

Chapter 2

Detection and Quantitation of Antifungal SIgA Antibodies in Body Fluids

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Abstract

The measurement of antibodies in the external secretions that bathe mucosal surfaces is important in understanding the host response to the opportunistic pathogen, *Candida albicans* and its determinants of pathogenesis at these sites. The principal immunoglobulin isotype in mucosal secretions is secretory immunoglobulin A (SIgA). Unlike the circulatory system, mucosal surfaces are open systems in which the concentrations of immune factors are affected by diurnal variation, changes in flow rate, complex formation with mucins, and other variables. Thus, it is necessary to control these factors if meaningful data are to be obtained. This chapter outlines methods for the measurement of anti-*Candida* SIgA antibodies in primary units and shows how to control the factors that influence antibody measurement in external secretions.

Key words: SIgA, SIgA antibodies, *Candida* antigens.

1. Introduction

In studies of the pathogenesis of fungal infections or for the development of vaccines, it is frequently necessary to be able to detect and quantitate antibodies directed against the organism and/or its antigens in various body fluids.

Body fluids can be divided into two types, those in closed systems such as blood, cerebrospinal fluid, peritoneal fluid, etc., and those in open systems, that is, mucosal secretions such as tears, nasal secretions, saliva, milk, genitourinary secretions, etc. While detection of antimicrobial antibodies in blood is generally quite straightforward, the detection and quantitation of antimicrobial antibodies in external secretions is altogether a different proposition. The reasons for this are several and include diurnal variation, the inverse relationship

between flow rate and antibody concentration, complexation of antibodies with high-molecular-weight mucins and other factors found in secretions, and proteolytic and glycolytic activity of the resident microbiotas that colonize mucosal surfaces. Detection of antibodies was revolutionized by the invention of the enzyme-linked immunosorbent assay (ELISA) by Engvall and Perlmann (1). This method is very versatile and sensitive, and is widely employed in both research and clinical care settings (2, 3). One limitation of the measurement of antimicrobial antibodies by ELISA is that the data are output in optical density units. While this may be satisfactory for making comparisons within individual laboratories, it makes comparisons with data from other laboratories difficult as optical density is affected by incubation time, source of antibody reagents, nature of the plastic plate, to name but a few. It is the purpose of this chapter to describe the application of ELISA to the detection of anti-*Candida* antibodies in external secretions in which the read-out is in primary units, rather than optical density. The method described combines the measurement of antimicrobial secretory IgA (SIgA) antibodies and total SIgA immunoglobulin on a single 96-well-microtitration plate. The described method is applicable to the detection and measurement of almost all antimicrobial antibodies that are induced in the secretions of experimental animals or humans. The inexperienced reader is advised to familiarize themselves with the basic principles of solid-phase assays before embarking on the assay described in this chapter. A free technical handbook on ELISA and the related technique ELISPOT is available from Pierce Chemical at <http://www.pierce.net.com/Objects/View.cfm?Type=Page&ID=AF5B61C9-9149-41F4-B63D-DA4C46CD9446>

2. Materials

2.1. Collecting Mucosal Secretions

1. One piece 3.0 mL sterile disposable transfer pipette (Fisher Scientific, Pittsburgh, PA, USA).
2. Sterile phosphate-buffered saline (PBS), pH 7.4.
3. 500 mM ethylenediamine tetracetic acid (EDTA) solution. Dissolve 14.61 g of EDTA (Sigma-Aldrich, St. Louis, MO, USA) in 50 mL of deionized-distilled water (ddH₂O) and filter sterilize using a 0.45 µm disposable vacuum filter unit.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

1. 96-Well flat bottom microtiter plates (Imm luxTM or Imm luxTM HB, Dynex Technologies, Chantilly, VA, USA).
2. Coating buffer: 0.05 M carbonate buffer, pH 9.6, containing 0.02% NaN₃. Dissolve 1.6 g of Na₂CO₃ (anhydrous)

(MW = 105.99), 2.9 g of NaHCO₃ (MW = 84.01), and 0.2 g of NaN₃ (all from Sigma) in a final volume of 1 L of ddH₂O. Store at 4°C. Discard after 2 weeks.

3. Blocking reagent: 0.1% (1.0 g/L) bovine serum albumin (BSA) fraction V (Sigma) containing 0.02% (0.2 g/L) NaN₃ in PBS, pH 8.0.
4. PBS-Tween: 0.1% (1 mL/L) Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma) containing 0.02% (0.2 g/L) NaN₃ in PBS, pH 8.0.
5. Horseradish peroxidase (HRP)-conjugated antibody diluent: 0.1% (1.0 g/L) BSA in PBS, pH 8.0.
6. Citrate-phosphate buffer: Mix four parts of 0.1 M citric acid (dissolve 1.92 g of anhydrous citric acid (Sigma) in 100 mL of ddH₂O) with six parts of 0.2 M Na₂HPO₄ (dissolve 2.84 g of Na₂HPO₄ (Sigma) in 100 mL ddH₂O). Adjust pH to 4.5, if necessary, with either solution.
7. HRP substrate solution: Dissolve 1 mg of *o*-phenylenediamine (Sigma) in 1.0 mL of citrate-phosphate buffer, pH 4.5 and add 0.012% H₂O₂ (4 µL of 30% H₂O₂ per 10 mL of buffer) (*see Note 1*). Prepare substrate solution immediately prior to use. Substrate is light sensitive; use dark bottle or wrap foil around the vessel.

3. Methods

The method described below is designed to quantitate *Candida*-reactive SIgA antibodies in external secretions and output the data in primary units by interpolating optical density into a SIgA standard curve (ng/mL). In this application, *Candida* cells or purified *Candida* antigens form the solid phase, that is, they are immobilized on the plastic surface of the well of one-half of a 96-well-microtiter plate. Here they serve to capture SIgA antibodies reactive with them in the external secretion. On the other half of the plate, an antibody to human secretory component (SC) forms the solid phase and serves to capture a series of dilutions of purified SIgA, thus forming an SIgA standard curve.

3.1. Determination of the Dry Weight of *Candida* Cells

1. Dry a 25 mm, 0.4 µm Whatman Nuclepore® polycarbonate filter (Fisher Scientific) to constant weight. Place the filter inside a P₂O₅ dessicator, evacuate the dessicator and place it overnight in a 60°C incubator.
2. Weigh individual filters on an analytical balance accurate to 10 µg, and record weight on Petri dish.

3. Set up a 25-mm fritted glass filter base with stopper in 125 mL Erlenmeyer side-arm flask (Millipore XX15 047 00 All-Glass Filter Holder).
4. Place a 0.45- μ m nitrocellulose filter (Sigma) on the fritted glass filter base and wet with ddH₂O; carefully place the polycarbonate filter on top of the nitrocellulose filter, making sure that there are no air bubbles between the filters.
5. Accurately pipette an aliquot of a *Candida* suspension (\sim 200 μ L) onto the central portion of the polycarbonate filter while a vacuum is applied to the side-arm flask.
6. Wash the cells with several hundred microliters of ddH₂O.
7. Place the filter back in the same Petri dish, and evacuate the dessicator and warm to 60°C overnight as before.
8. Reweigh the filter, determine weight of cells in the volume applied to the membrane, and calculate the total weight of cells based on the volume of the cell suspension.

3.2. Determination of the Concentration of Antigens

1. The concentration of protein antigens can be determined using commercially available protein assay kits such as the BCA (bicinchoninic acid) Protein Assay or the Coomassie (Bradford) Protein Assay, both obtainable from Pierce Biotechnology, Rockford, IL, USA.

3.3. Collecting Mucosal Secretions

1. Aspirate secretion and dispense into a graduated tube. Measure the volume of the secretion and add sufficient EDTA solution to give a final concentration of 5 mM (*see Note 2*).
2. Store the secretion at -70°C until use.

3.4. ELISA to Detect Binding of SIgA Antibodies Against *Candida* Cells or Antigens (*see Note 3*)

1. Coat one half of the wells of the microtiter tray (48 wells) with either *Candida* cells or purified antigen by dispensing 100 μ L of the stock cell or antigen preparation diluted in coating buffer into each well. The coating concentration routinely used for whole fungal or bacterial cell suspensions is 100 μ g dry weight/mL of coating buffer. For purified protein and carbohydrate antigens, the coating concentrations used are usually between 1.0 and 10 μ g/mL of coating buffer.
 - a. Coat the remaining 48 wells by dispensing 100 μ L of a 10 μ g/mL dilution of a murine monoclonal antibody to human SC (Hybridoma Labs., Baltimore, MD, USA) in coating buffer.
2. Seal the plate using adhesive plastic film sealer (Fisher).
3. Place microtiter tray on an orbital shaker (Bellco Technology, Vineland, NJ, USA, or similar) and incubate overnight (\sim 16 h) at 4°C.

4. Manually tip out the coating reagent by inverting the plate. Drain residual liquid by tapping the plate, upside down, on a pile of paper towels.
5. Cover all the naked plastic surface in each well with an irrelevant protein. This step is termed 'blocking' (*see Note 4*). To block the wells, wash them three times with blocking reagent filling the entire well ($\sim 350\ \mu\text{L}$). Automatic plate washers are available commercially to perform the wash steps. Alternatively, an 8- or 12-channel multichannel pipette (Eppendorf or equivalent) or a wash bottle may be used. If a wash bottle is used, cut the tip off the spout of the wash bottle such that a gentle stream, rather than a forceful one, is dispensed.
6. Next dispense $300\ \mu\text{L}$ of blocking reagent into each well, seal plate, and incubate for 1 h at room temperature.
7. Manually tip out the blocking reagent by inverting the plate. Drain residual liquid by tapping the plate, upside down, on a pile of paper towels.
8. Dilute secretion samples in PBS-Tween and dispense $100\ \mu\text{L}$ in duplicate or preferably triplicate into the wells coated with the *Candida* cells or antigen (*see Note 5*).
 - a. Dispense $100\ \mu\text{L}$ of duplicate or triplicate dilutions of purified SIgA (Cappel-MP Biochemicals, Solon, OH, USA) in PBS-Tween over the range of 10–100 ng/mL. Make dilutions on the day they are to be used.
9. Seal plate and incubate on an orbital shaker at room temperature for 1 h.
10. Manually tip out the content of the wells by inverting the plate. Drain residual liquid by tapping the plate, upside down, on a pile of paper towels.
11. Wash wells three times with PBS-Tween.
12. Make a 1:10,000 dilution of HRP-conjugated affinity purified rabbit IgG antibody to human α -chain (Jackson ImmunoResearch Labs. Inc., West Grove, PA, USA) with HRP diluent (*see Section 2* above) (*see Note 6*). This is often termed as the 'reporter' antibody. Prepare only as much as is required ($\sim 10\ \text{mL}/\text{plate}$) immediately before use.
13. Dispense $100\ \mu\text{L}$ in each well, seal the plate, and incubate for 1 h on an orbital shaker at room temperature in the dark (cover microtitration plate with aluminum foil).
14. Drain and wash wells as in **steps 9 and 10**. Do not leave PBS-Tween in wells for more than a few minutes because HRP is inactivated by NaN_3 .
15. Prepare substrate solution (*see Section 2*) and dispense $100\ \mu\text{L}$ in each well. Seal the plate.

16. Place plate on an orbital shaker and incubate at room temperature.
17. Read optical density at 450 nm in a dedicated microtiter plate spectrophotometer once the colorless substrate turns a yellow-brown color.
 - a. Subtract the absorbance of the background control wells (wells that do not contain secretion sample or SIgA standard).
 - b. Construct a standard curve by plotting the concentration of SIgA (ng/mL) on the x -axis versus optical density (OD₄₅₀) on the y -axis.
18. Take the mean of the duplicate or triplicate optical density readings from the *Candida*-cell- or antigen-coated wells and read them off the SIgA standard curve.

4. Notes



1. The substrate *o*-phenylenediamine is a carcinogen and the powder should be handled with care to prevent aerosolization. Substrate tablets (20 mg) are available from Sigma.
2. The quantitation of antimicrobial SIgA antibodies in external secretions presents a number of issues that are not faced when measuring antimicrobial antibodies in blood. The circulatory humoral immune system is a closed system; that is, immunoglobulin molecules continuously recirculate through the vasculature and interconnected lymphatics, and their concentrations are held at a steady state determined by their rate of degradation (half-life) and rate of new synthesis. In addition, immunoglobulins in blood are not subject to circadian rhythms. In contrast to the closed circulatory system, the mucosa-associated immune system is open, which means that antibody, primarily SIgA, is continuously exported in the local secretions that bathe mucosal surfaces by local exocrine glands. Consequently, the concentrations of SIgA in external secretions are low because the secreted volumes are high. Moreover, the concentration of SIgA in secretions is inversely related to flow rate. Flow rate is influenced by the level of hydration, hormones, drugs, and circadian rhythm. In addition, SIgA forms heterotypic calcium-ion-dependent complexes with mucins and several innate immune components present in secretions. In order to control these variables as much as possible, try to collect secretions at the same time of day. The immediate addition of the chelating agent EDTA upon collection of the secretion inhibits protease activity and

the formation of Ca^{2+} -dependent heterotypic complexes of SIgA with other factors. Whenever possible, measure the flow rate of the secretion or, if this is not possible, normalize the data to the protein concentration of the secretion.

3. The optimal concentrations of each reagent used in the ELISA must be established empirically before executing the assay. Optimal concentrations are determined by performing a 'checkerboard' titration in which the concentrations of two reagents, for example, the capture antibody or antigen and the reporter antibody are varied in a single microtiter tray. Serial dilutions of one reagent are dispensed down columns 1–12 of the plate, and serial dilutions of the second reagent are dispensed across rows A–H. Readers not familiar with performing checkerboard titrations are advised to consult the manual described in the introduction and listed in the references.

The solid phase, that is, the *Candida* cells, *Candida* antigen, or antibody to human SC, must always be in excess, i.e., they must not be saturated by antibodies in the secretion being tested or by the authentic SIgA that is used to construct the standard curve. This is one such variable that is examined during a checkerboard titration.

Proteins bind readily to polystyrene from which the microtiter plates are constructed. However, there are cases where the antigen may have to be modified to provide charged groups or the plastic surface treated to permit binding. Microtiter trays having wells specially treated for various applications are available from several commercial sources. Antibodies are useful molecules to 'present' antigens on the plastic surface. Antibodies not only bind well to plastic but also act to hold antigen off the plastic surface, which can be useful to avoid steric hindrance.

The standard assay volume for ELISA is 100 μL , but volumes between 50 μL and 200 μL can be used depending on scarcity of the sample and the level of antibody in the sample.

4. Blocking the uncoated plastic surface of the well is critical to prevent nonspecific binding of sample or reporter antibodies. As an alternative to BSA used above, which is expensive to purchase, a solution of nonfat dried milk may be used.

If incubation with antibody is not to be performed the same day as blocking, the plate can be stored at 4°C with the wells filled with blocking reagent.

5. If a large number of samples are to be dispensed into several plates, the incubation period should be increased in order to take account of the difference in incubation time between the first and last samples dispensed.

6. HRP is inactivated by NaN_3 . Note that the antibody diluent does not contain sodium azide. Although the wash solution does contain azide, the HRP is unaffected by short exposure during washing of the wells.

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