

Potato Dextrose Agar With Rose-Bengal and Chloramphenicol: A New Culture Medium to Isolate Pathogenic *Exophiala dermatitidis* From the Environment

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ABSTRACT

Objective: The objective of this study is to describe the potatodextrose agar (PDA) with rose-Bengal and chloramphenicol as a new and simple medium (R-PDA chloramphenicol agar) to facilitate the detection of black yeast, *Exophiala dermatitidis* compared to ready-made conventional medium, namely rose-Bengal chloramphenicol agar.

Methods: We prepared a new medium by adding chloramphenicol and rose-Bengal to ready-made PDA.

Results: This medium proves better growth of the black yeast in terms of increased colony size compared to commercial rose-Bengal chloramphenicol agar. The increase in colony size aids for distinguishing the slow growing black yeast from all the other filamentous fungi.

Conclusion: Compared to traditional rose-Bengal chloramphenicol agar, R-PDA chloramphenicol agar is superior to isolate *Exophiala dermatitidis* among other fast growing filamentous fungi which are present in the environment samples.

Key Words: Black yeast, environment, *Exophiala dermatitidis*, pigeon, rose-Bengal.



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INTRODUCTION

Exophiala Dermatitidis is an ascomycete dematiaceous fungus (1). This black yeast causes phaeohyphomycosis which can take place in both normal and immunocompromised hosts (1,2). Infection can take place on and under the skin, in the lungs and also in the nervous system (3). The neurotrophic nature of this black yeast can cause fatal infections (4). 17 out of 23 species of Exophiala are pathogenic to humans and animals. The three main pathogenic species are E. dermatitidis, E. xenobiotica, and E. oligosperma (1). Despite being infectious to human hosts, this opportunistic black yeast can be isolated from environment including tropical fruit surfaces (4), creosoted railway ties (5), saunas (2), dishwashers (6), land farming areas contaminated with waste petrol hydrocarbons (7) and faeces of frugivorous birds and flying foxes (4)

The isolation of this pathogenic black yeast from the environment samples can be challenging because of the existence of other fast-growing fungi which may lead to competition for space and nutrients among the population growing on the plate (8). Exophiala spp. form brown to black colonies on the agar due to the presence of melanin in its cell wall (9). One of the common media used for the isolation of this black yeast is Sabouraud's dextrose agar (SDA) with antibiotics to prevent bacterial growth (5). Commercial media such as Mycosel™ agar were also used as they facilitate the detection of the pathogenic fungi from samples with dense amount of other fungi and bacteria due to the inhibitor effect of cycloheximide and chloramphenicol (7). Traditional rose-Bengal chloramphenicol agar with the addition of dichloran has also been used for the isolation of the black yeast from environment rich in volatile aromatic hydrocarbon (10).

We propose a new isolation medium, potato dex-

trose agar (PDA) with rose-Bengal and chloramphenicol (R-PDA chloramphenicol agar) by taking into consideration of the melanin production by the yeast.

METHODS

Culture Media: R-PDA chloramphenicol agar was made by suspending 39 g of ready-made PDA (CM0139, Oxoid, Basingstoke, Hampshire, England) in 1 L of distilled water, then by adding 0.033 g of rose-Bengal by Martin (11), and 0.25 g of chloramphenicol.

Rose-Bengal chloramphenicol agar was made by adding 5 g of mycological peptone, 10 g of glucose, 1 g of dipotassium phosphate, 0.5 g of magnesium sulphate, 0.05 g of rose-Bengal and 15.5 g of agar with 0.25 g of chloramphenicol to prepare 1 L of medium.

The plates were deep filled, approximately 20 ml of culture media was poured to each Petri dish. The control plate was PDA amended with chloramphenicol.

Molecular Method: Home-made potato dextrose broth (PDB) was made from boiled fresh potatoes with distilled water. Dextrose was added to the potato broth and autoclaved. Two loops of pure culture of Exophiala dermatitidis which were initially isolated from pigeon droppings were inoculated to PDB in a flask and allowed to shake for 24 hours. The suspension was then centrifuged to separate the cells and supernatant. The DNA extraction procedure was carried out following the instructions of the i-genomic BYF DNA Extraction Mini Kit (iNTRON Biotechnology Inc., Kyungki-Do, Korea). ITS1F as forward primer and ITS4 as reverse primer were used for the PCR reaction (12). The steps in the cycle were as follows: Initial denaturation 95°C (5 min.), denaturation 95°C (30 sec.), annealing 58°C



FIGURE 1 Observation of the result of gel electrophoresis. Left row shows the 1 kb ladder and right lane shows band formed at 600 bp.

(30 sec.), extension 72°C (60 sec.) and finally extension 72°C (5 min.). The PCR product was run in 1x agarose gel with 1x TAE buffer for 30 min. at 100V before soaking in ethidium bromide and viewing under UV light. The PCR product was purified according to MEGAquick-spin™ (iNtRON Biotechnology Inc., Kyungki-Do, Korea). The purified PCR product was sent for sequencing. The obtained sequence was aligned and identified for its homology in the GenBank database (<http://www.ncbi.nlm.nih.gov/BLAST/>) at National Center for Biotechnology Information (NCBI, Bethesda, MD, USA).

Plating the Black Yeast: Inoculation test was conducted using pure culture of *E. dermatitidis* (KX964623) and not directly from the pigeon's droppings. Two loops of pure culture *E. dermatitidis* were aseptically inoculated into a 100 ml of PDB in a flask and allowed to shake for 24 hours. After 24 hours, serial dilution was performed using sterile distilled water from the dilution 10⁻¹ to 10⁻⁵. Universal bottles were prepared earlier containing 9 ml of sterile distilled water each. 1 ml was transferred from the broth containing *E. dermatitidis* preculture to the first universal bottle labelled 10⁻¹ and the dilution continues. 100 µl from the last three dilutions were plated to five R-PDA chloramphenicol agar and traditional rose-Bengal chloramphenicol agar plates each. The plates were then incubated at 37 °C for 3-5 days. *Candida albicans* was also streaked on R-PDA chloramphenicol agar to observe the growth.

Statistical Analysis: t-test was done to determine whether there were any significant difference between both the RPDA chloramphenicol agar and traditional rose-Bengal chloramphenicol agar.

RESULTS

Molecular Identification: DNA was successfully extracted from the yeast cells and amplified. A band was formed at 600 base pair (Figure 1). The sequencing result showed that the strain (BY1) was 100% identical with *E. dermatitidis* (KP76113.1), which is the black yeast that was growing on the plate from supernatant of pigeon droppings. Our strains (BY1) and (BY2) were deposited as (KX964623) and (KX964624), respectively.

Plating the Black Yeast: The plates were observed after 5 days (Figure 2). The starting culture was 4.76x10⁴. The plates were scanned and the colony area was measured using ImageJ (13) for both types of media. Figure 2A and Figure 2B shows the plates

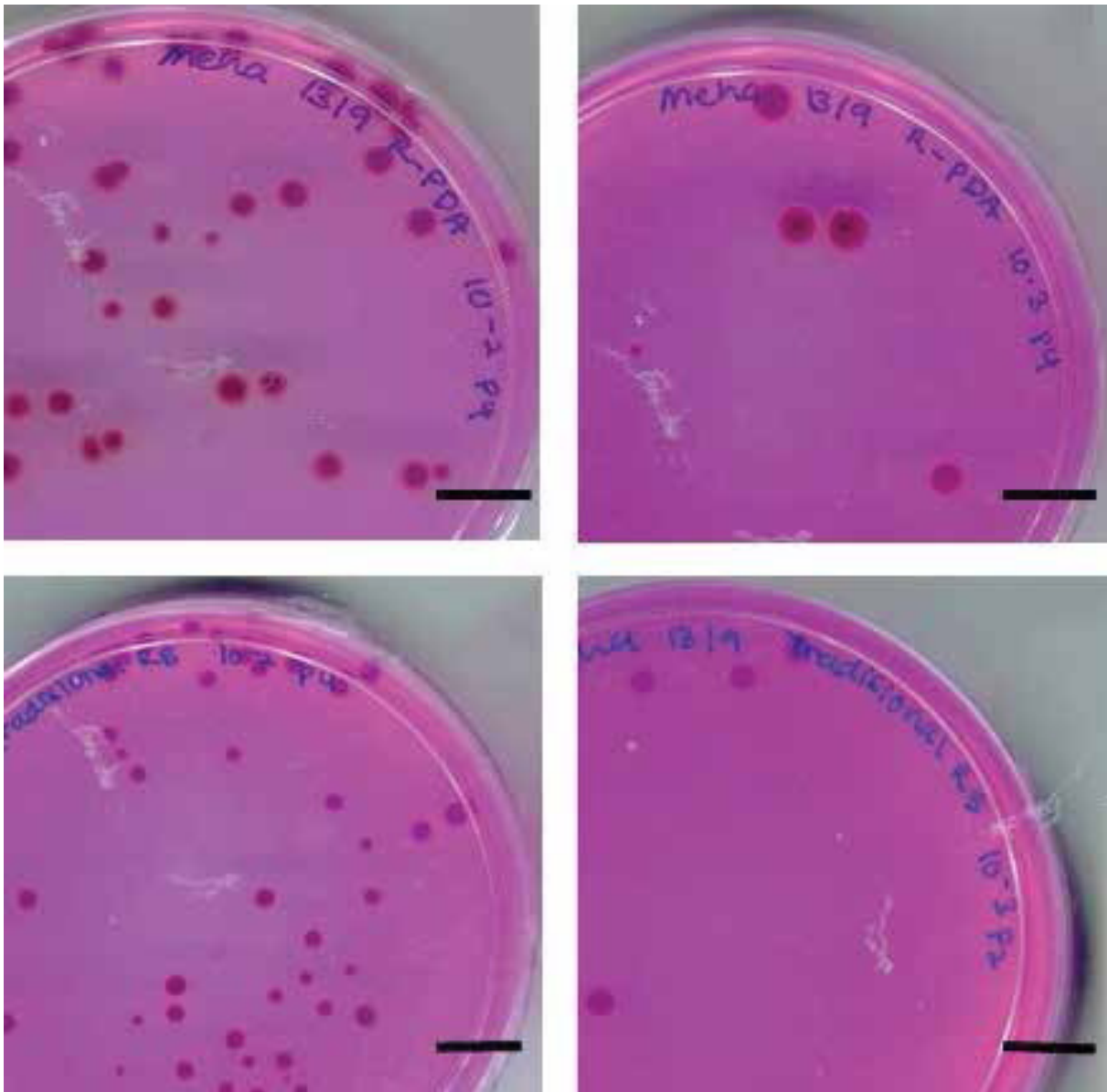


TABLE 1 Limitations Figure 2. The colony diameter for R-PDA chloramphenicol agar (A) for 10-2 dilution and (B) 10-3 appeared bigger by observation compared to rose-Bengal chloramphenicol agar (C) for 10-2 dilution and (D) 10-3 dilution (scale bar=10 mm).

R-PDA chloramphenicol agar from dilution 10-2 and 10-3 respectively whereas Figure 2C and Figure 2D shows the plate of traditional rose-Bengal chloramphenicol agar from dilution 10-2 and 10-3, respectively. The colony sizes of the black yeast in both media were compared and tabulated (Table 1). The colony diameter of the black yeast was bigger in R-PDA chloramphenicol agar than those on the

traditional rose-Bengal chloramphenicol agar. The colony area in R-PDA chloramphenicol agar was significantly bigger than those on the traditional rose-Bengal chloramphenicol agar ($\alpha=0.05$). *C. albicans* was not able to grow on the R-PDA chloramphenicol agar and the inoculum turned into pink after 3 days of incubation at 37°C.

TABLE 1 The Comparison of *E. dermatitidis* (KX964623) Mean Colony Area (mm²) Between R-PDA Chloramphenicol Agar and Rose-Bengal Chloramphenicol Agar With 10⁻² and 10⁻³ Dilutions

	R-PDA Chloramphenicol Agar 10 ⁻² * (mm ²)	Rose-Bengal Chloramphenicol Agar 10 ⁻² * (mm ²)	R-PDA Chloramphenicol Agar 10 ⁻³ † (mm ²)	Rose-Bengal Chloramphenicol Agar 10 ⁻³ † (mm ²)
Colony size	13.79±4.07‡,§	5.53±1.98‡	16.07±5.37‡,§	5.71±2.07‡

R-PDA chloramphenicol agar: Potato dextrose agar with rose-Bengal and chloramphenicol.

* One hundred colony areas were measured using ImageJ from 5 plates of the 10⁻² dilution

† Twenty colony areas were measured using ImageJ from the 5 plates of the 10⁻³ dilution.

‡ The standard deviation was indicated after the mean value.

§ The colony size on both media was significantly different ($\alpha=0.05$).

DISCUSSION

Dichloran rose-Bengal chloramphenicol (DRBC) medium (10) was used to isolate black fungi from environment rich in volatile aromatic hydrocarbon. *E. dermatitidis* survives in a habitat that is occupied by competitors (2), therefore a medium similar to DRBC medium can be used to isolate this black yeast from environmental samples easily. *E. dermatitidis* has the ability to produce dark brown to black colonies because they have the ability to produce melanin naturally on their thick cell wall. This feature defends them from solar radiations and other environmental treats (14).

E. dermatitidis was initially isolated from pigeon droppings which were plated on this R-PDA chloramphenicol agar. As the sample was collected from the environment, there were other filamentous fungi present on the plate. However, the detection of this black yeast on R-PDA chloramphenicol agar was easy compared to on rose-Bengal chloramphenicol agar.

Rose-Bengal was used as it has the ability to suppress filamentous fungi which aids in the visual distinction of the yeast colonies on the medium (11). The size of the colony on the agar plate is im-

portant so that the colony can be detected easily as there are some fast growing fungi on the plate. Traditional rose-Bengal chloramphenicol agar gave smaller colony growth compared to R-PDA chloramphenicol agar. Apart from that, traditional rose-Bengal chloramphenicol agar requires addition of peptone in the medium (15), however in our R-PDA chloramphenicol agar no peptone was added and it was still effective to detect the black yeasts. The black yeast grows better in potato extract compared to the medium containing mycological peptone.

The usage of rose-Bengal chloramphenicol agar is also suitable when higher and prolonged incubation temperatures are needed especially when isolating human pathogens which grow on temperatures up to 37°C. The suitable concentration of rose-Bengal can sustain its colour and inhibitory action. Based on King et al. (16), the designated DRBC medium used gives approximately 0.025 g/L of rose-Bengal. From our experiment we proposed usage of 0.033 g/L which is higher than the designated DRBC medium by King et al. (16) to inhibit growth of *Mucor* and *Rhizopus* spp. The smaller amount of rose-Bengal by King et al. (16) was effective because dichloran was also added in that medium (17). In our R-PDA chloramphenicol agar, dichloran

was not used, while a slightly higher concentration (0.033 g/L) was used for 1x of R-PDA. Based on the material safety data sheet of the traditional rose-Bengal chloramphenicol agar, the amount of rose-Bengal was 0.05 g/L which is definitely higher than the R-PDA chloramphenicol agar. However, this concentration of the rose-Bengal definitely did not suppress the growth of the black yeast. In fact, the colony growth size appeared much better in a lesser concentration of rose-Bengal (Table 1).

The agar plates were deep filled to decrease the effects of drying during prolonged incubation and to ensure uniform colony size between the plates.

Besides, the more concentration of rose-Bengal increased, the more intensity of the pink colour in the medium thickened. This made the identification between dark brown yeast colonies and dark pink yeast colonies difficult. Therefore, we propose a concentration of rose-Bengal between 1x to 1.48x for the easy detection of the dark brown yeast colonies.

Some yeasts are initially white in colour when it is allowed to grow on PDA but it appears pink on R-PDA chloramphenicol agar as it uses the colour of the media to appear pink. *C. albicans* was not able to grow in this medium proving that not all the yeast is able to grow in this medium making it selective for the isolation of *E. dermatitidis*. However, the shelf life of this agar is short as rose-Bengal can undergo some oxidation reaction which can be toxic to cells if used after 1 month. This was because there was no single colony observed from the plates prepared one month earlier before the experiment was conducted. As the photon reactive pigments in rose-Bengal produce reactive oxygen under light condition, it can cause decrease in colony count because of the photon degradation of rose-Bengal to toxic chlorinated derivatives. However, the exposure to light may not be beneficial for fungal growth but can be useful

for bacterial elimination (18). Therefore, the usage of our medium under laboratory conditions would not be a problem as we would like to eliminate the bacterial colonies as well as filamentous fungal colonies to aid in the detection of the black yeast.

Based on the t-test, as the colony size between R-PDA chloramphenicol agar and traditional rose-Bengal chloramphenicol agar was significantly different, the null hypothesis is not rejected. It means that the R-PDA chloramphenicol agar is much better to be used for identification compared to traditional rose-Bengal chloramphenicol agar.

As R-PDA chloramphenicol agar has the ability to suppress filamentous fungi, the slow growing yeast will not need to compete with other microbiota on the medium for space and nutrients. The procedure for preparation is also much easier and faster compared to traditional rose-Bengal chloramphenicol medium. *E. dermatitidis* is rarely isolated from the environment but is able to produce melanin naturally without using phenolic compounds from the media to turn dark brown or black. We propose a new medium which can be easily prepared for the isolation of this black yeast, *E. dermatitidis*.

Conflict of Interest: The authors declare that there are no conflicts of interest.

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REFERENCES

- 1** Sutton DA, Rinaldi MG, Sanche SE. Dematiaceous fungi. In: Anaissie EJ, McGinnis MR, Pfaller MA, eds. *Clinical Mycology*. 2nd ed. New York: Churchill Livingstone Elsevier, 2009: 330-4.
- 2** Sav H, Ozakkas F, Altunbas R, et al. Virulence markers of opportunistic black yeast in *Exophiala*. *Mycoses*. 2016; 59(6): 343-50.
- 3** Seyedmousavi S, Badali H, Chlebicki A, Zhao J, Prenafeta-Boldú FX, De Hoog GS. *Exophiala sideris*, a novel black yeast isolated from environments polluted with toxic alkyl benzenes and arsenic. *Fungal Biol*. 2011; 115(10): 1030-7.
- 4** Sudhadham M, Prakitsin S, Sivichai S, et al. The neurotropic black yeast *Exophiala dermatitidis* has a possible origin in the tropical rain forest. *Stud Mycol*. 2008; 61:145-55.
- 5** Zhao J, Zeng J, De Hoog GS, Attili-Angelis D, Prenafeta-Boldú FX. Isolation and identification of black yeasts by enrichment on atmospheres of monoaromatic hydrocarbons. *Microb Ecol*. 2010; 60(1): 149-56.
- 6** Zalar P, Novak M, De Hoog GS, Gunde-Cimerman N. Dishwashers—a man-made ecological niche accommodating human opportunistic fungal pathogens. *Fungal Biol*. 2011; 115(10): 997-1007.
- 7** Satow MM, Attili-Angelis D, De Hoog GS, Angelis DF, Vicente VA. Selective factors involved in oil flotation isolation of black yeasts from the environment. *Stud Mycol*. 2008; 61: 157-63.
- 8** Castro e Silva Dde M, Santos DC, Pukinskis SR, et al. A new culture medium for recovering the agents of cryptococcosis from environmental sources. *Braz J Microbiol*. 2015; 46(2): 355-8.
- 9** Nyaoke A, Weber ES, Innis C, et al. Disseminated phaeohyphomycosis in weedy seadragons (*Phyllopteryx taeniolatus*) and leafy seadragons (*Phycodurus eques*) caused by species of *Exophiala*, including a novel species. *J Vet Diagn Invest*. 2009; 21(1):69-79.
- 10** Isola D, Selbmann L, de Hoog GS, et al. Isolation and screening of black fungi as degraders of volatile aromatic hydrocarbons. *Mycopathologia*. 2013; 175(5-6): 369-79.
- 11** Martin JP. Use of acid, rose Bengal, and streptomycin in the plate method for estimating soil fungi. *Soil Science*. 1950; 69(3): 215-32.
- 12** White TJ, Bruns T, Lee SJ, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Shinsky JJ, White TJ, eds. *PCR Protocols: A Guide to Methods and Applications*. New York: Academic Press, Inc., 1990: 315-22.
- 13** Bylesjö M, Segura V, Soolanayakanahally RY. LAMINA: a tool for rapid quantification of leaf size and shape parameters. *BMC Plant Biology*. 2008; 8(1): 82.
- 14** Gunde-Cimerman N, Grube M, de Hoog GS. The emerging potential of melanized fungi: black yeast between beauty and thebeast. *Fungal Biol*. 2011; 115(10): 935-6.
- 15** Deak T. *Handbook of Food Spoilage Yeasts*. 2nd ed. Boca Raton: CRC Press, 2008.
- 16** King AD Jr, Hocking AD, Pitt JI. Dichloran-rose Bengal medium for enumeration and isolation of molds from foods. *Appl Environ Microbiol*. 1979; 37(5): 959-64.
- 17** Henson OE. Dichloran as an inhibitor of mold spreading in fungal plating media: effects on colony diameter and enumeration. *Appl Environ Microbiol*. 1981; 42(4): 656-60.
- 18** Chilvers KF, Reed RH, Perry JD. Phototoxicity of rose Bengal in mycological media--implications for laboratory practice. *Lett Appl Microbiol*. 1999; 28(2): 103-7. 2017.