Comparison of quantitative and qualitative tests for glucose-6-phosphate dehydrogenase deficiency in the neonatal period

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SUMMARY

Introduction: Considering the high prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency among newborns, different screening methods have been established in various countries. In this study, we aimed to assess the prevalence of G6PD deficiency among newborns in Rasht, Iran, and compare G6PD activity in cord blood samples, using quantitative and qualitative tests.

Methods: This cross-sectional, prospective study was performed at five largest hospitals in Rasht, Guilan Province, Iran. The screening tests were performed for all the newborns, referred to these hospitals. Specimens were characterized in terms of G6PD activity under ultraviolet light, using the kinetic method and the qualitative fluorescent spot test (FST). We also determined the sensitivity, specificity, negative predictive value, and positive predictive value of the qualitative assay.

Results: Blood samples were collected from 1474 newborns. Overall, 757 (51.4%) subjects were male. As the findings revealed, 1376 (93.4%) newborns showed normal G6PD activity, while 98 (6.6%) had G6PD deficiency. There was a significant difference in the mean G6PD level between males and females ($P = 0.0001$). Also, a significant relationship was detected between FST results and the mean values obtained in the quantitative test ($P < 0.0001$).

Conclusion: According to the present study, FST showed acceptable sensitivity and specificity for G6PD activity, although it appeared inefficient for diagnostic purposes in some cases.
INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD) deficiency [1] is the most common X-linked recessive enzyme deficiency [2], affecting 400 million people worldwide [2–5]. Individuals with diminished G6PD activity are sensitive to cellular oxidative damage and present with symptoms, such as hemolytic anemia and jaundice [5, 6]. According to the World Health Organization (WHO), 2.9% of people around the world and 10–15% of Iranian men suffer from G6PD deficiency [7–10].

During the neonatal period, G6PD deficiency may manifest as neonatal jaundice, which can cause severe neurological complications and even death in some populations [11, 12]; therefore, early diagnosis of G6PD deficiency can help prevent these serious complications. WHO has endorsed screening of cord blood samples for all neonates to detect this condition. Overall, the prevalence of G6PD deficiency has been estimated at 3–5% or higher in males [6].

All screening tests have been used and commercialized in different countries and can simply detect male patients; however, these tests are not highly efficient in diagnosis of heterozygous females. Considering the high prevalence of G6PD deficiency among newborns, different screening methods have been established in various countries. The semi-quantitative method described by Beutler and Mitchell [13] and the modified versions of this method [4, 14] are the most common screening tools for this condition.

Glucose-6-phosphate dehydrogenase status is usually determined by measuring enzyme activity in the lysate from whole red blood cells, using either quantitative or qualitative assays. However, assays using whole-cell lysate may classify women who are heterozygous for G6PD as normal, even if G6PD-deficient cells are detected in large amounts [15–18]. The only strategy to accurately identify heterozygous females for G6PD is through genotyping or cytochemical staining for intracellular G6PD activity.

Quantitative assays allow for the discrimination of intermediate to normal levels of G6PD deficiency with fine resolution; however, these available tests require advanced laboratory equipments and skilled personnel. A commonly used qualitative assay is the fluorescent spot test (FST), which can be performed in some low-resource areas to identify severe G6PD deficiency. Although FST can identify severe deficiencies, discrimination of intermediate levels is more difficult.

With this background in mind, development of a robust, quantitative, point-of-care G6PD test for field studies in low-resource areas is a priority for malaria control and elimination [5, 19]. Therefore, in this study, we aimed to assess the prevalence of G6PD deficiency among newborns in Rasht, Iran, and to compare G6PD activity in cord blood samples, using the quantitative gold standard and qualitative tests.

MATERIALS AND METHODS

Study setting and population

We performed this cross-sectional, prospective study at Alzahra, Golsar, Aria, Famili, and Rasoul-Akram hospitals in Rasht, Guilan Province, Iran. All normal newborns, admitted to these hospitals, were included in this study, and screening tests were performed. Cord blood specimens were transferred to 2-mL glass ethylene diamine tetra acetic acid (EDTA) anticoagulant venipuncture vacuum tubes on cold packs and stored at 2–8 °C. Specimen processing was carried out one or two days after blood collection.

Study design

To calculate the sample size, a previous study performed in Guilan Province was used as the reference [20], and G6PD deficiency rate was considered at 6.4% with an error of 0.05. Finally, the sample size was calculated at 1500 subjects. In all the specimens, G6PD enzyme level was measured, using both qualitative and quantitative methods.

Analytical method for G6PD evaluation

Quantitative G6PD test

All specimens were characterized in terms of G6PD activity under ultraviolet light, using the kinetic method (Man Co., Tehran, Iran). Both quantitative and qualitative methods were run, using the same kinetic method. The quantitative assays were performed, based on the kit instructions. The enzyme activity was determined at 37 °C, using a temperature-regulated spectrophotometer (Hitachi 717;
F. Hoffmann-la Roche Ltd, Basel, Switzerland) by measuring changes in the absorbance rate at 340 nm over 5 min for reaction solutions maintained in disposable UV cuvettes.

In this study, all G6PD activity rates are presented in U/gHb. The cord blood samples were dissolved in the lysing solution, and the calorimetric function of the solvent was measured through auto-analysis. The results of auto-analysis were inserted in the following formula:

\[
\text{Enzyme activity (IU/gHb)} = \frac{\text{Amount of Auto-analysis Device}}{\text{Hb (g/dL)} \times 10}
\]

The results were categorized as follows: enzyme activity ≤1.5 IU/gHb (deficient); 1.5 IU/gHb < enzyme activity <6.5 IU/gHb (moderate deficiency); and enzyme activity ≥6.5 IU/gHb (normal).

**FST**

Each specimen was tested, using the qualitative G6PD FST kit (Kimia Pajouhan Co., Tehran, Iran), according to the manufacturer’s instructions. This method could detect the fluorescence of nicotinamide adenine dinucleotide phosphate (NADPH), which is proportional to G6PD activity under long-wave UV light (365 nm). Then, 2 mL of the blood was added to 200 mL of the reagent mixture and spotted on the filter paper at time 0. Each sample was incubated at 37 °C and spotted again after 5 and 10 min of incubation, respectively. Fluorescence was observed at the specified time intervals after the samples had dried out. Fluorescence intensity was used to classify specimens into three groups in terms of enzyme activity: normal (moderate to strong fluorescence after 5 min and strong fluorescence after 10 min), intermediate (weak fluorescence after 5 min and weak to moderate fluorescence after 10 min), and deficient (very faint or no fluorescence after 10 min).

**Statistical analysis**

Data were entered to SPSS version 18 and analyzed. The mean and standard deviation (SD) of the quantitative G6PD assay were determined for all the specimens. The mean difference, standard deviation, and 95% tolerance interval were calculated. To determine the correlation between the qualitative test results and FST findings, independent t-test, chi-square, and descriptive tests were performed. Also, we determined the sensitivity, specificity, negative predictive value (NPV), and positive predictive value for the qualitative assay.

**Ethical considerations**

All ethical considerations were respected using 2 mL cord blood samples. The study objectives were explained to the participants, and informed consent forms were obtained. In case of G6PD deficiency, the parents received complete information about the disease and were given some suggestions for the future. This study was approved by the Ethics Committee of Guilan University of Medical Sciences.

**RESULTS**

**Study population**

Blood samples were collected from 1474 newborns. Overall, 757 (51.4%) and 717 (48.6%) infants were male and female, respectively. Most of the infants were born in summer (n = 588, 39.9%), followed by autumn (n = 566, 38.4%), winter (n = 265, 18%), and spring (n = 55, 3.7%). All G6PD activity tests were performed regardless of the patient’s G6PD status. Table 1 presents the gender distribution, based on the reference G6PD test and the qualitative assay.

**Quantitative measurement of G6PD activity**

Evaluation of all quantitative results was performed at 37 °C. The reference values of G6PD enzyme activity in the study population, based on the kinetic quantitative test, are presented in Table 2. The median G6PD activity in the study population was 9.50 U/gHb (range: 0.20–17.00 U/gHb). The results indicated that 1376 (93.4%) newborns had normal G6PD activity, while 98 (6.6%) showed G6PD deficiency. Overall, 86 (11.4% of the males) and 12 (1.7% of the females) male and female neonates had G6PD deficiency, respectively.
Qualitative measurement of G6PD activity

Fluorescent spot test was performed on all the collected specimens (n = 1474). Among these specimens, 1380 (93.6%) showed normal G6PD activity, 56 (3.8%) showed G6PD deficiency, and 38 (2.6%) exhibited intermediate G6PD deficiency, according to the manufacturer’s instructions. The range of G6PD activity in the specimens was 0.7–6.4 U/gHb (classified as intermediate in FST) with the mean activity of 2.14 ± 1.01 U/gHb.

Based on the findings (Table 3), six subjects with intermediate G6PD deficiency on FST showed G6PD activity <10% of the normal level, while 23 newborns showed G6PD activity <20% of the normal activity. Moreover, 30 newborns with intermediate G6PD deficiency on FST showed <30% of the normal activity, 36 cases had <60% of the normal activity, and 38 newborns with intermediate G6PD deficiency on FST showed <70% of the normal activity.

In the subsequent analysis of the data, intermediate G6PD deficiency was evaluated in two ways, that is, it was classified alongside deficient or normal G6PD activity to assess the test performance against the reference quantitative assay (Table 2). By considering intermediate G6PD deficiency as G6PD deficiency, 94 (6.4%) subjects were classified as deficient. On the other hand, by grouping intermediate G6PD deficiency alongside normal G6PD activity, 56 (3.8%) subjects were found to be G6PD-deficient.

Table 3 presents a summary of the performance of the qualitative assay against the kinetic quantitative test. Fluorescent spot test showed 100% sensitivity and 100% NPV for 10% and 20% cutoff values, while its sensitivity dropped to 89.5% when the cutoff value was 70% of the normal level (NPV remained 100%). Based on the FST results, 38 (2.6%) neonates were partially deficient, while 56 (3.8%) cases were severely deficient in G6PD. Deficiency was not detected in four patients, although G6PD level was below 6.5 U/gHb. Also, none of the samples were misdiagnosed. Although G6PD deficiency could be detected by FST, 2.6% of the healthy samples reported cases were reported as partially deficient.

There was a significant relationship between FST results and the mean values obtained in the quantitative test (P < 0.0001; Figure 1). The qualitative FST confirmed that 48 (77.4%) patients had severe deficiency, as demonstrated by the quantitative test, while 14 (66.6%) cases had partial deficiency. Table 4 demonstrates the mean G6PD activity in the two groups. Also, there was a significant difference in the mean G6PD level between males and females (P = 0.0001).

**DISCUSSION**

The present findings showed that FST can detect all cases of G6PD deficiency, similar to the quantitative test. However, this condition could not be detected in 22.5% of cases with severe G6PD deficiency (14:62)

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**Table 1.** Demographic data in newborn infants screened for G6PD activity

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Number</th>
<th>Quantitative (U/g Hb) (Mean ± SD)</th>
<th>Qualitative G6PD activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>757</td>
<td>8.78 ± 3.13</td>
<td>673, 88.9</td>
</tr>
<tr>
<td>Female</td>
<td>717</td>
<td>9.64 ± 1.94</td>
<td>707, 98.6</td>
</tr>
</tbody>
</table>

**Table 2.** Reference values for G6PD activity in the study population by quantitative test

<table>
<thead>
<tr>
<th>Reference values (U/g Hb)</th>
<th>Total N = 1474</th>
<th>Female N = 717</th>
<th>Male N = 757</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>9.20</td>
<td>9.64</td>
<td>8.78</td>
</tr>
<tr>
<td>SD</td>
<td>2.65</td>
<td>1.94</td>
<td>3.13</td>
</tr>
<tr>
<td>Median</td>
<td>9.50</td>
<td>9.60</td>
<td>9.30</td>
</tr>
<tr>
<td>Range</td>
<td>0.20–17.00</td>
<td>0.70–17.00</td>
<td>0.20–16.60</td>
</tr>
</tbody>
</table>

### Table 3. Clinical performance of the Kinetic quantitative test compared with FST qualitative test for detection

<table>
<thead>
<tr>
<th>Cutoff value, U/g Hb (percent of normal median value = 9.50)</th>
<th>10% Activity cutoff</th>
<th>20% Activity cutoff</th>
<th>30% Activity cutoff</th>
<th>60% Activity cutoff</th>
<th>70% Activity cutoff</th>
<th>Ref. Activity cutoff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cutoff value, U/g Hb</td>
<td>0.950</td>
<td>1.90</td>
<td>2.85</td>
<td>5.70</td>
<td>6.65</td>
<td>6.5</td>
</tr>
<tr>
<td>No. of samples with G6PD levels below cutoff (%) (no. of M + F)</td>
<td>35 (2.4) 32M + 3F</td>
<td>74 (5) 68M + 6F</td>
<td>85 (5.8) 78M + 7F</td>
<td>93 (6.3) 85M + 8F</td>
<td>105 (7.1) 90M+ 15F</td>
<td>101 (6.9) 88M+ 13F</td>
</tr>
<tr>
<td>FST qualitative test*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity % (95% CI)</td>
<td>100</td>
<td>100</td>
<td>98.8</td>
<td>98.9</td>
<td>89.5</td>
<td>93.0</td>
</tr>
<tr>
<td>Specificity % (95% CI)</td>
<td>95.8</td>
<td>98.5</td>
<td>99.2</td>
<td>99.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PPV % (95% CI)</td>
<td>37.2</td>
<td>78.7</td>
<td>89.3</td>
<td>97.8</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NPV % (95% CI)</td>
<td>100</td>
<td>100</td>
<td>99.9</td>
<td>99.9</td>
<td>99.2</td>
<td>99.4</td>
</tr>
<tr>
<td>FST qualitative test†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity % (95% CI)</td>
<td>50.0</td>
<td>68.9</td>
<td>63.5</td>
<td>60.2</td>
<td>53.3</td>
<td>55.4</td>
</tr>
<tr>
<td>Specificity % (95% CI)</td>
<td>99.5</td>
<td>99.6</td>
<td>99.8</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>PPV % (95% CI)</td>
<td>82.8</td>
<td>91.0</td>
<td>96.4</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>NPV % (95% CI)</td>
<td>97.9</td>
<td>98.3</td>
<td>97.8</td>
<td>97.3</td>
<td>96.5</td>
<td>96.8</td>
</tr>
</tbody>
</table>

F, female; M, male.

*Fluorescent spot test: intermediate test results combined with deficient test results.
†Fluorescent spot test: intermediate test results combined with normal test results.
and 22.2% of those with partial G6PD deficiency (8:36). Also, four (2.6%) neonates, who were identified as G6PD-sufficient, had partial G6PD deficiency. Overall, this tool was introduced as a successful screening test for G6PD deficiency in both males and females.

The FST results were in line with the quantitative test results when intermediate G6PD deficiency was considered as G6PD deficiency (100% sensitivity and 100% NPV at 10% and 20% cutoff values, respectively). However, FST accuracy reduced, as intermediate deficiency was classified as normal G6PD. Also, further analysis of the data indicated reduced sensitivity for qualitative FST at 10% cutoff level. FST results were found to be quite close to the reference values on the quantitative test for the majority of validity indices when intermediate G6PD deficiency was categorized as G6PD deficiency (Table 3).

Poor standardization of the evaluation criteria is a challenge in the assessment of currently available G6PD deficiency tests. One example is the problem of defining normal G6PD activity, which is typically described as the mean or median activity for a given population (including deficient and heterozygous values). However, the prevalence of G6PD deficiency can significantly influence the normal value in a population [21]. The results of different studies using different methods are presented in Table 5.

In a study by Kosaryian et al., some neonates with partial G6PD deficiency could not be detected by FST [22]. Moreover, Anioon et al. showed that FST could only detect 7.5% of heterozygotes [15]. In addition, similar to the present study, Wang et al. showed that the quantitative test could detect G6PD deficiency more efficiently than other tests [23].

Development of standardized criteria in G6PD tests [24] includes a quantitative test to measure the activity level and establish a normal G6PD reference value for a study population. The standardized reference value for a study population is established by considering the median G6PD activity of the male population, while excluding males with severe deficiency to minimize the impact of severely deficient individuals on the normal reference value [21].

In the present study, the prevalence of partial G6PD deficiency was estimated at 2.4%, while the
### Table 5. Comparison of studies in detection of G6PD deficiency and their methods

<table>
<thead>
<tr>
<th>Study</th>
<th>Age/Gender</th>
<th>Quantitative method</th>
<th>Qualitative method</th>
<th>G6PD measurement method</th>
</tr>
</thead>
<tbody>
<tr>
<td>LaRue et al. (2014) [21]</td>
<td>214 subjects (19–59 years), Male = 107, Female = 107</td>
<td>Six males had activity levels 10% of the median value for all males in the study population: The adjusted male median = 7.18 U/g Hb (range = 0.84–12.26 U/g Hb); Sensitivity: 100%, NPV: 100% for the 10%, 20%, and 30% activity cutoff values, but sensitivity dropped to 82.6% when the cutoff level was 60% of normal, and NPV fell to 97.8%</td>
<td>182 (90.5%) subjects had normal G6PD activity levels, 19 (9.5%) subjects were deficient. Sensitivity: 91.3%, NPV:98.9% FST accuracy fell for these parameters when intermediate values were added to normal values</td>
<td>BinaxNOW qualitative test, Trinity Biotech quantitative G6PD test, quantitative test, Trinity Biotech, FST</td>
</tr>
<tr>
<td>Kosaryan et al. (2015) [22]</td>
<td>365 newborns, Male = 174, Female = 191</td>
<td>19 male neonates were deficient (10.9%; 95% CI: 6.27–15.53), Sensitivity: 95%, Specificity: 99.7%, PP: 100%, NPV: 99%</td>
<td>13 male neonates were deficient (7.5%; 95% CI: 3.59–11.41), sensitivity: 68%, specificity: 100%, PP: 100%, NPV: 96%</td>
<td>Decolonization test, quantitative test, FST</td>
</tr>
<tr>
<td>Wang et al. (2009) [23]</td>
<td>82 male infants</td>
<td>Detects 35.1% of severe hyperbilirubinemia The prevalence of G6PD deficiency among the male neonates was 5.1% (26/976) by both the FST and the enzyme assay method. The G6PD activity levels of all 26 cases of G6PD-deficient male neonates were &lt;20% normal (severe enzyme deficiency). In the female neonate group, the frequency of G6PD deficiency was 1.3% (6/472) by the FST and 9.35% (44/472) by enzyme assay. The 6 cases diagnosed as deficient by the FST showed severe enzyme</td>
<td>Detects 25.7% of severe hyperbilirubinemia The overall prevalence of G6PD deficiency was 3.28% by the semi-quantitative FST and 7.17% by enzyme assay. In the female heterozygote group, G6PD deficiency was diagnosed in 53% (35/67) by enzyme assay and in 7.5% (4/67) of cases by the FST</td>
<td>Quantitative test enzyme levels, FST and detection Semi-quantitative FST and enzyme activity</td>
</tr>
<tr>
<td>Ainoon et al. (2003) [15]</td>
<td>976 neonates and 67 known female heterozygotes</td>
<td>The prevalence of G6PD deficiency among the male neonates was 5.1% (26/976) by both the FST and the enzyme assay method. The G6PD activity levels of all 26 cases of G6PD-deficient male neonates were &lt;20% normal (severe enzyme deficiency). In the female neonate group, the frequency of G6PD deficiency was 1.3% (6/472) by the FST and 9.35% (44/472) by enzyme assay. The 6 cases diagnosed as deficient by the FST showed severe enzyme</td>
<td></td>
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</tbody>
</table>

(continued)
Table 5. (Continued)

<table>
<thead>
<tr>
<th>Study</th>
<th>Age/Gender</th>
<th>Quantitative method</th>
<th>Qualitative method</th>
<th>G6PD measurement method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reclos et al.</td>
<td>2000 samples from neonates (50% male; 50% female)</td>
<td>Females, who were classified as having normal enzymatic activity with the semi-quantitative test, were classified as partially deficient with the quantitative test. Using the quantitative test, the percentage of G6PD deficient neonates in this population was 5.5%, compared with 3.17% reported in routine screening using the semi-quantitative method. High risk population: the quantitative method detected 28 cases of total or partial G6PD deficiency in sisters of males with known total deficiency</td>
<td>A considerable amount of partially G6PD deficient female neonates (heterozygotes) are undetected and classified as having normal enzymatic activity using the semi-quantitative method, which uses a cutoff of 2.1 U/g hemoglobin (Hb). The use of a fully quantitative G6PD screening kit is proposed, employing the automated hemoglobin normalization and a cutoff of 6.4 U/g Hb</td>
<td>Quantitative evaluation of G6PD enzyme activity and semi-quantitative method</td>
</tr>
</tbody>
</table>

NPV, Negative Predictive Value, PPD, Positive Predictive Value, FST, Fluorescent Spot Test, CI, Confidence Interval, G6PD, Glucose 6-Phosphate Dehydrogenase.
prevalence of severe G6PD deficiency was 4.2% in the quantitative test. Also, the prevalence of partial and severe G6PD deficiency was 2.6% and 3.8%, respectively. The total prevalence of G6PD deficiency was 6.6% and 6.4% in quantitative and qualitative tests, respectively. Also, 11.4% of male and 1.7% of female subjects in our study suffered from G6PD deficiency in the quantitative study, while the corresponding values were 11.1% and 1.4% in the qualitative test, respectively.

Several studies have been conducted in Iran to determine the prevalence of G6PD deficiency. Zahed Pasha et al. showed that 12.5% of male newborns were G6PD deficient, based on FST [1]. Other studies in Iran have also shown a high rate of 8.65–16.4% in northern regions (Mazandaran and Guilan provinces), 12% in southern regions (Shiraz), and 19.3% in south-eastern regions of Iran [7].

A previous study in Tehran, Iran, in 2000 on cord blood screening of newborns for G6PD deficiency revealed a relatively lower rate in two hospitals (2.1% of the total population and 3.6% of males), compared with other reports [7]. In addition, evaluation of a screening program for 115,622 newborns in Iran showed a deficiency rate of 6.1%. In this study, the male-to-female ratio was 6:1 and 86.1% and 13.9% of the subjects were male and female, respectively [25]. Overall, the difference between the prevalence rates reported in Iran could be due to variations in the genetic type of G6PD in different social and ethnic groups.

Iran is a large country (population of approximately 80 million people) with different ethnicities residing in northern, southern, and central provinces. In most areas with a high prevalence of G6PD deficiency, several G6PD mutations have been detected. According to the literature, the first and most common variant of G6PD in the coastal provinces of Caspian Sea (northern Iran) and Kurdistan (western Iran) is the G6PD Mediterranean variant [26, 27].

CONCLUSION

In the present study, FST results were in line with the findings of the quantitative test, although FST tended to provide false negative values for mild to moderate G6PD deficiencies. The present study indicated the acceptable sensitivity and specificity of FST for G6PD activity, although some cases remained undiagnosed. Although FST is a cost-effective and available test, in regions with a high prevalence of G6PD deficiency, it is logical to use the quantitative test for a more accurate diagnosis and limit the undiagnosed cases as much as possible. The results of this study suggest that G6PD enzyme assay should be replaced instead of FST, as a screening tool to improve the detection of G6PD deficiency. For a more definite conclusion, further DNA tests are recommended in our population.

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