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In Vitro Inhibition of Oral *Candida albicans* by Chicken Egg Yolk Antibody (IgY)

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Abstract This study was conducted to measure *Candida albicans*-specific chicken egg yolk antibody (IgY) inhibition of fluconazole-sensitive and resistant strains of *C. albicans* in order to assess potential use in the prevention and treatment of oral candidiasis. In this study, laying hens were immunized, and IgY was extracted by water dilution. The Minimal Inhibitory Concentrations (MICs) of IgY for inhibiting *C. albicans* growth were determined using the broth microdilution method from the CLSI M27-A2 protocol. Fluconazole (FLC) was used as the control. The results were analyzed with the χ^2 test. The anti-*Candida* titer of anti-*C. albicans* IgY was 1:12,000. The concentration of the IgY extract that effectively inhibited the growth of *C. albicans* was between 1.25 g/l and 5.0 g/l, and the efficacy rate was 82.98% during the observed 24–48 h time period. No correlation was recorded between the drug resistance of FLC and growth inhibition by IgY. It was concluded that anti-*C. albicans* IgY inhibited the growth of *C. albicans* in vitro and there was no correlation between the drug resistance of FLC and the growth inhibition by IgY ($P > 0.99$).

Keywords Antibodies · *Candida albicans* · Chickens, Egg yolk · Minimal inhibitory concentration · Stomatitis, Denture

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Introduction

Candida albicans composes up to 72% of clinically isolated *Candida* strains [1], and is commonly found on the skin and mucous membranes. It is an opportunistic human pathogen that causes infection only when immune resistance becomes compromised. *C. albicans* infection of the oral cavity is common and frequently associated with wearing dentures, diabetes mellitus, endocrine disorders, anemia, malnutrition, tumors, antibiotic treatment, oral contraceptives,

corticosteroid use, xerostomia, radiation therapy, chemotherapy, and HIV infections [2]. About 66% of patients in the latter stages of cancer are oral *Candida* carriers and 30% have actively an infection [3]. Similarly, 66% of elderly people who wear removable dentures are infected with oral *Candida* spp. [4]. In older people, factors such as drug administration and depressed immune systems allow opportunistic pathogens like oral *Candida* spp. to initiate disease. Although a number of antifungal drugs are available, many have side effects or interact negatively with other drugs [2]. Thus, for patients who are immunocompromised or require long-term treatment with other medication, drug options are limited. Antifungal therapy is further complicated by the increased presence of resistant strains [5]. Our study examined a new approach for inhibiting the growth of oral *C. albicans* and assesses the potential use in the prevention and treatment of oral *C. albicans* infection.

Chicken egg yolk antibody (IgY) has received much attention in recent years because it can be easily prepared in high concentration and is both affordable and safe [6]. IgY is successfully used in medical immune testing, diagnosis, heterografts, and therapy [7–10] and especially, with particular attention to the application on passive immune protection in both animals and humans [11]. IgY antibodies have been studied against *Escherichia coli*, rotavirus, and *Helicobacter pylori*, but rarely against *C. albicans* [12–14]. In this study, we assayed the efficacy of anti-*C. albicans* IgY at preventing and treating oral *C. albicans* infections by measuring the ability to inhibit the in vitro growth of *C. albicans* using the CLSI M27-A2 protocol [15].

Materials and Methods

Preparation of Chicken Egg Yolk Antibody Against *C. albicans* (IgY)

The Experimental Animal Center of Animal Husbandry and Veterinary Medicine College of Jilin University China provided ten 25-week-old Hy-line egg laying hens. The standard strain ATCC 10231 of *C. albicans* (China Materia Medica Biological Product Inspection Institute) was inactivated by sonication for 15 min [14], and emulsified in complete Freund's adjuvant (CFA) or incomplete Freund's adjuvant (IFA), for use as the primary immunogen and booster immunogen, respectively. Before immunization and two weeks after the third immunization, the eggs were collected and stored at 4°C. The water dilution method was used to isolate and extract IgY, using Akita and Nakai's technical path as a reference [16]. Distilled water and egg yolks were mixed at a ratio of 9:1 and the pH was adjusted to 5.0. The mixture was placed at 4°C overnight, centrifuged at 12,000 rpm for 20 min at 4°C, and the water-soluble fraction (WSF) of the egg yolk was precipitated twice with 50% and 33% saturation of ammonium sulfate. IgY was obtained after demineralization through dialysis and stored at −20°C. Nasster's reagent was used to determine that demineralization was complete.

Coomassie Brilliant Blue Staining (Pierce Biotechnology, Inc. USA) measured the protein concentration in the antibody extract at 7.9 g/l, and the purity of IgY was approximately 91.5%. Immunohistochemical staining showed that anti-*C. albicans* IgY was specific for *C. albicans* in vitro. ELISA results illustrated that the anti-*Candida* titer of anti-*C. albicans* IgY was 1:12,000 [17].

Source of Test Strains

Isolation of Clinical Oral Candida *Strains*

A total of 45 oral *C. albicans* strains were isolated from saliva cultures of patients with oral candidiasis in the School and Hospital of Stomatology, Peking University and identified as *C. albicans* using the serum germ tube test, CHROMagar color medium, growth on Sabouraud's Dextrose Agar (SDA) at 45°C, and the API 20C AUX system.

Source of Standard strains

Standard strains of *C. albicans*, ATCC 10231 and AS 2.538, (Institute of Microorganisms, Chinese Academy of Sciences) were also used in this study.

Quality Control Strains

Standard strains, ATCC 22019 and ATCC 6258, (The Fungus Center, First Hospital of Peking University, Beijing) were used in this study as quality controls and IgY inhibition controls.

IgY-Induced Inhibition of Oral *C. albicans*

Control Drug

The antifungal drug, fluconazole (FLC; 200406040) was provided by Shanghai Sunve Pharmaceutical Company.

Test Methodology

The broth microdilution method from the M27-A2 protocol developed by the Clinical Laboratory Standard Institute (CLSI) in 2002 was used in this study [15].

Preparation of Candida Culture Medium RPMI 1640 liquid medium was made by adding 10.4 g RPMI 1640 powder containing L-glutamine and no sodium bicarbonate (Gibco) and 34.53 g MOPS (Sigma, USA) to 900 ml distilled water and adjusting the pH to 7.0 with 1 mol/l sodium hydroxide. The medium volume was 1 l, then filtered using a 0.22 μm filter, and stored at 4°C until use. Sabouraud culture medium was purchased from the Tianjin Jinzhang New Medical Technology Research Institute in China.

Preparation of Antifungal Drugs The stock solution of FLC was prepared by diluting 1,280 mg FLC powder into a liter of distilled water and stored at −20°C, until use. The IgY supernatant was extracted and concentrated by embedding dialysis tubing filled with IgY in saccharose, in order to prepare the IgY stock solution. Protein concentration was determined using Coomassie Brilliant Blue Staining, adjusted to 10 g/l and stored at −20°C, until use.

Preparation of Antifungal Drug Susceptibility Testing Plates The stock solutions were thawed at 4°C for 1 h, and the drugs were diluted in RPMI 1640 liquid medium. The prepared drugs were added to sterile 96-well U bottomed plates (Costar, USA). For the FLC plates, 100 μl FLC was added to each well with the concentration decreasing gradually from the 1st to the 10th wells. The concentration of FLC in every left well was twofold of the right one, with an initial concentration of 128 mg/l and a final concentration of 0.25 mg/l. 100 μl RPMI 1640 liquid medium was added to the 11th and 12th wells in the absence of drugs. The 11th well was used as a control of fungus growth, and the 12th was used as a control for asepsis. The plates were sealed with a plastic bag and stored at −20°C. For the IgY plates, 1 through 9, the 11th and 12th wells were set up in the same format as the FLC plates, with an initial concentration of 10 g/l and a final concentration of 0.039 g/l. 100 μL of 0.85% physiological saline was added to the 10th well also as a control of fungus growth. IgY was thawed and purified using a 0.22 μm filter and IgY plates were prepared on the day when they were used.

Preparation of Fungus Suspensions to be Tested All test strains (45 clinical isolates, two standard *C. albicans* strains and two quality control strains) were streaked twice on Sabouraud Agar medium and incubated at 35°C for 24 h to ensure purity and vitality. Five colonies with a diameter exceeding 1 mm were harvested, placed in 5 ml of 0.85% physiological saline, vortexed for 15 s to mix evenly and counted under a microscope at 200× using a hemocytometer. The fungus suspension was adjusted to $1-5 \times 10^6$ cfu/ml with 0.85% physiological saline and diluted 1,000× with RPMI 1640 to a final concentration of $1-5 \times 10^3$ cfu/ml.

In Vitro Drug Sensitivity Test The FLC drug sensitivity plates were thawed for an hour each at 4°C and 24°C. The IgY drug sensitivity plates were prepared on the day of use. The fungus suspensions were vortexed for 15 s before being added to the drug sensitivity plates. 100 μl of each culture was added consecutively from the first well to the 11th well in duplicate. The 12th well received 100 μl of RPMI 1640 liquid medium as the negative control.

IgY extracted from the non-immunized eggs was also tested with the same method.

Culture and Determination of Results The inoculated drug sensitivity plates were stored in an incubation box that was placed in a constant-temperature incubator at 35°C for 24–48 h incubation. If the growth control well had a good amount of growth, the minimal inhibition concentration (MIC) value of the quality control strain was determined to be within the scope stipulated by the CLIS M27-A2 protocol (i.e., ATCC 22019 MIC0.5 mg/l–4.0 mg/l and ATCC 6258 MIC8.0 mg/l–64 mg/l), and the readings were taken visually. In addition to the visual end point readings, samples were

measured with UV at 405 nm using a microplate reader (ELx 808, Absorbance microplate reader, BIO-TEK, USA) after shaking. Each microdilution well was compared to the control and given a value according to the following observations: (4) no reduction of turbidity, (3) slight reduction in turbidity, (2) prominent decrease in turbidity, (1) slightly hazy, and (0) optically clear. A minimal drug concentration of 2 was considered the MIC value for each strain.

Statistical Analyses The results were analyzed using the SPSS 10.0 software.

Results

MIC Measurements for FLC and IgY-Induced Inhibition of *C. albicans*

For drug sensitivity and resistance values of the FLC antifungal drug, see Table 1 [15].

The MIC measurement of 47 *C. albicans* test strains was repeated three times, and the scope of MIC fluctuation did not exceed the concentration gradient of this drug. To determine the drug sensitivity of each strain, the most frequent MICs observed after three measurements were recorded as the MICs of *C. albicans* (Tables 2, 3). The results of visual reading and spectrophotometric MICs were identical.

In the 47 *C. albicans* test strains examined, nine showed drug resistance to FLC, and 38 showed drug sensitivity. Of these, two strains had MICs values between 16 and 32 mg/l and were dose dependent susceptible. A total of 39 strains were effectively inhibited by anti-*C. albicans* chicken egg yolk antibody (IgY) at concentrations between 1.25 and 5.0 g/l. The efficacy rate was 82.98%, and the confidence interval (at 95%) was between 69% and 92%. Eight *C. albicans* strains and the quality control strains (ATCC 22019 and ATCC 6258) were not inhibited when the IgY concentration was 5 g/l and the anti-*Candida* titer was 1: 12 000. IgY from non-immunized eggs did not inhibit *Candida* growth.

Table 1 Interpretative guideline for in vitro susceptibility testing of *Candida* species

Antifungal agent	MIC* (mg/l) Distribution of MICs (mg/l)		
	S[a]	SDD[b]	R[c]
Fluconazole	≤8	16–32	≥64

* Minimal inhibition concentration

[a] Susceptible; [b] Susceptible-dose dependent; [c] Resistant

Correlation Between Drug Resistance to FLC and Growth Inhibition by IgY

A correlation test of a matched fourfold table was performed using the Fisher's Exact Test (Table 4). $P > 0.99$, illustrates that there was no correlation between drug resistance to FLC and the growth inhibition by IgY.

Discussion

Oral fungal infections have a long disease course, are not easily cleared, and are prone to relapse. Although most oral fungal infections are superficial, the number of deep infections, which can be life threatening, is on the rise. Many studies illustrate that patients with dentures are more susceptible to *Candida* infection and are more likely to be carriers of the fungus. Thus, prevention of normal human symbionts like *Candida* spp. from mutating or progressing into opportunistic pathogens is very important.

Strain-epitope specific antibody can be protective in murine, hematogenously disseminated, candidiasis [18], but it is not convenient and is time-consuming. In this research, we attempted to develop an anti-*C. albicans* IgY as a novel method to prevent oral candidiasis. We wanted to find a general antibody

Table 2 Distribution of MIC ranges in 47 strains of *Candida albicans* for Fluconazole

Antifungal agent	MIC* range (mg/l)	MIC (mg/l) distribution (the number of isolates)									
		≥64	32	16	8	4	2	1	0.5	0.25	≤0.125
Fluconazole	0.125–64	9	1	1	1	1	1	10	16	7	0

* Minimal inhibition concentration

Table 3 Distribution of MIC ranges of IgY for 47 strains of *Candida albicans*

Antifungal agent	MIC*range (g/l)	MIC (g/l) distribution (the number of isolates)									
		>5	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02
IgY**	j-a	8	33	5	1	0	0	0	0	0	0

* Minimal inhibition concentration; ** Chicken egg yolk antibody

Table 4 Matched fourfold table of Fisher's Exact Test

		FLC		Total
		Drug sensitivity	Drug resistance	
IgY	Inhibition	31	8*	39
	No inhibition	7	1	8
Total		38	9	47

* $P > 0.99$

that would neutralize most *C. albicans* strains that would be more suitable for clinical applications.

IgY can be extracted using water dilution (WD), polyethylene glycol (PEG) precipitation, dextran sulfate (DS) precipitation, and xanthan precipitation. The WD method had the greatest IgY production levels and degrees of purity, as well as being the most convenient for application and use in large-scale production [16]. We successfully extracted anti-*C. albicans* IgY using this method, and the protein content was 7.9 g/l, which was higher than the 5.5 g/l of anti-*H. pylori* IgY obtained by Lee et al. [14], but lower than the 9.8 g/l anti-*E. coli* IgY obtained by Akita and Nakai [16]. The purity of the antibody was relatively high and relevant to the strain and age of the chicken and the type of antigen used. Our study and that by Lee et al. [14] both utilized Hy-line chicken, but the extraction procedures and reagents were distinct, which may account for differences in antibody content and purity. In the WD method used in this study, WSF was isolated by centrifugation. The IgY extract was precipitated twice using ammonium sulfate, while in the WD method by Lee et al., WSF was isolated but not by salt precipitation.

Use of IgY for fungi inhibition in accordance with the CLSI protocol has not been previously reported. Using other published results as a reference, the protein concentration was approximately 10 g/l, and the maximal initial concentration of IgY was specified as 5 g/l. If the antibody extract concentration was higher than 10 g/l, then it was diluted according to the measured concentration, and if it was less than 10 g/l, then it was concentrated by embedding dialysis tubing filled with IgY in saccharose. These procedures are easy and the financial burden is low, thus making it commercially viable.

Trailing growth makes some isolates, that appear susceptible after 24 h, seem to be resistant after 48 h. To avoid this, the M27-A2 methodology for *Candida* and others [15,19] recommended an end point reading at 24 h, at which time isolates are deemed clinically susceptible to FLC. In the present study, the end points were read between 24 and 48 h, if the growth controls had good viability and the MICs of the quality control strains were within the specified range.

The anti-*Candida* titer of the ELISA test was 1:12,000. This may differ depending on the kind of antigen, the strain of chicken [20], the immunization protocol, and the time of egg collection. Inhibition was dependent on the protein concentration of the antibody extract, such that low concentrations failed to inhibit the growth of *C. albicans*, as well as the titer of the antibody. In this study, we found that IgYs of low titer were unable to inhibit the growth of *C. albicans* when used at the same concentration as high potency IgY. The optimal IgY concentration needs to be examined further to determine whether IgY can prevent growth of the eight isolates that were not inhibited in this study.

In our previous study (not published), formalin-killed yeast cells were used as the antigen to immunize the hens, but it's anti-*Candida* titer of the ELISA test was low (1:6,000) and it did not inhibit the growth of *C. albicans*. As Arseculeratne et al. [21] reported, *Candida* cells treated with Triton-x100 were used as the antigen and the titer of anti-*Candida* antibody was over 800. In their study, strain-specific anti *C. albicans* and *C. krusei* antibody did not inhibit these organisms in vitro. That level of antigen induced low titer antibody.

It is not fully understood why IgY inhibits the growth of different strains of *C. albicans*.

Anti-*Streptococcus mutans* MT8148(*c*) IgY has a remarkable inhibitory effect against rat carries that are caused by *S. mutans* serotype-*c* [22]. IgY against EAF(+) enteropathogenic *Escherichia coli* inhibited the growth of the *E .coli* EAF(+) but not the EAF(−) strain [23]. According to Moragues et al. [24], Mab C7, a monoclonal antibody against the *Candida albicans* cell wall mannoprotein exhibited candidicidal effect with a 73.4% reduction in the *Candida* growth. This type of fungicidal activity has been studied in a number of fungi, including *C. lusianiae*, etc., because it reacted with similar antigens expressed in a variety of fungi. We speculate that IgY may also react with antigens expressed in the cell wall of different strains of *C. albicans*.

FLC is one of the most common anti-fungal drugs in use today, so we chose it as the drug control. In this study, the effective rate of IgY-induced inhibition of *C. albicans* was 83% of the isolates tested. We found no correlation between drug resistance to FLC and *C. albicans* inhibition by IgY. This may be because they have different mechanisms of action. IgY had an inhibitory effect on *C. albicans* strains with sensitivity and resistance to FLC. Furthermore, since it has an excellent biological safety profile, IgY may be used without inducing side effects in all individuals except those sensitive to eggs. In this study, the production, purity, potency, and specificity of biologically active anti-*C. albicans* IgY were all relatively high. IgY may be used for preventing and treating oral *C. albicans* infection, which could lead to establishing the foundation for further clinical applications. The inhibitory effect of IgY in a complex oral microenvironment where dentures, body fluids, bacteria, and biofilms may be present, requires further study. Future studies will focus on the effect of IgY in vivo in the near future.

Conclusions

Anti-*C. albicans* IgY inhibits the growth of *C. albicans* in vitro. There was no correlation between the drug resistance to FLC and the growth inhibition by IgY ($P > 0.99$).

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