Original Article

Automation of serum (1→3)-beta-D-glucan testing allows reliable and rapid discrimination of patients with and without candidemia

Florian Prüller[1], Jasmin Wagner[2], Reinhard B. Raggam[1], Martin Hoenigl[2], Harald H. Kessler[3], Martie Truschnig-Wilders[1] and Robert Krause[2,*]

[1]Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz, Austria, [2]Section of Infectious Diseases and Tropical Medicine, Department of Internal Medicine, Medical University of Graz, Austria, and [3]Institute of Hygiene, Microbiology and Environmental Health, Medical University of Graz, Austria

*To whom correspondence should be addressed. Robert Krause, Section of Infectious Diseases and Tropical Medicine, Department of Internal Medicine, Medical University of Graz, Auenbruggerplatz 15, A-8036 Graz, Austria. Tel: +004331638581796; Fax: +004331638514622. E-mail: robert.krause@medunigraz.at or florian.prueller@medunigraz.at

Abstract

Testing for (1→3)-beta-D-glucan (BDG) is used for detection of invasive fungal infection. However, current assays lack automation and the ability to conduct rapid single-sample testing. The Fungitell assay was adopted for automation and evaluated using clinical samples from patients with culture-proven candidemia and from culture-negative controls in duplicate. A comparison with the standard assay protocol was made in order to establish analytical specifications. With the automated protocol, the analytical measuring range was 8–2500 pg/ml of BDG, and precision testing resulted in coefficients of variation that ranged from 3.0% to 5.5%. Samples from 15 patients with culture-proven candidemia and 94 culture-negative samples were evaluated. All culture-proven samples showed BDG values >80 pg/ml (mean 1247 pg/ml; range, 116–2990 pg/ml), which were considered positive. Of the 94 culture-negative samples, 92 had BDG values <60 pg/ml (mean, 28 pg/ml), which were considered to be negative, and 2 samples were false-positive (≥80 pg/ml; up to 124 pg/ml). Results could be obtained within 45 min and showed excellent agreement with results obtained with the standard assay protocol. The automated Fungitell assay proved to be reliable and rapid for diagnosis of candidemia. It was demonstrated to be feasible and cost efficient for both single-sample and large-scale testing of serum BDG. Its 1-h time-to-result will allow better support for clinicians in the management of antifungal therapy.

Key words: candidemia, laboratory diagnosis, (1→3)-beta-D-glucan, single-sample testing, invasive fungal infection.

Abbreviation: 1–3BDG = (1→3)-beta-D-glucan; CV = coefficient of variation; NPV = negative predictive value; PPV = positive predictive value.

Introduction

Invasive fungal infections including candidemia are serious and potentially life threatening in critically ill patients. Diagnosis is often difficult due to weak or absent clinical signs and symptoms, and current diagnostic methods, which are not sufficiently sensitive or specific, often contribute to the presentation of results that are too late to be clinically useful [1–3]. Many patients with clinical suspicion of candidemia are treated empirically with antifungal therapy [4]. This approach increases selective pressure for the development of antimicrobial resistance, potential risk of adverse drug reactions, and costs [5]. Blood cultures are currently considered to be the "gold standard" for diagnosis of candidemia [2]. However, blood cultures are usually positive after 2 days of incubation (range, 0–10 days) in patients with fungemia or remain negative for *Candida* spp., as shown in autopsy-proven cases of disseminated candidiasis [6–8]. Recently, it was shown that administration of antifungal therapy within 12 h after drawing blood for cultures reduced mortality in patients with multiple morbidities [2,9]. This clearly demonstrates the need for a reliable and rapid diagnostic test for candidemia that can be applied on a daily basis [10,11].

Detection of the fungal cell component $(1\rightarrow3)$-beta-D-glucan (BDG), which is present in the cell wall of most fungi, was reported to be a meaningful approach in laboratory diagnostics of fungal infections [12,13]. Assays are usually based on the modified *Limulus* amebocyte coagulation pathway and are performed manually; this includes the Fungitell assay (Associates of Cape Cod, East Falmouth, MA, USA). This assay format is designed to test 21 samples in parallel per run, making single-sample testing unfeasible.

In our study, the US Food and Drug Administration–cleared and under the European In Vitro Diagnostics Directive (98/79/EC) certified Fungitell assay was adapted for automation on the BCS® XP (Siemens Healthcare Diagnostics, Marburg, Germany) coagulation analyzer, allowing for both rapid single-sample and large-scale testing of BDG lasting 45 min. The automated protocol was evaluated in the routine laboratory setting using clinical samples obtained from patients with culture-proven candidemia and from culture-negative controls. All samples were tested in parallel using the standard assay protocol.

Materials and methods

Clinical samples

A total of 109 samples, 15 obtained from patients with culture-proven candidemia and 94 from culture-negative patients, were tested. The serum samples from candidemic patients were obtained on the day that yeast colonies were observed in blood cultures. *Candida albicans* (10 isolates), *C. parapsilosis* (2), *C. tropicalis* (2), and *C. glabrata* (1) were recovered from the positive blood cultures. All control patients with culture-negative samples had been admitted for elective plastic surgery procedures (eg, Dupuytren's contracture). These individuals had neither clinical signs and symptoms nor laboratory parameters (eg, elevated leukocyte count or C-reactive protein) indicative for any infection. All samples were aliquoted and stored at −70°C for future testing.

The ethics committee of the Medical University of Graz, Austria, approved this study. Patients provided written informed consent.

Standard protocol of the Fungitell assay

The Fungitell assay for detecting BDG in serum samples was performed according to the manufacturer's instruction. Briefly, 5 µl of serum was pretreated with 20 µl of an alkaline reagent (0.25 M potassium hydroxide and 1.2 M potassium chloride, mixed 1:1) for 10 min at 37°C in order to convert triple-helix glucan to single-helix glucan and inactivate serine proteases and serine–protease inhibitors in the serum sample [14–16]. After this step, 25 µl of each of the five standards with corresponding concentrations (500, 250, 125, 62.5, and 31.25 pg/ml) and 25 µl of the blank included in the assay were transferred to the microplate, followed by addition of 100 µl Fungitell reagent to each well. Then, the microplate was inserted into an incubating (37°C) plate reader. Every serum sample was tested in duplicate. The assay was monitored at 405 nm kinetically for 40 min. According to the manufacturer, a BDG concentration of ≥80 pg/ml is considered to be positive, while a concentration of <60 pg/ml is considered to be negative [13,17]. The linear range for BDG was from 31.5 pg/ml to 500 pg/ml, and sample values interpolated by the Flexstation 3 Benchtop Multi-Mode Microplate Reader (Molecular Devices GmbH, Biberach an der Riss, Germany) were used for measurements (Fig. 1a).

Automated protocol of the Fungitell assay

The Fungitell assay was adapted to be automated using a routine BCS XP coagulation analyzer according to the manufacturer's instructions. The Fungitell reagent was reconstituted as recommended by the manufacturer, aliquoted, and stored at −70°C, allowing single-sample testing if needed.

After calibration and generation of an archivable standard curve using six corresponding concentrations (2000, 800, 322.5, 127.5, 51.5, and 20.5 pg/ml BDG), the linearity and precision were evaluated. The BDG standard preparation included in the kit was used for linearity and precision
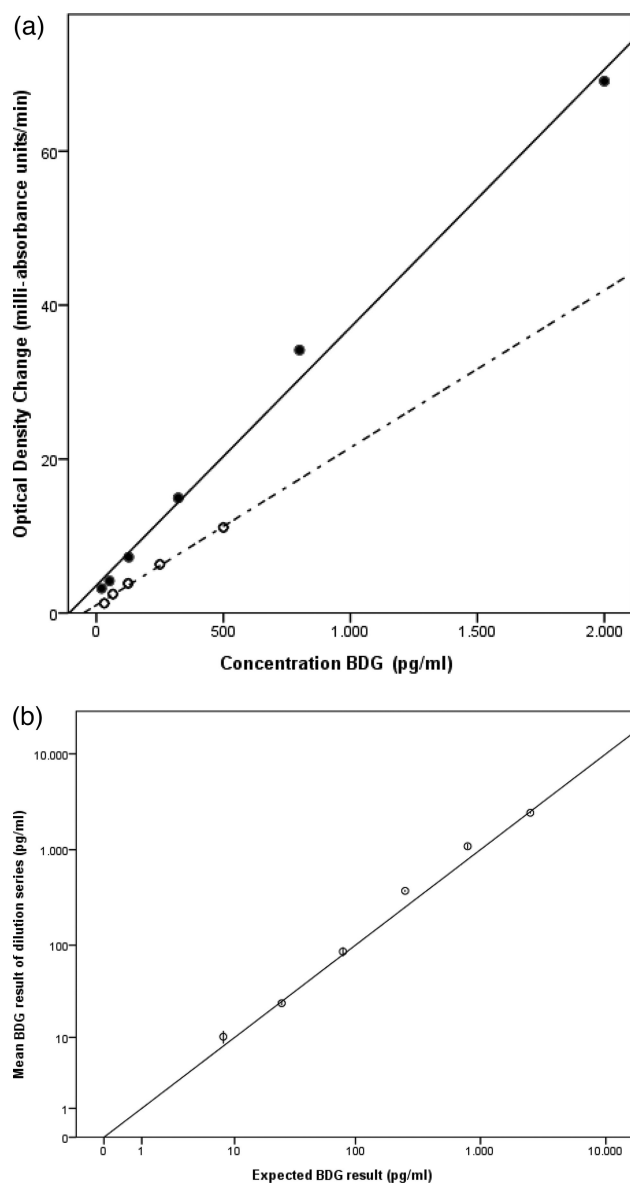
(a)

(b)

Figure 1. Assay characteristics. (a) Assay Calibration—Calibration curve based on the automated protocol (solid line) with 6 corresponding calibration points [2000, 800, 322.5, 127.5, 51.5, and 20.5 pg/ml 1–3BDG)] covering a measuring range with interpolation from 4000 to 15.4 pg/ml 1–3BDG ($R_2 = 0.998$). Calibration curve based on the standard manual method (dashed line) with 5 corresponding calibration points [500, 250, 125, 62.5, and 31.25 pg/ml 1–3BDG] and internal interpolation ($R_2 = 0.994$). (b) Assay linearity—Dilution series for a sample spiked with 2500 pg/ml of 1–3BDG. Mean of triplicate measurements ±3 SD.

testing. The dilution series (0.5-log steps, that is, 1:3.16 dilutions) was prepared using a serum sample obtained from a culture-negative control spiked with 2500 pg/ml of BDG. Each dilution was analyzed in triplicate, and the mean BDG concentration was determined. The precision was tested using three serum samples obtained from culture-negative control patients and spiked with BDG for final concentrations of 80 pg/ml (low), 350 pg/ml (medium), and 700 pg/ml (high). For between-day precision, five aliquots

of each sample were tested on five days. The within-run precision was determined by testing five replicates of each sample in a single run.

Comparison of results obtained with the two protocols

The clinical performance and time-to-result of the automated protocol in the routine diagnostic laboratory was evaluated by testing 109 clinical samples. Results were compared with those obtained using the manual standard.

Statistical methods

For statistical analysis, IBM SPSS Statistics for Windows, Version 19.0 (Armonk, NY, USA) was used. A Bland–Altman plot was constructed, and a correlation coefficient calculated in order to compare manual standard and automated protocols [18]. Validity data and predictive values were determined [19,20].

Results

Analytical performance of the automated protocol

The analytical measurement range showed a quasilinear curve from 8 to 2500 pg/ml (Fig. 1b). Determination of between-day precision showed coefficients of variation (CVs) ranged from 3.1% to 5.5%; within-run precision CVs ranged from 3.0% to 5.0% (Table 1).

### Clinical performance of the automated protocol

Compared with the manual protocol

With the Fungitell assay, a BDG concentration ≥80 pg/ml is considered to be positive and a concentration <60 pg/ml to be negative, according to the manufacturer. Of 109 clinical serum samples, 17 tested positive by both test systems and 91 were found to be negative by both systems (Fig. 2a). A discrepant result was obtained with one sample that was found to be positive (87 pg/ml) with the manual standard protocol and negative (29 pg/ml) with the protocol when adapted for automation.

Patients with culture-proven candidemia showed a mean BG level of 1247 pg/ml (range, 116–2990 pg/ml) when measured with the automated protocol and 852 pg/ml (range, 89–1853 pg/ml; interpolated values according to Fig. 1a) with the manual standard protocol. In the culture-negative control group, the mean BDG concentration was found to be 28 pg/ml (range, <15–124 pg/ml) when measured with the automated protocol and 34 pg/ml (range, <32–118 pg/ml) with the manual standard

Table 1. Between-day and within-run imprecisions obtained from spiked serum samples with the automated protocol (for both five replicates).

	(1→3)-beta-D-glucan concentration (pg/ml)	Mean (pg/ml)	Standard deviation	Coefficient of variation (%)
Between-day	80	83.1	4.6	5.5
	350	328.2	10.2	3.1
	700	714.0	34.6	4.9
Within-run	80	82.7	4.2	5.0
	350	339.8	10.3	3.0
	700	702.5	31.1	4.4
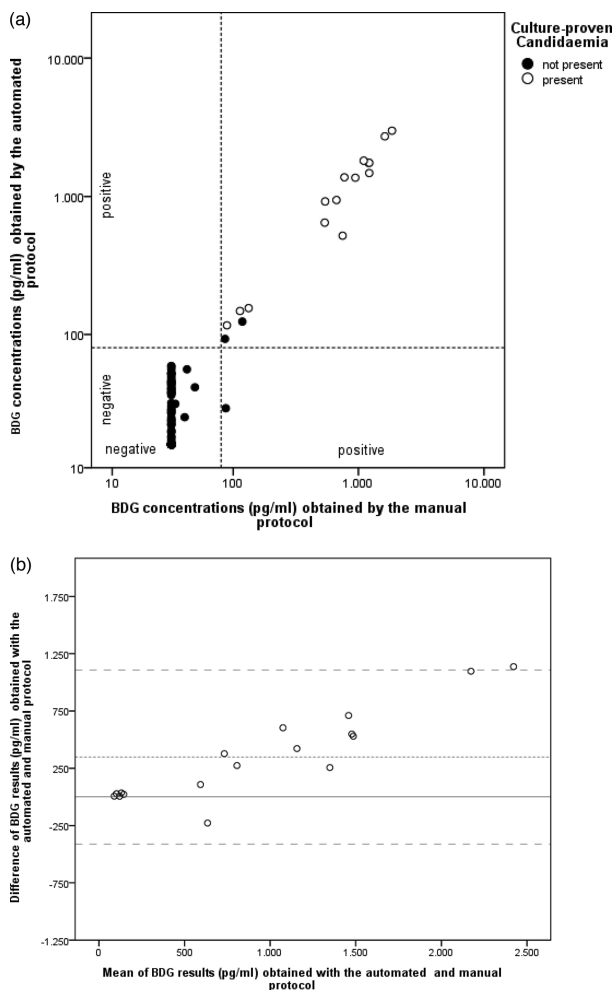
Figure 2. Assay comparison—concordance between the automated and the manual protocol. (a) Four quadrant plot—Distribution of concordant and discrepant results obtained by the standard manual and the automated protocols. 1-3BDG results (pg/ml) are in logarithmic scale and classified according to culture results. (b) Bland–Altmann plot—Agreement between 17 1–3BDG results (pg/ml) obtained with the standard manual and the automated protocols. Bland-Altman plot: the x-axis shows the mean concentration for each sample obtained by both of the protocols; the y-axis shows the difference between the values obtained with the 2 protocols. The solid line represents zero bias, the dotted line represents the mean difference between the values, and the dashed lines represents the mean difference plus or minus 1.96 SD (95% limits of agreement).

protocol. Twelve of 15 patients with candidemia showed BDG values >500 pg/ml with both the automated and manual standard protocol.

In the 17 clinical samples with positive results, the automated protocol revealed higher BDG levels (mean difference, 349 pg/ml) compared with the manual standard protocol (interpolated values according to Fig. 1a and Fig. 2b). For all 17 positive samples, a correlation coefficient (R^2) of 0.948 was obtained.

Compared with blood culture results

All culture-proven candidemic patients showed BDG values >80 pg/ml with both protocols and were classified as positive. Of 94 samples obtained from culture controls with culture-negative results, 91 had BDG values <60 pg/ml with both protocols and were classified as negative (Fig. 2a). Two samples were found to be positive with both assays and one sample gave discrepant results, as described previously (Fig. 2a).

Validity data and predictive values, based on a prevalence for candidemia of 13.76% (95% confidence interval, 7.92%–21.68%) in this study group, show very good agreement between the manual standard protocol and the automated protocol (Table 2).

Time-to-result

With the automated protocol, time-to-result was 45 min, including instrument setup (since the standard curve can be stored once calculated, it is reloadable on the coagulation analyzer), and hands-on-time was only 3 min irrespective of the number of samples tested in one run (1–18 samples, one positive and one negative control). With the manual standard protocol, time-to-result was a minimum of 90 min, including a hands-on-time of 50 min followed by 40 min of determination.

Costs

Reagent demand and costs can be reduced by up to 60% by adopting the automated assay. However, it should be noted

Table 2. Validity data and predictive values for both protocols.

Diagnostic accuracy	Manual standard protocol (%)	Automated protocol (%)
Sensitivity	100 (95% CI, 78.03% – 100%)	100 (95% CI, 78.03% – 100%)
Specificity	96.8 (95% CI, 90.94% – 99.3%)	97.8 (95% CI, 92.51% – 99.68%)
Positive predictive value*	83.3 (95% CI, 58.56% – 96.23%)	88.2 (95% CI, 63.52% – 98.2%)
Negative predictive value*	100 (95% CI, 96.03% – 100%)	100 (95% CI, 96.03% – 100%)

CI, confidence interval.
*Based on a prevalence for candidemia of 13.76% (95% CI, 7.92% – 21.68%) in the analyzed study group.

that single-sample testing is not feasible with the manual standard protocol and not performed routinely in clinical practice.

Discussion

Diagnosis of candidemia remains challenging as culturing patient blood samples remains the gold standard. However, time-to-results often exceeds 2 days, and false-negative results may be found, in particular, in patients with disseminated candidiasis [21]. Determination of BDG may therefore become a valuable diagnostic tool for patients with suspected invasive fungal infection [5,22–25]. In its present format, the Fungitell assay is designed to test 21 samples in duplicate, per plate, making single-sample testing not feasible. Alternatively, samples could be collected and referred to a reference laboratory that performs high-throughput testing, with results being received the next day(s) [2].

In this study, the existing manual standard protocol of the commercially available Fungitell assay was modified and evaluated on the automated BCS XP coagulation analyzer. This allowed both rapid single-sample and large-scale testing. The automated protocol demonstrated a sufficiently linear range (8–2500 pg/ml) and excellent precision, that is, <6%, which is significantly lower than reported for the manual standard protocol (>10%) [26].

When clinical samples were tested, all samples obtained from patients with culture-proven candidemia and two samples obtained from controls with culture-negative results showed concordant positive BDG values above the published threshold (≥80 pg/ml) using both the manual standard and automated protocols. False-positive BDG results were previously reported to occur frequently in patients on hemodialysis with cellulose membranes; in patients with a cardiopulmonary bypass; after administration of immunoglobulin, albumin preparation, or of coagulation

factors (due to processing with cellulose-containing filters); in the presence of serious bacterial infection; in patients with biofilm-containing devices (e.g. catheters); and after packing of serosal surfaces with glucan-containing gauze (e.g. following major surgery) [26–35]. The presence of a possible confounding factor should be taken into consideration when obtaining a positive BDG result, in particular, when the result is only slightly above the cutoff level (≥80 pg/ml).

In the present study, only one discrepant result was found with a sample obtained from a culture-negative control; this sample had a BDG concentration slightly above the threshold (87 pg/ml) when analyzed with the manual standard protocol. With the automated protocol, the value measured was found to be negative (<60 pg/ml). The automated protocol may contribute to more reliable results because samples and reagents are handled under a hood, which provides a closed system, minimizing airborne contamination as advised by the manufacturer. Impurity may only occur when samples are opened just before being placed on the closed automated instrument.

With the automated protocol, quantitation of BDG may be performed 24 h a day without additional staff training. In contrast to the manual standard protocol, single patient samples can be tested with reduced time-to-result, reduced hands-on-time, and reduced test costs. The possibility storing the standard curve for subsequent runs makes it possible to generate results more quickly compared with the manual standard protocol. However, single sample testing is unfeasible and is not performed with the manual standard protocol in routine clinical laboratory practice.

Single-sample testing with the automated protocol may provide clinicians with BDG results within hours rather than days, which was necessary in order to obtain a sufficient number of samples with the manual standard protocol, and may support the management decision of whether to initiate or withhold antifungal therapy. Since various

studies have shown that early initiation of antifungal therapy may improve survival by up to 80%, faster determination of BDG may further increase survival rates [9,36]. Additionally, the new automated protocol of this BDG assay may help to reduce unnecessary and expensive antifungal therapy in patients who do not have a fungal infection.

In summary, automation of the Fungitell assay allows reliable, rapid, and cost-efficient single-sample and large-scale testing of BDG and therefore appears to be meaningful for routine use in the clinical laboratory. The automated protocol of the test may therefore provide valuable, accelerated decision support for clinicians who treat patients at risk for invasive fungal infections. To better investigate the clinical value of having immediate BDG results available, implementation of BDG results in clinical diagnostic workflows is necessary and should be investigated in prospective studies.

Authorship Contributions
All authors have seen and approved the content and have contributed significantly to the work. The manuscript has not been submitted or accepted elsewhere.

Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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