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Adhesion of Candida parapsilosis to Bovine Serum Albumin under Fluid Shear

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TITLE:

Adhesion of *Candida parapsilosis* to Bovine Serum Albumin under Fluid Shear

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SUMMARY:

Adhesion is an important first step in colonization and pathogenesis for *Candida*. Here, an in vitro assay is described to measure adhesion of *C. parapsilosis* isolates to immobilized proteins under fluid shear. A multichannel microfluidics device is used to compare multiple samples in parallel, followed by quantification using fluorescence imaging.

ABSTRACT:

C. parapsilosis (Cp) is an emerging cause of bloodstream infections in certain populations. The *Candida* clade, including Cp, is increasingly developing resistance to the first and the second line of antifungals. Cp is frequently isolated from hands and skin surfaces, as well as the GI tract. Colonization by *Candida* predisposes individuals to invasive bloodstream infections. To successfully colonize or invade the host, yeast must be able to rapidly adhere to the body surfaces to prevent elimination by host defense mechanisms. Here we describe a method to measure adhesion of Cp to immobilized proteins under physiologic fluid shear, using an end-point adhesion assay in a commercially available multichannel microfluidic device. This method is optimized to improve reproducibility, minimize subjectivity, and allow for the fluorescent quantification of individual isolates. We, also, demonstrate that some clinical isolates of Cp show increased adhesion when grown in conditions mimicking a mammalian host, whereas a frequently used lab strain, CDC317, is non-adhesive under fluid shear.

INTRODUCTION:

Candida spp. are common commensal organisms on human skin and mucosae that can lead to invasive diseases among the immunocompromised with substantial associated morbidity, mortality, and cost[1-3]. Although *C. albicans* remains an important cause of these infections, non-*albicans* species such as *C. parapsilosis*, *C. glabrata, C. krusei*, *C. tropicalis*, and *C. auris* are being increasingly recognized, especially in vulnerable populations and with frequent resistance to

available antifungal drugs[4]. Non-*albicans* species present distinct elements of biology and pathogenesis that are under active investigation.

Adhesion is an important first step in colonization and pathogenesis. Interference with this step may, therefore, offer an opportunity to stop disease progression at an early stage. Studies of *Candida* adhesion and invasion have been predominantly focused on static conditions[5,6]. These studies have helped define the structure and functions of fungal adhesins in disease[7-9]. However, adhesion in the bloodstream, gastro-intestinal (GI) tracts, and urinary tracts, and in catheters must occur under conditions of fluid shear flow which places unique constraints upon adhesion. Adhesion under shear requires rapid catch bond formation and the ability to withstand strong pulling forces produced due to the movement of liquids[10,11]. The *C. albicans* adhesin, Als5 has been shown to facilitate shear dependent adhesion[12,13]. CpAls7 (CpALS4800) has been previously shown to mediate adhesion of Cp to epithelial cells, and a knockout showed decreased virulence in a urinary tract infection model[14]. We demonstrated that CpALS4800 promotes adhesion under physiologically relevant fluid shear conditions[15].

Candida colonization and pathogenesis have been extensively studied in the animal models[16-18]. The most frequently used models are murine mucosal and bloodstream infections but invertebrate models, such as *Galleria* larvae, are increasingly being used because of the low cost, rapid throughput, and simplicity. Animal models recapitulate many steps of the human disease process in both the pathogen and host, including the host adaptive and innate immune responses, interactions of yeast with tissues and the microbiota, and yeast responses to the host environment. In contrast, in vitro adhesion assays permit the focus specifically on the adhesion step, and on the experimental manipulation of variables such as shear force, growth conditions of yeast, and adhesion to specific substrates.

Because Cp is capable of growth in both humans and environmental sources, it is likely to be capable of sensing and responding to different environments. In support of this notion, multiple clinical isolates of Cp show low adhesion under fluid shear when grown in the standard yeast growth medium, yeast-peptone-dextrose (YPD), but switch to strong adhesion when grown for a few hours at 37 °C in the tissue-culture medium 199 (M199)[15,19]. A detailed protocol is provided here for a medium throughput assay that permits the measurement of adhesion of multiple yeast samples that run in parallel, under defined conditions of growth, fluid shear, temperature, and substrate. The assay has been designed to maximize reproducibility, and to allow for the use of clinical isolates of Cp, as well as strains that have been experimentally manipulated in the lab. The assay as described here, for Cp adhesion to a bovine serum albumin (BSA) substrate, demonstrates that clinical isolates exhibit a range of adhesion, whereas two commonly used lab strains, CDC317 and CLIB214 show poor adhesion.

PROTOCOL:

Candida spp. are classified as Biosafety Level 2 organisms and should be handled using appropriate precautions.

1. Growth and induction of clinical strains

1.1. Streak Cp strains on 1% (m/v) yeast extract, 2% (m/v) peptone, 2% (m/v) dextrose (YPD) 2% (m/v) agar plates, and grow at 22 °C.

NOTE: Plates may be stored on the lab bench and re-used over the following week.

1.2. The day prior to the adhesion assay, transfer approximately 6 colonies of each strain to a 250 mL Erlenmeyer (conical) flask containing 10 mL of YPD medium. Grow overnight in a microbiological shaker set to 250 rpm at 37 °C.

NOTE: Cp need to grow for 20–24 h to reach the stationary phase in liquid culture at 37 °C.

1.3. Perform the following steps on the day of the adhesion assay.

1.3.1. Transfer 1 mL of the liquid culture from the Erlenmeyer flask to a microfuge tube.

1.3.2. Centrifuge the culture at a maximum speed in a microfuge (16,000 x *g*) for 3 min. Resuspend the pellet in 1 mL of sterile water and repeat this step for a total of three washes.

1.3.3. At the end of the last wash, resuspend the pellet in 1 mL of sterile water. Use wide-orifice pipette tips of 1 mL and 200 µL sizes for handling yeast suspensions during this and subsequent steps.

NOTE: Many adhesive strains of Cp stick to the sides of the Erlenmeyer flask, and require mechanical scraping to remove.

1.4. Count yeast cells using a hemocytometer or equivalent device. Dilute the yeast culture to achieve a reasonably countable concentration of 50-200 yeast cells. Use a 500-fold dilution, putting 20 µL into 10 mL of water.

NOTE: Most Cp strains grow to 10^8–10^9 cells/mL in an overnight shaking culture at 37 °C.

1.5. Dilute the yeast culture with 2 mL of YPD or Medium 199 (M199), at a final concentration of 3 x 10^6 cells/mL in a 2 mL microfuge tube. Incubate in a 37 °C water bath for 3 h.

2. Coating of microfluidic channels

2.1. Prepare in advance a 2% (m/v) solution of BSA in Hank's Balanced Salt Solution containing calcium and magnesium (HBSS+). To do this, gently sprinkle 4 g of BSA powder on the surface of 200 mL of HBSS+ and incubate at 37 °C for 30–60 min undisturbed to allow the protein to wet and then dissolve. Filter-sterilize the solution and store at 4 °C.

2.2. Warm 2.5 mL of this solution to 37 °C for at least 1 h, keeping it sterile.

NOTE: Warming is necessary to reduce the bubble formation in the microchannel plate.

2.3. While the BSA is warming, move the microfluidics controller to the tissue culture hood, turn on the device and start the software.

2.4. Place the fluidics interface within the sterile field, and gently clean the silicone gasket with a lint-free tissue paper wetted with 70% (v/v) alcohol. Avoid prolonged or repeated contact of alcohol with the acrylic plate to prevent crazing or cracking of the plastic.

2.5. Air dry the interface (facing up) in the hood with an airflow system to remove all traces of alcohol.

2.6. Add 100 µL of the pre-warmed BSA solution as a droplet in the central indentation of each of the "outlet" channel (**Figure 1A**) of a 48 well, 24-microchannel plate.

[Place **Figure 1** here]

2.7. Place the interface on the top of the microchannel plate, aligning the four bolts of the interface with their corresponding sockets in the plate.

2.8. Tighten the bolts using gloved fingers. Be aware that the resistance to hand tightening indicates misalignment. If this occurs, lift the interface slightly and reseat it, and resume fastening the bolts.

2.9. When the bolts are finger tight, use the torque wrench to further tighten the interface.

2.10. Using the microfluidics software interface (**Figure 1B**), set the Mode to **Manual**. Use the default Fluid option (**Water@19degC**) for both sets of columns and set shear to **1 dyn/cm^2**.

2.10.1. Activate "outlet" columns (#2,4,6,8) to pump liquid towards "inlets". **Run** at room temperature for 30 min.

2.11. Visually inspect each "inlet" well by peering through the bottom of the microchannel plate to ensure that a droplet of the BSA solution has pooled in each "inlet". This confirms that all 24 channels were successfully wetted and filled.

2.12. Unfasten the interface and top up each well ("inlets" and "outlets") with 250 µL of HBSS+ without BSA to prevent drying out of channels and put the plate in a tissue culture incubator.

NOTE: A minimum of 48 h is required for proteins to be uniformly adsorbed to the channel surfaces of the microchannel plate. After protein coating, plates may be stored for up to two weeks in a humid environment (such as a tissue culture incubator with 100% saturating humidity) before the use in adhesion assays. For longer periods, wrap plates in plastic film to

prevent evaporation.

3. Adhesion assay

3.1. During yeast incubation in YPD and M199 (step 1.5), aspirate inlet and outlet wells of the BSA coated microchannel plate, without disturbing the channel that runs from the central indentation of each well (**Figure 1A**). Instead, aspirate from the edge of the well. Add 1 mL of HBSS+ to the "outlet" wells.

3.2. Attach the microfluidics interface as above (step 2.5). Use the default fluid setting Fluid at (**Water@19degC**) and Shear at **2 dyn/cm^2** at room temperature to wash the channel and remove any unbound BSA. Wash for 2-3 h at this flow rate.

3.3. Remove the interface from the microchannel plate. Turn on the plate heater unit of the microfluidics device and confirm that it is set to 37 °C.

3.4. Aspirate the medium from all wells on the plate and add 0.5 mL of induced yeast from step 1.5 to each pair of "outlets". Resuspend the yeast completely by inverting the 2 mL tubes 3-6 times, and gently pipette up and down immediately prior to the addition to wells. Leave the "inlet" wells empty.

3.5. Fasten fluidics interface to the microchannel plate as in step 2.5 above. Use device software to set Fluid to **HBSS@37degC**, and Shear to **5 dyn/cm^2**.

3.5.1. Activate "outlet" columns (#2,4,6,8) to pump liquid towards "inlets". Run on the plate heater at 37 °C for 30 min, to allow yeast to adhere to the BSA-coated channel.

3.6. During this time, prepare the wash buffer. To 30 mL of Dulbecco's phosphate-buffered saline containing calcium and magnesium (DPBS+) add 5 µM of calcofluor, which is included to render fluorescence in yeast cells for their detection. Warm in a bath at 37 °C.

3.7. At the end of the 30 min adhesion, **Pause** the flow using the software interface without altering other flow conditions. Unfasten the interface and aspirate all wells ("inlets" and "outlets").

3.8. Add 1 mL of calcofluor wash buffer to "outlets". Reattach fluidics interface, and resume the flow using the software for another 10 min. This step is designed to wash away non-adherent and loosely bound yeast, and simultaneously fluorescently stain yeast in the channel.

3.9. After 10 min, remove the fluidics interface and replace with a lid. Gently clean the bottom of the microchannel plate with a lint-free wipe and proceed to imaging.

4. Imaging and quantification

4.1. Place the microchannel plate in an appropriate microscope stage holder. Use a 20x objective lens, which will allow a field of view such that the channel fills approximately half of the image height. Locate the left end of channel 1 (in "inlet" 1). Adjust the stage so that the channel is positioned as in **Figure 1A**, position 1.

4.2. Acquire a single brightfield image of the channel. Measure the area of the channel area in μm^2, using the rectangle measuring tool in order to normalize area measurements during data analysis (see Discussion).

4.3. Switching to the DAPI fluorescent channel (excitation 395 nm, emission 440/40 nm) adjust the focus to adherent yeast on the bottom surface of the channel. Lock the autofocus at this plane. Set the fluorescence excitation intensity to 1.5% and camera exposure conditions (binning 2x2, 15 ms exposure, 12-bit, gain 4) to avoid saturation of the image sensor.

4.4. Using the motorized stage and **ND Acquisition | XY Imaging** in the microscope controller software, automatically capture a consecutive series of non-overlapping images spaced one field of view apart (666 µm) of first channel pair (1/2).

4.4.1. Collect 10 images from the left to the right for the upper channel, shift down 666 µm and collect another 10 from the right to the left for the lower channel (as shown schematically in **Figure 1A**).

4.4.2. Use the **Relative XY** option, so that once image positions are defined, a similar series can be triggered for each channel pair, after the start of the channel is manually defined.

4.4.3. Collect images in the DAPI channel (**ND Acquisition | λ | DAPI**).

4.5. Monitor images to confirm that the channel remains within the field of view as the motorized stage moves the plate.

NOTE: Each set of 20 images from a channel pair will be automatically saved with a consecutive file name by **ND acquisition**.

4.6. Use the autostep function (**XYZ Navigation | XY step**) to move 25750 µm down to "inlet" 3. Fine tune channel position manually as in step 4.1. Capture the next set of images (**ND Acquisition**) of the channel pair 3/4.

4.7. Repeat this process, until all 12 channel pairs have been imaged, with results saved in 12 files. Follow the manufacturer's ordering of channels from 1-24.

4.8. Merge all 12 sets of fluorescent images into a single file (**File | Merge ND Documents**). Confirm that the file order matches the order in which the 12 sets of images were captured.

4.9. Use **Binary | Threshold** to separate yeast from the background based on their fluorescence level. Apply the same threshold to the entire stack of images.

NOTE: A 12-bit greyscale intensity low threshold of 500, and a high threshold set to the maximum of 4095 is typical.

4.10. Measure the threshold area (**Measure | Perform Measurement | All Frames**). Open the report (**Analysis Controls | Automated Measurement Results**) and check data.

NOTE: This will generate a table of measurements for 240 images (20 images from each of 12 channel pairs), and threshold area for each image is listed in the column labeled **BinaryArea [μm²]**.

4.11. Export data to a tab delimited text file (**Export**).

REPRESENTATIVE RESULTS:

Using the methods described in the Protocol section, adhesion of 6 strains of Cp was compared (**Table 1**)

[Place **Table 1** here].

Four of the strains were recent clinical isolates at a low passage number[29,30] and CLIB214 and CDC317 are commonly used strains that have been in the laboratory culture for many years. A wide range of Adhesion Indices were observed, from 0.2% to 91% (**Figure 2**). Three clinical isolates (JMB81, JMB77 and Ro75) showed strong adhesion when grown in M199. Interestingly, both lab strains showed relatively poor adhesion in either growth medium. The third clinical isolate, WIH04, resembled the lab strains with relatively poor adhesion.

[Place **Figure 2** here]

The results shown here are from four consecutive experiments, run on different days. They demonstrate the reproducibility of the adhesion assay.

FIGURE AND TABLE LEGENDS:

Table 1. Strains of *Candida parapsilosis* used in this study.

Figure 1. Microfluidics assay layout. (**A**) A pair of channels, showing reverse fluid flow from the "outlet" to the "inlet". The consecutive tiled fields captured by the microscope are shown by dotted lines (1-10 for the upper channel, and 11-20 for the lower). (**B**) Setup of microfluidics controller software for reverse flow during BSA coating (Step 2.10). Screenshots reproduced here with permission from the manufacturer.

Figure 2. Adhesion assay comparing 6 isolates of *Candida parapsilosis*. Isolates were grown for

3 h in YPD or M199 medium prior to the adhesion assay as described in the protocol. Graph represents the mean, and error bars the standard error of the mean from four consecutive experiments, with duplicate channels in each experiment. Adhesion index represents the percentage of the flow channel surface that was covered with yeast. Comparisons were made with the analysis of variance (ANOVA). Between-group comparisons were made with the Holm-Sidak test. *, $P < 0.001$. YPD-M199 comparisons not significant for WIH04, CLIB214 and CDC317.

DISCUSSION:

The data resulting from the above protocol can be analyzed using a standard spreadsheet software. Data are expressed as "adhesion index", which is calculated as follows: The BinaryArea value for each set of 10 images (representing the yeast coverage for a single channel) is summed across the images, and the mean and standard deviation are calculated for the summed area of each channel pair. The channel area measured in step 4.2 represents the maximum possible area in a single field of view that might ever be covered in yeast. In this protocol, the entire length of the channel is recorded in 10 consecutive images, which represents an area of nearly 2.5 mm^2. This area from step 4.2 is multiplied by 10 to represent the length of the channel, and the value is used to normalize the means and standard deviations to express them as percentages of Adhesion Index. Effectively this measures the surface area of a channel that is covered in yeast, with an Adhesion Index of 100% indicating that the entire length of the channel was carpeted with yeast from edge to edge, an area of nearly 2.5 mm^2. An adhesion index of 0% would indicate that not a single pixel achieved the fluorescence threshold. This condition would suggest that calcofluor white was not added, or that the channel was blocked, and yeast cells failed to enter.

Cell adhesion under defined fluid shear was first measured using parallel plate flow chambers[20]. These custom-built flow chambers typically used microscope slides or cover slips as bases and offered a single channel for the measurement[21-23]. Using a commercial version of such a flow chamber, the yeast form of two *C. albicans* strains was found to bind more strongly to the endothelium than the hyphal or pseudohyphal form[24].

The development of a multichannel flow chamber system opened the possibility of higher throughput adhesion assays. The flow chamber hardware and disposable supplies are costly, but they offer a uniformity of manufacture that decreases experimental variability. Finkel et al. used this system to measure adhesion of *C. albicans* to the silicone material used to make one face of the channel as a model of intravenous catheter associated disease[25]. Another group used it to measure the adhesion of *C. albicans* and *Saccharomyces cerevisiae* to BSA-coated channels, followed by phase contrast (brightfield) imaging of a single field of view, followed by software-based cell counting[12]. A third group used the same multichannel system to assess the biofilm formation in *C. albicans* interacting with uncoated channels, also using brightfield imaging over several hours[26].

In the current protocol, the multichannel flow system was used to measure specifically the adhesion step of Cp. Several modifications help increase the accuracy of quantification, maximize the dynamic range, reduce the potential for biohazard spills, and reduce variation.

Importantly, the fluidics system is usually run in the forward orientation, with fluid flowing from "inlet" to "outlet". In addition to the viewing section of the channel, there is also a narrower serpentine region of the flow path which is used to offer hydraulic resistance to the flow. This region tends to trap yeast. In the current approach, yeast suspensions are run in reverse orientation, from "outlet" to "inlet" (**Figure 1A**). In this orientation, the serpentine region falls downstream of the viewing section, reducing concern of trapping of yeast cells.

Growth conditions of Cp immediately prior to the adhesion assay strongly influence adhesion, as does the coating of the channel with BSA or extracellular matrix proteins[13]. It was previously shown that Cp adheres to BSA, collagen, gelatin, fibronectin, and also to serum coated channels[15]. Adhesion to the uncoated channel surface was observed to be very weak. Growth in mammalian cell medium (M199) or serum led to an increased adhesion. It is important to note that unlike *C. albicans*, Cp did not exhibit a filamentous morphology in the 5 h duration of this assay. Shear rate strongly influences adhesion, with maximal shear observed at 5 dynes/cm^2, which is similar to that of blood in capillaries and post-capillary venules[27,28].

To reduce the potential for biohazard spills, yeast growth, adhesion under fluid shear, and washing steps are performed using Biosafety level 2 microbiology precautions at the laboratory bench. After these steps, the microfluidics plate is untethered from the hardware, the plate exterior is cleaned and covered, and the closed plate is imaged at the microscope stage. This approach reduces the risk of contamination of the microscope area.

This protocol uses calcofluor white to stain yeast cell walls. This approach is designed to allow fluorescent imaging of clinical isolates, without the need for genetic manipulation to add fluorescent protein tags such as GFP. Fluorescent thresholding allows for the measurement of the surface area that is covered with yeast cells. To reduce the potential interference with adhesion, the dye is added after the adhesion step, during washing. Chitin staining is the most intense at bud scars, however, for most strains of Cp, diffuse staining is present around the entire periphery. In the current assay system, use of the lower magnification, pixel binning, and careful adjustment of the fluorescence thresholding allows for the entire yeast cell to be measured. Nevertheless, it is important to take staining efficiency into account for strains that vary greatly in chitin content. This should be determined in preliminary experiments, or by examining a portion of the channel under a higher magnification prior to the image acquisition.

Adhesion along the length of the viewing channel may vary. By summing the fluorescence along the length of the channel, the variability and subjectivity offered by one or a small number of fields of view are reduced. The current approach instead extracts the maximum available information from the microfluidics plate by capturing the entire channel. Imaging is greatly facilitated by a motorized stage and autofocus mechanism for the rapid tiled imaging of the length of the channel (**Figure 1A**) with relatively little manual input. Use of lower power objectives result in an increased field of view, which in turn allows for the slight drift in position as the channel is scanned by the motorized stage. Because tolerances of the plate manufacture results in slight variation in the precise locations of channels, it is not possible to image all 24 channels/240 images in a single operation. The approach used here (2 channels and 20 images

at a time) is a compromise. Nevertheless, using this approach, it is possible to complete the imaging and quantification procedure (steps 4.1-4.11) in approximately 15 minutes.

Using this setup to measure adhesion of clinical isolates, a wide range of adhesion indices from 0.2% to 91% was observed. Interestingly, two frequently used Cp strains, CLIB214 and CDC317 showed weak adhesion (**Figure 2**). These observations indicate that there is a significant variation among Cp isolates, and that the assay can provide adhesion data across a wide dynamic range.

Potential variations or modifications of this assay include the use of different species of fungi. Virtually any species that stains fluorescently with calcofluor may potentially be used in this assay, although they may require different substrates or growth conditions. Fungi that differ significantly in the adhesion strength may be accommodated by altering the shear force, or duration of adhesion or washing steps. It is also possible to grow monolayers of endothelial cells or epithelial cells in the flow channels, and measure adhesion of yeast to host cells. Mammalian cells tend to exclude calcofluor, and generally offer low fluorescent background, so that specific detection of yeast is still possible. However, it should be noted that adhesion assays between two cell types require substantially greater effort to maintain both fungal and mammalian cells in optimal physiologic condition.

Limitations of the assay include difficulty of analysis of hyper-adhesive strains. Fungal strains that form large clumps may fail to enter the microchannel, or else obstruct flow within the channel. This difficulty is encountered with hyphae of *C. albicans*. If allowed to grow long, hyphal filaments form sticky mats that clog the channel. Another limitation is the identification of growth conditions that promote adhesion. These may vary for different species, growth forms, or even for specific adhesion pathways. Lastly, some adhesion molecules may require prolonged contact with ligands for strong binding to occur. For such instances a static adhesion assay, such as is frequently carried out in 96-well plates may be preferable.

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DISCLOSURES:

No disclosures.

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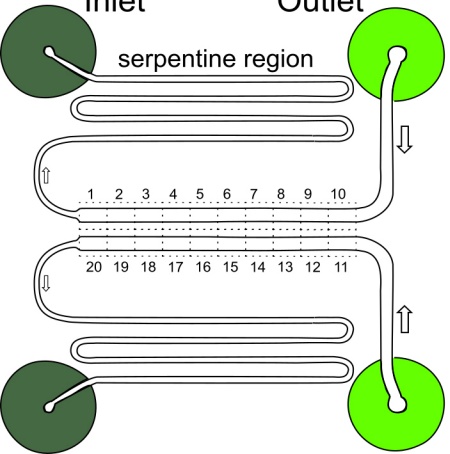
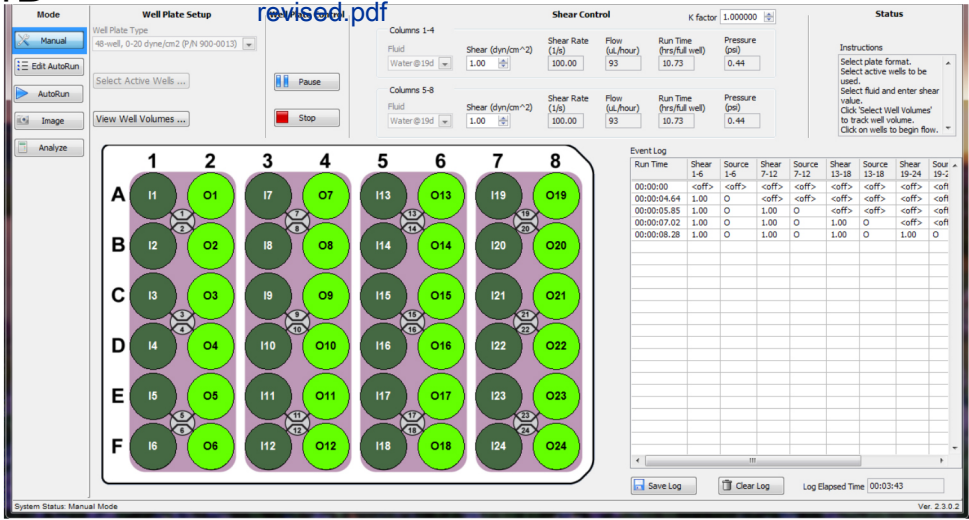
Figure 1

1A



1B

Click here to access/download;Figure;JoVE Fig 1 revised.pdf



Figure

2

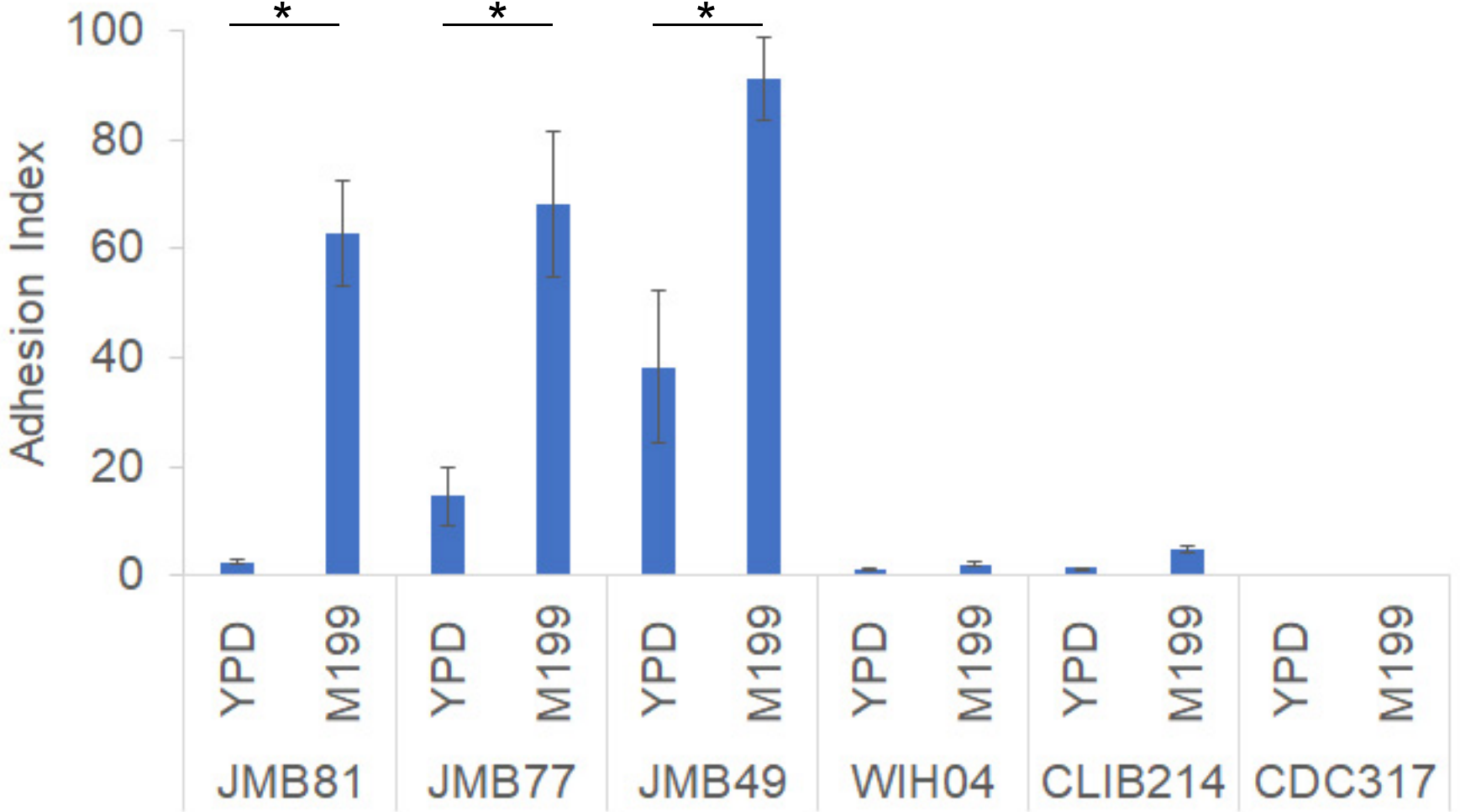


Table 1

Strain	Description	Reference/Source
JMB81	Invasive clinical isolate from infant blood culture	30
JMB77	Invasive clinical isolate from infant blood culture	30
Ro75	Commensal clinical isolate from colonized infant	29
WIH04	Invasive clinical isolate from infant blood culture	local
CLIB214	Case of Sprue, Puerto Rico	ATCC (#22019)
CDC317	Health care worker's hand	ATCC (#MYA-4646)

Name of Material/ Equipment	Company	Catalog Number
Bioflux 200	Fluxion	Bioflux 200
Bioflux Microfluidics plates, 48 well, low shear	Fluxion	910-0004
Bovine Serum Albumin (BSA) Fraction V	Fisher Scientific	BP1605
Calcofluor Fluorescent Brightener	Sigma-Aldrich	F3543
DAPI filter set 440/40	Nikon	
Dulbecco's Phosphate-Buffered Saline (DPBS+)	Corning Cellgro	21-030-CM
Hank's Balanced Salt Solution, 1X (HBSS+)	Corning Cellgro	21-023-CM
Inverted microscope with Perfect Focus	Nikon	Ti-E
M199 medium	Lonza	12-117Q
Motorized Stage	Nikon	Ti-S-E
Nikon 20x lambda Plan-Apo objective	Nikon	
NIS-Elements software 5.02	Nikon	
Spectra fluorescent LED light source	Lumencor	SPECTRA-X3
Zyla 4.2 sCMOS camera	Andor	Zyla 4.2

	Comments/Description
With calcium and magnesium	
With calcium and magnesium, without phenol red	
With Earle's salts and HEPES	

April 6, 2021

Amit Krishnan, Ph.D.
Review Editor
JoVE

Dear Dr. Krishnan:

Thank you for the opportunity to revise and resubmit our manuscript. We appreciate the careful review and have addressed the editorial and reviewer concerns. We agree that these suggestions have strengthened the manuscript. Point by point responses are included below.

Sincerely,

Sunil K. Shaw

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

The manuscript was thoroughly proofread.

2. Please provide an institutional email address for each author.

Email addresses provided.

3. Please revise the following lines to avoid previously published work: Lines 31-40.

This paragraph was revised as suggested (lines 34-40).

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Personal pronouns were removed throughout the manuscript.

5. Please define all abbreviations before use (BSA, HBSS, etc.)

Abbreviations were defined in the text.

6. Please remove the citations from the abstract and try including them in the introduction.

Citations were moved to the introduction.

7. Please ensure that all text in the protocol section is written in the imperative tense as if

telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Corrections were made as required.

8. Line 197-208: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

This information was moved to the Discussion (lines 272-286).

9. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Spacing and highlighting were adjusted.

10. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

The embedded Table was removed.

11. Please ensure that the discussion includes:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

The discussion was reviewed and appropriate changes were made to accommodate these points.

12. Please title case and italicize journal titles and book titles in the References. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

These corrections were made.

13. Figure 1B: Please consider removing the commercial name (BioFlux 200) from the figure.

Although we have written permission from the manufacturer to include screenshots of the software, we did not ask for permission to alter them. For this reason, we prefer not to remove the commercial name from the Figure.

14. Please ensure that the table includes all the essential supplies, reagents, and equipment. Sort the Table of Materials in alphabetical order.

The Table is complete and has been sorted in alphabetical order.

Reviewers' comments:

Reviewer #1:

In the manuscript "Adhesion of Candida parapsilosis to Extracellular Matrix Proteins under Fluid Shear" authors presented interesting method of the analysis of fungal cells adhesion under flow conditions. This is technique particularly useful for studying host-pathogen interactions in conditions mimicking some infectious niches and authors describe this method in the current manuscript in great detail.

However, I have some comments to consider by authors.

The protocol only describes the use of albumin, while the title of the manuscript mentions extracellular matrix proteins - whether the working conditions with these proteins will be similar? It is worth supplementing the protocol with this issue. Moreover, can the authors suggest a way to verify the immobilization efficiency of a particular protein in the system? How could the binding of fungal cells to the surface of the flow cells deprived of proteins be monitored?

These points have been added to the Discussion (lines 312-314).

Staining of chitin within the cell wall with Calcofluor White was given as the method of detection of fungal adhesion, while individual strains or species of fungi, if one would like to compare them, may differ in the level of chitin in the cell wall. Did the authors take into account possible differences between the chitin content during the comparative analysis between fungal cells? Can any control be proposed before the measurement under fluid shear is performed?

Although it is true that calcofluor staining can vary among different fungal species, staining among the cells and different strains of yeast in our assays is quite uniform. Nonetheless, the impact of variable staining would be quite limited in this assay as described. Because the "adhesion index" is calculated as the percent of surface area covered by yeast, the results are essentially binary; i.e. a given field will have fluorescence or not. The adhesion index is calculated as the fluorescent surface area divided by the entire available surface area. As long as the fluorescence threshold is set to distinguish between the presence and absence of any fluorescent yeast, variability of

fluorescence becomes less relevant. This assay would not be useful for measuring relative adhesion of different species in a comparative analysis within the same assay, as calcofluor does not provide a means to distinguish among different species. This is now addressed in the Discussion (lines 328-334).

Reviewer #2:
Manuscript Summary:
The manuscript describes a simple and medium-throughput assay to assess Cp adhesion to host proteins. It is an interesting, well organized, manuscript on a timely subject. A couple of issues, if addressed could, however, increase its impact and quality.

Minor Concerns:
Line 23 - what makes the method so specific: clinical isolates, alone? Cp alone?

The method is specific to Cp as described, but could be modified for other fungi. We agree that the mention of "clinical isolates" in this context is irrelevant and have deleted the phrase.

Line 54 - there is something wrong in this sentence. Infections are not models… please rephrase.

This sentence was rephrased (line 53).

Line 77 - "room temperature"? I would say its preferable to specify! Stored at the "lab bench"? Shouldn't 4ºC be preferable?

Room temperature was specified as suggested (22°C). Our stocks are not stored at 4ºC based on previous, unpublished work in our laboratory suggesting that cold temperatures lead to genetic instability on Candida *stocks. This is the main reason that plated strains are not used for longer than one week.*

Lines 80-81 - Why such a large flask for such a small volume? Why the need for such high surface /volume ratio?

We have not tested culture flasks of different volume, but our selection was to ensure optimal aeration during growth. It is possible that smaller flasks would behave similarly, but we have not tested that possibility.

Line 91 - "will be" instead of "will"

This phrase was deleted as the sentence was restructured.

Line 126-127 - what is meant by "humid environment"? Please exemplify.

This clarification has been added to the protocol section (lines 143-144).

Section 4 - It's not clear where the fluorescence signal is coming from. Is it natural cell or biofilm matrix fluorescence? Can't that change from strain to strain? In line 280 finally the authors mention calcofluor white, but the staining procedure is not detailed. Please do so. Still, please discuss the possible limitation of using this dye, as it binds chitin, whose thickness in the cell wall may vary from strain to strain.

The inclusion of calcofluor in the wash buffer is included in Step 3.5. We have added the phrase "which is included to render the yeast fluorescent for detection" to make it explicit that this wash buffer also provides the fluorescent staining. As described in the comments to Reviewer #1, variability of staining among strains has not been our experience, and would not impact the data as collected and analyzed.

Lines 300-301 - This sentence is highly speculative! What makes the authors think that the 6 used strains are representative of the "theoretical" maximum variation. Please tone the sentence down.

We have revised the sentence to make it less speculative. "These observations indicate that there is significant variation among Cp isolates, and that the assay can provide adhesion data across a wide dynamic range."(lines 350-351)

Reviewer #3:
Manuscript Summary:
The manuscripts describes a protocol that will be of interest to Candida investigators.

Major Concerns:
1. Include in the abstract that a protocol is described specifically to investigate the adhesion of C. parapsilosis to BSA. Explain in the introduction better the significance of adhesion of C. parapsilosis to BSA.
2. In the protocol section, I recommend including an introductory paragraph where the protocol steps are summarized in a more general way.
3. Describe also how the "Adhesion Index" was calculated in section 5 "Data Analysis".

Minor Concerns:
Line 44: "… helped define the structure and functions …". Here additional recent references (e.g. reviews) could be added (e.g. Willaert RG. Adhesins of Yeasts: Protein Structure and Interactions. J Fungi (Basel). 2018 Oct 27;4(4):119. doi: 10.3390/jof4040119. PMID: 30373267; PMCID: PMC6308950.)

Reference was added.

Line 76: specify the composition of YPD

The composition of YPD was added.

Line 78: 2 organism => organisms

Corrected.

Line 83: "Yeast...in liquid culture …" => "C. parapsilosis....in liquid YPD culture …"

Corrected.

Line 91: It will … => It will be …

This phrase was deleted as the sentence was restructured.

Line 98: 2% => 2% (m/v)

Corrected.

Line 105: 70% => 70% (v/v)

Corrected.

Line 169: ND Acquisition => This is specific for Nikon software NIS Elements. Can the procedure be generalized for other microscope brands?

Many of the details concerning imaging conditions are specific to the hardware and software used. Settings such as camera setup and software details will vary according to manufacturers or even arrangement of light path. To make the protocol less specific, a general descriptor is included in each step.