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Development and Validation of Benomyl Birdseed Agar for the Isolation of *Cryptococcus neoformans* and *Cryptococcus gattii* from Environmental Samples

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Abstract

One of the difficulties of isolating *Cryptococcus neoformans* and *Cryptococcus gattii* from environmental samples is the abundant overgrowth of other yeast and mold species on the plates. Here we report the application of benomyl to *Guizotia abyssinica* seed extract growth medium to improve the isolation of *C. neoformans* and *C. gattii* from environmental samples. We validated this medium by recovering *C. neoformans* and *C. gattii* from convenience soils and swabs from an endemic US region.

Keywords

Cryptococcus gattii; *Cryptococcus neoformans*; benomyl; birdseed agar

Introduction

Cryptococcus neoformans and its closely related sibling *Cryptococcus gattii* are encapsulated basidiomycete yeasts that cause a spectrum of potentially fatal diseases collectively known as cryptococcosis. The most common manifestations of cryptococcosis are meningitis and primary pulmonary infection, but these organisms can also cause both disseminated and cutaneous infections in humans and animals [1]. It is believed that this disease is primarily contracted through the inhalation of aerosolized yeast or basidiospores from the environment [2,3,4].

The main reservoirs of *C. neoformans* and *C. gattii* are in the environment. Although highly related, these two saprobic species occupy distinct ecological niches. *Cryptococcus neoformans* has been isolated from water, soil, and plants globally, although the main reservoirs of *C. neoformans* in the environment are believed to be pigeon guano and soil contaminated with pigeon and other bird guano [5,6]. Unlike *C. neoformans, C. gattii* is associated with trees and soil surrounding trees and was first discovered on *Eucalyptus* trees

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Disclosure of Conflict of Interest

All authors, no conflicts.

[7]. Prior to the recent emergence in the Pacific Northwest (PNW) of North America, [8,9,10] *C. gattii* was thought to reside predominantly in tropical and subtropical regions of the world. *Cryptococcus gattii* has since been associated with many different species of tree and it has been isolated from every continent but Antarctica [11]. In an effort to establish the geographical range of *C. gattii* as well as to determine ecological niches, environmental surveillance has been performed [12,13,14].

Previous environmental surveillance for *C. neoformans* and *C. gattii* has relied primarily on *Guizotia abyssinica* birdseed extract agar [15,16,17] and Sabouraud dextrose agar with antibiotics. These two growth media are non-selective and allow the overgrowth of fast-growing and abundant environmental molds and yeasts, which can obscure and obstruct the isolation of *C. neoformans* and *C. gattii*. Over the years, there have been numerous improvements in differential media for these two species of *Cryptococcus* [18,19,20]. However, selective medium for these organisms can be improved. Here, we report a novel formulation of selective media, which includes antibiotics, benomyl, and *Guizotia abyssinica* seed extract, to improve the isolation of *C. neoformans* and *C. gattii* from environmental samples. *Guizotia abyssinica* seed extract allows the differentiation of *C. neoformans* and *C. gattii* from other yeasts through the production of melanin which gives *Cryptococcus* colonies a brown color. Benomyl is a benzimidazole fungicide that inhibits many genera of ascomycete but does not inhibit the growth of many basidiomycetes, including *C. neoformans* and *C. gattii* [21,22]. Together they establish a selective and differential medium, “benomyl birdseed agar”, for isolation of these two *Cryptococcus* species from the environment.

Materials and Methods

Preparation of growth media

Sabouraud dextrose agar (SDA) and *Guizotia abyssinica* seed, or birdseed extract (BSE) agar were supplemented with 50 μg gentamycin and chloramphenicol per milliliter of medium. BSE was prepared as described previously [15,16,17]. Benomyl birdseed agar (BBA) was prepared by adding various concentrations of benomyl to BSE plus antibiotics. A benomyl stock was prepared by dissolving benomyl (Sigma Aldrich, St. Louis, MO) in dimethyl sulfoxide (at 5 mg/ml concentration) and was added to the molten medium after cooling to 55°C.

Environmental sample processing and imaging

Environmental samples used in this study include soil (sometimes mixed with decomposed plants and leaves), and swabs of shoes and vehicles that were collected in an endemic region of Washington state during routine sampling. Swabs were processed individually by placing each swab into a 50 ml conical tube that contained 5 ml of distilled water and vortexing for 1 minute. For soil samples, approximately 2 grams of each sample were transferred to a 50 ml conical tube that contained 10 ml of distilled water and were vortexed for 1 minute. All suspensions were allowed to settle for five minutes. Two hundred microliters of the supernatant was spread on each plate in duplicate. Plates were incubated at 30°C–37°C for

up to two weeks. Images of the plates were taken using an Apple IPhone camera and Casio Exilim digital camera.

Assessing the inhibitory effects of benomyl on environmental fungi

For determination of benomyl tolerability, reference strains of *C. neoformans* and *C. gattii* from our laboratory were cultured on BSE medium containing various working concentration of benomyl ranging from 0 to 2.5 μg/ml. Freshly grown cultures were diluted in sterile-distilled water to an OD_{600} of 0.2. The cell suspensions were further serially diluted in sterile-distilled water at a 1:10 dilution. Twenty microliters of the cell suspensions were spot-plated onto BSE.

To demonstrate that benomyl could inhibit the growth of many molds and other yeasts found in the environment, we plated convenience environmental samples on antibiotic-containing SDA and BSE media that contained benomyl at concentrations of 0, 1.5, 2.0, and 2.5 μg/ml. To demonstrate the selective property of benomyl for *C. neoformans* and *C. gattii*, we spiked 1 ml of soil suspension with 25–50 *C. neoformans* or *C. gattii* cells. One hundred microliters of the soil suspension was plated on both BSE and BBA plates. The plates were incubated at various temperatures ranging from 30°C to 37°C. Growth was recorded for up to 9 days.

Identification of *C. neoformans* and *C. gattii*

All environmental isolates of *C. neoformans* and *C. gattii* from this study were identified by microscopy, and by their growth characteristics on L-DOPA [23] medium and canavanine glycine bromothymol blue medium [24]. All *C.gattii* were also molecularly identified by DNA sequence analysis as previously described [25].

Results

Benomyl inhibits the growth of environmental molds but not *C. neoformans* or *C. gattii*

Following inoculation with *C. neoformans* and *C. gattii*, growth as brown pigmented colonies could be seen on BBA medium after overnight incubation. Growth and pigment formation could be observed in individual colonies after 2–3 days of incubation at all temperatures tested. After two days of incubation, cell growth on BBA and BSE plates was comparable. Benomyl showed no inhibition of the growth of *C. gattii* or *C. neoformans* isolates at any of the tested concentrations (Figure 1 and data not shown).

By contrast, benomyl was able to inhibit many environmental molds. When the environmental samples were plated, abundant mold growth was observed on both BSE and SDA growth media without benomyl, but when benomyl was added to BSE and SDA, the growth of mold and some yeasts was reduced substantially. The effect of benomyl on mold and other yeast growth can be seen at benomyl concentrations as low as 1.5 μg/ml. Benomyl did not inhibit the growth of all molds. Colony types consistent with Mucormycetes were able to grow at all benomyl concentrations tested.

BSE supplemented with benomyl can serve as a selective and differential medium for the isolation of *C. neoformans* and *C. gattii* from environmental samples

BBA displayed both selective and differential characteristics for *C. neoformans* and *C. gattii* when tested against spiked soils. When soils spiked with *C. gattii* or *C. neoformans* cells were plated on various media, we observed abundant mold growth on media without benomyl. The molds outgrew both *C. neoformans* and *C. gattii* on BSE plates and rendered the yeasts unrecoverable. In contrast, only brown yeast colonies were observed on BBA plate (Fig. 2).

As a proof of concept for the usefulness of this media in isolating *C. neoformans* and *C. gattii*, we used BBA to screen 93 environmental samples collected in the state of Washington, a known *C. gattii* endemic region. Sixteen environmental samples, all of them from swabs, were positive for *Cryptococcus*. Biochemical analysis confirmed that the recovery of *C. neoformans* (n=14) and *C. gattii* (n=2) in 16 of these samples. Based on MLST analysis, all *C. gattii* isolates were molecular type VGIIa the type expected from this region. We typically recovered 1–3 *Cryptococcus* colonies in each positive sample, and one sample contained both *C. gattii* and *C. neoformans*.

Discussion

The emergence of *C. gattii* in the PNW has raised concerns among public health officials and positive cases are being tracked [8,26,27]. Environmental sampling for *C. gattii* has been utilized to determine the ecological niches and geographical boundaries of this opportunistic pathogen in the PNW [12,13]. Although environmental sampling is being used more often to define the geographic boundaries of *C. gattii*, one of the remaining challenges has been the isolation of *Cryptococcus* in the context of an overgrowth of other environmental molds and yeasts. In the past, biphenyl has been used to inhibit the growth of some molds and yeasts but it is also toxic to *Cryptococcus* and human at higher levels [28], and growth of *Cryptococcus* in the presence of biphenyl, in our experience and the experience of others (Anastasia Litvintseva, personal communication), is slow and many other species of mold show breakthrough growth [17,29]. This study demonstrated that the amount of benomyl required to inhibit most molds is 0.00015%, which is about 700 fold lower than the amount of biphenyl that is typically used. The differential effect of benomyl on fungal growth has been reported previously [21,22]. Fokkema and colleagues reported that benomyl was highly effective against *Cladosporium* spp. while showing no effect against *Cryptococcus* ssp. in their study on rye leaves [21]. In addition, Summerbell reported that many filamentous fungi, including the ascomycete genera *Aspergillus* and *Penicillium* more commonly encountered in environmental samples, were largely unable to grow at a benomyl concentration of 2 μg per milliliter of medium [22]. In contrast, many medically important yeasts including *C. neoformans* showed low sensitivity to benomyl.

The growth media most commonly employed for the isolation of *C. neoformans* and *C. gattii* is BSE (or another L-3,4-dihydroxyphenylalanine containing medium such as DOPA). However, BSE is a non-selective growth media that allow excessive growth of molds and yeasts. These fast-growing molds and yeasts rapidly outgrow the slower-growing *C. neoformans* and *C. gattii* and make isolation difficult. To mitigate this problem, we

supplemented BSE with 1.5 μg/ml of benomyl to make a selective and differential isolation agar for *C. neoformans* and *C. gattii* (although other species of *Cryptococcus* may be recovered as well). The results demonstrate that benomyl, at 1.5 μg/ml concentration, did not affect the growth *C. gattii* or *C. neoformans*. More importantly, this concentration of benomyl effectively eliminated almost all environmental molds and yeasts. Using this isolation medium, we were able to isolate either *C. gattii* or *C. neoformans* from 16 of 93 environmental samples.

In conclusion, BBA medium provides an additional tool for the isolation of *C. neoformans* and *C. gattii* from the environment. Utilizing this isolation medium will assist in the public health effort to detect *C. gattii* in environmental samples not only from the Pacific Northwest but from throughout the world. The ability to detect *C. gattii* reliably from environmental samples will assist in defining possible exposures as well as in determining the current endemic regions for this fungus.

The findings and conclusions of this article are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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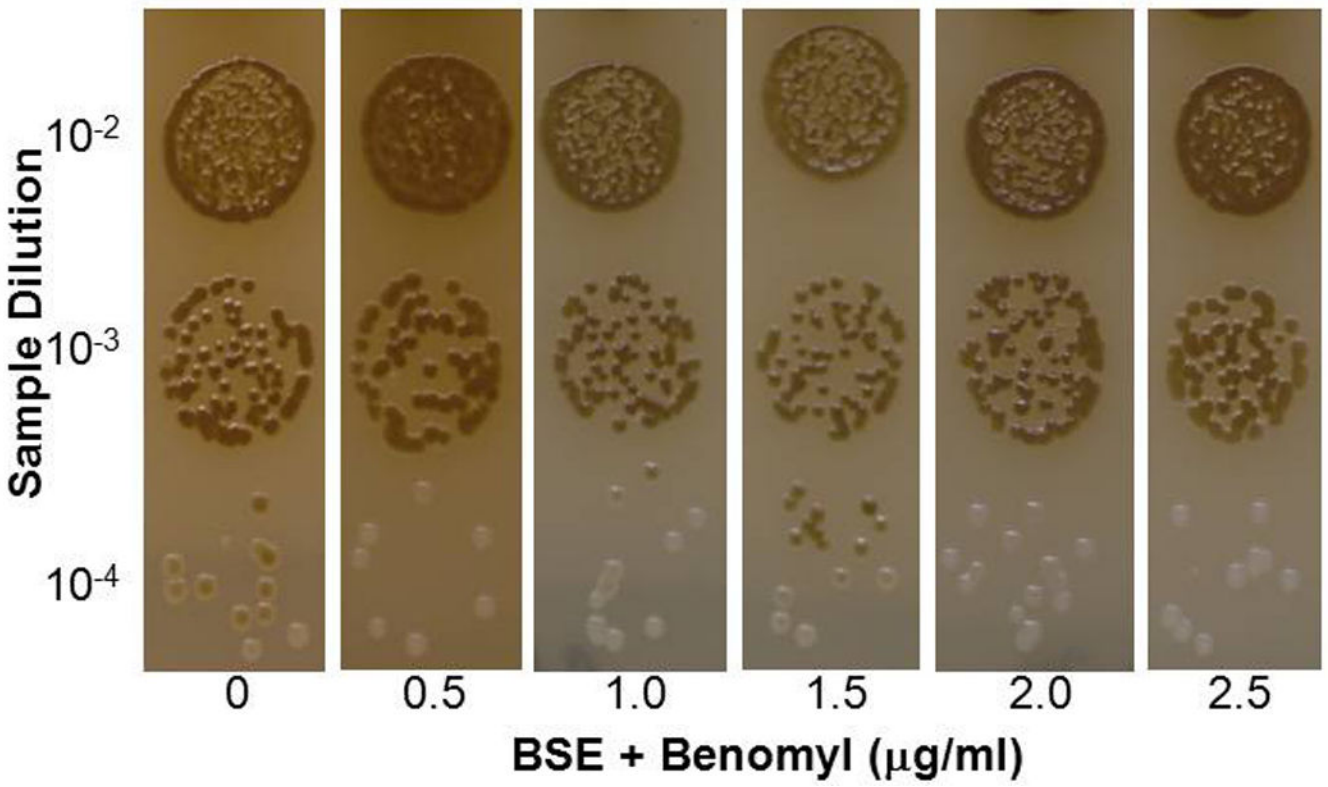

Figure 1. *Cryptococcus* exhibits high tolerance to benomyl

Three concentrations of *C. gattii* cells were spot-plated onto BSE medium supplemented with benomyl at concentration ranging from 0 to 2.5 μg/ml. The cells were incubated at 37°C for 48hr post inoculation.

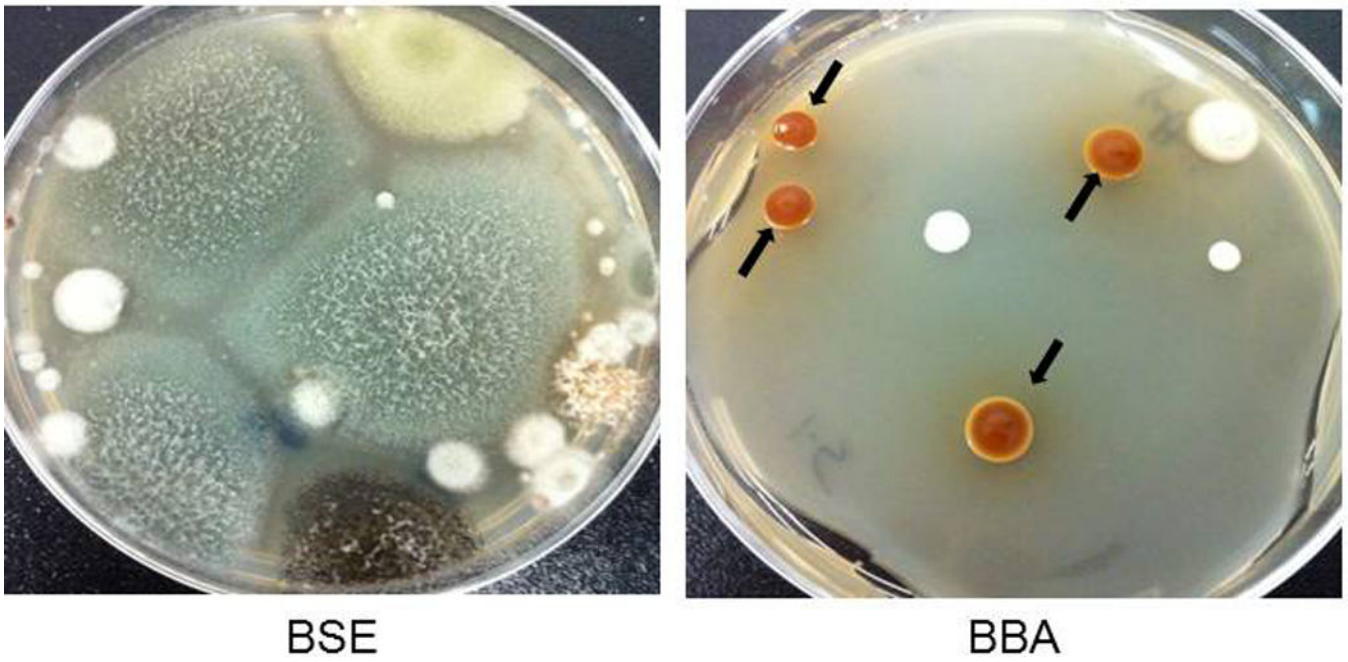

Figure 2. Selective and differential properties of BBA medium

A soil suspension was spiked with *C. gattii* and *C. neoformans* cells and plated onto BSE and BBA media. The plates were incubated at 37°C for 5–9 days. The black arrow indicates *Cryptococcus* colonies.

Table 1
Isolation of *Cryptococcus* from Environmental Samples

Environmental samples from 6 different sites were screened for *C. neoformans* and *C. gattii* using BBA medium. Vehicle samples were either from the wheels or the wheel wells following sampling.

Sampling Locations	Sample Type	Sample Types	# of Samples	Result (n)
1	Vehicle	Swab	2	Negative
2	Vehicle	Swab	4	*C. neoformans* (3)
	Vehicle #1	Swab	4	Negative
	Investigator #1 (shoes)	Swab	2	*C. neoformans* (1)
	Vehicle #2	Swab	4	*C. neoformans* (1)
	Investigator #2 (shoes)	Swab	2	Negative
	Highway Rest Area	Soil	2	Negative
3	Local recreational area #1	Soil	1	Negative
	Vehicle #3	Swab	4	*C. neoformans* (2)
	Investigator #3 (shoes)	Swab	2	*C. neoformans* (1)
	Local recreational area #2	Soil	1	Negative
	Vehicle #4	Swab	4	*C. neoformans* (1)
	Investigator #4 (shoes)	Swab	2	Negative
	Veterinarian #1 (office)	Soil	1	Negative
	Vehicle #5	Swab	4	Negative
	Investigator #5 (shoes)	Swab	2	Negative
	Veterinarian #2 (office)	Soil	1	Negative
	Vehicle #6	Swab	4	*C. neoformans* (1)
	Investigator #6 (shoes)	Swab	2	Negative
4	Investigator (shoes)	Swab	2	Negative
	Vehicle #1	Swab	4	Negative
	Investigator #1 (shoes)	Swab	2	Negative
	Local recreational facility	Soil	2	Negative
	Vehicle #2	Swab	4	*C. gattii* (1) and *C. neoformans* (2)
	Investigator #2 (shoes)	Swab	2	*C. gattii* (1)
	Local recreational area	Soil	1	Negative
5	Vehicle #3	Swab	4	*C. neoformans* (1)
	Investigator #3 (shoes)	Swab	2	Negative
	Local road stop	Soil	1	Negative
	Vehicle #4	Swab	4	Negative
	Investigator #4 (shoes)	Swab	2	Negative
	Office space	Soil	1	Negative
	Vehicle #5	Swab	4	*C. neoformans* (1)
	Investigator #5 (shoes)	Swab	2	Negative
	Local road stop	Soil	1	Negative
	Vehicle	Swab	4	Negative
6	Investigator (shoes)	Swab	2	Negative

Sampling Locations	Sample Type	Sample Types	# of Samples	Result (n)
	Vehicle Floor Matt	Swab	1	Negative