Research Article

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Development of a Gold Immunochromatographic Assay Method Using *Candida* Biofilm Antigen as a Bioreceptor for Candidiasis in Rats

https://doi.org/10.1515/chem-2019-0146
received November 27, 2018; accepted June 28, 2019.

Abstract: The gold immunochromatography assay (GICA), a new immunochromatography technique, uses a nitrocellulose membrane as a carrier and a colloidal gold-labeled antigen or antibody as a tracer (bioreceptor). This technology has many advantages over other immunoassays, including its simplicity, rapidity, cheapness, and the lack of a requirement for special training or expensive equipment, and it can be used to detect either antigens or antibodies. Therefore, we chose to develop this method for the diagnosis of candidiasis in Indonesia. The objective of the present study was to develop a diagnostic test for *Candida albicans* in rats using the GICA method. GICA bioreceptors were developed from biofilm antigens isolated from biofim Candida albicans grown on the surface of nitrocellulose membranes. The formation of biofilms was confirmed using scanning electron microscopy. Antigen levels as bioreceptors were optimized by using the immuno dot method. The samples to be analyzed were antibody serum, in the form of blood serum samples from heart mice that have been induced to become candidiasis. To this end, we optimized the antigen and antibody volumes necessary to make this diagnosis. The results show that the optimum concentration of antigen to be used in the test is 2.5 μg/μL and the optimum volume of antibody is 10 μL. The control rats produced a single red stripe on the control line and the candidiasis rat samples produced a double red stripe, with the bottom line being the control line and the upper line the test line. The test chip was successfully used for the diagnosis of candidiasis in rats and given the name "Candiday Kit.". We anticipate that this test will be suitable for the diagnosis of candidiasis in humans

Keywords: candidiasis; rat; gold immunochromatography assay; antigen; biofilm; *Candida albicans*.

1 Introduction

Candidiasis is an infectious disease caused by an overgrowth of *Candida* species. It may occur in the mouth, vagina, skin, or lungs, and may also cause septicemia, endocarditis, or meningitis [1]. Candidiasis or *Candida* infection occurs as a result of an imbalance between immunity and the pathogenicity of *Candida*. Several types of *Candida* infection can cause serious problems, especially in immunocompromized individuals [2]. One of the *Candida* species that can cause disease in animals and humans is *Candida albicans*.

In less favorable conditions for *C. albicans*, such as a lack of nutrition or the presence of factors that jeopardize its survival, *Candida* forms an association referred to as a biofilm [3]. This biofilm is a protective mechanism for microbes that is activated to cope with immune responses and anti-microbial substances. The pathogenicity and resistance capabilities of most microbes, including *Candida*, are determined by their abilities to form biofilms.

Early and accurate diagnosis of candidiasis is important for the provision of timely antifungal therapy to patients. Until now, blood culture has been considered the gold standard method for the diagnosis of candidiasis [4]. However, this method has a low sensitivity of ~50% [5] and it takes up to 8 days [6]. Thus, non-culture methods of diagnosis, such as PCR, which is used to detect *Candida* cell components, including β-D-glucan (BDG) and mannan, have been developed, and are now commercially available. However, the detection of mannan and BDG

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is associated with frequent false positive results [4, 7–9]. Therefore, a serologic diagnostic method has been developed that detects host antibodies against *Candida* components. In a previous study, extracts of *Candida albicans* (PIB) biofilm intracellular proteins were used as antigens that could be detected using anti-*Candida* sera in a dot blot immunoassay [10].

In the present study, we developed a gold immunochromatography assay (GICA) method using a biofilm antigen from *Candida albicans* as a bioreceptor, for the diagnosis of candidiasis in rats. This method uses a nitrocellulose membrane as a carrier and colloidal gold-labeled antigen or antibody as a tracer (bioreceptor). GICAs have many advantages over other immunoassays, including their simplicity, rapidity, cheapness, and lack of requirement for special training or expensive equipment, and can be used to detect either antigens or antibodies.

2 Materials and Methods

Candida albicans isolate strain used in this study were ATCC 10231, acquired from the Laboratory of Microbiology, Faculty of Dentistry, Airlangga University. The study has been approved by the The Animal Care and Use Committee (ACUC), Faculty of Veterinary Medicine, Airlangga University no 457-KE. Animals were monitored daily for distress from environmental factors that affect the immune system.

The animal model used in this study were male wistar rats (*Rattus novergicus*). In total of 20 rats, 2-3 month-old, weight of 160-170 g were divided into two groups i.e the control and treatment groups

GICA bioreceptors were biofilm antigens isolated from biofim Candida albicans grown on the surface of nitrocellulose membranes. Antigen levels as bioreceptors were optimized by using the immuno dot method. The samples to be analyzed were antibody serum, in the form of blood serum sampled from mice that have been induced to become candidiasis.

2.1 Preparation of Candida albicans biofilm antigen

Biofilms were prepared according to Samaranayake (2005) and Merrit (2005), with several modifications. A biofilm of *C. albicans* was grown on a nitrocellulose membrane with a diameter of 25 mm and a pore size of 0.22 µm in Spider medium. The sterilized membranes were positioned on the surface of the Spider medium in petri dishes using sterilized tweezers, then 0 µL of *C. albicans* inoculum with an optical density of 0.5 at 469 nm was dropped onto it. The medium was then incubated at 37°C for 1 h, and the petri dishes were inverted and incubated again for 24 h. The biofilm formed on the membrane was resuspended in sterilized phosphate-buffered saline and the suspension centrifuged at 3,000 g for 15 min. The biofilm pellet was then resuspended in 100 mL of 0.6 M KCl, containing *A. fulica* crude enzyme, and sonicated for 2 min, followed by centrifugation at 10,000 rpm for 15 min, after which the precipitate was discarded. The supernatant was concentrated using a freeze dryer and used as the antigen in the assay [11].

2.2 Induction of candidiasis in rats

The rats were acclimated for 1 week, then allocated randomly to control and treatment groups. The treatment group was orally administered with streptomycin (20 mg/kg), tetracycline (25 mg/kg), and gentamycin (7.5 mg/kg) daily for four days. On the fourth day, they were injected with cortisone acetate (225 mg/kg), and on the fifth day, they were inoculated orally with *C. albicans*. During this treatment, the rats were orally administered with the Spider medium. On the 35th day after *Candida* inoculation, blood samples were taken and centrifuged at 5,000 rpm for 15 min to obtain serum samples. These serum samples were kept refrigerated prior to use in the diagnosis of candidiasis.

2.3 Optimization of antigen concentration and serum volume

Antigen was tested at concentrations of 1.0, 1.5, 2.0, 2.5, and 3.0 ug/uL. Serial dilutions of antigen were performed in carbonate buffer (pH 9.6). Three microliters of each concentration of antigen were dropped onto a polystyrene surface in triplicate. After drying, the antigen was fixed using methanol for 5 min and then dried. To stabilize the antigen, the spots were dipped in 10% sucrose solution and then dried. The antigen-covered polystyrene was stored at 2°C before use.

20-microliter aliquots of serum were pipetted into wells of flat-bottomed microplates containing Tris-HCl buffer (pH 7.2). Dipsticks were then dipped into each well and incubated at room temperature for 30 min, washed with Tris-HCl buffer, dipped into wells containing 200 µL of colloidal gold linked to protein A, and incubated for 15 min at room temperature. The dipsticks were then washed

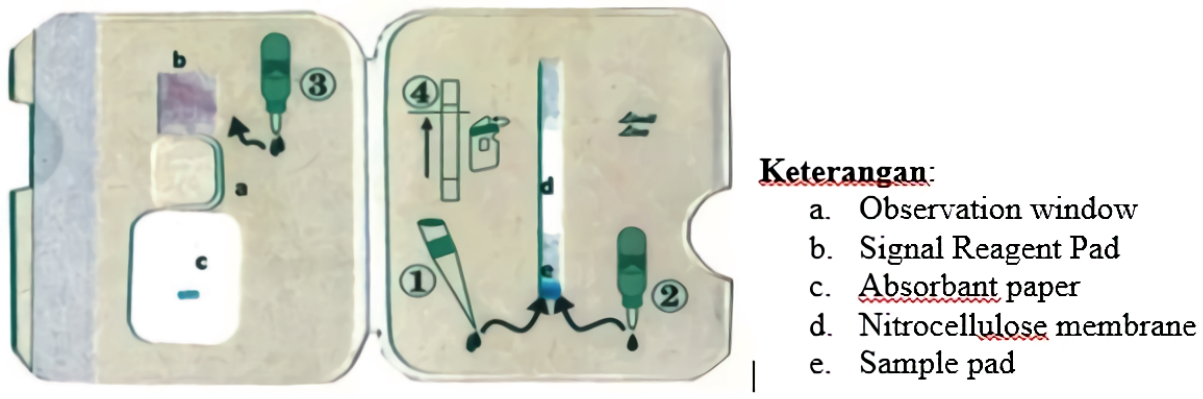

Figure 1: The components of the "Candiday Kit" test chip.

three times and observed for the appearance of a red spot. The result was interpreted as positive if a fully round red spot appeared and negative if no red spot appeared or the spot was not fully rounded.

The serum volume was optimized using positive control rats, testing 5, 10, 15, and 20 µL volumes and an antigen concentration of 2.5 µg/µL, using the same method.

2.4 Design and construction of the test chip

The test chip consisted of four parts, as shown in **Figure 1**. Part D, the immunochromatography layer, consisted of nitrocellulose membrane, to the upper and lower sides of which 1 cm-thick polyester layers were attached. Antigen was applied linearly using a BioJet™3000 to the upper part of the nitrocellulose membrane as a test line and conjugated goat anti-rat-IgG was applied as a control line. The nitrocellulose membrane was cut into 5-cm strips using a Kinematic Automation PS-360.

2.5 Diagnosis of candidiasis using the GICA test chip

The test chip was left on a flat surface for ~20 min until it reached room temperature, then removed from its aluminum sachet. (**Figure 1**). Ten microliters of serum were dropped onto the blue-colored sample pad (**Figure 1**). A drop of buffer was added to the same pad (2), followed by two drops of signal reagent colloidal gold (3). If the sample was absorbed by the test line, the test chip was then closed (4) and the test result observed *via* the observation window after 15–20 min (part a). The test result was interpreted as positive if two red lines appeared, negative if only one red line appeared, and invalid if no line appeared.

2.6 Data analysis

The results of antigen optimization and candidiasis diagnosis using the immunochromatographic test chip were analyzed qualitatively.

3 Results and Discussion

3.1 Animal model of candidiasis

The model used was of *in vivo* intestinal biofilm formation by *C. albicans* colonizing the intestinal mucosa of rats (**Figure 2)**. The formation of biofilms was confirmed using scanning electron microscopy [12, 13].

3.2 Optimization of antigen concentration and serum volume

The optimization of antigen concentration and serum volume were performed using immunodot dipsticks. Positive results were indicated by the appearance of full red-colored spots. *C. albicans* biofilm antigen optimization was performed using 20 µL of rat serum as positive control and antigen concentrations of 0.5, 1.0, 2.0, 2.5, and 3.0 µg/µL in triplicate. The results of this optimization are shown in **Figure 3**. Antigen concentrations of 0.5 and 1.0 µg/µL did not result in the appearance of red spots (negative

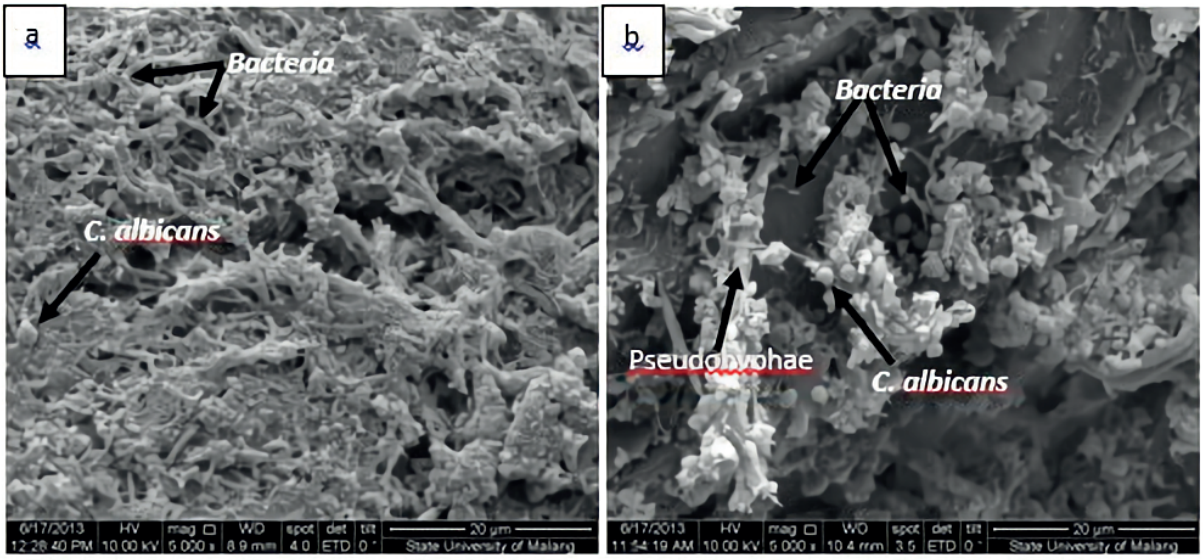

Figure 2: Scanning electron microscopy images of intestinal membrane mucosa. (a) control rat, (b) rat with candidiasis. 5,000× magnification.

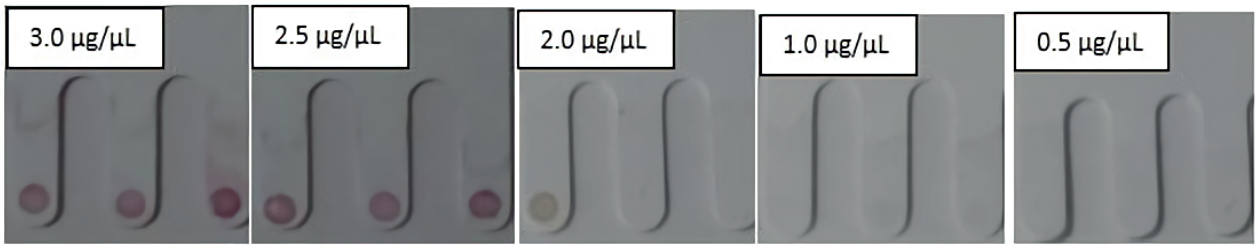

Figure 3: Optimization of antigen concentration, with a serum volume of 20 µL.

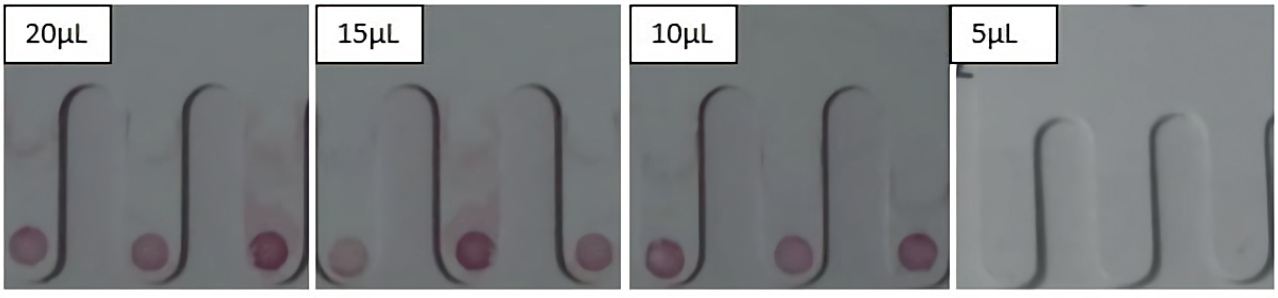

Figure 4: Optimization of serum volume, with an antigen concentration of 2.5 µg/µL.

results), an antigen concentration of 2.0 µg/µL produced one red spot out of three, and concentrations of 2.5 µg/µL and 3.0 µg/µL produced three full red spots. Therefore, the optimal concentration of *C. albicans* biofilm antigen was determined to be 2.5 µg/µL.

The serum volume optimization was performed using rat serum as a positive control and a blank, and volumes of 5, 10, 15, and 20 µL, and 2.5 µg/µL antigen, in triplicate. The results of this optimization are shown in **Figure 4**. The antigen gave a positive response to the positive control serum at volumes of 10, 15, and 20 µL. Therefore, we used 10 µL serum in the study, as the lowest effective volume.

3.3 The diagnosis of candidiasis using the "Candiday Kit" chip

The diagnostic results obtained using the GICA test chip in negative control (–) sample K1 and treatment sample P1 are shown in **Figure 5**.

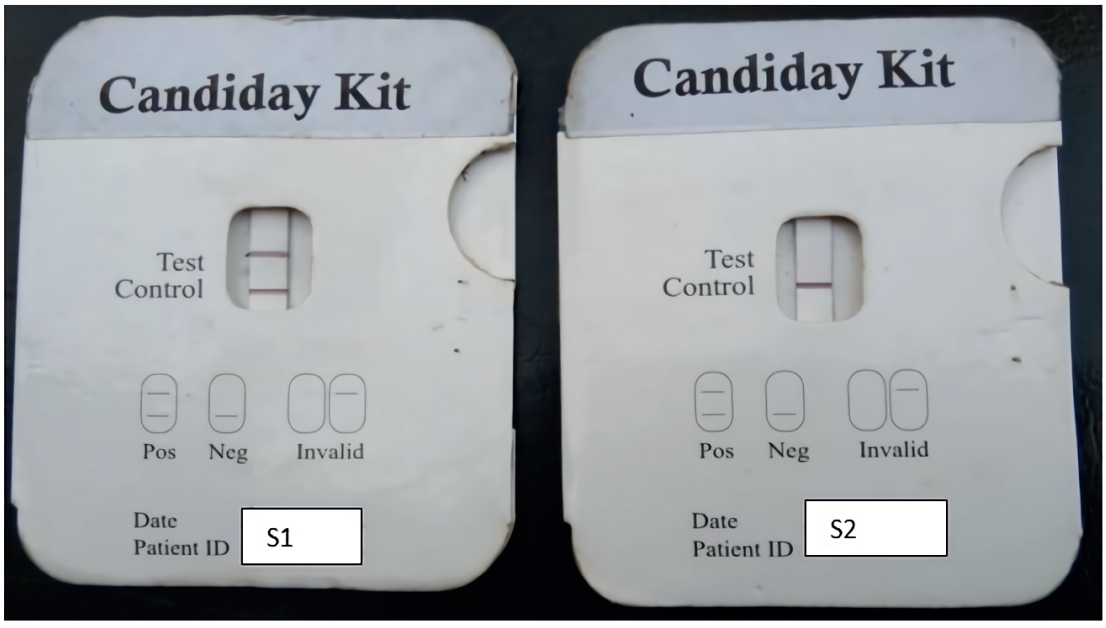

Figure 5: Diagnosis of candidiasis using a "*Candiday Kit*" chip.

Figure 5 shows the kit results through the observation windows of the "*Candiday Kit*." The K1 sample produced a single red stripe on the control line and the P1 sample produced a double red stripe, with the bottom line being the control line and the upper line the test line. The complete results are presented in **Table 1**. The negative control sample did not produce a red color on the test line, due to the absence of an antibody to *Candida*. The absence of a red stripe on the test line implies a negative result in the test or the absence of a *C. albicans* biofilm in the digestive tract of the animal.

The GICA method developed in this study is based on the principle of reverse flow immunochromatography. This differs from the immunochromatographic method used in pregnancy tests that generally uses lateral flow. The principles of the reverse flow immunochromatography used in this study are:

- The test starts when a serum sample is placed on the sample pad (right end) and is allowed to flow along the nitrocellulose membrane through the two lines: the control line, carrying the goat anti-mouse IgG, and the test line, carrying the *C. albicans* biofilm antigen (**Figure 6a**).
- In parallel, signal reagent is allowed to flow in the opposite direction from the signal reagent pad (left end), diffusing through the same test and control lines. Signal reagent contains protein A gold, a secondary polyclonal rabbit antibody conjugated with colloidal gold (**Figure 6a**).

Table 1: Results of candidiasis testing using the "*Candiday Kit*".

Sample code	Observation results		Test conclusion
	Test line	Control line	
P1	+	+	+
P2	+	+	+
P3	+	+	+
P4	+	+	+
P5	+	+	+
P6	+	+	+
P7	+	+	+
P8	+	+	+
P9	+	+	+
P10	+	+	+
P11	+	+	+
P12	+	+	+
P13	+	+	+
P14	+	+	+
P15	+	+	+
K1	-	+	-
K2	-	+	-
K3	-	+	-
K4	-	+	-
K5	-	+	-

- In the test line, the anti-*Candida* antibody contained in the serum samples forms complexes with the antigens and signal reagent, which are indicated by the appearance of a red line (**Figure 6b**).
- In the control line, A gold protein forms a complex with anti-mouse IgG, which is indicated by the appearance of a similar red line (**Figure 6b**).

Zakoskina [14] performed immunochromatography testing for brucellosis and concluded that the colloidal gold-labeled particles could form gold-antibody complexes.

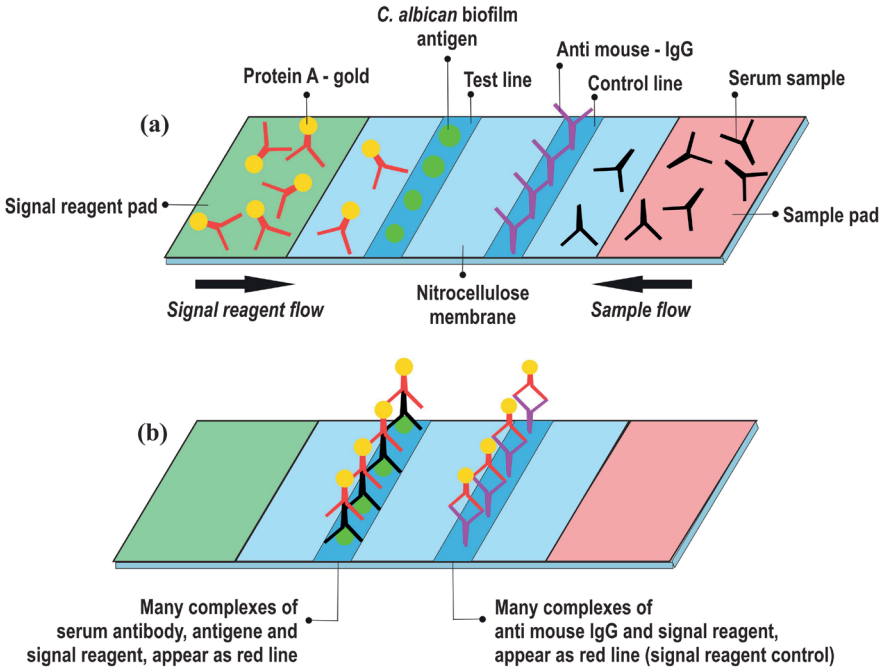

Figure 6: Immunochromatographic principle for the assessment of antigen-antibody reactions.(a) Flow of serum sample and signal reagent along a nitrocellulose membrane strip, (b) Antigen-antibody reactions on the test and control lines.

These complexes diffused along the nitrocellulose membrane and formed a complex with the immobilized antigen on the test line, generating a stripe. The appearance of a second stripe on the control line, viewed through the observation window, indicated that the test functioned well.

Immunochromatography testing was also used by Marot-Leblond [15] to diagnose vulvo-vaginal candidiasis, using the lateral flow principle. This test had a specificity of 98.6%, sensitivity of 96.6%, and efficiency of 98%. This method was used to detect antigen on a swab sample. These previous findings further suggest that an immunochromatographic method may be suitable for the diagnosis of candidiasis, using either antibody or antigen samples.

4 Conclusion

We have successfully used the GICA method to detect antibody against *C. albicans* biofilm antigen from rats with candidiasis. This method used antigen at a concentration of 2.5 µg/µL and a serum volume of 10µL.

Acknowledgments: The authors are grateful to the Direktorat Jenderal Pendidikan Tinggi (DIKTI), Ministry of National Education, for their financial support, through Hibah Kompetensi research funding in 2017.

Conflict of interest: Authors declare no conflict of interest.

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