.9)
G6PD Heterozygotic (AG)	74 (43)	--
G6PD Homo/Hemizygotic (GG)	25 (14.5)	52 (36.1)
Missing	1 (0.6)	--
Total	172 (100)	144 (100)

RESULTS

This study comprised 316 individuals, from which 136 were included in HL case group while the other 180 represent the control group. The most representative *G6PD* gene variants from Sub-Saharan Africa were studied, carrying-out their genotyping to evaluate a possible association between them and the high incidence of hearing loss in this country. Table 1 describe the main characteristics of the populations under study. No significant differences were found between both groups, exception for oral language stratification ($P<0.0001$).

Concerning the genotype distribution for each variant under study, the results obtained are shown in Table 2. These results were stratified by gender and can be observed the higher frequency of each allele in men, the main reason is because men are hemizygotic (only have one allele of each variant, since they only have one X-chromosome). However, the allelic frequency determined for each allele was very similar between both genders, except for c.202 G>A variant (Table 3).

Table 3 Allelic Frequencies of both *G6PD* SNPs under study stratified by gender

SNP ID	Nucleotide substitution	Variant group	Allele Frequency (%)	
			Male	Female
rs1050829	c.376A>G	B (376A)	63.9	63.7
	c.376A>G	A+ (376G)	36.1	36.3
rs1050828	c.202G>A	A- (202A)	13.2	19.3

Our data was analyzed according the most common sub-Saharan *G6PD* variants, which could be correlated accordingly their phenotypic relevance (B-normal; A+ -intermediate; A--deficient). Table 4 presents the *G6PD* variants distribution. The normal phenotype described by B variant group corresponds to the most frequent one (51.1% in control group

and 50.7% in case group), while the intermediate phenotype corresponds to around 12% in both populations. Full enzymatic-deficient individuals, represented by A-group, which included males and homozygous females for both SNPs under study, correspond to 7.2% and 8.2% in control and cases populations, respectively.

Table 4 Distribution of *G6PD* variants between STP populations under study

***G6PD* Variants**	**Control n (%)**			**Case n (%)**		
	Global	**Male**	**Female**	**Global**	**Male**	**Female**
B (376A)	92 (51.1)	57 (65.5)	35 (37.6)	68 (50.7)	33 (58.9)	35 (44.9)
A+ (376G)	22 (12.2)	19 (21.8)	3 (3.2)	17 (12.7)	15 (26.8)	2 (2.6)
A- (202A+376G)	13 (7.2)	10 (11.5)	3 (3.2)	11 (8.2)	8 (14.3)	3 (3.8)
Heterozygous group*	51 (28.3)	-	51 (54.8)	38 (28.4)	-	38 (48.7)
B include AA genotyped women for 376 variant and A genotyped men; A+ include GG genotyped women and G men for variant 376; A- include women carrying both AA genotype for 202 variant and GG for 376 variant and men carrying both variant alleles; *All heterozygous women were grouped together independently						

Our results were stratified according clinical or phenotypic relevance of *G6PD* variants (Tables 5, 6 and 7).

Table 5 *G6PD* Variant Groups distribution in case (n=136) and control (n=180) populations

Variables	**Crude OR (95%) CI**	**Adjusted OR (95% CI) [a]**
Gender Group		
Female	1 (Reference)	1 (Reference)
Male	0.771 [0.492-1.208]	0.717 [0.397-1.296]
Age Range		
[2-14]	1 (Reference)	1 (Reference)
[15-35]	0.812 [0.520-1.269]	0.650 [0.394-1.072]
Malaria Infection		
NO	1 (Reference)	1 (Reference)
YES	1.552 [0.964-2.497]	**1.867 [1.107-3.148] ***
***G6PD* Variant Groups**		
B	1 (Reference)	1 (Reference)
A+	1.045 [0.516-2.119]	1.259 [0.598-2.649]
A- (376A/202G)	1.145 [0.483-2.711]	1.328 [0.547-3.222]
Heterozygous group	1.008 [0.597-1.703]	0.904 [0.481-1.701]
[a] ORs were adjusted for age, malaria, and gender. Data in bold highlights the statistic significant results (P<0.05) *P Adjusted=0.019 (P values are adjusted by unconditional multiplicative logistic regression)		

Table 5 presents the observed results after logistic distribution analysis of each *G6PD* variant group, as well as the distribution of the potential risk factors inherent to this study: gender, age, and malaria infection.

Table 6 *G6PD* Variant Groups distribution in male population

Variables	**Crude OR (95%) CI**	**Adjusted OR (95% CI)**
Age Range		
[2-14]	1 (Reference)	1 (Reference)
[15-35]	0.881 [0.452-1.721]	0.516 [0.234-1.139]
Malaria Infection		
NO	1 (Reference)	1 (Reference)
YES	**2.798 [1.351-5.798]** †	**3.721 [1.631-8.489]** ‡
***G6PD* Variant Groups**		
B	1 (Reference)	1 (Reference)
A+	1.364 [0.612-3.039]	1.378 [0.596-3.184]
A- (376A/202G)	1.382 [0.496-3.847]	1.424 [0.485-4.184]
Data in bold highlights the statistic significant results (P<0.05). †P Crude = 0.006 (P values are adjusted by unconditional multiplicative logistic regression). ‡P Adjusted = 0.002 (P values are adjusted by unconditional multiplicative logistic regression)		

The genotypic analysis was also performed between each population; however, the results did not reveal any association between each *G6PD* genotype and the incidence of HL (data not shown). None of the two SNPs were in agreement with the expectation of the Hardy-Weinberg law (P<0.0001, exact probability test).

Table 7 *G6PD* Variant Groups distribution in case (n=136) and control (n=180) populations

Variables	Crude OR (95%) CI	Adjusted OR (95% CI)[a]
Age range		
[2-14]	1 (Reference)	1 (Reference)
[15-35]	0.763 [0.418-1.393]	0.765 [0.393-1.487]
Malaria infection		
NO	1 (Reference)	1 (Reference)
YES	0.914 [0.478-1.748]	1.065 [0.529-2.141]
***G6PD* variant groups**		
B	1 (Reference)	1 (Reference)
A+	0.667 [0.105-4.238]	0.783 [0.120-5.107]
A- (376A/202G)	1.000 [0.189-5.299]	1.093 [0.203-5.870]
Heterozygous group	0.745 [0.397-1.398]	0.857 [0.447-1.642]
[a]ORs were adjusted for age, malaria, and gender		

The global analysis present on Table 5 showed an increased risk for those cases that have reported malaria infection (OR 1.867, CI 95% [1.107-3.48], P=0.019). However, and after stratification by gender, in male population the same effect of increased risk was found (OR 3.721 CI 95% [1.631-8.489], P=0.002).

DISCUSSION

The enrolled population was recruited during humanitarian missions occurred during 2012-2014. The high prevalence of hearing impairment in STP open the door to study the possible risk factors inherent to HL disorder in this country. To answer questions about the most relevant risk factors were considered to be studied in this population: 1) their genetic background 14 through the study of the role of the DFNB1 locus (GJB2 and GJB6 genes); 2) the putative role of Beta globin mutation-sickle cell trait and HL [15]; and 3) to estimate the rate of infected people with rubella and its correlation with HL [16]. These previous studies helped us to characterize STP population, and especially for rubella infection, allowed to implement the rubella vaccination in this country [14-16].

The high frequency of polymorphic variants has arisen because *G6PD* deficiency gives a relative protection against severe malaria hypothesizing about its selective advantageous in malaria endemic areas consequence of evolutionary pressure [5,8,9]. Many variants have been described, however the main focus of this work was on *G6PD* type B, type A+ and type A-described as the most prevalent in Africa by several studies [6-10,17].

The distribution and prevalence of *G6PD* variants have shown a distinct geographical pattern, reflecting correlations with epidemiology of malaria, such as malaria-endemic and malaria eliminating countries [6,12]. *G6PD* A-is a common variant in sub-Saharan Africa that reaches frequencies of 20% in populations living in malarial areas. This variant is characterized by two nonsynonymous changes relative to the normal allele (*G6PD* B), which decrease enzyme activity ≈12% of normal and confer ≈50% reduction in risk of severe malaria in both females and males [8]. It follows that the *G6PD* A-variant is beneficial in the presence of malaria caused by *Plasmodium falciparum*, while in the absence of malaria this variant is deleterious [18]. For *G6PD* A+ the prevalence is almost the same as *G6PD* A-, but with a decreased enzymatic activity, with 80% of normal activity and without reduction of risk of severe malaria [18].

In fact, our results reveal an increased risk to develop HL in global STP population for those who have stated to have had a malaria infection episode (OR: 1.867, 95% CI [1.107-3.148]). We also found an association between malaria infection and HbS in STP population [15].

The epidemiological profile of Malaria in São Tomé and Príncipe reveals a significant decrease of malaria admissions and deaths over 2006-2007 [19]. Since then, the high proportion of all patients pointed to children as the most affected. Some preventive actions to control the disease have started in 2003, including indoor residual spraying (IRS) to intermittent preventive treatment (IPT) and also, through change the antimalarial policy [19].

The *G6PD* deficiency is an X-linked trait, and is often stated as being more common in males, however, this is incorrect. As a result of X-chromosome inactivation, heterozygous females are genetic mosaics. However, could not be state the mathematical average of alleles' distribution, 50% *G6PD* normal and 50% *G6PD* deficient, in females. The random distribution of heterozygous genotype defines females in which the enzyme activity phenotype overlaps with normal, whereas in others it overlaps with the *G6PD* deficiency prevalent in homozygous. Of course, this mosaicism interferes in clinical implications [6].

G6PD deficiency is normally associated to haemolytic anaemia, which could manifest during infections, after treatment with certain drugs or after eating fava beans (known as favism) and also, kernicterus. Mechanism of protection is more prone to induce acute haemolytic anaemia and early phagocytosis of infected cells. Males have more severe haemolytic anaemia than female [20,21], this can probably increase the risk of having HL when associated to malaria and *G6PD*, as we have when analyzing the male gender. In our sample, male group presents a high risk to develop HL (OR: 3.721 95% CI [1.631-8.489]). This could be explained by the fact that, male gender is usually more exposed to malaria infection as described by Cardoso, et al. and Prettz, et al. inherent to their job activity being more exposed to *P. falciparum* infection, leading to haemolytic anaemia [21,22]. In opposite, female gender, even with more susceptibility to anaemia [23] is probably more protected to severe anaemia with malaria and *G6PD* even because female gender presents more *G6PD* variants which randomly interfered with enzymatic alterations.

In the general sample of São Tomé and Príncipe we expected a high prevalence of *G6PD* variants and because of it, we expected a high incidence in hyperbilirubinemia neonatal and HL consequently.

Prevention should be emphasized, screening newborns in regions with a high frequency of *G6PD* deficiency, educating the population to avoid the drugs and fava beans that precipitate attacks of haemolytic anaemia. At the same time, it's essential being alert to kernicterus in newborns, preventing sequel in future, as HL and mental retardation [23,24].

The effect of the *G6PD* deficiency in the ear is not known unless during neonatal period [25,26]. It is well known that *G6PD* deficiency is associated with hyperbilirubinemia and kernicterus, which is a bilirubin induced neurologic dysfunction (BIND), associated with auditory neuropathy spectrum disorder (ANSD), acute and chronic haemolytic anaemia [25,27,28].

Hyperbilirubinemia occurs commonly in neonates and is usually mild and transient, with no long-lasting sequel. However, bilirubin-induced neurologic damage may occur in some children [28]. The auditory pathway is the most sensitive part of the central nervous system to bilirubin-induced toxicity, and permanent sequel may result from only moderately elevated total serum/plasma bilirubin levels. The damage to the auditory system occurs primarily within the brainstem and cranial nerve VIII, and manifests clinically as auditory neuropathy spectrum disorder. Around 30% of jaundice infants who have permanent neurological damage are *G6PD* deficient [24].

CONCLUSIONS

The results reveal an adaptive response of population of São Tomé and Príncipe, to an increase in virulence of *P. falciparum* in sub-Saharan Africa. In the sample, we found that malaria infection has significant implication in developing HL increasing the risk in general population, being more representative in male gender. Our results did not allow us to correlate any specific variant of *G6PD* gene with HL. However, they emphasize the hypothetical correlation between malaria infection and the increased risk for HL.

This work alerts the need in screening these variants in early age (neonatal), with the objective to prevent serious complications during life which are not only HL.

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Authors' contributions

CC, SNS, PC, contributed significantly to the experimental design, its implementation, analysis and interpretation of the data. CC, SNS have been involved in the writing of the manuscript, PC, HC, JP to revision stages, and CC, PC, HC, JP and SNS have read and approved the final version.

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Ethics approval and consent to participate

The project was submitted and approved by the Medical Ethics Committee of STP 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 written consent process was applied for all participants. Consent to use the survey data was also obtained.

Competing interests

The authors have no competing interests to declare.

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