Chapter 10

In Vitro and *Ex Vivo* Assays of Virulence in *Candida albicans*

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Abstract

The measurement of virulence using *ex vivo* and *in vitro* models is discussed in the context of the human pathogenic yeast, *Candida albicans*. The models described are of two types. First, reconstituted tissues of various sorts are used that are derived from human carcinomas. The tissues are grown *in vitro* in complex media, attain a three-dimensional tissue structure, and retain cell-surface antigens typical of the specific tissue. Both adherence and invasion of tissues can be studied following infection with strains of *C. albicans (1, 2)*. Further, one can increase the level of complexity by providing infected tissues with host phagocytes or cytokines such that an immune contribution to protection can be followed *(3–5)*. The second model employs *Drosophila melanogaster* larvae that are infected with *C. albicans (6)*. In this model, the progression of virulence is followed after injection of strains of a pathogen of interest into the fly abdomen. Thus, in the case of human pathogenic fungi, the recognition of host tissues and invasion by the specific pathogen can be studied *in vitro* and correlations developed for human disease. The obvious advantage to using animal models (e.g., mice) is reduced cost, such that large numbers of *C. albicans* strains can be assessed for their virulence properties. Additionally, another application of these models is in drug discovery. It is clear that there are both advantages and disadvantages of the use of alternate models other than a murine model, to evaluate disease, and this is discussed below.

Key words: Virulence assessment, nonanimal models, *Drosophila*.

1. Introduction

First, I will suggest my definitions of *in vivo*, *ex vivo*, or *in vitro* models that are used to evaluate microbial virulence. Also, a relatively new application to the study of microbial virulence has been reported (see below) using the fruit fly, *Drosophila melanogaster*, termed a mini-host model, in obvious reference to the difference in size among this creature versus a mouse *(6)*. That designation will be used in this chapter. An *ex vivo* assay usually refers to the removal of cells, from a test animal after an infection,

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e.g., alveolar macrophages following intranasal infection for various time periods with *Aspergillus fumigatus* conidia *(7)*. In this example, virulence/survival of the test organism is then determined by culturing lung lavage fluids on media that support the growth of the fungus and measuring the number of viable fungi recovered from the animals. The viability of a set of strains (and the killing of infected mice) in which a gene-deleted mutant is compared to other strains (wild-type and gene-reconstituted strains) can be compared for virulence differences, drug treatments, and drug efficacy, or even immune responses. In the latter instance, one can compare virulence in immune knockout mice or mice treated in ways to depress or activate specific arms of the immune response. This information is valuable to understand broad concepts of immunity or even to identify the activity of specific host cell populations, each through the use of "knockout" mice. Corticosteroid-treated animals, for example, were infected intranasally with *A. fumigatus*, a respiratory pathogen, to determine the consequence of that treatment with fungicidal activity of alveolar macrophages *(7, 8)*. So, herein, as the examples above describe, *ex vivo* refers to studies in which host cells are removed following infection of an animal host, and the particular model described above provides a way to evaluate virulence of fungal strains that infect the alveoli following their inhalation.

In vivo models are easy to define: animals are infected and assessed for the same types of endpoints just described, only always within the context of the animal. *In vitro* models are defined as those in which the host (be it an animal or a cell-line) is infected and that interaction is then studied in the context of a nonhost environment. In summary, the definitions of these types of models reflect where the interaction is studied i.e., in infected animals, in cells removed from infected animals, or in a host–fungus relationship strictly investigated in the laboratory.

The selection of an *in vitro*, *in vivo*, or *ex vivo* assay to evaluate some parameter reflects two primary considerations – cost and time – both of which are related. For example, to evaluate drug efficacy in an animal model requires a dose–response determination for the drug under study. In addition, the response of different pathogens, size of inocula, and the required controls must all be investigated. Such experiments become more complicated when additional parameters are evaluated, such as how the drug is delivered (i.v., peritoneal, intrathecial, etc.) and in what form (e.g., nonencapsulated or liposomal encapsulation). The number of mice required for such a study approaches several hundred, at a significant financial burden to the investigator (mice, housing per diem, salary of the investigators, etc.). Further, time is money. Of the many invasive animal models of candidiasis, the most commonly used model is infection through tail-vein injection, a time-consuming procedure. Ethical considerations also play an

important role. Must animals die? Procedures need to be included in protocols that will minimize pain or alleviate pain. Animal death is not acceptable as an endpoint to an experiment; so increased surveillance of infected animals must be undertaken and parameters established to assess morbidity. The oversight provided by institutional reviews of protocols is absolutely essential. In addition, an animal model must mimic as closely as possible the disease in humans, a requirement that often goes unfulfilled. Likewise, *in vitro* models have their limitations since data from such studies only suggest correlations with disease. Infection and virulence of a selected mutant of *C. albicans* in a fruit fly model or in reconstituted tissue *in vitro* fits with candidiasis in a patient only as much as can be suggested by the experimental conditions. Attempts to circumvent this problem by creating a "host-like" environment include the addition of multiple host components such as serum to phagocytosis assays, the addition of phagocytes or other immune cells to studies of interactions *in vitro* of the fungus with cultured tissue, among other possibilities *(3–6)*. Two *in vitro* models are described below, one using cultured tissue derived from human carcinoma cells and the other using *D. melanogaster*.

2. Materials

2.1. In Vitro *Tissue Models*

1. *C. albicans* strains SC5314, CAF2-1, CAI4, or stains appropriate to the issue under investigation (*see* **Note 1**).
2. Human reconstituted tissues, Skinethic Laboratory, Nice, France (*see* **Note 2**).
3. Culture medium, brain heart infusion agar, 35 g/L, prepared one-day ahead of the tissue assay, stored at 4°C until needed.
4. Washing buffer, phosphate-buffered saline (PBS): 0.01 M phosphate, containing 0.86% NaCl, final pH 7.2.
5. Fixation of infected tissues. At appropriate time intervals, tissues and filters are removed from the microtiter plates, washed with PBS, and then prepared for fixation: 10% formalin in PBS. Following dehydration, tissue-filters are embedded in paraffin at room temperature. Sections of 4 μm are obtained, transferred to glass slides, and stained with periodic acid-Schiff (PAS) stain (*see* **Note 3**).
6. Scanning electron microscopy. PBS to remove nonadhering organisms; fixed with 2.5% glutaraldehyde and 2% formaldehyde, washed three times with PBS, postfixed with osmium tetroxide, washed again similarly with PBS, dehydrated in a graded series of ethanol solutions, and gold-coated after critical point drying.

2.2. In Vitro *Tissue Models: Transcription Studies*

1. RNA isolation. Infected or uninfected epithelial tissues are shock-frozen and total RNA is isolated using RNAPureTM (Peqlab, Erlangen, Germany). A number of similar kits are available for use.
2. cDNA synthesis is done using Superscript reverse transcriptase (Gibco, but this enzyme can be obtained from a number of distributors).
3. Quantitative RT-PCR. cDNAof 20 ng, amplification in real time in a Light-Cycler SYBR Green I kit (Roche), containing 3 mM Mg^{2+}, final concentration. Primer pairs, annealing temperature, and elongation time is optimized for each primer pair (*see* **Note 4**).
4. TES-phenol buffer for extracting RNA. 10 mM Tris-HCL, pH &.5, 10 mM 0.5 M EDTA, 0.5% SDS in DEPC H_2O, and 400 μL of phenol, pH 4.5.

2.3. *Drosophila melanogaster – A Mimi-Host Model for Testing Virulence and Antifungal Drug Efficacy*

1. *D. melanogaster* stocks – 2–4-day-old female, 30 flies used per treatment.
2. Yeast cells – overnight cultures in yeast-peptone-dextrose (YPD) medium.
3. Infections are initiated by injecting 1×10^7–1×10^{10} yeast cells into the thorax of individual flies.

3. Methods

It is important to realize that measuring the amount of organism adhered to tissues in this system is only semiquantitative. Visualization of adhering organism is accomplished by light microscopy of fixed/stained tissue sections or by scanning electron microscopy. Nevertheless, general conclusions can be made on the order of events in timed experiments for specific sets of mutants. For example, wild-type strains were observed more adherent than two-component signal transduction mutants as well as secreted aspartyl protease (SAP) mutants over a period of 24 h *(1, 2, 5)*. In addition, strains can be evaluated for penetration into the tissue by following the infection for longer periods of time (24–48 h). The other major observations that can be measured include host responses, i.e., cytokines, β-defensins, or in the case of fungal products, SAP antigen or transcript *(5)*. The tissue model allows the investigator to broaden the objectives of the experiments for either host or fungus.

3.1. In Vitro *Tissue Models*

C albicans SC5314 or CAF2-1 or other suitable strains as the specific investigation dictates can be used. As mentioned above, the model is useful for evaluating the virulence of any single gene-deleted strain and its set of controls. In the case of *C. albicans*,

strain sets need to include a single gene-deleted strain as well as the gene-reconstituted strain in addition to the companion null strain lacking both genes. As the selection of correct strains is done via Ura3$^-$ conversion to prototrophy, then loss of *URA3* in transformants provides a selection for the second gene knockout; *URA3* positional affects have been described that may influence phenotypes since the *URA3* gene is not in its native locus *(9)* (*see* **Note 5**).

1. Wild-type strains are grown in one's favorite medium (BHI or yeast-peptone-glucose are the most common), and then adjusted to an inoculum of 10^6–10^7 cells by hemocytometer counts. This somewhat tedious method can be bypassed by instead using a cell concentration that corresponds to a known optical density reading.
2. There are several types of reconstituted tissues provided by Skinethic Laboratory, Nice, France, that are of either epidermis or endodermis origins. Most of the tissues are derived from human cancer cell lines. The tissues are provided on polycarbonate filters in a microtiter well format that is also available as a 96-well high throughput screen (HTS) that may be useful for antifungal drug testing, although this objective has not been reported as yet.
3. The reconstituted tissue must be cultured for at least 24–72 h after arrival to the lab. For the reconstituted human esophageal tissue, the company provides a chemically defined medium (MCDB 153 medium, containing gentamicin (25 μg/mL), 5 μg/mL of insulin, and 1.5 mM Ca^{2+}) for growth and maintenance of the tissue. This medium is sufficient to allow additional growth of tissues as needed. Instructions from the producer are well-defined and help in planning the timing of experiments. Some variation in the medium may reflect growth requirements of different tissues. For inoculation, protocols vary in regard to inoculum size, as indicated above, but tissues (per well) can be infected in a volume of 50 μL of PBS, pH 7.2.
4. For measuring the amount of organism adhered to tissues at designated time points, the infected tissues are washed three times with PBS and fixed in 10% formalin in PBS for at least 24 h.
5. Fixed cells are then dehydrated, sectioned, the sections transferred to glass slides, and stained with the PAS. Light microscopic visualization allows for qualitative inferences in the amount of organism adhering to tissues, and at later times, the invasion of the tissue can be analyzed.
6. Scanning EM allows for an analysis of interactions at the surface of the tissues and at greater magnification. Such analysis can also be useful in antigen tagging experiments, as for example those reported for Sap proteins *(2)*. Tissue damage caused by the organism can be measured by the release of lactate

dehydrogenase using spectrophotometric measurements of NADH oxidation following the conversion of pyruvate to lactate. Several assays are available, e.g., see the Wroblewski—La Due method *(2)*.

3.2. In Vitro *Tissue Models: Transcription Studies*

1. In these experiments, measurements of tissue responses that are associated with immunity to the organism can be accomplished. For instance, the interaction of *C. albicans* with oral epithelial tissue was studied in order to understand the role of the cytokine interleukin-8 (IL-8) in protection against candidiasis *(3)*. The reasoning is that IL-8 provides an important role in T-helper 1 responses, which is critical to protection in oral candidiasis. In these experiments, epithelial tissues were infected with a specific inoculum of washed yeast cells of several species of *Candida*. At various times postinfection, infected or uninfected tissues were collected and centrifuged. The amount of secreted IL-8 in the supernatant of the infected tissues was detected using a sandwich ELISA method with optimized amounts of a mouse antihuman IL-8 monoclonal antibody (G265-8, PharMingen, San Diego, CA, USA). For assays, this antibody is coated in microtiter wells overnight, washed, nonspecific binding prevented, the tissue-fungus supernatants are added, and allowed to incubate overnight. Then, the primary antibody supernatants are washed several times with PBS, and a biotin-labeled anti-IL-8 monoclonal is added. Again washing is critical to remove nonbound antibody and the amount of IL-8 is determined using an avidin-biotin-alkaline phosphatase complex (DAKO) followed by the addition of the phosphatase substrate that is compared to assays with increasing concentrations of IL-8. Optical densities at 405 nm are obtained from experimental samples and the amount of IL-8 determined from the linear range of standard curves with the recombinant IL-8. The authors were able to correlate tissue damage by *C. albicans* with increased responses of IL-8, while *C. glabrata* and *C. tropicalis* produced less damage and also less IL-8. Heat-killed *C. albicans* failed to induce an IL-8 response.
2. cDNA synthesis is performed using standard methods as described above in **Section 2.2**.
3. RT-PCR. Infected tissues are processed as described next *(5)*.

Each tissue, infected or otherwise, is transferred to RNase/DNase-treated 1.5 mL microcentrifuge tubes containing a standard buffer. The samples are centrifuged at 13,000 rpm for 1 min and the supernatant decanted, tissues are suspended in 400 µL of TES (10 mM Tris·HCl pH 7.5, 10 mM 0.5 M EDTA, 0.5% SDS; in DEPC H_2O) and 400 µL of phenol pH 4.5. After vortexing for 10 s, the samples are incubated at 67°C for 1 h, vortexing every 10–15 min, placed on ice for 5 min, and then centrifuged for

5 min at 13,000 rpm. The top layer is transferred to a 1.5-mL microcentrifuge tube and 400 μL of phenol (pH 4.5) added. The mixture is vortexed and incubated on ice for 5 min. After centrifuging for 5 min at 13,000 rpm, the top layer is transferred to a new 1.5-mL microcentrifuge tube, and 400 μL of chloroform:isoamyl alcohol (24:1) is added, vortexed, and placed on ice for 5 min. After vortexing and centrifuging as above, the top layer is transferred to a new 1.5-mL microcentrifuge tube. DEPC-treated sodium acetate of 40 μL and 1 mL of ice-cold 100% ethanol are added to the mixtures, which are again vortexed and incubated on ice for 5 min. After centrifugation for 5 min at 13,000 rpm, the supernatant is removed and 500 μL of ice-cold 70% ethanol is added. Again, after centrifugation, the supernatant is discarded, the pellet air-dried, and suspended in 100 μL DEPC H_2O. The concentration and purity of the RNA was determined at wavelengths of 260 and 280 nm using a spectrophotometer.

4. Quantification. The RT-PCR reactions to quantify gene transcription are performed according to the protocol for the Qiagen One-Step RT-PCR Kit.
   (a) A 50-μL reaction mixture included 25 μL of RNase-treated H_2O, 10 μL of 5X buffer, 2 μL of dNTP mix (10 mM each), 0.5 μg each of primers Ssk2 NB 5′ and Ssk2 NB 3′, 1 μg of RNA, and 2 μL of enzyme mix.
   (b) The reactions are set up in duplicate to detect both transcripts for all samples. The amplification conditions for detecting the transcript can vary by individual design but may follow this program: 30 min at 50°C (reverse transcription), 15 min at 95°C (activation of the HotStarTaq Polymerase), followed by 25 cycles of 1 min at 94°C, 30 s at 56°C, and 55 s at 72°C, concluding with a final extension step of 10 min at 72°C.
   (c) The amplification conditions for detecting the *ACT1* transcript (internal control) are as described above, except annealing is performed for 30 s at 59°C and extension for 45 s at 72°C. A gel imager (Alpha Imager 2000, Alpha Innotech Corp.) is used to quantify the intensity of transcripts.
   (d) The levels of transcripts are expressed as a ratio and calculated by dividing the band intensity of each gene by the band intensity of *ACT1* for each RNA sample. A fold-difference is determined and calculations are performed for all six samples and three independent experiments. The two-tailed Student's *t*-test was utilized to determine the significance of the values.

3.3. D. melanogaster Model: Inoculation

1. Oregon R flies are used as wt flies. Manipulation, feeding, and housing of flies is described *(10)*.
2. Yeast cells are grown and maintained on YPD agar.

3. Inoculation into the *thorax* is done using a thin, sterile needle (0.25 mm diameter) dipped into a suspension of 1×10^7–1×10^{10}, and it is then used to inject the organism into the thorax. Alternatively, for ingestion assays, fly-food vials containing YPD agar on which 1×10^7 cells are grown for 24 h may be used. Flies are fed for 48 h, and then switched to fly-food and maintained at 28°C. After inoculation (*see* **Section 3.3.3**), flies are maintained at 29°C. If death occurs within 3 days, such flies should not be included in data analysis. During the infection process, flies are changed to fresh vials containing food every 2 days. Survival is assessed daily for a total of 8 days (30 flies per strain). Quantification of the organism used for inoculation is determined by dipping the inoculum needle used for infection in sterile saline and determining colony-forming unit (CFU) by plating dilutions of this suspension onto YPD plates, which were incubated at 30°C for 48 h. Likewise, for the ingestion model, flies are evaluated for survival, daily for 8 days. For measurements of tissue burden, groups of 20 flies are collected a 0, 12, 24, and 36 h after infection, then transferred to plastic tubes, and ground in 1.0 mL of physiological saline. Tissue loads of the organism are determined by plating aliquots on YPD medium and CFU determined as described above.

Histopathological assessment of infection is determined as follows: following either type of assay (ingestion or thorax inoculation), at day 2 postinfection, flies are fixed with 10% (vol/vol) formaldehyde, stained with Grocott-Gomori methenamine silver nitrate, and sections examined for fungal growth by microscopy.

The assay can also be evaluated for drug-efficacy testing using antifungals such as fluconazole. In these experiments, flies are first starved for 6–8 h and then transferred to vials containing their food supplemented with fluconazole (1 mg/mL). After 24 h, flies are infected by injection as described above, and then transferred to new vials containing fluconazole. Control flies (uninfected) are treated similarly but not fed with fluconazole. Fluconazole protection was monitored daily for 8 days.

4. Notes

1. Strain CAI4 is Uri^- and, therefore, is not used in comparison to gene-deleted strains in which *URA3* has been restored either to the locus of the gene that was deleted or its own locus.
2. Tissues that are infected should be evaluated for adherence and invasion within a reasonable amount of time (0–4 days) since, especially in wt strains germination and growth of the organism occur quickly and overwhelm the microscopic fields with extensive hyphal development.

3. We find that the PAS stain gives much better contrast in microscopic examination; the fungus retains a reddish color against the colorless background.
4. Real-time PCR is preferred to the standard RT-PCR that is more often reported. Real-time PCR allows one to evaluate transcription at different times within the PCR amplification steps.
5. Ura3-positional affects on phenotype are reported using the Urablaster method to obtain specific gene-deleted mutants. This problem can often present itself if phenotypes do not match gene dosage. For example, a single gene-carrying strain should have a phenotype that matches a single gene copy compared to wet strains, which have two copies of the specific gene.

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