Biofilms are microbial communities that are associated with solid surfaces such as intravascular catheters. *Candida* species are a major cause of medical device–associated infections. Twenty percent to 70% of all candidemias are associated with this biofilm process. Diagnosis and effective treatment of *Candida* device-associated infections requires removal of the involved device. The ability to identify a biofilm device infection before catheter removal may obviate removal of a substantial number of devices. Prior studies in our laboratory identified cell wall changes (specifically, increased β-1,3 glucan) associated with biofilm, compared with planktonic *C. albicans*. Both in vitro and in vivo (catheter) biofilm models were used to determine whether biofilm cells secreted more β-1,3 glucan and whether these differences could be used to discern the presence of a *Candida* biofilm infection with 3 species (*C. albicans*, *C. glabrata*, and *C. parapsilosis*). A limulus lysate assay was used to quantify β-1,3 glucan in supernatants from planktonic or biofilm cultures and in the serum of rats with an intravascular catheter biofilm infection or disseminated candidiasis. β-1,3 glucan was detected from both in vitro and in vivo models from each condition. However, the concentrations of β-1,3 glucan from the biofilm conditions were 4–10-fold greater in vitro (P < .001) and were 10-fold greater in vivo (P < .001), despite equal or fewer numbers of cells in the biofilm conditions. These results suggest the secreted polysaccharide β-1,3 glucan may serve as a useful tool for the diagnosis of *Candida* biofilm and device-associated infections.

Vascular catheter infections are common and are associated with high mortality and morbidity [1–3]. Nearly 5 million vascular catheters are used annually in the United States, and it is estimated that 2%–12% of these result in sepsis [4–7]. Among central venous catheter (CVC)–related infections, those due to *Candida* species are associated with an attributable mortality approaching 40% [8–10]. *C. albicans* device infections are difficult to treat and often require removal of the device for cure. Current treatment guidelines recommend device removal for treatment of candidemia, although removal may be technically difficult and associated with significant risk [7].

The majority of hospital-acquired infections, including *C. albicans* infections, are associated with biofilms [11]. A biofilm is a sessile microbial community embedded in polymeric matrix, with organisms irreversibly attached to a substratum or to each other [11, 12]. The cells in this environment exhibit an altered phenotype with respect to antimicrobial resistance [12–16]. *Candida* biofilms can be up to 1000-fold more resistant to conventional antifungal agents, making the infections very difficult to control in the medical setting.

No laboratory methods exist to rapidly detect biofilm infections in vivo. Blood cultures require several days for incubation, are only positive in 50%–70% of patients with disseminated candidiasis, and do not differentiate between biofilm and nonbiofilm infection. Both semiquantitative and quantitative catheter cultures have been used for diagnosis of bloodstream infections, with greater specificity for catheter infection.
than blood culture alone [17–19]. Both methods involve awaiting culture results. However, quantitative cultures do not require removal of the catheter. To avoid having to remove a catheter for diagnosis of infection, the differential time to positivity of catheter and peripheral blood cultures has also been used [19]. This method utilizes the higher burden of organisms in a blood culture obtained from an infected catheter and measures the earlier time to positivity, compared with that of a peripheral culture. Although this method could circumvent the need for catheter removal to diagnose infection, 24–72 h may be spent awaiting culture results. The only Food and Drug Administration–approved, non–culture-based test for diagnosis of invasive candidiasis is an assay for β-1,3 glucan [20, 21].

Both in vitro and CVC in vivo C. albicans models have been used to examine drug susceptibility and pathogenesis [12–16, 22]. Using these models, the current studies identified differences in carbohydrates surrounding biofilm cells. Findings suggest a dramatic increase in secreted carbohydrates, including β-1,3 glucan. We have demonstrated the ability to differentiate between biofilm and planktonic cells in vitro and in vivo using an assay for this material.

**MATERIALS AND METHODS**

**Organisms and culture conditions.** Four clinical Candida strains obtained from patients with bloodstream infection were chosen for these investigations. C. albicans strains K1 and 98-17, C. glabrata 5376, and C. parapsilosis 5986 were stored in 15% (vol/vol) glycerol stock at −80°C. Before experiments, strains were maintained on yeast extract–peptone–dextrose medium (YPD;1% yeast extract, 2% peptone, and 2% dextrose). Fresh colonies were used for inoculation of YPD or RPMI 1640 with morpholine propanesulfonic acid (MOPS), and cultures were allowed to incubate for 16 h at 37°C with orbital shaking at 250 rpm before inoculation.

**In vitro biofilm formation.** Flat, sterile medical-grade silicone disks were utilized as the biofilm substrate. Before infection, the disks were coated with mouse serum and incubated at 37°C for 30 min. C. albicans K1 was grown in YPD at 37°C on an orbital shaker to late logarithmic phase. After the conditioning period, 150 μL of culture (106 cells/mL) was inoculated on the disk and spread evenly. The disks were then incubated with 1 mL of RPMI/MOPS for 45 min at 37°C. After the adherence period, disks were rinsed in sterile water and then placed in 10 mL of RPMI/MOPS and incubated at 37°C with gentle shaking for varying periods of time (detailed below).

**Planktonic culture conditions.** C. albicans K1 was grown overnight in YPD at 37°C on an orbital shaker at 250 rpm and used for inoculation of planktonic culture. Planktonic cultures were grown in 10 mL RPMI/MOPS in a glass flask at 37°C on an orbital shaker at 250 rpm until late logarithmic phase (106 cfu/mL).

**In vitro glucan determination and microbiologic enumeration.** Supernatants from C. albicans in vitro biofilm cultures and planktonic cultures were collected for determination of β-1,3 glucan concentration. Viable cell number was used as a normalization factor for both the in vitro planktonic and biofilm systems. Viable cell burdens were measured using plate counts to ensure the cultures contained a similar number of cells and were in a similar phase of growth. Both adherent and non-adherent cells were included in microbiologic enumeration of biofilm culture. Culture supernatants were collected from both the planktonic and biofilm systems at 4, 8, and 12 h. The cultures were centrifuged at 3000 g for 20 min, and the cell-free supernatant (confirmed by light microscopy) was removed and stored at −20°C until glucan analysis. Biofilm formation was confirmed by visual inspection and by confocal microscopy. Glucan concentration was determined using a limulus lysate assay, the Glucatell (1,3)-β-D-Glucan Detection Reagent Kit (Associates of Cape Cod), in accordance with the manufacturer’s directions. The culture experiments were repeated on 2 occasions. Three glucan assay replicates were performed for each experiment per time point.

**In vivo venous catheter biofilm model.** A C. albicans CVC biofilm model was used for in vivo experiments as described elsewhere [22]. Briefly, animals were anesthetized, placed in supine position, and prepped in a sterile fashion. A vertical incision was made in skin of the anterior neck just right of midline. The external jugular vein was identified and exposed with blunt surgical dissection, and a longitudinal incision was made in the vein wall. A sterile, heparinized (100 U/mL) polyethylene catheter (PE 100 [inner diameter, 0.76 mm; outer diameter, 1.52 mm]; Intramedic catalog no. 427430; Becton Dickinson) was placed in the opening and advanced to a site above the right atrium (~2 cm). The catheter was secured with 3-0 silk ties. The proximal end of the catheter was then tunneled subcutaneously and secured on the subscapular skin via a button. The anterior neck site was closed with surgical staples (Ethicon Endo-Surgery), and the midscapular button was secured with a 2-0 Ti-Cron suture. Recovery of the animal after the catheter surgery was assessed according to a standard protocol approved by the Veterans Administration Animal Committee. After 24 h, 106 cells/mL of C. albicans strains K1 or 98-17, C. glabrata 5376, or C. parapsilosis 5986, in late logarithmic phase and suspended in normal saline, were instilled in the catheter lumen for 4 h and then flushed with heparin. After a 12-, 24-, or 48-h incubation, blood samples were obtained from tail vein and catheter.

**Tail-vein candidiasis model.** A tail-vein candidiasis model was used to represent disseminated candidiasis in the absence of biofilm growth on an infected medical device [23, 24]. Animals without catheters in place were infected by tail vein with 106 cells/mL of C. albicans strains K1 or 98-17, C. glabrata.
Figure 1. Confocal micrographs of an in vitro *Candida albicans* biofilm. *C. albicans* biofilms were grown on silicon disks and stained with FUN-1 and concanavalin A (ConA) fluorescent dyes. Metabolically active yeast cells are stained red by FUN-1, a fluorescent viability stain. ConA binds carbohydrates and results in green fluorescence of the cell walls of both yeast and hyphae. Extracellular green fluorescence is due to ConA binding to carbohydrate components of the matrix.

5376, or *C. parapsilosis* 5986, in late logarithmic phase and suspended in normal saline. After 12, 24, or 48 h, serum samples were obtained for determination of \( \beta-1,3 \) glucan content. To exclude the possibility of the catheter material alone causing an elevation of serum \( \beta-1,3 \) glucan, several animals with catheters in place were infected by the tail-vein route. These animals had a nonbiofilm infection and a catheter in place.

**In vivo glucan assay and microbiologic enumeration.** Serum samples (1 mL) were frozen at \(-20^\circ\text{C}\) until analyzed for \( \beta-1,3 \) glucan content using a limulus lysate assay, according to the manufacturer’s instructions (Fungitell (1,3)-\( \beta\)-d-Glucan Detection Reagent Kit; Associates of Cape Cod). Assays were performed in triplicate. Kidneys were harvested and collected at various time points (detailed above) from animals with CVC infections and tail-vein infections for microbiologic viable burden counts as an estimate of total-body organ burden [24].

**Statistical analysis.** Two biological replicates and 3 assay replicates were performed at each time point for in vitro glucan assay experiments. Three assay replicates were performed for in vivo glucan determination. Analysis of variance and Stu-
dent’s t test were used to statistically compare glucan concentrations. Viable burden counts from both in vitro and in vivo studies were performed in duplicate. Viable counts from planktonic and biofilm conditions were compared using a Student’s t test to determine significance.

**Biofilm scanning electron microscopy (SEM).** SEM was used to image catheters of animals with CVC infections to confirm biofilm formation and assess the extent of biofilm on the catheter luminal surface [22]. Animals with CVC infections were killed at 12, 24, or 48 h, and catheters were collected for SEM. Catheters were harvested and transected perpendicular to catheter length. Segments were then placed in fixative (4% formaldehyde and 1% glutaraldehyde in PBS) for 20 h, washed for 5 min in PBS, and placed in 1% osmium tetroxide for 30 min. After a series alcohol washes, final drying was performed by critical point drying. Catheter segments were mounted on stubs and gold coated. Samples were imaged in a scanning electron microscope (JEOL JSM-6100) in the high-vacuum mode at 10 kV. The images were assembled using Adobe Photoshop (version 7.0).

**Confocal microscopy.** Confocal microscopy was used to confirm in vitro Candida biofilm formation. Biofilms were grown on silicone disks as described above. Disks were cut into small sections and placed in PBS with fluorescent dyes (the FUN-1 [50 μmol/L] component of the LIVE/DEAD yeast viability kit [Molecular Probes] and concanavalin A [ConA] conjugate [200 mmol/L; Alexa Fluor 488 conjugate]) and processed according to the manufacturer’s instructions, as described elsewhere [22]. Imaging of FUN-1–stained cells was accomplished by using a protocol with an excitation wavelength of 488 nm and an emission band at 617 nm for red. The ConA protocol included excitation and emission wavelengths of 490 and 528 nm, respectively. Biofilms were observed with a Nipkow disk-based confocal microscope (Axiovert 200; Zeiss) equipped with a mercury arc lamp. The objectives used included ×20 and ×40 differential interference contrast oil immersion objectives. Confocal images of green (ConA) and red (FUN-1) fluorescence were conceived simultaneously using the Z-stack mode. Images were processed for display by using Axiovision software (version 3.x; Zeiss).

**RESULTS**

**In vitro biofilm confocal microscopy.** We used confocal microscopy to confirm and characterize the presence of biofilm on the silicone disks in our in vitro biofilm model (figure 1). We selected FUN-1 and ConA fluorescent dyes to stain yeast cells, hyphae, and matrix. At 24 h, yeast cells, hyphae, and matrix components were present on the silicone disk. Live, metabolically active yeast cells processed the FUN-1 viability stain, resulting in red fluorescence within intravacuolar structures. Glucose and mannose residues of cell wall polysaccharides were visualized as a green fluorescence due to ConA binding. The cell walls of both yeast and hyphae were visualized by this dye. The carbohydrate component of the matrix was also visualized by ConA dye and was seen as an extracellular green fluorescence. Because of colocalization of both fluorescent dyes, the metabolically active cells appeared yellow to orange during multichannel image capture.

**In vitro biofilm glucan assay.** We collected supernatants from the planktonic culture and the in vitro biofilm model described above to compare concentrations of β-1,3 glucan. Glucan was detected in supernatants from both cultures and temporally increased with rising cell numbers (figure 2). Using microbiologic viable counts, we confirmed that cultures contained the same number of viable cells. Although microbiologic...
viable counts demonstrated similar numbers of cells in each culture, biofilm culture supernatants were found to contain significantly higher concentrations of β-1,3 glucan (P < .001). At each of the time points, the supernatant from the biofilm system contained 2–10-fold more glucan. The difference was most pronounced at the 8-h time point, when biofilm supernatant contained >150 pg/mL β-1,3 glucan and the planktonic supernatant content was <25 pg/mL (P < .001).

**In vivo biofilm SEM.** Our next experiments were intended to determine whether the enhanced release of β-1,3 glucan from biofilm cells observed in vitro occurs in vivo as well. We used our in vivo CVC model to mimic device-associated infection involving formation of a biofilm. We utilized SEM to verify biofilm formation and extent on the infected CVCs. We captured images of the luminal surface of multiple catheter segments 24 h after inoculation (figure 3). *C. albicans* strains K1

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Figure 3. Scanning electron micrographs (SEMs) of in vivo *Candida* species biofilms. Catheter segments from the rat venous catheter model were prepared for SEM. Panels A–D show biofilm on the luminal surface of the catheter segments from *C. albicans* K1, *C. albicans* 98-17, *C. glabrata* 5376, and *C. parapsilosis* 5686 at ×50 magnification. Panel E shows the *C. glabrata* biofilm at ×1000 magnification.

Figure 4. Serum β-1,3 glucan concentrations (picograms per milliliter) in *Candida albicans* biofilm and disseminated (nonbiofilm) infections. Central venous catheters were surgically implanted in rats and infected with *C. albicans*. The viable burden of cells (log_{10} colony-forming units/organ) from the kidneys of animals with disseminated and biofilm infections were determined at various time points to estimate systemic burden of infection (A). Serum samples were obtained at 12 and 48 h, and β-1,3 glucan content was determined using a limulus lysate assay (B). Black vertical bars represent β-1,3 glucan content in picograms per milliliter in serum samples obtained from the catheter. Light gray bars represent β-1,3 glucan content in the peripheral serum sample (tail vein) obtained from an animal with a biofilm catheter infection. Dark gray bars represent β-1,3 glucan content of a serum sample from an animal with a disseminated infection. Error bars represent 1 SD.
and 98-17 formed extensive biofilms consisting of a network of hyphae and yeast cells embedded in a heterogeneous matrix. *C. glabrata* and *C. parapsilosis*, however, formed biofilms consisting primarily of yeast cells. Like *C. albicans*, these biofilms also were heterogeneous and contained abundant matrix material.

**In vivo biofilm glucan assay.** To determine whether biofilm formation is associated with elevated concentrations of β-1,3 glucan in vivo, we compared the CVC (biofilm) rat model to a traditional disseminated candidiasis (nonbiofilm) model. Animal catheters were inoculated with *C. albicans* K1, and biofilm formation was confirmed using SEM at 24 h (figure 3A). After catheter inoculation, serum samples were collected both directly from the catheter and the tail vein at 12 and 48 h. In a second group of rats, infection was produced by injection of identical inoculum via the tail vein. Blood samples were collected at the various time points. We measured serum glucan content as well as the viable cell burden in kidneys. As anticipated, the viable burden in the kidneys, as an estimate of the systemic burden of cells, was greater in the disseminated model (figure 4). Despite the in vivo presence of more cells, the serum from the biofilm catheter infection contained nearly 10-fold more β-1,3 glucan (*P* < .01) (figure 4). Also, tail-vein serum samples taken from animals with a biofilm infection contained high concentrations of β-1,3 glucan, indicating a systemic release of glucan during biofilm infection. Animals with catheters were also infected by tail-vein route. Serum samples from these animals did not have elevation of β-1,3 glucan, indicating that the catheter material does not result in the elevated serum glucan levels without the presence of a biofilm (data not shown).

Next, we extended our investigated of β-1,3 glucan secretion to other *Candida* species. We used clinical isolates of *C. glabrata*, *C. parapsilosis*, and an additional *C. albicans* isolate for infection of the biofilm CVC model. SEM confirmed the presence of biofilm on the luminal surface at 24 h (figure 3B–3E). Similar to our previous results, animals infected by biofilm route had 2–10-fold higher serum β-1,3 glucan concentrations (figure 5). However, the magnitude of the difference varied by species. Biofilm infection with *C. glabrata* was associated with the highest serum β-1,3 glucan content. The disseminated model produced >10-fold less glucan using this same strain. Interestingly, a serum sample taken from a site distant from the biofilm infection contained only low levels of glucan. The *C. parapsilosis* and *C. albicans* biofilm infections produced 2–8-fold higher serum β-1,3 glucan concentrations, compared with the respective disseminated models.

**DISCUSSION**

*Candida* infections are common in hospitalized patients, particularly those with implanted medical devices [4, 6, 7]. CVCs become infected at an estimated rate of 1.2–4.5/1000 patient-catheter days [25, 26]. The diagnosis of a *Candida* CVC infection is problematic, and the decision to remove a catheter is difficult. Replacement of a CVC is costly, associated with patient discomfort, and carries a mechanical complication rate of 6.3%–19.4% [25].

The *Candida* cells growing as biofilms are different from their free-floating counterparts [11]. Sessile and embedded in a ma-
matrix, they exhibit a drug-resistant phenotype, making removal of an infected catheter often necessary for cure [7, 10–12, 15]. The ability to detect a Candida biofilm could have a great impact on the treatment on candidemia. Of patients diagnosed with candidemia, 30%–70% are reported to have a catheter-related bloodstream infection [9, 10, 27]. However, studies including high numbers of patients with neutropenia have demonstrated lower incidence of CVC-associated candidemia and, thus, biofilm infection (18%–47%) [6, 28]. Many devices in these patients are surgically implanted, making removal and replacement more difficult. Current guidelines recommend device removal for all patients with candidemia [7]. A test allowing detection of a biofilm device-associated infection would identify patients who would benefit most from device removal. Those with candidemia and a negative biofilm test would be able to avoid device removal.

In these investigations, we have proposed β-1,3 glucan as a biofilm marker for Candida. Previous studies have demonstrated differences in secreted carbohydrate composition between C. albicans biofilm and planktonic cultures [29, 30]. Our recent laboratory study has demonstrated a greater concentration of β-1,3 glucan in the cell wall of Candida biofilm cells [30]. We reasoned that the unidentified matrix carbohydrate could be a β-glucan molecule. To test this hypothesis, we compared the C. albicans biofilm conditions to planktonic conditions and demonstrated increased β-1,3 glucan concentrations surrounding Candida biofilms both in vitro and in vivo. Using an in vivo model, which closely mimics a patient CVC infection, we identified significantly higher serum levels of β-1,3 glucan, compared with animals with disseminated infections. Our findings suggest that serum β-1,3 glucan may be a potential target to differentiate biofilm and nonbiofilm infections in patients.

The limulus lysate assay used in the present studies is approved for diagnosis of invasive fungal infection including Candida [20, 31–34]. A cutoff of 60 pg/mL glucan has been used for diagnosis of an invasive fungal infection [20, 34]. However, in one study, the serum β-1,3 glucan concentration in patients with candidemia ranged from 60 to >2000 pg/mL [20]. Although a difference in systemic viable burden may account for variation in serum glucan concentrations in these patients, it is also possible that patients with higher values had a biofilm catheter infection. In the present experiments, β-1,3 glucan served as a useful marker for in vitro and in vivo biofilm formation. Using the glucan assay as an indicator of biofilm infection may markedly improve the specificity of this test. Further investigation of the β-1,3 glucan assay in patients with Candida biofilm infections would be important to determine whether this assay, or a modified glucan assay, may be able to diagnose a biofilm or device-associated infection in a heterogeneous patient population.

References


