Scientific Research Publishing

Open Journal of Blood Diseases, 2023, 13, 24-32
https://www.scirp.org/journal/ojbd
ISSN Online: 2164-3199
ISSN Print: 2164-3180

Glucose-6-Phosphate Dehydrogenase Deficiency: Difficulties in Diagnosis at the Souro Sanou University Hospital, Burkina Faso

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How to cite this paper: Sawadogo, S., Nébié, K., Kouldiati, J., Traoré, C., Sawadogo, S., Kissou, A.S. and Kafando, E. (2023) Glucose-6-Phosphate Dehydrogenase Deficiency: Difficulties in Diagnosis at the Souro Sanou University Hospital, Burkina Faso. *Open Journal of Blood Diseases*, **13**, 24-32.
https://doi.org/10.4236/ojbd.2023.131004

Received: November 23, 2022
Accepted: February 21, 2023
Published: February 24, 2023

Abstract

Glucose-6-phosphate dehydrogenase deficiency is the most common enzymopathy worldwide. The precise prevalence of G6PD is unknown in Burkina Faso. The objective of the study was to describe the difficulties to diagnose this disease at the Souro Sanou University hospital (CHUSS) in Bobo-Dioulasso. It involved five patients comprising one child with homozygous SS sickle cell disease, one adolescent screened following a family investigation, and three adults including a man and two women. Blood smear stained with May Grunwald Giemsa was performed to look for specific signs of G6PD-deficient red blood cell and brilliant cresyl Blue for Heinz Bodies. A microscope Olympus BX53 equipped with a Camera (XC10) and connected to a computer was used to read blood smears and capture images. Genes sequencing by Sanger method were performed in a specialized laboratory in molecular genetics. For each analysis, the protocol and instructions of the equipment and reagent manufacturer were applied. Of the five patients, three had anemia and only one had hyperreticulocytosis. Two patients had biological signs of hemolysis and one patient had an elevated CRP. Blood smear stained with MGG and cresyl blue showed specific signs of G6PD-deficient red blood cells and Heinz bodies in all patients. Biochemical analysis and molecular typing confirmed G6PD deficiency. The presence of G6PD-deficient red blood cells in the blood smear guides the diagnosis of G6PD deficiency. The diagnosis is biochemical and is based on the combined measurement of G6PD plus pyruvate kinase and/or hexokinase.

Keywords

G6PD Deficiency, G6PD-Deficient Red Cell, Diagnosis Difficulties, Burkina Faso

1. Introduction

G6PD is an enzyme that allows the elimination of free radicals and protects the cell, mainly the red blood cell, against damage caused by hydrogen peroxide. G6PD deficiency is the most common enzymopathy, affecting more than 400 million people worldwide [1]. It is an X-linked recessive genetic disorder that affects males preferentially. It is due to mutations in the NADP and/or 6PG binding sites [2] [3] [4] and/or the dimer or tetramer subunit binding sites [5] in the coding region of the enzyme gene.

G6PD deficiency, if unrecognized, can trigger a severe hemolytic crisis following the use of certain drugs, the best known of which are Primaquine and Dapsone [6] [7]. This hemolytic crisis can also occur following the consumption of foods such as beans [8]. This hemolysis is responsible for the morbidity and various encephalic complications due to kernicterus because of the hepatic immaturity of neonates [9]. G6PD deficiency was classified either by electrophoretic mobility (A-, B-), by the intensity of the enzyme deficiency and the severity of hemolysis into three classes (I, II, III), or by molecular variant [8].

The prevalence of G6PD deficiency is particularly high in areas of high malaria endemicity and in populations with high endogamy. In Burkina Faso, this prevalence is not known, but it is estimated to be low [9]. The diagnosis of G6PD deficiency is not always accessible in our country. Indeed in practice, the clinical context and specific hematological signs allow to evoke the diagnosis. However, enzymatic tests for the diagnosis of certainty and molecular biology for confirmation are not available. This study aimed at the describing the specific hematological signs, the biochemical profile for diagnosis of certainty and confirmation by molecular diagnosis in five cases at the hematology laboratory of the Sanou Sourô University hospital (CHUSS) of Bobo-Dioulasso, Burkina Faso.

2. Material and Methods

2.1. Patients and Materials

It was a cases series report including a 3-year-old child with homozygous sickle cell disease who routinely screened for the G6PD deficiency, an 18-year-old adolescent who was screened following a family investigation, a 64-year-old woman with homogeneous splenomegaly and hemolysis following diarrhea, a 42-year-old man and a 51-year-old woman diagnosed following an episode of hemolysis.

Samples were collected in EDTA K3 vacutainer tubes and 4 mL heparinized tubes. The automated Mindray BC 6800 was used for blood count.

For each patient, blood smears were prepared on microscope slides and stained

with May Grunwald and Giemsa from VWR international, Brilliant Cresyl Blue. The smears were observed with an Olympus BX53 microscope equipped with a camera (XC10) to capture images that were transferred to a computer for processing.

The evaluation of hemolysis was performed using the Architect and Mindray BioSentec Bs-480 automated systems; for the quantitative determination of G6PD and pyruvate kinase (PK), we used the Mindray BioSentec Bs-480 automated system with the DiaSys G6PDH Kit and pyruvate kinase (PK) kits respectively.

The key reagents and material for the PCR included the Qiagen FlexiGene DNA Kit, Thermocycler Applied Biosystems Veriti, agarose and trays for electrophoresis. Sanger sequencing and sequences analysis were performed at Serge Pissard's molecular genetics laboratory at hospital Henri Mondor, University Paris-Est, Créteil.

2.2. Methods

To detect Heinz bodies, one volume of brilliant cresyl blue was mixed with two volumes of the patient's whole blood in a hemolysis tube sample and incubated for twenty minutes in a 37˚C water bath. After this time (T0), the tube was homogenized by inverting and returned to the water bath. Then, blood smears were prepared at different incubation times: 30 minutes, 1 hour, 2 hours, 4 hours and 24 hours. The smears were then read under a microscope for golf ball-like inclusions in the red blood cells or juxta-membrane inclusions.

Fresh blood smears from patients stained with MGG were read in microscope to seek specific signs of G6PD-deficient red cells.

Blood counts with reticulocytes, biochemical assessment of hemolysis and G6PD and Pyruvate Kinase assays were performed according to instructions of manufacturers of the automated equipment and reagents kits.

The molecular variants of G6PD sought were as follows: c376A > G, c202G > A, c542A > T, c563C > T, c968T > C. When these variants were not found, Sanger sequencing of exons 3 to 6 and 9 was performed.

The extraction of the DNA with the FlexiGene kit was carried out by the following steps interspersed with centrifugation and oven incubation: cell lysis, denaturation and precipitation of the DNA with isopropanol and ethanol, drying to obtain a pure DNA. The extracted DNA is used to amplify the G6PD-specific gene using dNTPs, and sense and antisense primers in the Thermocycler. The amplified product is first digested with four restriction enzymes and then subjected to agarose gel electrophoresis along with a ladder to determine the types of mutations present. If no known mutations are detected he product is sequenced using the Sanger method.

The aspects studied were hematological signs of deficient red blood cells in the blood smear (ghost red cells, bitten red cells, fungus-like red cells, ...), biological signs of hemolysis (total and free bilirubin, haptoglobin, lactate dehydrogenase, ...), enzymatic assays (Pyruvate kinase, G6PD) and mutations on the G6PD gene.

3. Results

A total of five patients were identified including a 3-year-old child with homozygous sickle cell disease who are been routinely screened for the G6PD deficiency, an 18-year-old adolescent who was screened following a family investigation, a 64-year-old woman with homogeneous splenomegaly and hemolysis following diarrhea, a 42-year-old man and a 51-year-old woman diagnosed following an episode of hemolysis.

Blood counts with reticulocytes of patients were shown in Table 1. Among the five patients, three were anemic and one had hyperreticulocytosis.

Haemolysis and inflammatory assessment prior to the biochemical assay were shown in Table 2.

On blood smear examinations, we found images of specific (Figures 1(a)-(c)) and non-specific (Figure 1(d)) cytological signs of G6PD deficiency and an image of fungus-like red cell (Figure 1(e)).

The result of the MGG and brilliant cresyl blue stained smears are shown in Table 3. Phantom or semi-phantom red blood cells, bitten red cells were found in all the patients. Patient 1 had fungus-like red cells and schizocytes were noted in patients 1 and 4.

Table 1. Characteristics of the patient's complete blood count.

PAT	RBC $10^6/\mu L$	HGB g/dL	HCT %	MCV fL	MCH pg	MCHC g/dL	Retic $10^{12}/L$	WBC $10^3/\mu L$	NEUT %	EO %	BASO %	LY %	MONO %	PLT $10^3/\mu L$
1	4.42	11.7	36.1	81.7	26.5	32.4	0.0508	12.06	41.8	0.5	0.01	5.9	5.3	84
2	5.01	14.2	44.4	88.6	28.3	32	0.0701	8.95	51.4	5.1	0.8	32.5	10.4	224
3	3.08	8.2	23.4	76	26.6	35	0.2603	11.31	37.3	1.8	0.6	46.1	14.3	497
4	3.34	10.7	33.1	99.2	31.9	32.2	0.0171	2.58	29.2	2..11	0.2	67.7	0.8	85
5	5.39	13.7	43.5	80.6	25.5	31.6	0.0455	4.38	37.8	4.3	0.5	51.3	6.1	191

PAT = patient, Retic = reticulocyte. Patient 1 and 4 thrombocytopenia are false because there are platelets clumps on their blood smear. The patient 4 myelogram was normal the leukocytes demargination test was positive 2 hours after injection of adrenaline into his skin: WBC was $10.9 \times 10^3/\mu L$

Table 2. Assessment of hemolysis and inflammation in patients before enzyme testing.

Patients	Total Bilirubin µmol/L	Free Bilirubin µmol/L	Haptoglobin g/L	LDH U/L	CRP mg/L
1	23.7	17.7	0.44	219	<6
2	23	16	<0.08	221	5.44
3	51.1	42.2	<0.08	582	4
4	244.99	235.77	<0.10	524	28
5	6.3	3.8	<0.14	156	2

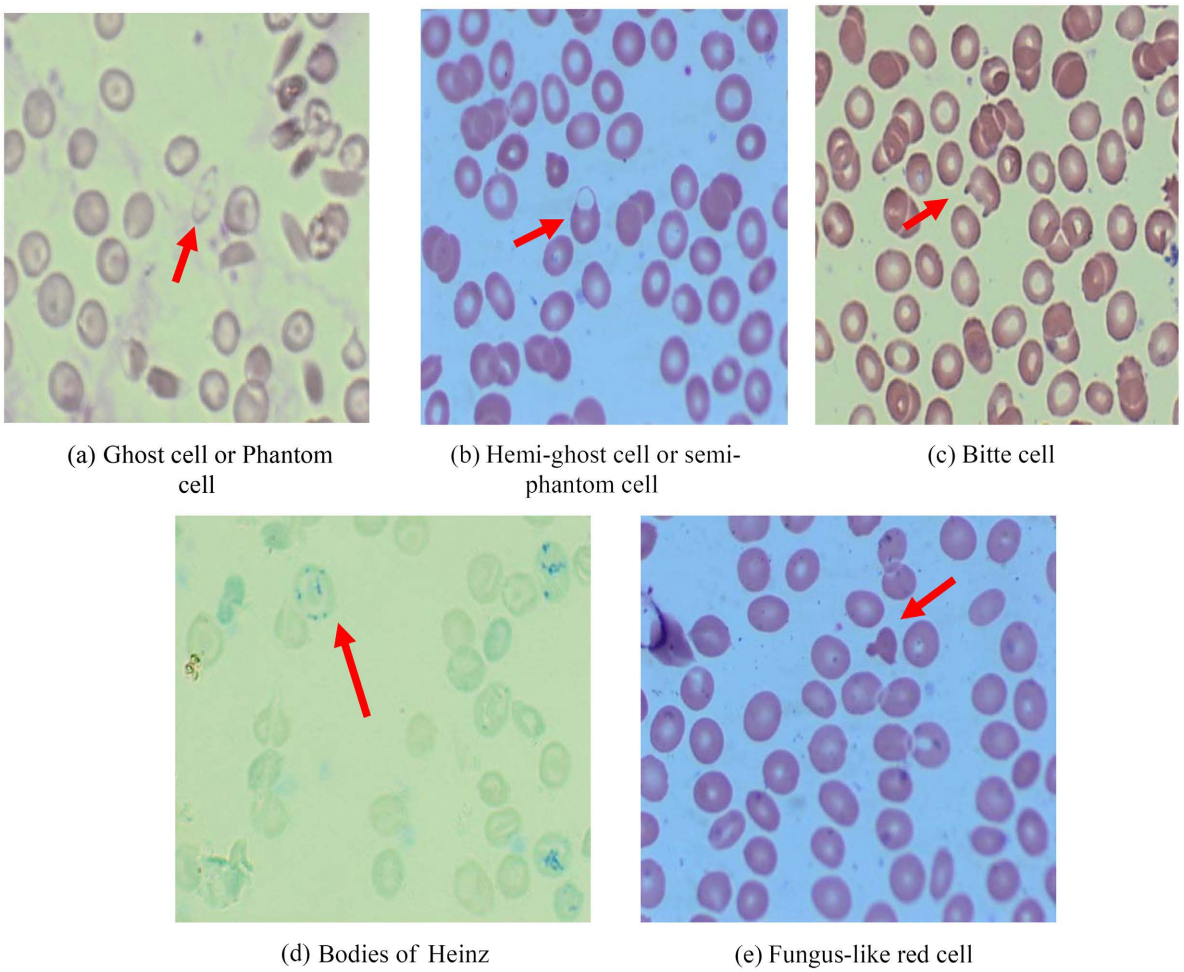

Figure 1. Cytological abnormalities on smears stained with May Grunwald Giemsa and brilliant cresyl blue.

Table 3. Results of smears stained with May Grunwald Giemsa (MGG) and brilliant cresyl blue.

Patient	Cytological signs at smear stained with MGG	Search for Heinz bodies
1	Phantom red blood cells, semi-phantom red blood cells, biting red blood cells, spherocytes, schizocytes, fungus-like red blood cells.	Positive
2	Phantom red blood cells, semi-phantom red blood cells, bitten red blood cells.	Positive
3	Phantom red blood cells, semi-phantom red blood cells, bitten red blood cells.	Positive
4	Phantom red blood cells, semi-phantom red blood cells, schizocytes.	Positive
5	Red blood cells, semi-phantom, phantom, bitten red cells.	Positive

The positivity of Heinz bodies varied between 0.5% and 5% depending on incubation time.

The biochemical assays showed G6PD deficiency in all five patients (Table 4). Molecular typing of the variant c376A > G, c202G > A, 2 were heterozygotes and the 3 hemizygotes (Table 4).

4. Discussion

In this study, we described the hematological, biochemical and molecular

Table 4. Results of enzymatic and molecular analyses for G6PD.

Patient	Pyruvate kinase (Normal: 11 - 41 UI/g HB)	G6PDH (Normal: 8 - 22 UI/g HB)	Type G6PDH
1	23 UI/g HB	4.94 UI/g HB	c376A > G, c202G > A; Heterozygote
2	18 UI/g HB	<1.0 UI/g HB	c376A > G, c202G > A; Hemizygote
3	29 UI/g HB	<3.0 UI/g HB	c376A > G, c202G > A; Hemizygote
4	25 UI/g HB	<2.55 UI/g HB	c376A > G, c202G > A; Heterozygote
5	20 UI/g HB	<1.0 UI/g HB	c376A > G, c202G > A; Hemizygote

characteristics of five patients with G6PD deficiency. The study is one of the first in our country. It has limitations related to its design. Indeed, it is a series of 5 cases, thus not representative of the general population.

G6PD deficiency is a clinically asymptomatic disease apart from its chronic hemolytic form. It can be life threatening if unknown. Its prevalence in Burkina Faso is unknown in general and particularly in the region covered by the Souro Sanou University Hospital in Western Burkina Faso. The indirect Hematological signs of G6PD deficiency are Ghost cell (**Figure 1(a)**, **Table 3**), Hemi Ghost Cell (**Figure 1(b)**), bitten cell (**Figure 1(c)**), highlighted by the May Grunwald Giemsa stain but also by the brilliant cresyl blue stain. These are main cytological signs of diagnostic. However, the lack of equipment (no automatic machines for the preparation and staining of blood smears) and lack of qualified personnel in quantity constitutes an obstacle for the diagnosis of G6PD deficiencies. Indeed, only two technicians and one part-time biologist carry out, on average, 150 blood samples daily.

Patients 1 and 4 were discovered by chance by these cytological signs, whereas in the other cases they were systematically sought. These signs indicate the presence of G6PD-deficient red blood cells or the G6PD deficiency gene, but not G6PD deficiency. In the case of haemolysis, hyperreticulocytosis hides the deficiency. The presence of Heinz bodies (**Figure 1(d)**) is also an indirect but non-specific sign of G6PD deficiency because it is also found in thalassemia and unstable haemoglobins. But in thalassemia the inclusions are dispersed throughout the red blood cell cytoplasm. On the G6PD deficiency smear, spherocytes are described [10], however, these are not true spherocytes but remnants of semi-phantom red blood cells. Hence, they are referred to as non-spherocytic haemolytic anemia. On the patient 1 blood smear, fungus-like red blood cells were found (**Figure 1(e)**), which are specific for hereditary spherocytosis due to band 3 deficiency [11]. This explains its splenomegaly, for which no other etiology has been found. Similar images were found on smears of blood sample from one son of the patient.

Only one type of molecular variant, c376A > G, c202G > A was found (**Table 2**). The same type of variant was previously reported in Burkina Faso and Mali [12] [13].

G6PD deficiency in women is generally due to a homozygous or heterozygous

composite mutation of the gene, which was not the case in patients 1 and 4. They are Hemizygous, so the deficiency could be due to asymmetric inactivation of healthy X chromosome in the red blood cells [14] [15] or by direct inactivation of G6PD by a mutated p53, which may indicate the existence of cancer [16].

Molecular diagnosis apart from its high cost is feasible at any time and gives the type of lesion. However, it cannot assert the G6PD deficiency in hemizygotes.

Biochemical assays are not easy in cases of hyperreticulocytosis because laboratories do not generally perform any quantification after microcentrifugation as recommended by some authors [17] [18]. Some do not couple the assay with the pyruvate kinase or hexokinase assay, making it difficult to interpret results. For the sickle cell patient and the adolescent, the biochemical diagnosis was easy at the first sampling. However, for the others, several hemolysis tests and hemograms with reticulocytes (**Table 3** and **Table 4**) were required before the G6PD deficit could be detected. Hemizygote-deficient or non-deficient should be diagnosed, not only for themselves, but also to prevent their condition from being a trigger factor for their potential hemizygote-breeding children.

Some authors reported that G6PD deficiency linked to X protects hemizygotes but not heterozygotes against severe malaria [19] while others [20] claim that it protects both Hemizygous male and heterozygous female. These viewpoints must be nuanced since in heterozygotes with a proven deficiency, there is a protection against severe malaria as in male hemizygotes. The treatment of G6PD deficiency is mainly preventive, and hopes of curative treatment besides medullar allotransplantation are emerging [21].

5. Conclusion

G6PD deficiency in its non-chronic form is a sneaky condition. If undiagnosed, it can quickly become life-threatening. Its exact prevalence in Burkina Faso is not known yet, and clinicians should guide biologist to look for specifics signs if necessary. Specifics Hematological signs indicate the presence of G6PD-deficient red blood cells and not deficiency in G6PD. The diagnosis is biochemical and must be made with help of a Blood cells count and one or two other red blood cell enzymes like pyruvate kinase and or erythrocyte hexokinase.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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