Short Communication

Detection of fungal DNA in peritoneal fluids by a PCR DNA low-density microarray system and quantitation of serum (1-3)-β-D-glucan in the diagnosis of peritoneal candidiasis

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Abstract

Microbiological documentation of peritoneal candidiasis (PC) is hampered by the low numbers of yeasts observable by direct microscopic examination and recoverable by culture methods. The performance of a polymerase chain reaction (PCR) DNA Low-Density Microarray System (CLART STIs B) was compared to that of BACTEC FX automated culture method for the detection of Candida spp. in 161 peritoneal fluids (PF) from patients with peritonitis. The clinical utility of (1–3)-β-d-glucan (BDG) antigenemia in the diagnosis of PC was evaluated in 42 of these patients. The overall agreement between the PCR assay and the culture method was good (κ = 0.790), and their sensitivities were 93.5% and 74.19%, respectively. Serum BDG levels in patients with Candida spp. in PFs (median, 200.3 pg/mL; Range, 22.0–523.4 pg/mL) was significantly higher (P = 0.002) than those found in patients without the yeast (median, 25.3 pg/mL; Range, 0–523.4 pg/mL). Our study demonstrates the potential clinical utility of molecular methods and the measurement of serum BDG levels for the diagnosis of PC.

Key words: Antifungal therapy, BACTEC culture, PCR array assay, peritoneal candidiasis, peritoneal fluid, serum (1–3)-β-D-glucan.

Introduction

Intra-abdominal Candida spp. infection develops in 20%–40% of patients with secondary or tertiary peritonitis and represents a frequent cause of mortality [1]. In addition, Candida spp. are being increasingly recognized as a relatively common cause of spontaneous peritonitis in cirrhotic patients [2]. The microscopic observation (DME) of compatible fungal structures in Gram stained smears and/or the recovery of the organism in cultures of peritoneal fluid (PF) obtained during surgery have been classically considered sufficient for diagnosis [1], although this
assumption is not without controversy [3]. In fact, discrimination between Candida spp. colonization and infection is seldom straightforward, particularly in the context of polymicrobial infections [3]. Both DME and conventional culture may fail to demonstrate the presence of Candida spp. in PF in autopsy-proven cases of peritoneal candidiasis (PC) [1]. Furthermore, recovery of Candida spp. from blood cultures is rather uncommon in this clinical setting [1]. In this context, molecular methods may become irreplaceable in the near future, as preliminary data indicate that they may increase the diagnostic yield in comparison with conventional culture methods [4–6].

Recently, the clinical utility of (1–3)-β-d-glucan (BDG) antigenemia measurement in the diagnosis of blood-culture negative intra-abdominal candidiasis in a selected cohort of critical care unit patients was demonstrated [7]. In this proof-of-concept study we assessed the potential utility of molecular methods in the diagnosis of PC by comparing the performance of a PCR DNA Low Density Microarray system (CLART STIs B, Genomica, Madrid, Spain) with that of the BACTEC automated culture method in the detection of Candida spp. in PFs from patients with spontaneous, secondary and tertiary peritonitis. In addition, we evaluated the clinical utility of BDG antigenemia quantitation in the diagnosis of PC.

Materials and methods
Specimens and patients
The current observational study included 142 patients (80 males and 62 females; median age 66 years; range 19–93 years) admitted to the Surgery Critical Care Unit between 2012 and 2013. Forty-two of these patients were prospectively enrolled for evaluation of the diagnostic performance of BDG antigenemia, and the remaining 100 patients were retrospectively included in the investigations. A total of 161 PFs and 42 sera from these patients were available for analysis. Sixty-two patients had a tertiary peritonitis due to anastomotic leakage or repetitive surgery, and 46 had a secondary peritonitis. Of these 108 cases, 58 were of supracolic origin. Thirty-four patients had spontaneous peritonitis. All patients with secondary or tertiary peritonitis had a PF specimen obtained during surgery. Nineteen patients had also a second specimen obtained by surgical drainage within 24 h following surgery. PF from patients with spontaneous peritonitis was obtained by paracentesis. All patients had blood samples submitted for culture, and all received intra-operative antibiotic prophylaxis following guidelines in use at our center. No patient was under prophylactic or empiric antifungal treatment at the time of sampling. Systemic antifungal treatment was started in the following scenarios: (i) At least one specific risk factor for intra-abdominal Candida spp. Infection [1]; (ii) yeasts in intra-abdominal specimens (obtained surgically or within 24 h from external drainage), irrespective of fungal concentration and the associated bacterial growth; (iii) the presence of candidemia.

Microbiological studies
DME by Gram staining was performed for 131 samples. Microscopic observations were made with oil-immersion objective during a minimum of 5 min (up to 10 min) examination period. Noncentrifuged PFs obtained during surgery (one specimen/patient) were directly inoculated into a pair of bottles of BACTEC media (PLUS aerobic/F and PLUS anaerobic/F; BD Diagnostics, Sparks, MD, USA) (1–3 ml/bottle, depending on total sample volume submitted), which were incubated for a maximum of 7 days, and analyzed using the automated continuous blood culture monitoring system BACTEC FX system (BD Diagnostics) as previously described [8]. The broth was aspirated for Gram staining when microbial growth was detected. The broth was then subcultured on chocolate blood agar, Sabouraud cloramphenicol agar, or Schaedler agar, as appropriate. Microbial identification was achieved by following established and approved laboratory procedures [9].

PCR DNA low-density microarray system
The molecular testing was performed retrospectively on clinical specimens that had been stored at −70°C. A volume of 400 μl of noncentrifuged specimens was used for DNA extraction with the EZ1 Virus Mini Kit v2.0 (Qiagen, Valencia, CA, USA) in the EZ1 biorobot (Qiagen), according to the manufacturer’s instructions. The eluate volume was 60 μl Candida spp. were detected and identified using the CLART STI B assay according to the manufacturer’s protocol. The procedure is similar to that of CLART HPV2 (Genomica, Madrid, Spain), which has been previously described in detail [10]. Briefly, polymerase chain reaction (PCR) was performed using 5 μl of the eluate volume. Prior to visualization, the PCR products were denatured at 95°C for 10 min. Visualization was performed using 10 μl of the denatured PCR products on the CLART microarray. Hybridization between the amplicons and their specific probes on the microarray resulted in formation of an insoluble precipitate of peroxidase when adding a streptavidin conjugate that binds to the biotin labeled PCR products. The precipitate was analyzed automatically on the Clinical Array Reader (Genomica). This assay allows the detection of the following species of Candida: C. albicans, C. glabrata, C. parapsilosis, C. krusei,
tropicalis, C. guilliermondii, and C. dubliniensis. According to the manufacturer, the limit of detection (LOD) for C. albicans and C. glabrata is 100 UFC/5 μl but the LOD for the other species is not contained in the company literature.

Quantitation of serum (1–3)-β-D-glucan

The Fungitell assay (Associates of Cape Cod, MA) was used for BDG measurement in serum specimens, with each specimen tested in duplicate. Samples with BDG levels above 500 pg/ml were diluted and retested. BDG levels below 31 pg/ml (lower validation limit) were calculated by extrapolation. The analyses were repeated if BDG duplicates differed by more than 20%. Mean BDG values of duplicates were used for data analyses.

Statistical analysis

The data were analyzed with the aid of the statistical package SPSS version 20.0 (SPSS, North Chicago, IL). Differences between medians were compared using the Mann–Whitney U-test. Two-sided exact P values are reported. A P value < 0.05 was considered statistically significant. Receiver operating characteristics (ROC) curve analysis was also performed with the SPSS program. For the calculation of the sensitivities of both the culture method and the PCR assay, positive results obtained by both methods or by either one were considered true positives. For the calculation of the sensitivity and the specificity of the BDG detection method (values above or below the established cut-off) true positives were considered those in which Candida spp. was detected in PFs by either method or both, and true negatives were those in which Candida spp. was not detected in PFs by both methods.

Results

PCR array assay vs. culture method

DME revealed the presence of yeast structures in six out of 131 specimens (4.5%) and as shown in Table 1, Candida spp. were recovered from 23 out of 161 specimens (14.2%) (from 21 patients with secondary or tertiary peritonitis) by using the BACTEC automated culture system. Three out of the 21 patients (14.2%) concomitantly had the same Candida spp. isolated from blood. Candida albicans was most frequently recovered (n = 19). Candida spp. was the only microorganisms recovered from 11 specimens (47.8%; Candida albicans, n = 10; Candida glabrata, n = 1). In the remaining samples, one or more bacterial species (Enterococcus faecium, n = 3, Enterococcus faecalis, n = 2; Staphylococcus aureus, n = 1; Escherichia

| Table 1. Performance of a PCR DNA Low-Density Microarray System in comparison with the BACTEC FX automated culture method in the detection of Candida spp. in purulent fluids from patients with peritonitis. |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| PCR DNA Low-Density Microarray result (no. of specimens) |  BACTEC culture result (no. of specimens) |
| Candida albicans | Candida glabrata | Candida parapsilosis | Candida famata | Negative | Inconclusive |
|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| Candida albicans | 0 | 0 | 1 | 0 | 4 |
| Candida glabrata | 1 | 0 | 0 | 0 | 15 |
| Candida parapsilosis | 1 | 0 | 1 | 0 | 1 |
| Candida famata | 1 | 0 | 0 | 0 | 0 |
| Total (161) | 127 | 4 | 19 | 1 | 128 |

PCR, polymerase chain reaction. *Negative for Candida spp. in one of the patients. Candida famata is not included in the PCR DNA Low-Density Microarray panel.
coli, n = 2; Klebsiella pneumoniae, n = 2; Pseudomonas aeruginosa, n = 1; Proteus mirabilis, n = 1) were isolated.

The PCR DNA microarray system was able to detect the presence of *Candida* spp. in 29 specimens from 27 patients (18.0%) (Table 1), *Candida albicans* was the species most frequently found. Of the remaining 132 samples, 128 were found to be negative, and four were categorized as ‘inconclusive’ (these specimens were retested using a different aliquot yielding the same result), based upon the manufacturer’s interpretative criteria. A single *Candida* spp. was detected in 24 samples, and two *Candida* spp. were detected (C. albicans + C. glabrata, n = 4, and C. albicans + C. parapsilosis, n = 1) in five specimens. The BACTEC culture system missed eight positive samples (27.5%) detected by the PCR DNA microarray system (Table 2), whereas the molecular method failed to demonstrate *Candida* spp. in two specimens that tested positive by culture (8.6%). One of these yielded an inconclusive result. In the other specimen, *Candida famata*, which is not included in the PCR DNA microarray panel, was recovered in culture. Dual *Candida* spp. infections were apparently missed by the BACTEC system, but we cannot be certain about this extent, as broths were not subcultured on chromogenic yeast media. The overall agreement between the culture method and the PCR DNA microarray was good (κ = 0.79; 95% confidence interval [CI], 0.63 to 0.94).

The sensitivities of the PCR DNA microarray system and the BACTEC culture method for the detection of *Candida* spp. were 93.5% and 74.19%, respectively. For the above calculations, inconclusive results in the former assay were categorized as negatives.

**Serum (1–3)-β-D-glucan**

Forty-two patients had a single serum specimen drawn within 48 h after ICU admission (22 males and 20 females; median age, 67 years; range, 21–90 years; 26 patients had tertiary peritonitis caused by anastomotic leakage or recurrent GI perforation, and 16 had secondary peritonitis due to GI perforation). Quantitation of BDG antigenemia was performed in 13 sera from 13 patients with *Candida* spp. in PF. *Candida* spp. was isolated by culture in six PFs (in all cases *Candida albicans*) and detected by the PCR DNA assay in all 13 patients (*Candida albicans*, n = 9; *Candida parapsilosis*, n = 1; *Candida guilliermondii*, n = 1; *Candida albicans + Candida glabrata*, n = 2). Mixed intestinal bacterial flora was recovered in culture from seven out of the 13 PFs. Candidemia was only found in one out of these 13 patients. The remaining 29 PFs tested negative by both methods. Serum BDG levels in patients with *Candida* spp. in PFs by culture and/or detected by PCR (median, 200.3 pg/ml; range, 22.0–523.4 pg/ml) was significantly
higher ($P = 0.002$) than those found in patients in whom *Candida* spp. was not recovered or detected in PFs (median, 25.3 pg/ml; range, 0–523.4 pg/ml) (Fig. 1). ROC curve analysis of BDG levels in sera indicated that the optimal cut-off level for discriminating between patients with and without *Candida* spp. in PF was 82.6 pg/ml (Fig. 2), with a sensitivity of 84.6% and a specificity of 76%. In addition, serum BDG levels in patients with *Candida* spp. in PFs detected exclusively by PCR (median, 252.1 pg/ml; range 62.0–523.4 pg/ml), were not significantly different ($P = 0.21$) from those measured in patients with positive culture results (median 144.4 pg/ml; range, 22.0–422 pg/ml).

**Clinical outcome of patients testing positive by PCR and negative by culture**

Eight patients with PF samples that did not yield the yeast in culture had *Candida* spp. detected in the peritoneal fluid by PCR (Table 2). None of these patients had candidemia, and only one received antifungal therapy (Micafungin) on the basis of baseline risk factors for the occurrence of systemic candidiasis. Five patients died during intensive care unit (ICU) stay due to multiple organ dysfunctions. These patients were not treated with antifungals and displayed significantly higher levels of serum BDG than those who survived (median 269.1 pg/ml vs. 93.7 pg/ml; $P = 0.025$).

**Discussion**

There are no reference standards for the etiological diagnosis of PC. The latter is largely based upon the observation of compatible fungal structures in the DME and/or recovery of *Candida* spp. in culture from PFs obtained during surgery in patients at high risk for intra-abdominal *Candida* infection [1]. Nevertheless, consistent criteria for discriminating between colonization and infection are lacking [1,3,11,12]. In addition, candidemia is seldom observed in these patients.

Both DME and culture are deemed to display suboptimal sensitivities for the observation of *Candida* spp. in or recovery from PFs in proven cases of PC [1,3]. Molecular methods are being increasingly used for the diagnosis of deep-seated candidiasis as they have been shown to increase the rate of detection of *Candida* spp. in PFs or blood in comparison with conventional culture methods [4–6]. Nevertheless, molecular assays cannot be currently designed to cover all microorganisms possibly involved in these processes.

In this study, the performance of a PCR DNA Low Density Microarray System was compared to that of the BACTEC automated culture method in the detection of *Candida* spp. in PFs from patients with peritonitis. Of note the fact that the above molecular test has been marketed for the detection of pathogens commonly involved in sexually transmitted infections employing urogenital specimens. Nevertheless, given its relatively good sensitivity for the detection of *Candida* spp. commonly implicated in PC, we decided to employ it in this proof-of-concept study. In our experience, the PCR DNA Low-Density Microarray method displayed a substantially higher sensitivity than the BACTEC culture method for the detection of *Candida* spp. The BACTEC method performs comparably to standard culture methods for *Candida* spp. recovery from both blood
and PFs [7]. In fact, the molecular method was able to detect Candida spp. in eight specimens (from eight patients) that resulted negative by culture.

The data presented in the current study are remarkably similar to those of Tissot et al. [7] in that serum BDG levels in patients with Candida spp. in PFs was significantly higher ($P = 0.002$) than those found in patients without it. The ROC curve analysis of BDG levels in sera indicated that the optimal cut-off level for discriminating between patients with and without Candida spp. in PF was 82.6 pg/ml, despite the fact that our study differed from the above in one major aspect. A PCR DNA assay, in addition to the BACTEC automated culture method, was employed for the detection of Candida spp. in PFs, and as a result the possibility exists that some patients in the study by Tissot et al. [7] could have been miscategorized due to the low sensitivity of culture methods for recovery of Candida spp. from PFs.

The potential clinical utility of serum BDG measurements is somewhat limited by two main facts: (i) It does not provide information about the Candida species involved and about their susceptibility to antimicrobial agents. Thus, preemptive antifungal therapy administered on the basis of serum BDG analyses may not be optimal in all cases. (ii) Serum BDG levels may be highly elevated in a number of clinical situations other than invasive candidiasis [1]. Thus, serum BDG measurement should be considered, at best, ancillary to culture (or molecular) methods for appropriate therapeutic management of PC.

Whether the sole detection of Candida spp. DNA in PF in the absence of fungal recovery in culture has any clinical significance remains to be determined. Nevertheless, our data seemed to indicate that it may be: (i) Serum BDG levels in patients with Candida spp. in PFs detected exclusively by PCR were not significantly different from those measured in patients with positive culture results. In fact, serum BDG levels were >90 pg/ml in all but one of these patients. (ii) Five out of the eight patients died during ICU stay. These patients were not treated with antifungals and displayed significantly higher levels of serum BDG than those who survived. Nevertheless, large prospective studies including a sufficient number of proven cases of PC are necessary to gauge the diagnostic value of PCR methods for the diagnosis of PC.

In summary, our study further suggested that both molecular methods and the measurement of serum BDG levels are of potential utility in the diagnosis of PC.

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**Declaration of interest**

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

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