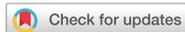


Original Article



Basic Verification of β -D Glucan in Leukocyte-Rich Plasma for the Diagnosis of Deep Mycosis

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OPEN ACCESS

Received: Oct 31, 2020

Accepted: Feb 14, 2021

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Funding

This study was partially supported by a 2016-2018 grants-in-aid for scientific research [grant number 16K19616].

Conflict of Interest

No conflicts of interest.

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ABSTRACT

Background: Currently, supplementary serological testing for β -D glucan (BDG) is often selected to diagnose deep mycosis in care covered by the health insurance in Japan. The Wako method used by our center has low sensitivity, and different studies have used different cut-off values due to factors that cause false positives and false negatives. One possible cause of false negatives is the use of platelet-rich plasma (PRP) as the sample material. Because phagocytic white blood cells (WBC) are precipitated by centrifugation and only plasma is measured, it seems unlikely that the actual amount of BDG is being measured when using PRP. Further, a frequent cause of false positives is contamination from blood products and gauze containing BDG. To resolve these issues, the blood cell separator, hydroxyethyl starch, is used to precipitate only the red blood cells to obtain leukocyte-rich plasma (LRP). We hypothesized that it might be possible to improve the diagnostic rate of deep mycosis by measuring the BDG content of plasma containing WBC and fungal components and by comparing the BDG content of PRP and LRP measured simultaneously.

Materials and Methods: Healthy human blood, albumin-added blood, wrung-out gauze fluid-added blood, and fungal solution-added blood were prepared, and PRP and LRP were prepared using hydroxyethyl starch. The BDG content of each sample was measured using the Wako method and compared. In addition, PRP and LRP of fungal-added blood were Gram-stained and examined under a microscope, and the number of WBCs and phagocytosed fungi was counted visually and compared.

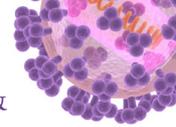
Results: Measuring the BDG content of LRP confirmed that there were no false positives with LRP, and *in vitro* experiments comparing albumin-added false-positive blood to fungal-added blood showed significant differences between PRP and LRP only in the fungal-added blood.

Conclusion: Calculating the BDG-ratio (LRP/PRP) by measuring both LRP and PRP may eliminate false positives and false negatives of true deep mycosis and improve the diagnostic rate.

Keywords: Leukocyte; Platelet-rich plasma; Hydroxyethyl starch; Beta-glucan

INTRODUCTION

Currently, β -D glucan (BDG) is often selected for supplementary serological testing of deep mycosis in care covered by health insurance in Japan [1]. In emergency settings or intensive


Author Contributions

Conceptualization: KS, SK, KI, SE. Data curation: KS, GM. Funding acquisition: KS, SK. Investigation: KS, GT. Methodology: KS. Project administration: KS, KI. Resources: KS. Validation: KS. Writing - original draft: KS, YY. Writing - review & editing: KS, YI.

care units, patients with severe conditions are often susceptible to infection. While the possibility of deep mycosis needs to be kept in mind, the various causes of false positives and false negatives indicates that measuring BDG has low diagnostic accuracy. Without an accurate diagnosis, antifungal agents cannot be initiated. Methods of measurement include the Wako, Fungitec G test MK (G-MK). and Maruha methods; however, various testing accuracies have been reported depending on the cut-off value [2]. The current methods use platelet-rich plasma (PRP), a form of centrifuged plasma, as the sample material; however, fungi bind to or are phagocytosed by white blood cells (WBC), thereby increasing the risk of false negatives because only the plasma is measured and not the fungal BDG. Further, it is known that false positives can be caused by factors such as the administration of albumin and contamination from medical materials [3]. In the present study, we used hydroxyethyl starch (HES), a blood cell separator, to precipitate only red blood cells (RBC) and create leukocyte-rich plasma (LRP). We hypothesized that measuring the BDG content of plasma containing WBCs and fungal components would suppress false negatives and that comparing the BDG content of PRP and LRP would allow us to distinguish false-positive specimens, thus, improving the diagnostic rate of deep mycosis.

In this study, we performed basic experiments to confirm (1) that there are no false positives due to nonspecific reactions with HES itself, (2) that there are no false positives in LRP by mixing HES with healthy blood, and (3) that false-positive and true-positive samples *in vitro* are valuable to compare the differences in the BDG content between PRP and LRP.

MATERIALS AND METHODS

This study was reviewed and approved by the ethics review committee of the facility (st-2019-001).

1. Materials

1) Healthy human blood

Informed consent was provided for the use of blood, and among volunteers who obtained ethical approval, adults with no particular history were randomly selected and blood was collected.

2) HES

HES physiological saline solution (6%, Voluven[®], Otsuka Pharmaceutical Co., Tokyo, Japan) was used.

3) Wako method

Plasma pretreatment solution and Limulus reagent: plasma pre-treatment solution (0.02% Triton X-100, Wako Pure Chemical Industries, Osaka, Japan) and β -D Glucan Test Wako[®] (freeze-dried product, Wako Pure Chemical Industries) were used. The Toxinometer MT-5500[®] (Wako Pure Chemical Industries) was used for the measurements. The procedure was performed according to the manufacturer's instructions.

4) PRP samples

To remove only RBC with as little fungal centrifugation as possible, we followed the protocol published by Inada et al. [1, 2] and obtained PRP by centrifuging heparinized whole blood for 40 s at 1,200 \times g (3,000 rpm). The Wako method using this type of sample was referred to as the PRP method.

5) *LRP samples*

To precipitate RBC, 0.5 mL HES was added to the same amount of 3 heparinized whole blood samples from healthy people, mixed slightly, and left for 15 min. The supernatant was collected to obtain LRP without any RBC. We previously reported on the verification and examination of LRP samples [4]. The Wako method using this type of sample was referred to as the LRP method.

6) *Correction of measurements using the LRP method considering the hematocrit*

Because the plasma was diluted when adding an equal amount of HES to the blood, the LRP measurement needed to be corrected by taking into account the hematocrit (Ht) when comparing the values from the PRP and LRP methods. When x mL of Ht (%) blood was used, the amount of PRP to be measured was $x(1-Ht)$ mL. The plasma portion of LRP was diluted to $x(1-Ht) + x$ mL because the same amount of HES was added. Therefore, as a correction formula for this dilution, we multiplied the measured LRP value by $(2-Ht) / (1-Ht)$ [4, 11]. In other words, the upper limit of measurement using the PRP method is 900 pg / mL, and the lower limit of measurement is 0.6 pg / mL; however, the upper and lower limits can be increased by multiplying the measured value by the correction coefficient.

7) *Albumin-added blood*

An albumin preparation (0.06 mL, 25% Red Cross Albumin®, Red Cross, Tokyo Japan.) was added to 0.49 mL of healthy human blood and mixed at 37°C for 2 h.

8) *Wrung-out gauze fluid*

This was prepared by immersing medical gauze (3 Sterilized Square Gauze Blisters®, Hasegawa Menko Co. Nagoya, Aichi Prefecture, Japan) in 20 mL of 9% physiological saline, then wringing out the gauze into a sterilized test tube.

9) *Fungal solution-added blood*

Using a cotton swab, a small sample of *Candida albicans* was taken from colonies in our bacterial laboratory, and mixed with 10 mL of 9% physiological saline to prepare a fungal solution. Since the hyphae of the cells may form a mass and cause a difference in concentration in the dispersed sample, the mixture was mixed for 1-min.

2. Methods

1) *Examination of BDG content in HES only*

Pre-treatment was performed directly in 0.1 mL HES, and the BDG content was measured using the Wako method.

2) *BDG content of PRP and LRP from healthy human blood*

The BDG content of PRP and measured LRP was measured using 0.5 mL of whole blood from healthy volunteers ($n = 10$) and compared.

3) *Comparison of BDG content between PRP and LRP from albumin-added blood*

Albumin (0.06 mL, 25% Red Cross Albumin) was added to 0.49 mL heparinized whole blood from healthy volunteers ($n = 10$) and mixed for 2 h at 37.0°C. Then, the BDG content was measured using the PRP and LRP methods and compared.

4) *Comparison of BDG content between PRP and LRP from wrung-out gauze fluid-added blood*

Wrung-out gauze fluid (0.01 mL) was added to 0.49 mL heparinized whole blood from

healthy volunteers (n = 10) and mixed for 2 h at 37.0°C. Then, the BDG content was measured using the PRP and LRP methods and compared.

5) *Comparison of BDG content between PRP and LRP from fungal-added blood*

The fungal solution (0.01 mL) at the prepared concentration was added to 0.49 mL heparinized whole blood from healthy volunteers (n = 10) and mixed for 2 hours at 37°C. Then, the BDG content was measured using the PRP and LRP methods and compared.

6) *Comparison of BDG ratios*

The BDG ratio was defined as the LRP-BDG value divided by the PRP-BDG value. The BDG ratios were calculated and compared.

7) *WBCs and phagocytosed fungi*

PRP and LRP (10 μL each) were collected from the fungal-added blood, gram stained, and inspected. The number of WBCs and phagocytosed fungi was counted visually and compared.

8) *Statistical analyses*

SPSS(version 22; IBM Corp., Armonk, NY) was used for the statistical analysis. The Man-Whitney *U* test and multiple comparisons were used, with $P < 0.01$ indicating statistical significance.

RESULTS

1. BDG content in HES only

In HES alone, all the values were low, and there were no abnormally high values (Fig. 1).

2. Comparison of BDG content in PRP and LRP from healthy people

The difference between PRP and measured LRP from healthy human blood was not significant, and no abnormally high values were observed (Fig. 2) (n = 10, $P = 0.143$, Mann-Whitney *U* test).

3. Comparison of BDG content in PRP and LRP from albumin-added blood

The difference in BDG between PRP and corrected LRP from albumin-added blood was not significant (Fig. 3) (n = 10, $P = 0.971$, Mann-Whitney *U* test).

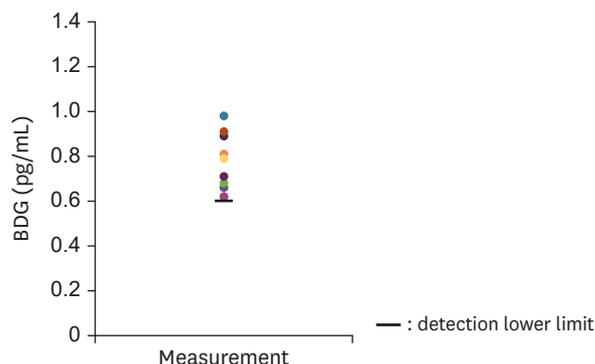


Figure 1. BDG content in HES only. Lower limit of detection, 0.6pg/mL. BDG, β-D glucan; HES, hydroxyethyl starch.

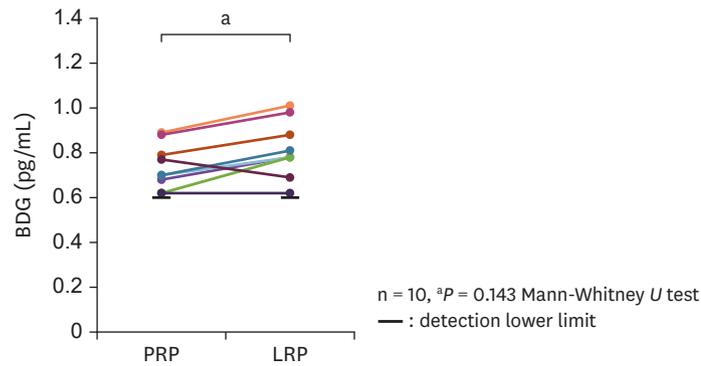


Figure 2. BDG in PRP and LRP from healthy people. Lower limit of detection, 0.6 pg/mL. BDG, β-D glucan; PRP, platelet-rich plasma; LRP, leukocyte-rich plasma.

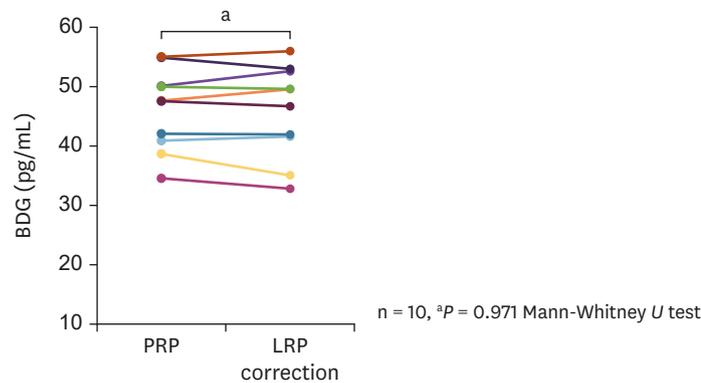


Figure 3. BDG content in PRP and LRP from albumin-added blood. Lower limit of PRP detection, 0.6 pg/mL. Corrected lower limit of LRP detection (hematocrit 40%), 1.6 pg/mL. BDG, β-D glucan; PRP, platelet-rich plasma; LRP, leukocyte-rich plasma.

4. Comparison of BDG content in PRP and LRP from wrung-out gauze fluid-added blood

The difference in BDG between PRP and corrected LRP from wrung-out gauze fluid-added blood was not significant (**Fig. 4**) ($n = 10, P = 0.222$, Mann-Whitney U test).

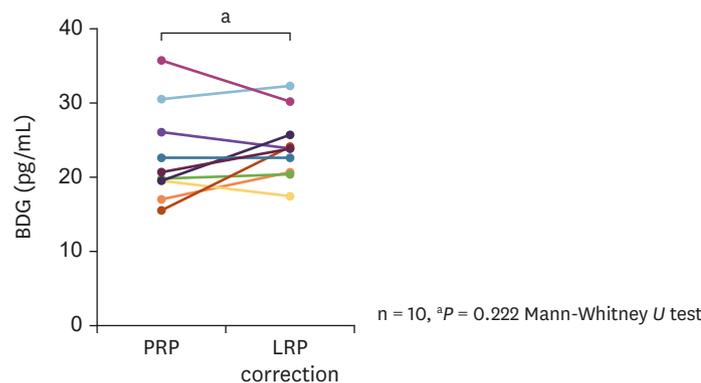


Figure 4. BDG content in PRP and LRP from wrung-out gauze fluid-added blood. Lower limit of PRP detection, 0.6 pg/mL. Corrected lower limit of LRP detection (hematocrit 40%), 1.6 pg/mL. BDG, β-D glucan; PRP, platelet-rich plasma; LRP, leukocyte-rich plasma.

5. Comparison of BDG content in PRP and LRP from fungal-added blood

The BDG content of corrected LRP was significantly higher than that of PRP in fungal-added blood (Fig. 5) (n = 10, $P < 0.001$, Mann-Whitney *U* test).

6. Calculation and comparison of BDG ratios

The values for albumin-added blood and wrung-out gauze fluid-added blood were 0.99 ± 0.04 and 1.10 ± 0.22 , respectively, while that for true-positive samples was 1.83 ± 0.36 . The BDG ratio of true-positive samples was significantly higher than that of false-positive samples (Fig. 6) (n = 10, $P < 0.001$, multiple comparisons, Bonferroni method).

7. Comparison of phagocytosed fungi count in PRP and LRP samples from fungal-added blood

Gram staining and examining PRP and LRP from fungal-added blood showed that LRP contained more fungal and WBC components than PRP, as well as more phagocytic WBC components (Fig. 7) (n = 10, $P = 0.002$ and, 0.001 , respectively Mann-Whitney *U* test).

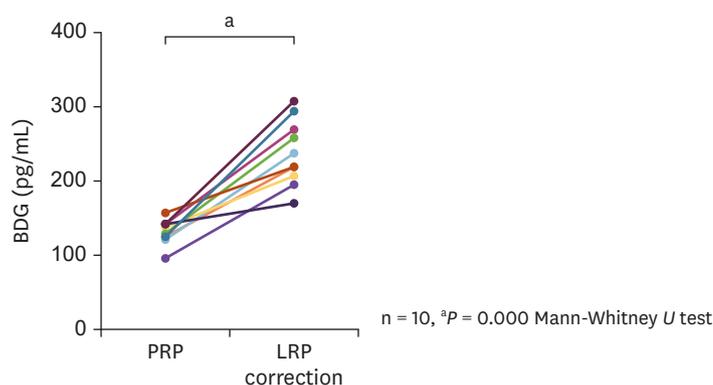


Figure 5. BDG content of PRP and LRP from fungal-added blood. Lower limit of PRP detection, 0.6 pg/mL. Corrected lower limit of LRP detection (hematocrit 40%), 1.6 pg/mL. BDG, β-D glucan; PRP, platelet-rich plasma; LRP, leukocyte-rich plasma.

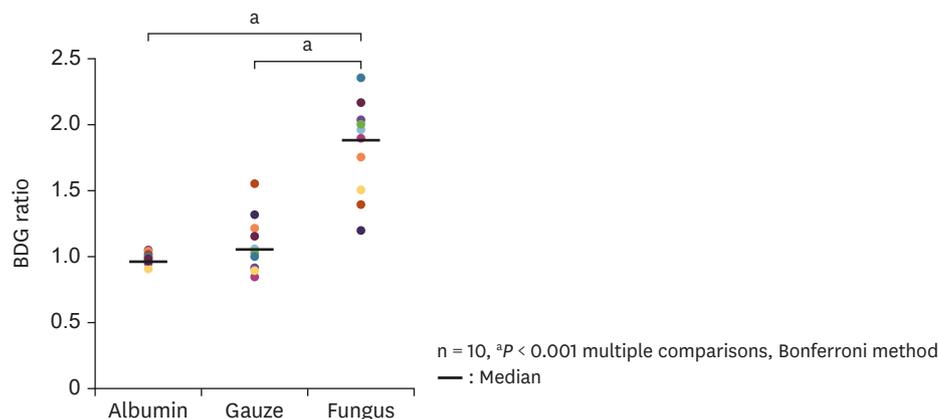
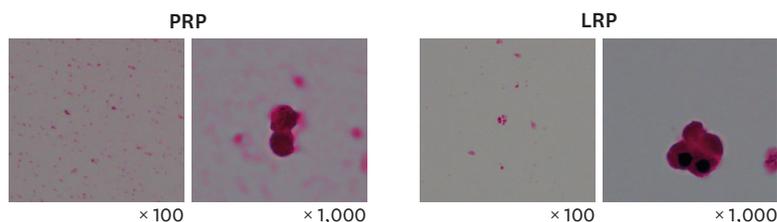
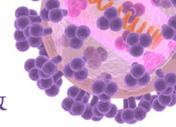


Figure 6. Calculation and comparison of BDG ratios. BDG-ratio: corrected LRP-BDG / PRP-BDG. BDG, β-D glucan; PRP, platelet-rich plasma; LRP, leukocyte-rich plasma.



	PRP	LRP	P-value
WBC components (pieces/10 mm ³)	116.70 ± 52.21	287.30 ± 167.41	0.002
Phagocytic WBC components (pieces/10 mm ³)	7.90 ± 5.95	53.90 ± 20.37	<0.001

n = 10, P = 0.002, <0.001, Mann-Whitney U test.

Figure 7. Inspection of PRP and LRP samples from fungal-added blood. PRP, platelet-rich plasma; LRP, leukocyte-rich plasma; WBC, white blood cell.

DISCUSSION

In clinical practice, deep mycosis, and particularly fungemia, is often seen in serious underlying diseases and systemic conditions and has a high mortality rate of approximately 40%. Depending on the facility, it can take approximately 3 days to get results which partially explains the high mortality rate of deep mycosis [2].

Although the Wako method used at our institution has higher specificity than other methods, its sensitivity is slightly lower, and different studies have used different cut-off values [5]. The G-MK method has high sensitivity; however, caution is needed when interpreting the results because of its low positive predictive value [5-8]. We believe the main reason for this is that PRP, a form of plasma obtained from centrifuging, is used as the sample material. As fungi and WBCs that have phagocytosed fungi are precipitated during centrifugation to obtain PRP, the current Wako method measures only plasma. Thus, it is unlikely to measure the actual amount of BDG and only quantifies the small amount that remains in plasma. However, with LRP, WBCs are not precipitated and, thus, BDG from phagocytosed fungi can be measured along with plasma BDG. This may suppress false negatives and improve the accuracy of the test. The same is true for endotoxins, and we have obtained similar results in basic research and clinical studies on endotoxemia [4, 9].

Factors known to lead to false positives include plasma formulations produced by passing through cellulose membranes (albumin, γ globulin formulations), dialysis using cellulose membranes, and contamination by BDG in gauze [10-12]. BDG is widely distributed in the cell walls of fungi such as yeast, mushrooms, and mold, as well as in algae and higher plants. BDG has been reported to have several physiological activities; however, unlike endotoxins, invasion does not cause acute toxicity, and there is no immune response to BDG [3]. Therefore, BDG itself is not phagocytosed by WBC. That is, because free BDG that remains in plasma components is measured with PRP, positive values can be obtained, including false positives in the absence of fungi.

Our results indicated that the HES preparation we used does not contain BDG. Even if HES is mixed with blood, there would be no difference between PRP and LRP and no physiological reactions that would create a false positive. Regardless of the presence or absence of WBC-phagocytosed fungi, the measurement of PRP or LRP alone can lead to positive values if false-positive factors are present, which makes clinical decisions difficult. However, when both were measured simultaneously, there was no difference between PRP and LRP from albumin-

added blood and wrung-out gauze fluid-added blood, and a significant difference was observed only with fungal-added blood. This indicates that because the BDG in albumin and gauze is not phagocytosed by WBCs, the amount of BDG will be the same in PRP and LRP despite the presence of WBC in LRP. However, in fungal-added blood, the BDG of fungi phagocytosed by WBCs was also measured with the LRP, and, thus, the results were higher than those for PRP.

In conclusion, when both PRP and LRP were measured and BDG ratios were calculated, the values were significantly higher for fungal-added blood, suggesting that the BDG ratio may be useful for distinguishing true positive cases of deep mycosis. Going forward, we plan to proceed with clinical research and report the results.

ACKNOWLEDGMENTS

We would like to express our sincere gratitude to the bacteriology laboratory at our university's school of medicine and to our hospital's bacteriology laboratory for their advice and cooperation in this study.

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