■ Original Article ■

Development of Modified Selective Media to Differentiate *Cryptococcus* Species Complex and its Serotypes using Natural Materials

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The formation of brown colonies due to phenol oxidase activity on classic agar media containing natural material extracts of *Helianthus annuus* or on medium containing L-3,4-dihydroxyphenylalanine has been used to identify *Cryptococcus* species complex. In this study, various natural materials were used to develop a modified medium and to identify five major serotypes of *Cryptococcus* species complex. Serotypes A, D, and A/D were pigmented on medium using *Perilla frutescens var. japonica Hara* (PerJ agar) after a three-day incubation. Serotypes B and C were pigmented on PerJ agar after four- and five-day incubations, respectively. Growth time and pigmentation of the five serotypes occurred more rapidly on PerJ agar than on the other media. In addition, colony morphology, size, and pigmentation were specific by serotype. In conclusion, PerJ agar should be used in clinic settings to identify *Cryptococcus* species complex and its serotypes rapidly.

Key Words: *Cryptococcus* species complex; HPLC; L-DOPA; Modified selective media; Serotype; Natural materials

INTRODUCTION

Encapsulated yeast of the genus *Cryptococcus* contains two major pathogenic species: *Cryptococcus neoformans* (*C. neoformans*) and *Cryptococcus gattii* (*C. gattii*). Among the more than 30 different species of *Cryptococcus*, only *C. neoformans* and *C. gattii* are considered major causative agents of cryptococcal infections. In particular, they are fatal, opportunistic pathogens that cause systemic fungal infection in human and animal hosts (Negroni, 2012). Cryptococcosis is one of the most common secondary infections in immune compromised hosts, particularly AIDS patients. Globally, there are approximately one million cases of meningitis each year in AIDS patients, resulting in approximately 624,700 deaths within three months after becoming infected with *Cryptococcus* species complex. As such, these fungi are defined as among the most important opportunistic fungal pathogens (Park et al., 2009).

Currently, *Cryptococcus* species complex are classified into two distinct species and three varieties, based on the different immunologic properties and epidemiology. *C. neoformans* (including two varieties: var. *grubii* and var. *neoformans*) is distributed worldwide, especially in bird droppings

and nests, while *C. gattii* has a more geographically restricted distribution and is prevalent in tropical and subtropical regions (Negroni, 2012). Although isolation and identification of these fungal pathogens from clinical sources is very important for patients, as cryptococcosis is a life-threatening infection, they are time-consuming and complicated processes. Diagnosis of *Cryptococcus* species complex from clinical materials is generally based on the observation capsule of yeasts by direct microscopy; culture and biochemical tests are subsequently required for isolation and identification of these fungal pathogens (Negroni, 2012). Classic media containing natural material extracts (such as *Guizotia abyssinica* [niger seed agar] or *Helianthus annuus* [sunflower seed agar]) or chemicals (such as caffeic acid or L-3,4-dihydroxyphenylalanine [L-DOPA]) are commonly used as the primary selective culture media for detecting typical brown pigment formation due to phenyl oxidase activity. These selective culture media are useful tools for isolating *Cryptococcus* species complex from environmental and clinical materials (Eisenman et al., 2011; Menezes et al., 2011). However, these selective culture media have some limits. For instance, the incubation step of *Cryptococcus* species complex takes too long to detect brown pigment formation, and it is very difficult to distinguish among the species of *Cryptococcus* species complex.

Therefore, in this study, we attempted to develop modified selective culture media by using various affordable natural material extracts. In addition, we compared the growth of *Cryptococcus* species complex strains on ten different natural material-based media.

MATERIALS AND METHODS

Yeast strains

In this study, four reference strains were purchased from the American Type Culture Collection (ATCC). ATCC 66031 (serotype D) and 48184 (serotype A/D) strains were used as reference strains for *C. neoformans* var. *neoformans* and ATCC MYA-4560 (serotype B) and 32608 (serotype C) strains were used as reference strains for *C. gattii*. One clinical strain, which was identified as *C. neoformans* var. *grubii* (serotype A) in a previous study, was used as a

reference strain for *C. neoformans* var. *grubii* (Chae et al., 2012). Non-*Cryptococcus* species complex strains—three *Candida* species (*C. albicans*, *C. tropicalis*, and *C. glabrata*) isolates—were provided by General Hospitals in Busan. And we used seventeen environmental samples of pigeon droppings which are confirmed as *Cryptococcus* species complex positive samples in previous study (Chae et al., 2012). All isolates were identified using the Vitek2 yeast identification system (bioMerieux, Marcy l'Etoile, France) and the genotyping method used in previous studies (Chae et al., 2012). All yeast strains were stored at -70℃ in a deep freezer until used.

Primary culture of yeast strains

To prepare the yeast culture suspensions, all yeast strains were recovered from frozen glycerol stocks. Briefly, a yeast suspension of 100 μl was inoculated onto Sabouraud dextrose agar (SDA). All plates were incubated in the dark at 25℃ under humid conditions for five days. The cultures were observed daily to monitor growth and formation of pure colonies. After five days, the recovered pure colonies of each strain were transferred into Sabouraud dextrose broth and incubated on a shaker for 24~48 hours at 25℃.

Preparation of SSA and various natural material media

Sunflower (*Helianthus annuus* seed agar (SSA), which is used as a typical selective agar for *Cryptococcus* species complex, was prepared as described previously [Khan et al., 2004]. Briefly, the natural materials were ground as finely as possible with a grinder. Then, 25 g of the ground natural materials were boiled in 500 ml of distilled water (DW) for 30 min and filtered through several layers of gauze. Next, 7.5 g of Bacto agar, 0.5 g of glucose, 0.5 g of KH_2PO_4, and 0.5 g of creatinine were added, and the volume was readjusted to 500 ml. The mixture was autoclaved at 121℃ for 15 min, after which the medium was dispensed into 50 mm-diameter petri dishes (10 ml per plate). To prepare the modified natural material media, various dried natural materials (including *Sesamum indicum*, *Heukimja*, *Perilla frutescens* var. *japonica Hara*, *Glycine max [L.]) Merrill*, *Pinus koraiensis*, *Ricinus communis L.*, *Arachis hypogaea*, *Juglans nigra L.*, and *Juglans sinensis Dode*) were purchased from

a local market. The same method used to prepare the SSA was followed.

Culture of yeast strains in various natural material media

All of the yeast suspensions were cultured on various natural material media. Five strains of *Cryptococcus* species complex (ATCC 66031, 48184, MYA-4560, 32608, and one clinical isolate) and four isolates of non-*Cryptococcus* species (*C. albicans*, *C. tropicalis*, and *C. glabrata*) were inoculated on SSA and various other natural material media. The plates were incubated in the dark at 25℃ under humid conditions and observed at 12-hour intervals. All experiments were performed in triplicate.

Culture of environmental samples in various natural material media

Culture method was described in previous study (Chae et al., 2012). Briefly, each samples were suspended in phosphate buffered saline (PBS) by vortexing, and centrifuged. Then 100 μl of aliquot from supernatant were inoculated onto SSA and various other natural material media. The plates were incubated in the dark at 25℃ under humid conditions for 2~8 days. All plates were examined daily to observe the specific color of yeast-form colonies.

HPLC analysis for natural material extract

To perform high-performance liquid chromatography (HPLC) analysis, aqueous natural material extract samples were prepared. The natural material extract samples were extracted from ground natural materials with different two solvents—ethanol and DW. The extraction process using DW was performed by boiling ten dried natural materials (5.0 g) in DW (100 ml) for one hour. The extraction and sample preparation processes using ethanol were as follows. Two dried natural materials (*Helianthus annuus* and *Perilla frutescens* var. *japonica Hara*) were dissolved in 80% ethanol (100 ml) and stored for 24 hours with the help of a shaker set at 25℃. The natural material extracts were filtered through several layers of gauze, after which the extract was centrifuged at 2,500 × *g* and the supernatant was decanted.

The standard material (L-DOPA) was purchased from Sigma-Aldrich (USA). All natural material extract samples and standard material were obtained at a professional facility (Koptri, Seoul, Korea). All samples were filtered through a 0.45 μm PTFE filter and analyzed with a Warters 600 pump, Warters 717 automatic sample injection system, Waters PDA detector, and Empower software. A TSKgel ODS-100V column (4.6×250 mm) was used at a flow rate of 1.0 ml/ min. The temperature of the column was set at 35℃, and the injection volume was 10 μl.

RESULTS

Phenotype characteristics of yeast strains on various natural material-based media

Five strains of *Cryptococcus* species complex (ATCC 66031, 32608, MYA-4560, 48184, and one clinical strain) and three clinical isolates of *Candida* species (*C. albicans*, *C. glabrata*, and *C. tropicalis*) were cultured on SDA and ten different natural material-based media, respectively. Three clinical isolates of *Candida* species produced no pigment (Table 2). Only white colonies were observed on all of the natural material-based media, with the exception of Pk (using *Pinus koraiensis*) and JjD (using *Juglans nigra L.* and *Juglans sinensis Dode*) agar (no growth). Table 1 shows pigment formation by five strains of *Cryptococcus* species complex on different natural material-based media. On the typical SSA selective media, all strains of *Cryptococcus* species complex were differentiated from the white colonies of *Candida* species by specific brown pigment formation. However, no differences were detected between serotypes of *Cryptococcus* species complex.

On the SeI (using *Sesamum indicum*) agar, five strains of *Cryptococcus* species complex were classified into two types of colonies according to colony color (light brown [LB] and white [W]). Serotypes A, B, D, and A/D produced LB pigment, whereas serotype C was W. On GlM (using *Glycine max [L.] Merrill*) agar, the strains were classified into three types of colonies according to colony color (dark yellow [DY], beige [Be], and W). Serotypes A, D, and A/D produced DY pigment, whereas serotype B was Be. The colony color of serotype C remained the same (W). On RiC (using *Ricinus communis L.*) agar, the strains were classified into two types of colonies according to colony color (DY and

Table 1. Different pigment formations on modified natural material-based culture agar according to serotypes of *Cryptococcus* species complex

Natural materials \ Strain	Cryptococcus species complex				
	Serotype A	Serotype B	Serotype C	Serotype D	Serotype A/D
Helianthus annuus L.	B	B	B	B	B
Sesamum indicum	LB	LB	W	LB	LB
Heukimja	LB	LB	W	LB	LB
Perilla frutescens var. japonica Hara	B	B	B	B	B
Glycine max (L.) Merrill	DY	Be	W	DY	DY
Ricinus communis L.	DY	DY	W	DY	DY
Arachis hypogaea	LY	LY	W	LY	LY
Prunus dulcis	LY	LB	W	LB	LY
Pinus koraiensis	LY	No growth	W	No growth	No growth
Juglans nigra L., Juglans sinensis Dode	No growth	No growth	No growth	No growth	No growth

*light brown; LB, brown; B, white; W, dark yellow; DY, light yellow; LY, and beige; Be.

Table 2. Pigment formations of *Candida* species on modified natural material-based culture agar

Natural materials \ Strain	Candida species		
	C. albicans	C. tropicalis	C. glabrata
Helianthus annuus L.	W	W	W
Sesamum indicum	W	W	W
Heukimja	W	W	W
Perilla frutescens var. japonica Hara	W	W	W
Glycine max (L.) Merrill	W	W	W
Ricinus communis L.	W	W	W
Arachis hypogaea	W	W	W
Prunus dulcis	W	W	W
Pinus koraiensis	No growth	No growth	No growth
Juglans nigra L., Juglans sinensis Dode	No growth	No growth	No growth

*light brown; LB, brown; B, white; W, dark yellow; DY, light yellow; LY, and beige; Be.

W). Serotypes A, B, D, and A/D produced DY pigment, whereas serotype C was W. On ArH (using *Arachis hypogaea*) agar, the strains were classified into two types of colonies according to colony color (light yellow [LY] and W). Serotypes A, B, D, and A/D produced LY pigment, whereas serotype C was white. On PrD (using *Prunus dulcis*) agar, the strains were classified into three types of colonies according to colony color (LY, LB, and W) (Fig. 1A). Serotypes A and A/D produced LY pigment, whereas serotypes B and D were LB. The colony color of serotype C remained the same (W). On SeI, GlM, RiC, ArH, and PrD agar, slight changes in colony color were observed in almost all *Cryptococcus* spp. cultured media. However, brown or other specific pigment formation were not observed in all strains of *Cryptococcus* species complex.

On Heu (using *Heukimja*) agar, the strains were classified into two types of colonies according to colony color (LB and W) (Fig. 1B). Serotypes A, B, D, and A/D produced
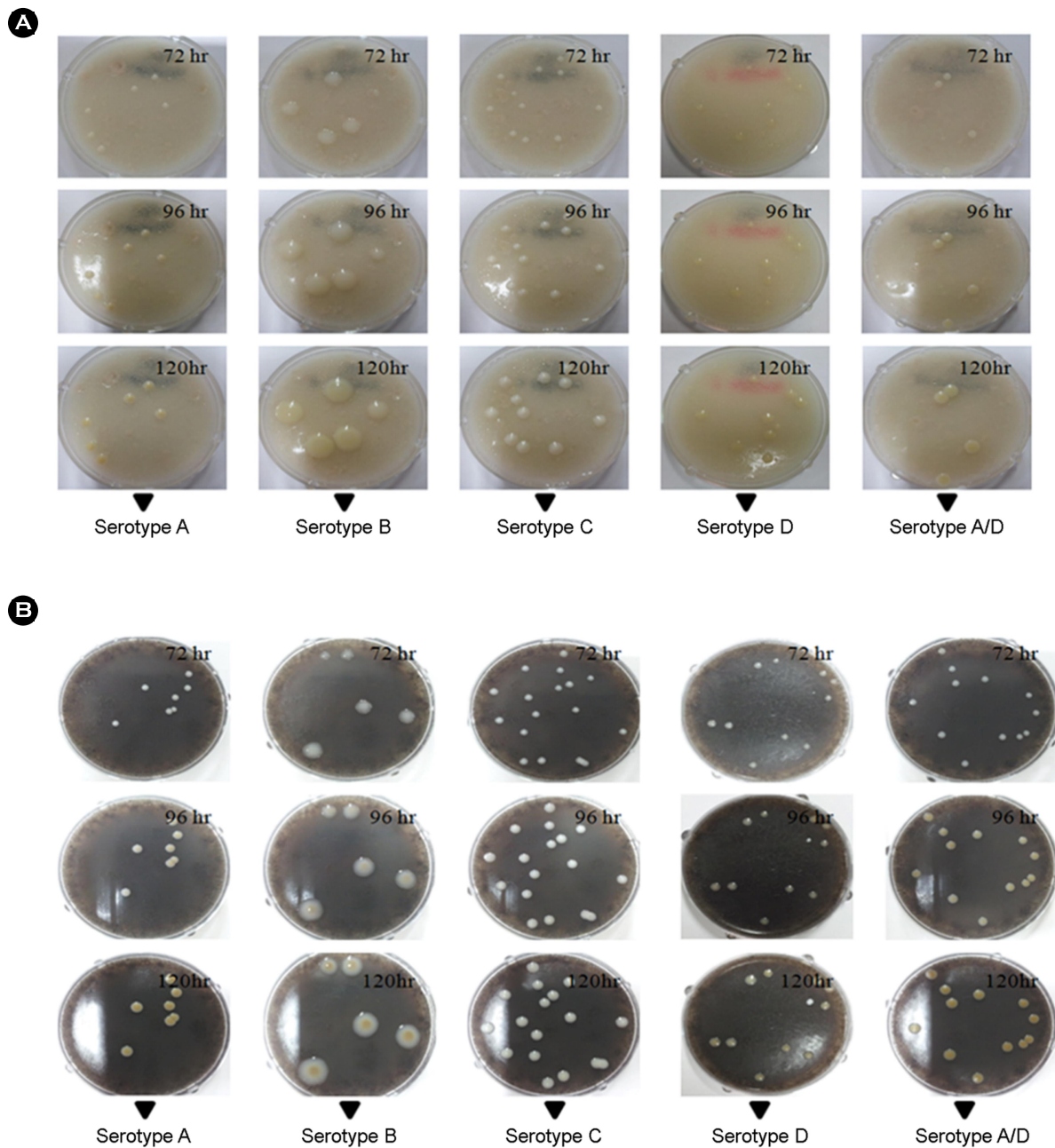
Fig. 1. Pigment formation of *Cryptococcus* species complex on natural material-based media according to incubation times. (A) Pigment formation of *Cryptococcus* species complex on PrD agar according to incubation times. (B) Pigment formation of *Cryptococcus* species complex on Heu agar according to incubation times.

LB pigment, whereas serotype C was white. Unusually, serotype B on Heu media was observed to have a different pigment formation than the other serotypes. LB pigment was observed exclusively in the center zone of the colony. This formation, which appeared in all serotype B colonies

cultured on Heu media, did not change over incubation time.

On PerJ (using *Perilla frutescens var. japonica Hara*) agar, brown pigment was observed in all strains of *Cryptococcus* spp. cultured media (Fig. 2). Colony growth and pigment formation occurred more rapidly than on the typical
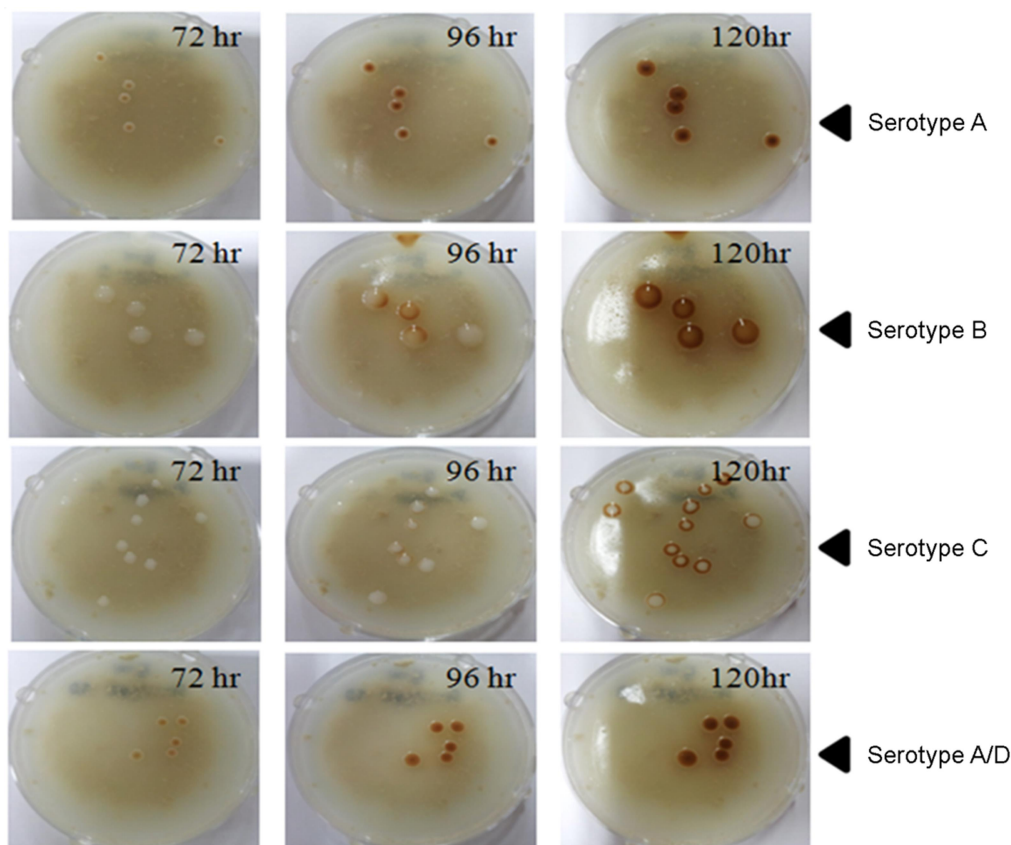
Fig. 2. Specific pigmentation of *Cryptococcus* species complex on PerJ agar according to incubation times.

selective medium, SSA. After incubating for 48 hours, only white colonies were observed on SSA, but brown pigmented colonies were observed in serotype A, D, and A/D strains cultured on PerJ agar. Furthermore, a specific pigment formation was observed in serotype B and C cultured media—brown pigment was produced in the rim of the colony first—which was different from the other serotypes on PerJ agar. On Pk and JjD agar, colony growth was not observed in almost all yeast strains on cultured media. In particular, colony growth was not observed at all on JjD media.

Isolation of *Cryptococcus* species complex strains from environmental samples on various natural material-based media

Specific brown pigment yeast-form colonies were observed on the SSA and PerJ agar. To identification of brown yeast-form isolates, we performed genotyping by using ITS

sequencing (Chae et al., 2012). As a result, all brown yeast-form isolates were identified as *Cryptococcus neoformans*. And we subcultured each brown isolates on PerJ agar for detection of the pigment formation pattern. The pigmentation pattern of all isolates was same as serotypes A, D, and A/D strains.

HPLC analysis to quantify L-DOPA

L-DOPA is one of the typical exogenous substrates required to synthesize melanin pigment in *Cryptococcus* species complex. To quantify L-DOPA content in ten different natural material extract samples, HPLC analysis was performed at a professional facility. To evaluate the calibration curve for L-DOPA, a standard solution of L-DOPA was prepared based on a concentration range of 1~100 mg/L (1, 10, 50, and 100 mg/L [ppm]) and evaluated at 305 nm. As a result, a sharp peak was detected at a retention time of

Table 3. The HPLC analysis of dried natural material extracts

Extracts	Area (μV*sec)	% Area	Height (μV)	Retention time (min)
Standard, L-3,4-dihydroxyphenylalanine	353044	100.0	21505	2.28
Helianthus annuus L. (DW)	–	–	–	–
Helianthus annuus L. (ethanol)	–	–	–	–
Perilla frutescens var. japonica Hara (DW)	402556	100.0	14223	2.3
Perilla frutescens var. japonica Hara (ethanol)	104159	100.0	3506	2.89
Sesamum indicum	270347	100.0	10114	2.21
Heukimja	134417	100.0	5327	2.24
Glycine max (L.) Merrill	764313	100.0	25208	2.31
Ricinus communis L.	885919	100.0	33015	2.21
Arachis hypogaea	575419	100.0	18995	2.21
Prunus dulcis	–	–	–	–
Pinus koraiensis	799158	100.0	31088	2.18
Juglans nigra L., Juglans sinensis Dode	664361	100.0	19404	2.29

2.28 min. The coefficient of correlation (R^2) was 0.999, and the equation of the calibration curve was Y=3576X-2282.4. Consequently, good linearity of the calibration curve for L-DOPA was established (data not shown). The quantity of L-DOPA content in the natural material extract samples was subsequently analyzed, and no L-DOPA was detected in any of the samples. In other words, the retention time of the peaks for the natural material extract samples was different from that of the standard compound, L-DOPA (Table 3). This result suggests that the ten different natural material-based media did not contain L-DOPA.

DISCUSSION

Culture media for microorganisms have played important roles in the primary isolation and identification of micro-organisms from various sources in many clinics and labora-tory fields. This method is simple, cost effective, and a useful tool for diagnosing infectious diseases. Many typical selective and differential media have specific indicators to differentiate among microorganism mixtures, such as certain inhibitory agents (e.g., antibiotics, chemicals, pH) and sub-strates. In particular, pigment formation is one of the typical indicators on culture media for isolating and identifying microorganisms. For instance, yeast strains obtained from environmental sources have been classified easily using colony color type (brown type, white type, beige type, and pink type) on SSA (Chae et al., 2012). These results suggest that good selective and differential media can be considered to be useful screening tools for identifying microorganisms.

The encapsulated pathogenic yeast *Cryptococcus* species complex has the ability to produce a specific dark brown pigment called melanin on media containing certain diphe-nolic compounds, such as L-DOPA. *Cryptococcus* species complex cannot produce melanin pigment without certain exogenous substrates (Casadevall et al., 2003; Eisenman et al., 2011; Garcia-Rivera et al., 2005). Based on this idea, several modified culture media were developed for the rapid identification of *Cryptococcus* species complex, such as mustard seed, chili pepper, Pinus halepensis seed, Eggplant, benomyl bird seed and blackberry agar (Menezes et al., 2011; Mseddi et al., 2011; Nandhakumar et al., 2006; Pedroso et al., 2007; Pham et al., 2014; Sengul et al., 2014). However, there are some limits. Almost all previously developed selec-tive media for *Cryptococcus* species complex have focused on rapid pigmentation, or melanization. Of course, it is an important request, but is not everything. Currently, *Crypto-coccus* species complex are classified into two species and five major serotypes. It is important to identify *Crypto-coccus* species complex accurately on selective media. As

such, we attempted to develop modified selective culture media to differentiate among *Cryptococcus* species using various affordable natural material extracts.

The results indicated that SeI, GlM, RiC, ArH, PrD, Pk, and JjD agar were not suitable for use as selective media for *Cryptococcus* species complex. On those media, either no specific pigmentation was observed (SeI, GlM, RiC, ArH, and PrD agar) or not all of the cryptococcal strains grew well (Pk and JjD agar). On the Heu agar, a specific pigmentation pattern was observed in only the serotype B strain (Fig. 1A), and this medium was also not suitable. On the other hand, PerJ agar was a specific medium for *Cryptococcus* species complex. On PerJ agar, a brown pigment formation on the colony was observed more rapidly than on the typical medium, SSA (Fig. 2). In addition, the various *Cryptococcus* species had different brown pigmentation patterns. In serotypes A, D, and A/D of the strains cultured on PerJ agar, brown pigment was produced in the center of the colony first, but it was produced in the rim of colony first in serotypes B and C. These results suggest that PerJ agar is a useful medium for the rapid identification and differentiation of the serotypes of *Cryptococcus* species complex.

It is well known that certain exogenous substrates are required to produce the brown pigment of *Cryptococcus* species complex on media. Therefore, HPLC analysis was performed to evaluate L-DOPA content in the natural material extracts. The retention times of all of the natural material extracts were different from that of the standard compound, L-DOPA (Table 3), indicating that none of the samples extracted from the ten different natural materials contained L-DOPA. These results suggest that the brown pigmentation mechanism of *Cryptococcus* species complex on SSA or PerJ agar might be different on typical L-DOPA-containing media. Thus, further studies regarding the exact brown pigmentation mechanism of *Cryptococcus* species complex on PerJ agar are needed. One problem with making PerJ agar is that it is a complicated procedure. Khan and colleagues previously described a simplified SSA (without creatinine and KH_2PO_4) (Khan et al., 2004); therefore, we attempted to make simplified PerJ agar. Five strains of *Cryptococcus* species complex were cultured on the simpli-

fied PerJ agar under the same conditions, and brown pigment was produced well (data not shown).

In this study, we attempted to develop modified selective media for *Cryptococcus* species complex in order to more rapidly identify and distinguish among their serotypes. Modified PerJ agar is a useful medium that is made with affordable ingredients. It is a simple and cost-effective medium for differentiating *Cryptococcus* species complex from clinical and environmental sources.

CONFLICT OF INTEREST

There was no conflict of interests between all authors.

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