



The Development of Polyclonal Antibody Against Glucose-6-Phosphate Dehydrogenase (G6PD) for G6PD Deficiency Newborn Screening in Indonesia

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Abstract

Background/Aim: Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an enzymatic disorder affecting carbohydrate metabolism, is a common cause of haemolytic anaemia and jaundice in newborns. Despite the proven benefits of G6PD screening, Indonesia faces significant challenges in implementing nationwide programs. Standard screening via dried blood spot (DBS) samples has limitations, prompting the need for improved diagnostic methods. This study aimed to develop a more sensitive and accessible screening method by creating polyclonal antibodies against G6PD.

Methods: G6PD protein was isolated from newborn cord blood and confirmed using western blot analysis. The rabbits were immunised with purified G6PD antigen. The polyclonal antibody with the highest titre was purified by saturated ammonium sulphate (SAS) method.

Results: Western blotting confirmed the presence of G6PD at approximately 56 kDa in the isolated protein fraction. Immunised rabbits showed a significant increase in antibody titre compared with the control group. This research successfully isolated G6PD and produced polyclonal antibodies against it. However, further work is required to fully characterise the antibody and evaluate its suitability for G6PD deficiency screening. This antibody development holds promise for creating a dipstick kit for G6PD deficiency screening, thus overcoming the limitations of the DBS method. Colloidal gold-based immunochromatographic strips could be a suitable platform for such a kit due to their ease of use and rapid results.

Conclusion: Presented findings constitute a solid foundation for introducing a novel immunochemical reagent and developing a rapidly scalable protocol for producing G6PD-specific antibodies.

Key words: Immunoglobulins; Antibodies; Antibody, polyclonal; Glucose-phosphate dehydrogenase; Deficiency; Neonatal screening.

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Introduction

Glucose-6-phosphate dehydrogenase (G6PD) deficiency, an enzymatic disorder affecting carbohydrate metabolism, is a common cause of haemolytic anaemia and jaundice in newborns.¹ The

pentose phosphate pathway that plays vital role in maintaining redox balance in erythrocytes will

be affected. The enzyme is predominantly found in the cytoplasm, but genetic mutations can significantly reduce its intracellular levels. This enzymatic defect renders red blood cells susceptible to lysis when exposed to oxidants, leading to a spectrum of clinical manifestations, ranging from mild fatigue and anaemia to severe kernicterus and neurological damage. In newborns, G6PD deficiency often presents as neonatal jaundice that is frequently overlooked or misdiagnosed. Globally, an estimated 400 million individuals suffer from G6PD deficiency, with a high prevalence in Eastern regions.² In Indonesia, the prevalence of G6PD deficiency has become more apparent in the past two decades. Incidence rates varied significantly across regions, ranging from 3.9 % in North Sumatra to 8 % in West Papua. A significant portion of the population remains unaware of the clinical symptoms or diagnostic methods for this condition.³

A major challenge is the lack of routine newborn screening for G6PD deficiency in Indonesia. Early detection is crucial for a timely intervention. A study in a private Surabaya hospital identified 37 cases of G6PD deficiency associated with adverse pregnancy outcomes (30 miscarriages and seven stillbirths/birth defects). Similarly, at Dr Soetomo Surabaya General Hospital and nine cases of G6PD deficiency were linked to five miscarriages.⁴ Recent research has established a correlation between G6PD deficiency and pregnancy complications, including miscarriages, foetal death and birth defects. Therefore, early detection can significantly reduce these risks. Currently, the most sensitive method for screening neonatal G6PD deficiency is quantitative enzymatic activity measurement using spectrophotometry or fluorometric assays. However, these methods are resource-intensive, requiring skilled personnel, specialised laboratory equipment and often necessitating sample transport to larger laboratories.^{5,6} The limited testing facilities pose delayed or missed diagnoses. To address the limitations, antibody-based detection methods offer a promising alternative for point-of-care (POC) applications. Rapid test development using native human G6PD specific antibodies will pose low-cost and field-deployable diagnostic tools, especially in rural area or region with limited laboratory access.^{7,8}

The objective of this study was to explore the immunogenic properties of Indonesian native protein for broad-based screening applica-

tions through the development of a polyclonal antibody against G6PD as the initial step. Therefore, the characterisation of polyclonal antibodies against G6PD is important for G6PD deficiency in newborn screening development in Indonesia. The rationale for this research was to expand the national G6PD screening coverage and develop easy-to-use detection kits, as foundational step toward larger screening coverage in Indonesia and supporting early intervention strategies to mitigate neonatal morbidity.

Methods

Research design

This study used both *in vivo* and *in vitro* laboratory exploratory designs. This study was conducted at the Biochemistry Laboratory, Faculty of Mathematics and Natural Sciences, Institute of Biosciences, Brawijaya University. The survey was held between May 2024 and October 2024. The research sample was newborn cord blood that was obtained by purposive sampling from Melati Husada Woman and Children Hospital, Malang. Blood samples were collected in a sterile container, obtained from the umbilical cord 15 to 20 cm long at the time of the birth of the baby. The experimental animal utilised for polyclonal antibody production was *Oryctolagus cuniculus*.

Human G6PD protein identification

Serum was separated from umbilical cord blood samples. Subsequently, protein characterisation was performed using SDS-PAGE to determine the molecular weight. Western blotting was used to confirm the presence of G6PD protein at approximately 56 kDa. Following western blot confirmation, the target protein band was excised using electroelution, followed by emulsification with an adjuvant in accordance with the target volume for animal immunisation.

Development of polyclonal antibodies for the detection of human G6PD

A 300 μ L volume of G6PD antigen was mixed with 100 μ L of complete Freund's adjuvant (CFA) /incomplete Freund's adjuvant (IFA) (three to one ratio) and vortexed at full speed for approximately two hours until a complete emulsion was formed. A 400 μ L volume of this emulsion was injected into each rabbit. The immunisation

schedule was as follows: 0 days (pre-immunised bleeding, antigen + CFA injection), 21 days (First bleeding, antigen + IFA First injection), 28 days (Second bleeding), 35 days (Third bleeding), 42 days (Forth bleeding), 49 days (Fifth bleeding, antigen + IFA second injection), 56 days (Sixth bleeding), 63 days (Seventh bleeding), 70 days (Seventh bleeding), 77 days (Eight bleeding) and 84 days (Nineth bleeding). Following the immunisation schedule, IgG antibodies were purified from rabbit serum using saturated ammonium sulphate (SAS) precipitation, followed by protein A/G affinity chromatography.

Exploring the immunogenicity of human G6PD protein

ELISA was performed to determine the highest antibody titre and monitor the immune response to the induced antigen in rabbits. To confirm that the immunisation produced antibodies against

G6PD, indirect ELISA was conducted as follows: 50 µL of G6PD antigen in coating buffer (pH 9.6) was added to each well and incubated overnight. After washing with phosphate buffered saline with Tween-20 (PBST), the wells were blocked with 1 % bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 90 min at room temperature. Subsequently, 50 µL of rabbit serum was added and incubated for two hours at room temperature, followed by washing with PBST. An anti-rabbit IgG-AP conjugate (1:2500 dilution in PBST-1 % BSA) was then added and incubated for two hours at room temperature. After washing, the p-nitrophenyl phosphate (PNPP) substrate was added and incubated for 30 min at room temperature. The reaction was stopped with 1N NaOH and the absorbance was measured at 405 nm. A dot blot assay was performed to visualise the target molecules on a nitrocellulose membrane.

Results

G6PD isolation and characterisation

Characterisation of proteins based on their molecular weight was performed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) after separation of serum from cord blood samples. Western blot analysis was performed to confirm the isolated protein fraction. A

single band at roughly 56 kDa was observed under non-reducing conditions. Following western blot confirmation, the target protein bands were extracted using the electroelution technique. Subsequently, the extracted protein was mixed with the adjuvant in accordance with the required volume to immunise the test animals.

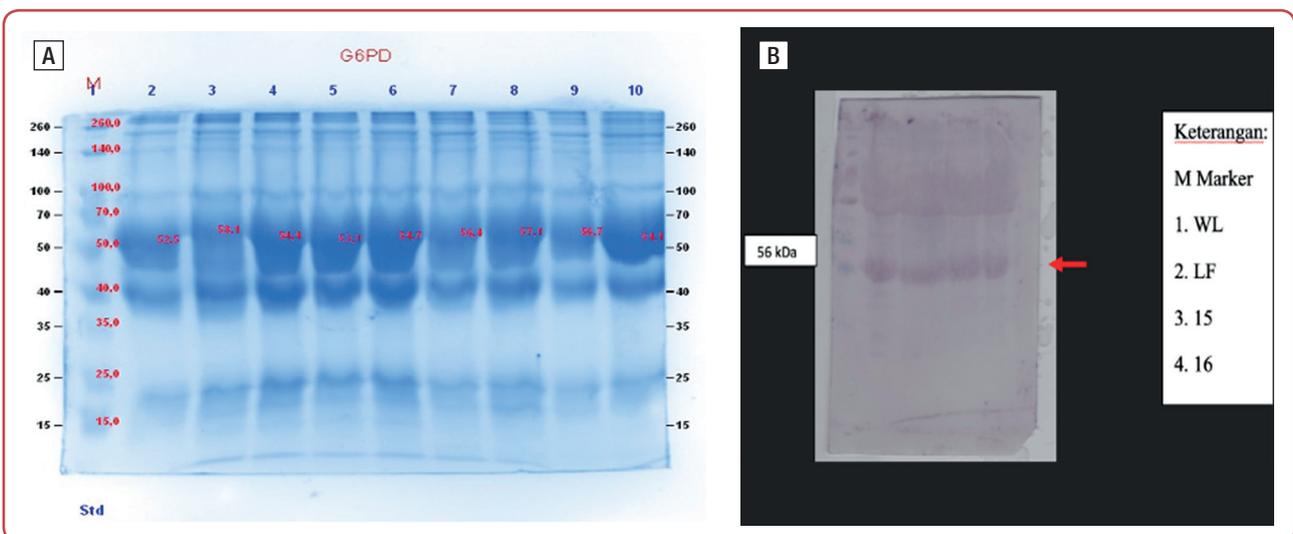


Figure 1: Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) showed multiple proteins from protein isolate with various molecular weights from 9 newborn cord; blood (A) and western blot (B) confirmed the existence and consistency of human glucose-6-phosphate dehydrogenase (G6PD) as a 56-kDa protein from 4 sample.

The blot was probed using anti-G6PD antibody, demonstrating successful isolation.

Production and characterisation of G6PD polyclonal antibody

Measurement of G6PD antibodies showed that the G6PD antigen present in the patient's serum was antigenic and capable of triggering antibody production. The control rabbit serum titre curve showed no significant changes in optical density (OD) values. The highest titre of polyclonal antibodies obtained from the G6PD protein induction was measured using ELISA. The aim of this methodology was to elucidate the production dynamics of polyclonal antibodies following the immunisation procedure (Figure 2). The graph shows the anti-G6PD titres in rabbit blood serum after ten bleedings induced with G6PD protein. The concentration of anti-G6PD increased gradually from the first week to the fifth week and reached its highest level at the second blood draw with an absorbance value of 0.466. The highest titre indicated an increase in the number of antibodies used for the primary immune response. The rabbit immune system recognises the human G6PD antigen, so the immune response continues to synthesise specific antibodies, forming anti-G6PD. A booster was administered in the 3rd and 7th weeks and in the seventh bleeding process, a trend of increasing G6PD antibody titre was found until the highest titre was obtained at the 8th bleeding with an absorbance value of 0.6805. This increase in absorbance is a secondary immune response, where B cells are reactivated to produce anti-G6PD after the rabbit is

re-immunised with G6PD and an incomplete adjuvant. In comparison to the absorbance values observed in the primary immune responses, the antibody titre exhibited in the secondary immune response was elevated.

Specificity and optimal titre of primary antibody against G6PD protein

Quantitative analysis of protein density, visualised by colour intensity, revealed varied response patterns among the four samples at different antibody dilution levels. Dot blot analysis employed G6PD antigen obtained from cord blood serum samples of four newborns coded as (PN, WR, WL and SN) (Figure 3). These sera were subsequently incubated with primary antibodies obtained from the purified antibody of the 8th week bleeding at dilutions of (1/10, 1/20, 1/40 and 1/80). Sample PR demonstrated a progressive increase in density, reaching an optimal point at a dilution of 1:40 with a density of 32,000 units, followed by a moderate decrease at a dilution of 1:80. Sample WR exhibited a different characteristic, showing high density stability in the dilution range of 1:10 to 1:40, but undergoing significant degradation at a dilution of 1:80. Meanwhile, sample WL exhibited a more gradual and regular pattern of increase up to a dilution of 1:40, with a more moderate decrease in density at subsequent dilutions. An interesting phenomenon was observed in sample SN, which showed a unique pattern of a drastic decrease in the early dilution phase until it

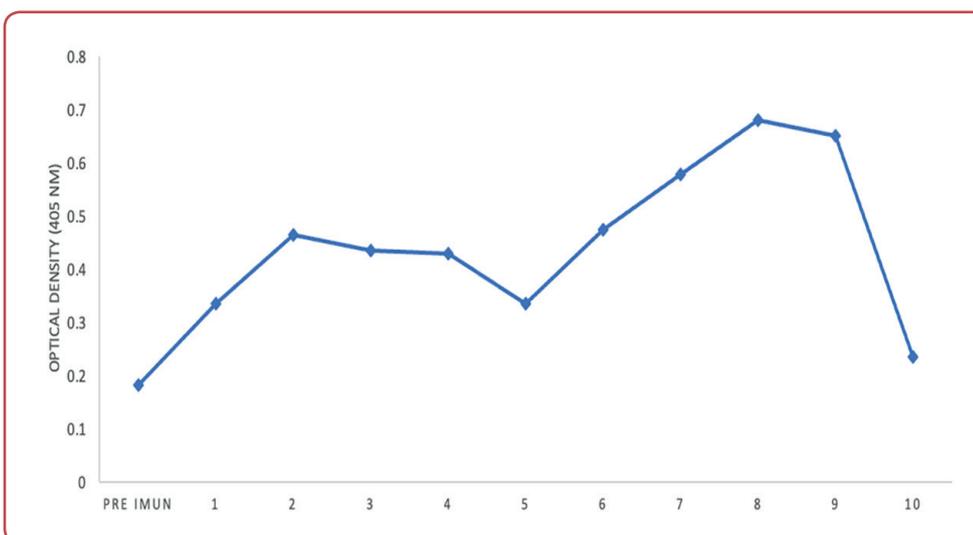


Figure 2: Antibody titre against glucose-6-phosphate dehydrogenase (G6PD) from indirect ELISA results. Kinetics of anti-G6PD antibody production. Serum samples were collected prior to immunisation and weekly for ten weeks post-immunisation. Optical density readings at 405 nm, reflecting G6PD-specific antibody binding, were recorded. This graph validates the immunogenicity of the human G6PD antigen and successful antibody generation.

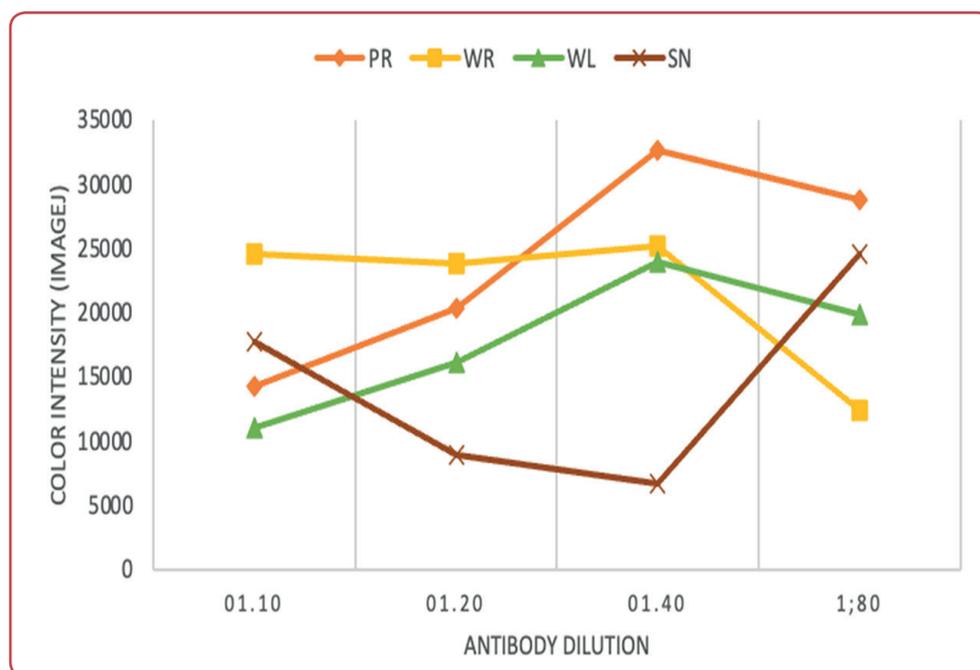


Figure 3: Graph of colour intensity value from dot blot assay.

The graph corresponding to glucose-6-phosphate dehydrogenase (G6PD) protein detection in four different cord blood serum samples (PR, WR, WL and SN) at varying antibody dilutions (1:10, 1:20, 1:40 and 1:80). Mean colour intensity values are quantitatively indicating antigen-antibody interaction strength, thus demonstrates the binding capacity and specificity of the produced antibody.

reached a minimum at a dilution of 1:40, followed by a significant elevation at a dilution of 1:80. The analysis results indicate that the optimal dilution for most samples was at a level of 1:40, with the exception of sample SN, which requires further evaluation regarding its different response characteristics.

Discussion

G6PD deficiency is a common worldwide condition caused by loss-of-function mutations in the G6PD gene. Individuals with this deficiency are more susceptible to oxidative stress, leading to classic acute haemolytic anaemia (favism).¹ In the absence of sufficient NADPH, red blood cells in individuals with G6PD deficiency are unable to produce the necessary enzymes for the detoxification of reactive oxidants that are present within their cells.^{1,9} A study has shown that 7% of infants with G6PD deficiency in the neonatal population experience jaundice, which can peak at 2–3 days post-birth and if untreated, progress to kernicterus and permanent neurological damage.¹⁰

G6PD status is typically determined by measuring the enzyme activity in whole red blood cells.

Cord blood screening for G6PD deficiency in neonates is a popular method, especially in low- and middle-income countries, with high prevalence rates. The most used screening method for G6PD deficiency is the fluorescent spot test (FST), a semi-quantitative assay. Although inexpensive and easy to perform, this method can only detect cases with 20% or less of normal G6PD activity and misses many cases of moderate G6PD activity (30–60%).¹¹ Ultraviolet (UV) spectrophotometry is the standard reference diagnostic method used to measure G6PD enzyme activity with labour-intensive method, which requires fresh samples and is not easily adaptable to POC settings.^{5,12}

Dried blood spot (DBS) samples are often used in neonatal screening due to their convenience and ease of transport, yet they suffer from some disadvantages.¹³ G6PD enzyme activity degrades after some periods in improperly stored DBS samples, causing false negatives. Haemolysis, poor quality blood sampling and contamination can affect the accuracy for the test. Collection of neonatal blood samples should be through the heel, placing a single, clean drop on filter paper and avoiding double spotting and contamination, as advised in the Clinical and Laboratory Standards Institute (CLSI). Blood drops should never be kept, put on wet surfaces, or be contaminated

with coffee, milk, or other liquids.^{13,14} If DBS samples are not tested promptly after collection, the G6PD enzyme activity may decrease due to degradation, leading to false-negative results.⁶

Currently, there are two G6PD screening POC tests (POCTs): BinaxNOW® and CareStart™. Both devices are commonly used for G6PD deficiency screening in malaria patients who received primaquine therapy.^{15, 16} POCTs use the enzymatic reduction principle, which converts colourless nitro blue tetrazolium into dark-coloured formazan. While useful, these devices have limitations: they are sensitive to temperature changes, function optimally only within a specific temperature range (18–32 °C) and cannot reliably identify heterozygous individuals with ≥ 30 % enzyme activity. Moreover, the absence of a control line limits result validation.⁶

In contrast, immunodiagnostic approaches, particularly those utilising antibodies against specific protein, provide a more stable and robust platform for detecting the presence of the enzyme, independent of its activity. In comparison to enzymatic assays, antibody-based methods offer several advantages. The antibodies are generally more resistant to sample degradation than enzymatic activity, which pose greater stability. Simplified interpretation with visual readouts will reduce the need for instrumentation and also provide rapid test format with minimal user training. This study explored the development of polyclonal antibodies against G6PD for use in rapid diagnostic tests. G6PD protein was successfully isolated from human cord blood and identified by western blotting, showing a band at approximately 56 kDa, consistent with its expected molecular weight under reducing conditions. Immunisation of rabbits with this purified protein induced a strong humoral response, confirmed through elevated ELISA absorbance values compared to controls injected with PBS. Antibody titres peaked around the 14th day, reflecting a robust primary immune response. A subsequent increase due to the secondary immune response suggested active memory B cell involvement, which enhanced antibody production. By the fifth week, antibody titres began to decline, consistent with the natural degradation of primary antibodies. These findings confirm the immunogenicity of the G6PD antigen and its capacity to induce specific polyclonal antibody production. The dot blot assay further demonstrated the functional-

ity of the produced antibodies, with optimal reactivity observed at a 1:40 dilution, indicating strong binding activity.

Although the antibodies were successfully generated and purified using dialysis, further characterisation is essential to assess their specificity and binding affinity. Techniques such as competition ELISA and immunoprecipitation will be necessary to confirm the reliability of these antibodies in detecting G6PD.⁷ Additionally, the clinical performance of these antibodies should be validated by comparing their ability to detect G6PD deficiency against established methods using DBS samples. The use of polyclonal antibodies against G6PD has significant potential for developing rapid diagnostic kits, especially in dipstick format. One promising platform is the colloidal gold-based immunochromatographic strip, a one-step method that allows for quick, visual detection without specialised equipment.⁸ Colloidal gold-based immunochromatographic strips, commonly known as lateral flow assays (LFAs), enables rapid, one-step, visual detection without the need for specialised laboratory infrastructure. Commercial applications utilising polyclonal antibodies include diagnostic tools for dengue NS1 (eg SD BIOLINE Dengue Duo), *Streptococcus A* antigen (eg BD Veritor™) and SARS-CoV-2 antigen tests widely deployed during the COVID-19 pandemic.¹⁷⁻¹⁹

These tests deliver results in less than 20 minutes and are suitable for both clinical and home settings. Studies have demonstrated that LFA platforms employing polyclonal antibodies can achieve sensitivities ranging from 85–95 % and specificities up to 98 %, depending on the antigen and target population.²⁰ Although LFAs for G6PD deficiency are not yet widely commercialised, several research prototypes —utilising antibody-based diagnostic tools— have demonstrated feasibility, particularly in Southeast Asian populations.²¹ These data support the potential translation of antibody into a lateral flow-based detection system for neonatal G6PD deficiency. The immunochromatographic strips offer distinct advantages for application in low-resource settings: ease of use, rapid results and minimal training requirements. A POC test of this nature could significantly enhance early detection and broaden screening coverage for G6PD deficiency in neonates, especially in high-burden regions such as Indonesia.¹⁶

Conclusion

The generation of polyclonal antibodies targeting G6PD represents a promising approach in overcoming current diagnostic limitations. These antibodies could serve as the foundation for POCT devices, improving detection rates and enabling timely intervention to prevent severe complications such as kernicterus. Further validation and optimisation are needed, but the groundwork laid in this study supports future development of accessible, rapid and reliable newborn screening tools for G6PD deficiency.

Ethics

All procedures have been approved by the Research Ethics Committee of Brawijaya University (decision No 146-KEP-UB-2024), dated 5 August 2024.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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