Comparative evaluation of (1, 3)-β-D-glucan, mannan and anti-mannan antibodies, and Candida species-specific snPCR in patients with candidemia

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Abstract

Background: Candidemia is a major infectious complication of seriously immunocompromised patients. In the absence of specific signs and symptoms, there is a need to evolve an appropriate diagnostic approach. A number of methods based on the detection of Candida mannan, nucleic acid and (1,3)-beta-D-glucan (BDG) have been used with varying specificities and sensitivities. In this retrospective study, attention has been focused to evaluate the usefulness of two or more disease markers in the diagnosis of candidemia.

Methods: Diagnostic usefulness of Platelia Candida Ag for the detection of mannan, Platelia Candida Ab for the detection of anti-mannan antibodies, Fungitell for the detection of BDG, and of a semi-nested PCR (snPCR) for the detection Candida species-specific DNA have been retrospectively evaluated using 32 sera from 27 patients with culture-proven candidemia, 51 sera from 39 patients with clinically suspected candidemia, sera of 10 women with C. albicans vaginitis, and sera of 16 healthy controls.

Results: Using cut-off values recommended by the manufacturers, the sensitivity of the assays for candidemia patients were as follows: Candida snPCR 88%, BDG 47%, mannan 41%, anti-mannan antibodies 47%, respectively. snPCR detected 5 patients who had candidemia due to more than one Candida species. The sensitivities of the combined tests were as follows: Candida mannan and anti-mannan antibodies 75%, and Candida mannan and BDG 56%. Addition of snPCR data improved the sensitivity further to 88%, thus adding 10 sera that were negative by BDG and/or mannan. In clinically suspected, blood culture negative patients; the positivities of the tests were as follows: Candida DNA was positive in 53%, BDG in 29%, mannan in 16%, and anti-mannan antibodies in 29%. The combined detection of mannan and BDG, and mannan, BDG and Candida DNA enhanced the positivity to 36% and 54%, respectively. None of the sera from Candida vaginitis patients and healthy subjects were positive for Candida DNA and mannan.

Conclusion: The observations made in this study reinforce the diagnostic value of snPCR in the sensitive and specific diagnosis of candidemia and detection of more than one Candida species in a given patient. Additionally, in the absence of a positive blood culture, snPCR detected Candida DNA in sera of more than half of the clinically suspected patients. While detection of BDG, mannan and anti-mannan antibodies singly or in combination could help enhancing sensitivity and eliminating false positive tests, a more extensive evaluation of these assays in sequentially collected serum samples is required to assess their value in the early diagnosis of candidemia.

Published: 4 September 2007
Received: 6 September 2006
Accepted: 4 September 2007

This article is available from: http://www.biomedcentral.com/1471-2334/7/103
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Background
Candidemia is a major infectious and steadily increasing complication of seriously immunocompromised patients that is associated with high mortality rates [1-5]. Non-specific clinical presentation, low positivity of blood cultures even in autopsy-proven cases and rapid course of the disease have necessitated the need for developing sensitive methods for the early diagnosis of invasive candidiasis [1,6]. Among the different approaches that have been developed and evaluated in the recent years include detection of Candida mannan [7,8], arabinitol [9-11], and nucleic acids [12-15]. However, all these methods have limitations of sensitivity and/or specificity. The recent introduction of BDG detection assay, a fungus-specific marker, has provided a new diagnostic tool with encouraging results [16-18]. This has encouraged the investigators to evaluate the usefulness of BDG in tandem of mannan and/or DNA for the early and specific diagnosis of invasive mycoses [19]. In this communication, we have evaluated the diagnostic value of Candida species-specific DNA, BDG, Candida mannan, and Candida anti-mannan antibodies in sera samples obtained from culture-proven candidemia patients and clinically suspected cases of candidiasis.

Methods

Patients and sera samples
Thirty-two sera from 27 culture-proven candidemia patients, 51 from 39 clinically suspected blood culture negative systemic candidiasis patients, 10 from Candida vaginitis patients and 16 from healthy subjects with no complaints of oral or vaginal candidiasis; their identity was reconfirmed by ID 32C assimilation profile. The patients had at least three of the following risk factors: extended period of hospitalization (> 2 weeks), isolation of Candida species from one or more anatomic sites, presence of intravenous catheter/line, recent history of a surgical procedure, and administration of immunosuppressive therapy. Sera of healthy subjects were included as controls to derive baseline values. All the sera were stored at -20°C until used. The study was approved by the Committee for Protection of Human Subjects in Research, Faculty of Medicine, Kuwait University and informed written consent was obtained from the patients involved in the study.

Candida species isolation and identification
Blood samples were processed for isolation of Candida species by BACTEC 9240 system (Becton Dickinson, Paramus, N.J. USA) using aerobic culture bottles. Aliquots from blood culture bottles yielding yeast growth were subcultured on Sabouraud dextrose agar plates with chloramphenicol (40 mg/L). A single representative colony was processed for identification by germ-tube test and Vitek 2 yeast identification system (bio Mérieux Marcy l’Etoile, France). In case of discrepant results with snPCR identification, their identity was reconfirmed by ID 32C assimilation profile.

Detection of Candida mannan
Mannan antigen was measured using a commercial sandwich immunoassay, Platelia Candida Ag (BioRad, Marnes La Coquette, France). The test was performed according to the instructions of the manufacturer. Briefly, each test serum (300 µl) was mixed with 100 µl of the treatment solution and placed in a boiling water bath for 3 minutes. After centrifugation, the supernatant was used for further testing. Fifty-µl of the conjugate and an equal amount of the treated serum supernatant was introduced into microtiter plate wells pre-coated with anti-mannan monoclonal antibody. After incubation at 37°C for 90 min and 5 washing steps, 200 µl of the substrate buffer was added to each well, and the plates were incubated for 30 min at room temperature. The enzymatic reaction was terminated by adding the stopping solution and the optical density was read at 450 nm using a Tecan Spectra (Austria) plate reader. The reactions were performed in duplicates and each experiment included positive and negative controls as well as a calibration curve, which was made with a pool of normal human serum supplemented with known concentrations of mannan ranging from 0.1 to 2 ng/ml.

Detection of Candida anti-mannan antibodies
Anti-mannan antibodies were measured using the Platelia Candida Ab/Ac/Ak kit, a two-stage indirect immunoenzymatic assay (Bio-Rad, Marnes La Coquette, France). The test was performed according to the manufacturer’s instructions. In brief, 100 µl of each test serum diluted 1/
Table 1: Particulars of 27 blood culture-positive candidemia patients and results on detection of species-specific Candida DNA, (1,3)-
beta-D-glucan, mannan and anti-mannan antibodies by diagnostic kit cut-off value

| Case no | Age/sex | Underlying condition                  | Blood culture          | Semi-nested PCR with serum | BDG (pg/ml) | Mannan (ng/ml) | Anti-mannan Abs (AU/ml) |
|---------|---------|---------------------------------------|------------------------|----------------------------|-------------|----------------|------------------------|
| 1       | 88/F    | Septicemia, Chest infection           | C. albicans            | C. albicans, C. parapsilosis | 35          | 0.32           | 9.8                    |
| 1b*     | 88/F    | Septicemia, Chest infection           | C. albicans            | C. albicans                | 53          | 0.32           | 11.2                   |
| 2       | 49/F    | Chronic renal failure                 | C. albicans            | C. albicans                | 40          | 0.26           | 0.8                    |
| 3       | 79/M    | Large bowel obstruction               | C. albicans            | C. albicans                | 172         | 0.8            | 13.2                   |
| 4       | 17/M    | Aseptic meningitis                    | C. albicans            | C. albicans                | 20          | 0.28           | 2.4                    |
| 5       | 34/M    | Gut surgery                           | C. albicans            | C. albicans                | 130         | 0.66           | 6.8                    |
| 6       | 50/M    | Pancytopenia                          | C. albicans, C. parapsilosis | 30          | 0.52           | 0.8                    |
| 7       | 56/M    | Appendicular mass                     | C. albicans            | C. albicans                | 44          | 1.3            | 10.6                   |
| 7b*     | 56/M    | Appendicular mass                     | C. albicans            | C. parapsilosis            | 69          | 0.38           | 9.6                    |
| 8       | 35/M    | -                                     | C. albicans            | C. parapsilosis            | 131         | 0.86           | 20.6                   |
| 9       | 42/M    | -                                     | C. albicans, C. tropicalis | 43          | 0.5            | 3                      |
| 10      | 26/F    | Small bowel obstruction, pancreatitis | C. albicans, C. parapsilosis | 32          | 0.48           | 8                      |
| 10b*    | 26/F    | Small bowel obstruction, pancreatitis | C. albicans            | -ve                        | 21          | 0.22           | 34                     |
| 11      | 22/F    | -                                     | C. albicans, C. tropicalis | 48          | 0.36           | 24.4                   |
| 12      | 78/F    | Laparotomy                            | C. albicans, -ve        | 28                         | 0.3          | 20.8                   |
| 12b*    | 78/F    | Laparotomy                            | C. albicans, -ve        | 22                         | 0.34         | 27.8                   |
| 13      | 71/F    | Laparotomy                            | C. albicans, C. parapsilosis | 127         | 1.44           | 2                     |
| 14      | 9mo/F   | Pneumonia                             | C. albicans, C. parapsilosis | 112         | 3.19           | 1                      |
| 15      | 68/M    | Bladder cancer                        | C. albicans             | 157                        | 0.36         | 35                     |
| 16      | 58/M    | Renal failure                         | C. albicans, C. parapsilosis | 254         | 1.54           | 7                      |
| 17      | 77/M    | Chest trauma                          | C. albicans             | 97                         | 0.64         | 1.6                    |
| 18      | 48/M    | Bronchopneumonia                       | C. albicans             | 321                        | 0.24         | 23.8                   |
| 19      | 20/F    | Meningitis                            | C. parapsilosis         | 32                         | 0.34         | 11.2                   |
| 20      | 80/M    | Urinary tract infection               | C. parapsilosis         | 120                        | 0.3          | 19.4                   |
| 21      | 54/F    | Diabetes mellitus, LVF                 | C. parapsilosis         | 65                         | 0.22         | 1.4                    |
| 22      | 04/M    | Head injury                           | C. parapsilosis         | 20                         | 0.28         | 2.2                    |
| 23      | 6mo/F   | Pneumonia                             | C. parapsilosis         | 101                        | 0.62         | 2.2                    |
| 24      | 80/M    | Chest infection                        | C. tropicalis, C. parapsilosis | 47          | 0.48           | 7                      |
| 25      | 41/M    | Polyarteritis nodosa                   | C. tropicalis           | 115                        | 2.8          | 1.4                    |
| 25b*    | 41/M    | Polyarteritis nodosa                   | C. tropicalis           | 108                        | 0.4          | 19.4                   |
| 26      | 74/M    | Jaundice, fever                        | C. parapsilosis         | 205                        | 1.88         | 12.4                   |
| 27      | 28/M    | Thrombocytopenia                       | C. krusei, C. albicans  | 132                        | 0.2          | 13.4                   |

Age is expressed in years unless indicated. mo: months, LVF: left ventricular failure, BAL: broncho-alveolar lavage, M: male, F: female, * repeated specimen of the respective patient, – : not grown/information not available

Interpretation of results:
- Mannan Ag: positive = > 0.5 ng/ml, intermediate = 0.25–0.5 ng/ml, negative = < 0.25 ng/ml
- Anti-mannan antibodies: positive = > 10 AU/ml, intermediate = 5–10 AU/ml, negative = < 5 AU/ml
- BDG: positive = = 80 pg/ml, equivocal = > 60 < 80 pg/ml, negative = < 60 pg/ml
- Positive results are shown in bold letters
6,400 was applied to each well of micro-titer plate wells sensitized with C. albicans cell wall mannann and the plate was incubated at 37°C for 1 h. After washing, 100 µl of the conjugate was added and the plate was incubated at 37°C for 1 h. After intensive washing, the reactions were revealed by 30 min of incubation in the dark with 200 µl of the substrate buffer. The enzymatic reaction was terminated by adding the stopping solution and the optical density was read at 450 nm using a Tecan Spectra plate reader. The reactions were performed in duplicates and each experiment included positive and negative controls as well as standard serum dilution to give four range points of 20, 10, 5, and 2.5 AU/ml for positive reference.

(1,3)-beta-D-glucan assay
The test was carried out using the glucan detection kit, Fungitell (Associates of Cape Cod Inc., E. Falmouth, MA, USA). The glucan standard provided in the kit was mixed with reagent water to give a 100 pg/ml concentration. This was further diluted to obtain glucan concentrations of 50, 25, 12.5 and 6.25 pg/ml. Five µl of the serum sample was transferred to the designated wells of the micro-titer plate and 20 µl of the blood treatment reagent (0.6 M KCl and 0.125 M KOH) was added to each well containing the serum samples. The plate was shaken for 5 seconds and incubated for 10 minutes at 37°C. Twenty-five µl of each of the glucan standards (100, 50, 25, 12.5 and 6.25 pg/ml) was added to the designated wells. The provided Fungitell reagent was reconstituted with reagent water and pyrosol reconstitution buffer and 100 µl of this solution was added to each well. The plate was shaken for 5 seconds in a Tecan Sunrise (Austria) plate reader before reading at 405 nm every 1 minute for 40 minutes. The concentration of BDG in the clinical samples was calculated in comparison with a kinetic curve derived from known concentrations of glucan.

PCR study
Reference Candida strains were obtained from American Type Culture Collection (ATCC, Manassas, Virginia, USA) and included C. albicans (ATCC 90029), C. parapsilosis (ATCC 10233), C. tropicalis (ATCC 750) and C. glabrata (ATCC 90030). All yeast strains were stored at -20°C in sterile distilled water. DNA was extracted from broth cultures by the method of Lee [20] with an additional step of DNA purification by extraction in phenol-chloroform (24:1). DNA from serum was extracted using the QIAamp DNA kit (QIAGEN) following the blood and body fluid spin protocol. All pan-fungal and species-specific forward and reverse primers as well as the DNA amplification method was the same as mentioned by Ahmad et al. [13]. To detect amplified DNA fragments, agarose gel electrophoresis was performed using 3% agarose gels as described previously [14]. The gels were exposed to UV light and photographed. The sizes of the amplified DNA fragments were identified by comparison with molecular size marker DNA (100-bp DNA ladder, Invitrogen).

Interpretation of results and statistical analysis
The cut-off values recommended by the manufacturers for each test were used for determining positive, equivocal/intermediate and negative tests and were as follows: BDG, ≥ 80 pg/ml as positive, 60–79 pg/ml as equivocal and < 60 pg/ml as negative; mannan, > 0.5 ng/ml as positive, 0.25–0.5 ng/ml as intermediate and < 0.25 ng/ml as negative; anti-mannan Candida antibodies, > 10 AU/ml as positive, 5–10 AU/ml as intermediate and < 5 AU/ml as negative. All positive assay results were considered as true positives in patients with candidemia and also in clinically suspected patients. Additionally, all values under the cut-off values including doubtful results were considered as negative for calculation of sensitivity of the assays. Agreement between the qualitative test results was assessed by use of the kappa statistics. The p-values for the different tests were calculated using the Independent samples T test, Pearson’s test and Z test for proportions using SPSS (Statistical package for social sciences) and Microstat.

Results
Detection and identification of Candida species by culture and snPCR
The particulars of 27 blood culture positive candidemia patients with respect to age, sex, underlying conditions, as well as results of detection of Candida DNA, BDG, and Candida mannan and anti-mannan antibodies are presented in Table 1. Eighteen were infected with C. albicans, 5 with C. parapsilosis, 3 with C. tropicalis and one with C. krusei. snPCR results were positive in 25 (92.5%) candidemia patients. Among 32 sera samples examined from 27 patients, 4 sera samples originating from three patients (Table 1, Case Nos. 10b, 11, 12 and 12b) were negative by snPCR. In 7 patients (Case Nos. 8,14, 15, 19, 22, 26, 27) Candida species isolated from blood cultures were identified differently by snPCR. The identity of Candida isolates showing discordant results was re-confirmed by Vitek2 yeast identification system and/or Chromagar Candida. In one patient (Case No. 7) while both the blood cultures yielded C. albicans, the second serum sample was snPCR positive for C. parapsilosis. In addition, snPCR detected 4 patients (Case Nos. 1, 6, 10, 13) with dual infection (3 with C. albicans and C. parapsilosis and one with C. albicans and C. tropicalis), and one patient (Case No. 24) was positive for C. albicans, C. parapsilosis and C. tropicalis.

Candida mannan
Using a cut-off value of > 0.5 ng/ml for a positive mannan test, the sensitivity and specificity in candidemia patients were 41% and 100%, respectively. The quantities of mannan in positive serum samples ranged from 0.5 to 3.19 (mean ± SD = 1.29 ± 0.88) ng/ml (Table 1).
(1,3)-beta-D-glucan

Fourteen (52%) patients with proven candidemia were positive for BDG test using a cut-off value of 80 pg/ml. The amount of BDG in 15 positive serum samples ranged from 97 to 321 pg/ml (mean ± SD = 152.13 ± 63.08 pg/ml) (Table 1). The sensitivity and specificity of BDG test were 47% and 100%, respectively.

Anti-mannan antibody

Using a cut-off value of > 10 AU/ml, the sensitivity of anti-mannan antibody test was 47% with 100% specificity. The antibody levels among the 15 positive sera samples from 14 patients ranged from 10.6 to 35 (mean ± SD = 19.81 ± 8.05) AU/ml. The 11 patients who yielded a negative test for mannan antigen were positive for anti-mannan antibodies (Table 1).

Comparative analysis of Candida DNA, BDG, mannan and anti-mannan detection

Using cut-off values recommended by the manufacturers, the comparative sensitivities of the assays for candidemia patients were as follows: Candida snPCR 88%, BDG 47%, mannan 41%, and anti-mannan antibodies 47% (Tables 1, 2 and Figures 1, 2, 3). Ten sera were positive for all the three markers that is, Candida DNA, BDG and Candida mannan (Figure 1) Fifteen sera samples positive for BDG were also positive by snPCR. None of the four sera samples that were negative by snPCR (Table 1; 1b, 11, 12, 12b), were positive for BDG or mannan, but all of them were positive for anti-mannan antibodies with values ranging from 20.8 -34 AU/ml (Table 1). Likewise, the 5 sera samples that were positive for BDG and negative for mannan were also uniformly positive for anti-mannan antibodies (Table 1; 15, 18, 20, 25b, 27). Only 4 of the 15 sera positive for anti-mannan antibodies were concomitantly positive for mannan. In general, increasing levels of BDG were associated with increasing levels of mannan. However, this correlation by Pearson test was not statistically significant (p = 0.078) (Figure 4). Candida DNA detection was found to be the most sensitive test (88%), followed by BDG and anti-mannan antibodies, 47% each, and mannan 41% (Figure 3). The combination of two tests improved the sensitivity for diagnosing invasive candidiasis/candidemia as follows: mannan and anti-mannan antibodies by ELISA 75%, mannan and BDG 56%, and mannan, BDG and Candida DNA 88% (Figure 3). The combined sensitivities for mannan and anti-mannan antibodies according to infecting Candida species were as: C. albicans (n = 22), 77%, C. parapsilosis (n = 5), 60% and C. tropicalis (n = 4), 75%, whereas for mannan and BDG, these were 55%, 40% and 75%, respectively (Table 2).

Clinically suspected candidemia patients

In the clinically suspected, blood culture negative category, 51 serum samples from 39 patients were tested. The positivities of the tests were as follows: Candida DNA was positive in 53%, BDG in 29%, mannan in 16%, and anti-mannan antibodies in 29% (Fig. 5). The combined detection of mannan and BDG, and mannan, BDG and Candida DNA enhanced the positivity to 36% and 54%, respectively (Figure 5). Serum samples of 10 patients were tested twice and of one patient thrice at different time intervals. In addition to C. albicans, 2 serum samples were positive for C. tropicalis and one for C. parapsilosis DNA. Of the 27 snPCR positive sera, 8 were also positive for anti-mannan, 5 for mannan and 6 for BDG (performed on 24 sera samples).

| Candida species in blood culture | No of sera tested | Number showing positive result by diagnostic kit value cut-off (%) |
|---------------------------------|------------------|---------------------------------------------------------------|
|                                 |                  | Mannan Ag | Anti-mannan Abs | BDG | Mannan + anti-mannan | Mannan + BDG |
| C. albicans                     | 22               | 10 (45)   | 10 (45)         | 9 (41) | 17 (77) | 12 (55) |
| C. parapsilosis                 | 5                | 1 (20)    | 2 (40)          | 2 (40) | 3 (60)  | 2 (40)  |
| C. tropicalis                   | 4                | 2 (50)    | 2 (50)          | 3 (75) | 3 (75)  | 3 (75)  |

Table 2: Comparative results of mannan antigen, anti-mannan antibodies and (1,3)-beta-D-glucan alone and in combination in proven candidemia patients according to the infecting Candida species
Figure 2
Comparative results on the detection of Candida mannan (A), BDG (B) and Anti-mannan antibodies (C) in culture proven, clinically suspected and colonized patients and healthy controls. The horizontal line in each plot represents the cut-off for a positive test.

Figure 3
Bar diagram showing the increasing sensitivity of the diagnostic tests to detect Candida infection in candidemia patients. A: Mannan Ag, B: BDG, C: Anti-mannan Abs, D: Mannan + BDG, E: Mannan + Anti-mannan Abs, F: Candida DNA, G: Mannan + BDG + Candida DNA

Figure 4
Regression analysis of glucan and mannan values in culture-proven candidemia patients. (n= 32, r² = 0.100, p = 0.078)

Figure 5
Bar diagram showing the increasing sensitivity of the diagnostic tests to detect Candida infection in clinically suspected candidiasis patients. A: Mannan Ag, B: BDG, C: Anti-mannan Abs, D: Mannan + BDG, E: Mannan + Anti-mannan Abs, F: Candida DNA, G: Mannan + BDG + Candida DNA
**Candida vaginitis patients and healthy controls**

Sera from Candida vaginitis patients were uniformly negative for Candida DNA, BDG, and mannan (Figure 2). Sera of two patients showed positive values for anti-mannan antibodies (10 and 16.2 AU/ml) (Figure 2). None of the test markers were positive in sera of 16 healthy controls (Figure 2).

**Discussion**

Diagnosis of invasive infections due to Candida species presents unique problems. Clinical and radiological signs are non-specific or develop late in the course of the disease. Conventional diagnostic tests are insensitive and the "gold standard" diagnostic procedures (histopathological examination and cultures from deep tissues) require aggressive approach, which is often not feasible due to thrombocytopenia, and the critical condition of these patients [21]. To overcome these limitations, assays for the detection of Candida antibodies, antigen, BDG and DNA have been developed and evaluated for the diagnosis of invasive candidiasis [8,16,22-28].

In the present study, we have retrospectively evaluated the diagnostic value of Candida DNA, Candida manan, and anti-mannan antibodies, and BDG individually and in comparison with each other in patients who yielded Candida species in blood cultures. snPCR has been successfully applied in the direct detection and species-specific identification of four clinically important Candida species (C. albicans, C. parapsilosis, C. tropicalis and C. glabrata) in sera samples. While species-specific Candida DNA was detected in 28 (88%) of the 32 sera samples obtained from 27 culture-proven candidemia patients, discordant results in comparison with Vitek 2 identification were obtained in eight patients (Case Nos. 7b, 8, 14, 15, 19, 22, 26 and 27) (Table 1). This discrepancy in the results may be attributed to the possibility that these patients probably had concomitant infection with two different Candida species and only one of the infecting species was processed for identification by Vitek 2 method. Since we did not use a differential medium, such as Chromagar Candida, for making sub-cultures from BACTEC blood culture bottles, the possibility of missing one of the infecting species (probably with fewer colonies) existed. Four of the discordant results occurred between C. albicans and C. parapsilosis and one each between C. tropicalis and C. parapsilosis and C. krusei and C. albicans. Barring C. krusei, the other three Candida species were included in the snPCR protocol. Since some delay occurred between blood culture positivity and collection of serum samples, it is possible that detectable levels of the DNA of one of the two infecting Candida species were not available in the circulation when the blood was drawn for snPCR testing. This may also explain the reason as to why sera of four culture-positive candidemia patients (Cases Nos. 10b, 11, 12, 12b) were negative by snPCR. On the other hand, snPCR detected 5 candidemic patients whose all tests were negative except snPCR (Cases 2, 4, 10, 22, 24) and 5 additional patients (Case Nos. 1, 6, 10, 13 and 24) (Table 1), where more than one Candida species was involved besides C. albicans, and included C. parapsilosis in 4 and C. tropicalis in 2. Case No. 24 yielded positive results for C. tropicalis and C. parapsilosis besides C. albicans. These results support the previous reports that a reasonable proportion of patients with candidemia may have infection with more than one Candida species [13,29,30]. Since Candida species vary in their antifungal susceptibility profiles, this observation may be useful in administering appropriate therapy.

Recent studies have suggested that the combined detection of mannan and anti-mannan antibodies considerably improves the diagnosis of candidiasis [8,25,26]. While individual sensitivity of the test for mannan and anti-mannan antibodies in our study was only 41% and 47% respectively, the combined detection increased the sensitivity to 75% (Fig. 3). Sendid et al. [25] concluded that irrespective of the Candida species causing the disease, the combined sensitivity of mannan and anti-mannan antibody detection in candidiasis patients was > 80%. In our study, the combined sensitivities of mannan and anti-mannan antibodies for C. albicans, C. parapsilosis, and C. tropicalis were 77%, 60% and 75% and for mannan and BDG, these were 55%, 40% and 75%, respectively (Table 2). Additionally, there was also an inverse relationship between mannan and anti-mannan antibody levels, but it was not statistically significant (p = 0.063; data not shown) perhaps due to limited number of samples tested.

Some recent studies have demonstrated the usefulness of BDG estimation in the early diagnosis and management of fungal infections including candidiasis [16,19,31-33]. In our study, the sensitivity and specificity of BDG at a cut-off level of 80 pg/ml were 47% and 100%, respectively. The positive sera samples showed a range of 97 to 321 pg/ml (mean value 152.13 pg/ml). The sensitivity of BDG detection for diagnosing invasive fungal infections in different group of patients has been reported to vary considerably (50 to 100%), largely because of use of different cut-off values (10 to 120 pg/ml) for a positive test [16-19,31,34]. In a recent study, Pickering et al. [17] evaluated 39 sera samples from 15 patients with blood culture positive yeast infections using a cut-off value of 80 pg/ml. Thirty (77%) samples were positive for BDG (range 84 to 1359 pg/ml), and 13 of the 15 patients had at least one specimen positive. In a recent multi-center study of 107 patients with proven candidiasis, 81% had a positive result for BDG at a cut-off of 60 pg/ml and 78% had positive results at a cut-off of 80 pg/ml [18].
Our study is noteworthy in that it compared the diagnostic value of BDG in comparison with mannan using a quantitative EIA test in blood culture positive candidemia patients. The combination of the two tests improved the sensitivity to 56%. Our observation is in agreement with an earlier study by Mitsutake et al. [32]. These authors compared the specificities and sensitivities of enolase antigen, mannan antigen, Cand-Tec antigen and BDG in the diagnosis of 39 patients with candidemia. Using a cut-off value of 60 pg/ml, the specificity and sensitivity of BDG test were 84.4 and 87.5% respectively. The authors suggested that combination of two diagnostic assays may increase the accuracy of diagnosis of candidemia. Recently, Ostrosky-Zeichner et al. [18] have investigated the utility of the BDG assay in the diagnosis of fungal infections using a case control methodology. Using a cut-off value of 80 pg/ml, sensitivity and specificity of 64% and 92% was reported, respectively, with a PPV of 89% and NPV of 73%. However, in a subsequent analysis of the data of this study [18], Upton et al. [37] suggested that sensitivity and specificity of the test could heavily be influenced by prevalence rate of the disease in the patient population. Therefore, the calculation of PPV and NPV results from a population of selected case patients and unmatched control subjects may not provide useful information about the efficacy of the test. In an another study, Pazos et al. [19] reported that BDG and galactomannan exhibited similar in vivo kinetics in patients with invasive aspergillosis, hence their combined detection not only improved the specificity and PPV to 100% without affecting the sensitivity and NPV, but was also useful in identifying false positive reactions in each test. It seems that same may also be true for Candida mannan and BDG kinetics in patients with invasive candidiasis, hence their levels may increase or decrease in tandem. While increased levels of BDG in individual patients in our study were generally associated with increased levels of mannan, this correlation, however, was not significant by Pearson test (p = 0.078, r² linear = 0.1) (Figure 2). Nevertheless, the combined detection of mannan and BDG may also be helpful in eliminating false positive reactions which may occur in hemodialysis or ICU patients who may also have been colonized with Candida species [35,36]. In this context, all our Candida vaginitis patients were negative for BDG as well as Candida DNA and mannan. This is consistent with previous reports suggesting that Candida colonization may not lead to a positive BDG assay [16,19].

Our study has several limitations. Apart from the small numbers of samples tested, most of the observations are based on a single serum specimen obtained before initiating antifungal therapy. Additionally, there is no data available about the delay that occurred in obtaining a positive blood culture or between blood cultures and serum withdrawal for snPCR.

Conclusion
The observations made in this study reinforce the diagnostic value of snPCR in the sensitive and specific diagnosis of candidemia. Additionally, in the absence of positive blood cultures, snPCR detected Candida DNA in sera of more than half of the clinically suspected patients. While detection of BDG, mannan and anti-mannan antibodies singly or in combination could help enhancing sensitivity and eliminating false positive tests, a more extensive evaluation of these assays in sequentially collected serum samples is required to assess their value in the early diagnosis of candidemia and also for monitoring response to antifungal therapy.

Abbreviations
BDG = (1, 3)-β-D-glucan
PCR = Polymerase Chain Reaction
SnPCR = Seminested PCR
DNA = Deoxyribonucleic Acid
PPV = Positive Predictive Value
NPV = Negative Predictive Value

Competing interests
The author(s) declare that they have no competing interests.

Authors’ contributions
ZUK and ASM conceived the study, supervised it and drafted the manuscript. FFA did the work which formed part of her Master thesis and contributed to writing of the manuscript. All authors have read and approved the final manuscript.

Acknowledgements
The work was supported by the College of Graduate Studies and Research Administration (grant MPI118), Kuwait University. The authors are thankful to Dr. Saeed Akhtar for statistical analysis of the data, and to Director, Mubarak Al-Kabeer Hospital for allowing access to hospital records of the patients.

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