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Study On The Variants Of Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency In The Indigenous Population Of Upper Assam, India

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Background**:** Most men around the world are afflicted with an inherited disease Glucose-6-phosphate (G6PD)**.** Around 400 million people in tropical and sub-tropical countries are affected globally. The main objective of the research is to have a comprehensive study on the frequency of G6PD deficiency and to investigate variants which are more dominant among the deficient population of Upper Assam.
Methods**:** At the onset, 2000 blood samples (1017 males and 948 females) from different Indigenous groups of Assam were screened for G6PD deficiency by Dichlorophenolindophenol (DPIP) dye decolonization method. DNA from the deficient subjects was amplified using polymerase chain reaction and was tested for the presence of the three common Indian mutations namely G6PD Mediterranean (563 C→T), G6PD Kerala Kalyan (949 G→A), and G6PD Orissa (131 C→G) by Amplification refractory mutation system (ARMS-PCR) method and PCR sequencing method.
Results: The DPIP screening test results revealed that out of 1017 males 29 (2.8 %) were found to be G6PD-deficient and out of 948 females, only 6 (0.6%) were found to be G6PD deficient with an overall prevalence rate of 1.75% respectively. Out of 35 deficient DNA samples, the mutation was identified in 8 G6PD deficient individuals. 27 of the 35 DNA samples remain uncharacterized. Out of the three Indian mutations, G6PD Orissa (131C>G) is commonly present in this population and also found that G6PD Orissa (131C>G) mutation is associated with both 1311C and 1311 T polymorphism.
Conclusion: The study's findings have provided new insight into the G6PD variation that is widespread among Upper Assam's indigenous population. Furthermore, we might anticipate that 27 samples out of the 35 deficient samples that have yet to be identified may include novel mutations. As a result, more research is needed to determine whether there are any novel mutations in this region.

Keywords: G6PD deficiency, Upper Assam, Mutation.

Introduction

In the red blood cells, pentose phosphate pathway begins with the breakdown of glucose catalyzed by the X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD). Deficiency due to G6PD remains the most common enzymopathies (1, 2). The G6PD gene (13 exons and 12 introns) is 18 kb and located in the long arm of the X- chromosome (locus q28)[3]. Males are hemizygous for this gene and females can be heterozygous or homozygous. Globally more than 200 mutations in the G6PD gene have been found (3). Many studies by Indian researcher have reported that the existance of G6PD Mediterranean (563C>T), Kerala-Kalyan (949G>A), and Orissa (131C>G) are common in Indian populations among different variants of G6PD deficiency (4,5). All tissues of the body express housekeeping enzyme G6PD, and clinical manifestations of this deficiency are solely and exclusively observed in red blood cells (RBC). The most common symptoms include severe hemolytic anemia and jaundice of the neonates that occur as a result of infection, eating fava beans, and taking medications with a high oxidation potential (3,6). Red blood cells are defended from the detrimental effects of reactive oxygen species by the G6PD enzyme. However, the main cause of hemolytic anemia is the absence of G6PD enzymes due to mutations in the G6PD gene (3). Previous studies have documented that the Assamese tribal population comprising of a diversity of castes and tribal groups, has a distinct lineation of the variants in G6PD deficient. The occurrence of G6PD deficiency in Assamese tribal groups has been thoroughly investigated; however, studies have not yet explored the molecular basis of G6PD deficiency in the Assamese ethnic groups. In addition, no such research has ever been done in the upper part of Assam. The main objective of the current investigation was characterization of mutation of genes leading to deficiency of G6PD and examining their distribution among the different ethnic groups in Upper Assam.The occurrence of G6PD deficiency in Assamese tribal groups has been thoroughly investigated; however, not many reports are available regarding the molecular basis of G6PD deficiency in Assamese ethnic groups.

Materials and Methods

Ethical Approval and Sample collection

This study has been approved by ICMR–Regional Medical Research Centre, N.E.Region's Institutional Ethics Committee (IEC) (Approval no.: ICMR-RMRC/Dib/IEC (Human)/2019-20/1176; dated: 08.07.2019). Initially, we have informed the study subjects about the importance of this study and duly signed in on the informed consent form. Thereafter, from every study subject, 5 ml of intravenous blood was collected in the vial containing EDTA, and a total of 2000 blood samples (1046 males and 954 females) were collected. After that, the G6PD deficiency of the enrolled subjects was examined by using the dichlorophenol–indophenol (DPIP) dye decolorization method. Subsequently, the G6PDH activity was estimated by G-SIX Kit according to the manufacturer's instructions.

Genomic DNA extraction and PCR amplification

DNA from G6PD deficient cases was extracted from whole blood using the Spin Column method using the QIAamp mini kit (QIAGEN, Germany) as per the manufacturer’s protocol. Genomic DNA was eluted in 200 µl of elution buffer and stored at -20°C until further study. Genomic DNA was amplified through optimized PCR conditions in the laboratory; briefly,

each PCR reaction contain 25 µl reaction volume viz 12.5 µl of 2X mastermix (Promega, USA), 0.2 µM of each forward and reverse primer (10 µM stock), and 2 µl of DNA template and nuclease-free water (added to make the volume 25µl). The optimized PCR cycling conditions as follows: Initial denaturation 94°C for 5 min; followed by 34 cycles of 94°C for 45 sec, 60°C for 30 sec, 72°C for 45 sec; and a final extension at 72°C for 10 min in a Thermal cycler (Applied Biosystems by Thermo Fisher Scientific, US). The PCR products were analyzed on 2% agarose gel with ethidium bromide staining.

The extracted DNA from the deficient subjects was amplified using the polymerase chain reaction (PCR) method. It was screened sequentially for the presence of the four common Indian mutations namely G6PD Mediterranean (563 C→T) (rs 5030868), G6PD Kerala Kalyan (949 G→A) (rs 137852339), G6PD Orissa (131 C→G) (rs 78478128) and silent (1311 C→T) (rs 2230037) mutation by Amplification refractory mutation system (ARMS-PCR) method.
In-house primers were designed using reference sequence NG 009015.2 for the rs id viz (rs 5030868, rs 2230037, rs 137852339, rs 78478128) respectively. For this "primer 3" (https://bioinfo.ut.ee/primer3-0.4.0/) software was used to design primers (Table 1) using default parameters in compassing the mutation sites. These were synthesized commercially by Eurofins Genomics India and reconstituted by using Nuclease Free water.

Statistical Analysis Used
Data were analyzed using Epi Info 7 software.

Sequencing
PCR was performed using designed primers and subjected to bidirectional nucleotide sequencing using respective primers. The sequences were edited manually using BioEdit software 7.2.5.

Sl. No.	Primer name	Primer Sequence 5' -3'	Length in bp	Nucleotide position	% GC content	Melting temp (Tm)	Amplicon Length
1.	rs 5030868 out F	ATGCAGCTCTGATCCTCACTCCCCGAAG	28	563 C→T	57.1	69.5	324 bp
	rs 5030868 out R	GAGTTCTGGAGGAATTCGTCCTCGGGGA	28	563 C→T	57.1	69.5	324 bp
	rs 5030868 in F (C)	GAGCTCTGACCGGCTGTCCAACCACATATC	30	563 C→T	56.7	70.9	209 bp

	rs 5030868 in R(T)	TAGATCTGG TCCTCACGG AACAGGGCG A	28	563 C→T	57.1	69.5	173 bp
2.	rs 2230037 FP	ATGATGACC AAGAAGCCG GG	20	1311 C→T	55.0	59.35	364 bp
	rs 2230037 RP	CTGAGGGGA CATGGTATG GC	20	1311 C→T	60.0	61.40	364 bp
3.	rs 137852339 FP	GAGCCCATT CTCTCCCTTG G	20	949 G→A	60.0	61.40	307 bp
	rs 137852339 RP	CAGTGCGTG AGTGTCTCA GT	20	949 G→A	55.0	59.35	307 bp
4.	rs 78478128 FP	GTGGCCCAG TAGTGATCC TG	20	131 C→G	60.0	61.40	577 bp
	rs 78478128 RP	GATTGGCGG AGAAAACGC AG	20	131 C→G	55.0	59.35	577 bp

Table 1

Results

The ubiquity of G6PD deficiency in the different community groups is tabulated in Table 4. Of all the 1017 males investigated for the deficiency, 29 (2.8 %) males showed to be G6PD-deficient, whereas, only 6(0.6%) females out of 948 females, showed G6PD deficiency (**Table 4**). Among 35 defective DNA samples, 8 G6PD-deficient people showed to have the mutated DNA. G6PD Orissa was found to be very common with an overall prevalence of 22.8%. In the Assamese community, this mutation was shown to be highly polymorphic. G6PD Orissa was more common among tribals, although it was also found in a few nontribal groups. None of the three variations were found in any of the 27 samples. The presence of the 1311 polymorphism is shown in **Table 5**. G6PD Orissa mutations were found to be associated with both 1311 C and T.

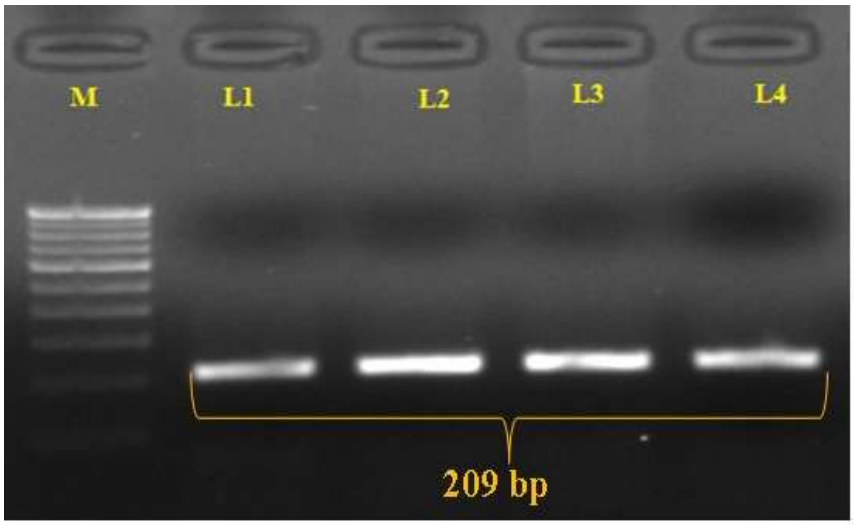

Figure 1 G6PD Mediterranean (C>T) at 209 bp run in 2% agarose Gel. Lane 1 represents a 100 bp ladder. L2-L5 clinical samples.

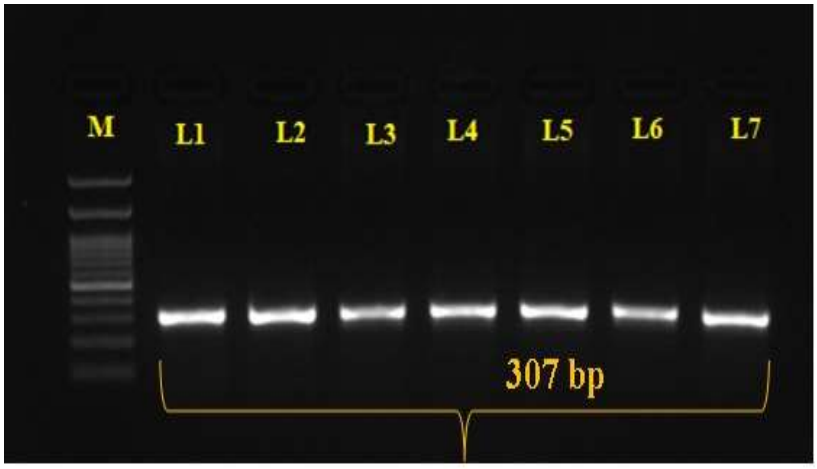

Figure 2 This gel image shows an amplification product of the G6PD gene encompassing SNP with rs 137852339 at 307 bp run in 2% agarose gel. Lane 1 represents a 100 bp ladder. L2-L8 clinical samples.

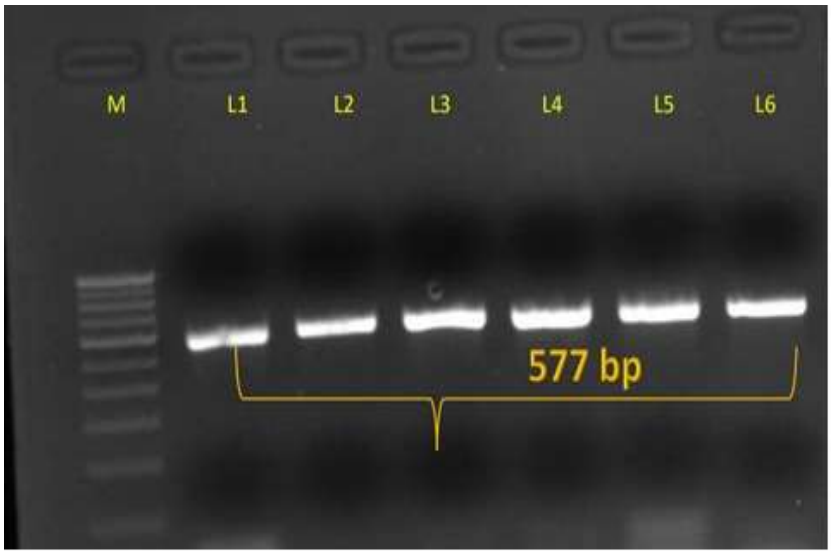

Figure 3 This gel image shows an amplification product of the G6PD gene encompassing SNP with rs 78478128 at 577 bp run in 2% agarose gel. Lane 1 represents a 100 bp ladder. L2-L7 clinical samples.

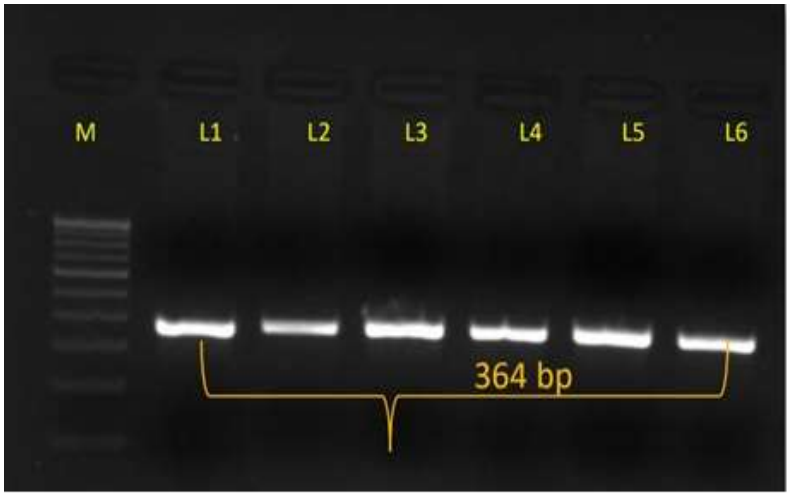

Figure 4 This gel image shows an amplification product of the G6PD gene encompassing SNP with rs 2230037 at 364 bp run in 2% agarose gel. Lane 1 represents a 100 bp ladder. L2-L7 clinical samples.

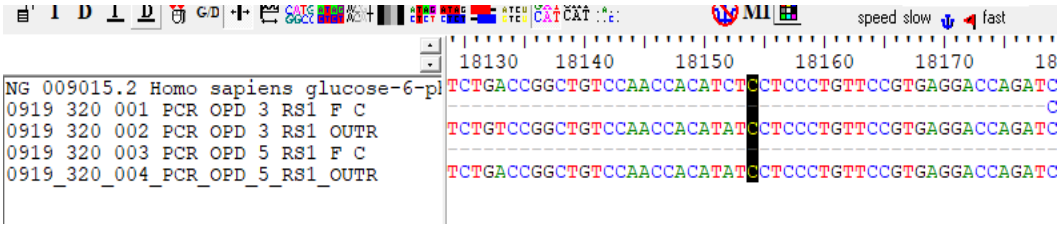

Figure 5 G6PD Mediterranean (C>T) at position 18154 C>T compared to genomic sequence NG_009015.2: g

Figure 6 G6PD 1311 (C>T) at position 20134 C>T compared to genomic sequence NG-_009015.2: g

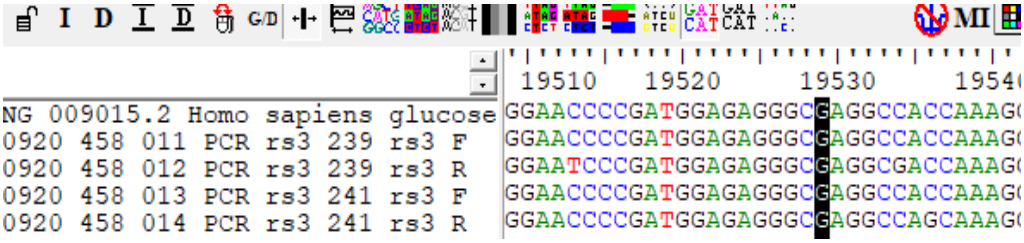

Figure 7 G6PD Kerela Kalyan (G>A) at position 19529 G>A compared to genomic sequence NG_009015.2: g

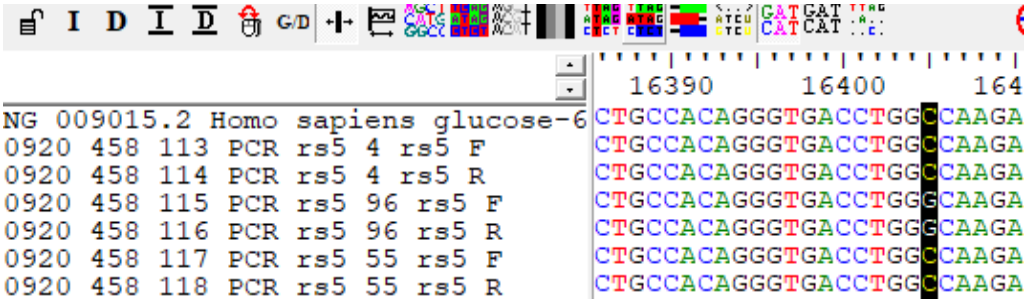

Figure 8 G6PD Orissa (C>G) at position 16405 C>G compared to genomic sequence NG-_009015.2: g

Sl No.	Mutation	Hemizygous male	Homozygous Female	Heterozygous Female

1	G6PD (1311 C>T)	29	5	1
2	G6PD Orissa	6	2	0

Table 2: Group wise distribution of G6PD Orissa and G6PD 1311 mutation.

Sl No.	**Mutation**	**n (Sample size)**	**Genotype Frequency (%)**	**Allele frequency**
1.	G6PD Mediterranean	35	CC=100%, CT= 0%, TT=0%	C=1
2.	G6PD (1311 C→T)	35	CC=48.6%, CT= 2.9%, TT= 48.6%	C=0.5, T=0.5
3.	G6PD Kerala Kalyan	33	GG=100%, GA=0%, AA=0%	G=1
4.	G6PD Orissa	33	CC=75.8%, CG=0%, GG=24.2%	C=0.7, G=0.3

Table 3: Showing Genotype and Allele frequency in G6PD deficient Groups

Sex	**No. of cases**		**P-value**
	G6PD Deficient	**G6PD Normal**	
Male	29 (2.8%)	1017	<0.05
Female	6 (0.6%)	948	
Total	**35 (1.75)**	**1965**	

Table 4: Sex Wise Distribution in G6PD Deficient vs. G6PD Normal Subjects.

Community	**Total number of individuals**	**G6PD Mediterranean**	**G6PD Kerala Kalyan**	**G6PD Orissa**	**131 1C**	**131 1 T**	**1311 C/T**
Different Caste Group	30	0	0	3	16	13	1
Tea Garden Labourer	5	0	0	4	1	4	0
Total	35	0	0	7	16	13	1

Table 5: Community-wise distribution of G6PD deficiency

Discussion

G6PD deficiency is a prevalent genetic hematological condition in India, affecting persons from Assam in particular. Assam is a state in northeastern India with an astonishing panorama of ethnic, linguistic, and genetic diversity and traditional socio-cultural customs and a huge indigenous population divided into caste, community, and religious groups. Malaria is more common in Assam because it is a subtropical region. As a result, we believe that G6PD deficiency is a beneficial mutation in this location. G6PD Orissa, G6PD Mediterranean, and G6PD Kerala Kalyan are the three most frequent mutations in the Indian population that cause G6PD deficiency (8). Exon 6 of the G6PD gene has the Mediterranean mutation (563 C>T),

the most frequent G6PD variant. This variety is widespread in Mediterranean countries and the Middle East and the Indian subcontinent (9,10). Exon 9 of the G6PD gene contains the Kerala–Kalyan (949 G>A) mutation. This mutation has been found in people from Maharashtra, Kerala, Punjab, and Indo-Mauritians from Andhra Pradesh and Tamil Nadu in Southern India (11). The G6PD Orissa (131 C>G) mutation, found in exon 3, has been discovered in the tribals of Orissa and Madhya Pradesh (12). This mutation is significant because it is linked to the NADP binding site (8). However, we discovered that this mutation was present in both tribal and caste groups in our research. The occurrence of this mutation in caste and tribal groups indicates that intercaste marriages are common in the population. Our research discovered G6PD Orissa (22.8 percent) in 5 tribal people and 3 people from other caste groups. 27 of the 35 DNA samples have yet to be identified. Due to a lack of DNA, further characterization of 3 samples was not possible. Orissa mutations and Kerala Kalyan mutations have an Indian origin because these mutations have been found mainly in the tribal populations which are considered India's aboriginal population. Furthermore, these mutations have not been reported from any other population in the world (8). In addition to these mutations, two silent mutations are proposed to be G6PD Mediterranean haplotypes. This silent G6PD mutation is widely prevalent in multiethnic, non-African populations (13). According to Beutler et al., European populations with this mutation have a T at 1311, whereas South Italy and India have a C at 1311 (14). According to our findings, the occurrence of both 1311 C and 1311 T is associated with the G6PD Orissa mutations. As a result, it's difficult to say if this mutation had a single origin or whether the two haplotypes resulted from an interallelic crossover (8).

Conclusion

The current research illustrated the occurrence of G6PD deficiency in the various ethnic groups of Upper Assam. Thus, it can be observed that out of the three polymorphic variants, G6PD Orissa, G6PD Mediterranean, and G6PD Kerala–Kalyan, G6PD deficiency in G6PD Orissa variation account for the most common among various ethnic groups of Upper Assam. This is the first study reported in this region. The documentation of the G6PD profile has both scientific and clinical relevance and needs further addressing. Therefore, further studies are required to investigate the characterization of the genetic nature of G6PD deficiency in the Assamese population whereby the findings will aid in the early detection, prevention, treatment, and management of the disease.

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