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Original Article

Immunomagnetic Isolation of *Cryptococcus neoformans* by Beads Coated with Anti-*Cryptococcus* Serum

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Abstract

Immunomagnetic separation (IMS) was utilized for the selective isolation of *Cryptococcus neoformans* from environmental sources, such as soils and pigeon droppings. Magnetic beads coated with anti-cryptococcal IgG (serotypes A and B) were used to isolate the fungus. In a modeled spiking experiment using *C. neoformans* serotype A strain and anti-serotype A antibody, the recovery rate of the cells was more than 47%. Specificity experiments using *C. neoformans* and *Candida albicans* showed that the beads, when coated with specific antibody for *C. neoformans*, were highly effective for the separation of *C. neoformans* strains from *C. albicans* (more than 97%). The IMS of serotype B cells with purified anti-serotype B antibody indicated a high specificity. When this IMS technique was applied to soils and pigeon droppings, *C. neoformans* cells were selectively isolated from 3 out of 8 samples, and *C. neoformans* DNAs were identified by PCR. Therefore *C. neoformans* cells were thus selectively isolated and the efficiency of the technique further confirmed.

Key words: *Cryptococcus neoformans*, immunomagnetic separation, magnetic beads, environmental isolate

Introduction

Cryptococcus neoformans is a cosmopolitan fungus and causes cryptococcosis in humans and animals. This infection is believed to be the most prevalent, life-threatening mycosis and to have worldwide distribution[1-4]. Human infection is thought to be acquired by the inhalation of infectious propagules from the environment[1-3]. The etiological niche of pathogenic *C. neoformans* has been viewed as soils, decaying wood, and avian habitats, particularly pigeon droppings. A strong ecological association of *C. neoformans* with pigeon droppings was reported by Emmons in 1955[3], and since this debris has been seen as a constant environmental reservoir of *C. neoformans*, it has been assumed that exposure to it, or its associated soils, is the most likely way for humans to develop cryptococcosis[1,2,5]. However, since there have been only a few confirmed incidences of pigeon-dropping-associated cryptococcosis, a strong association has not been confirmed and the role of this source in the disease remains controversial[5].

Immunomagnetic separation (IMS) techniques have been shown to be efficient for recovering specific eukaryotic cells from fluids, as well as for the separation of prokaryotic pathogens from heterogenous samples such as blood, food and fecal matter[6]. Integrated systems for IMS and PCR technology have recently been presented that facilitate the rapid DNA diagnosis of some bacteria, viruses, and other parasites[6-11]. In this paper, we investigated the usefulness of an immunomagnetic bead separation technique designed specifically for *C. neoformans* to facilitate

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Table 1. Selective isolation of *C. neoformans* from soils and pigeon droppings by IMS beads

Isolation no.	Day isolated	Sample collected	Detection [1] rate	Geographic location in Chiba
1	Sep. 30. 2002	Pigeon dropping	0/2	Chiba temple
2	Oct. 21. 2002	Pigeon dropping	0/1	Pet shop in Ichihara
3	Oct. 28. 2002	Pigeon dropping	2/2	Pigeon house in Toke
4	Sep. 30. 2002	Soil	0/1	Chiba temple
5	Oct. 28. 2002	Soil	1/1	Soil near pigeon house in Toke
6	Nov. 3. 2002	Soil	0/1	Hedge in front of this center [2]
Total			3/8	

1) Detection rate: number of positive samples/number of total samples.
2) This center: Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University.

the isolation of this fungus from its usual environmental sources such as soils and pigeon excrement.

Materials and Methods

Coating of the immunomagnetic beads with antibody:

Immunomagnetic beads were prepared by the method of Enroth and Engstrand[6]. Briefly, 1.5 μl of purified polyclonal rabbit anti-*C. neoformans* IgG (serotype A, 5.53 mg/m*l* protein, agglutination titers of 128×) was incubated with 500 μl of magnetic beads (6-7×10^8 beads per m*l*), which had been pre-coated with sheep anti-rabbit IgG (Dynabeads M-280, Dynal, Oslo, Norway), for 24 hr at 4℃ with gentle agitation. With the aid of a magnet, the beads were drawn to one side of the tubes and rinsed three times with 1m*l* of PBS containing 0.1% bovine serum albumin (BSA) for 30 min at 4℃ with gentle agitation. After the third wash, the beads were resuspended in 500 μl of PBS containing 0.1% BSA (PBS-BSA) and stored at 4℃.

Immunomagnetic beads for *C. neoformans* serotype B were prepared by the same method described above, and Crypto Check factor serum No.5 (Iatron Laboratories, Tokyo, Japan) was used for this experiment.

IMS method for the recovery of *C. neoformans* cells:

C. neoformans serotype A (ATCC 90113) strain was inoculated in potato dextrose agar (PDA, Difco, Detroit, Michigan, USA) slant and incubated for 2 or 3 days at 30℃. The fungal cells were suspended in 50 mM phosphate buffer (pH 7.2) and tenfold serial dilutions of the fungus were prepared: 10^1 to 10^3 fungal cells per m*l*. Twenty microliters of the coated beads was added to 1 m*l* of each dilution of fungal cell suspension and IMS was performed by the method of Enroth and Engstrand[6]. In brief, after incubation with the beads for 1hr at 4℃ with gentle agitation, the fungal cells were drawn to one side of the centrifugation tube (FALCON, Becton Dickinson and Co., Franklin Lakes, USA) with the magnet and the liquid layer was discarded. Then one milliliter of PBS-BSA was added and well mixed, and again the liquid layer was discarded with the help of the magnet. This washing was repeated three times. After the final washing, the beads were resuspended in 500 μl of PBS-BSA. One hundred microliter aliquots were spread onto 5 PDA plates. The colony numbers after incubation for 72hr at 30℃ were counted and compared with those of the control plate obtained without the IMS treatment.

Confirmation of combination of immunomagnetic beads and fungal elements:

Twenty microliters of the immunomagnetic beads was added to 1 m*l* of *C. neoformans* suspension (10^3cells/m*l*); the mixed suspension understood was incubated for 1hr at 4℃ and then was washed three times in PBS-BSA. The washed beads were resuspended in 500 μl of PBS-BSA. Ten microliters of resuspension was placed on a microscope slide and stained with a fluorescent reagent of FUNGIFLORA-Y (Biomate Co., Tokyo). After the addition of one or two drops of the reagent solution, *C. neoformans* cells attached to the beads were observed by a fluorescence microscope (excitation UV of 330-380 nm).

Competitive isolation of *C. neoformans* from the mixture of *C. neoformans* and *C. albicans*:

C. neoformans serotype A (ATCC 90113) and *C. albicans* (ATCC 90028) strains were also used for comparative studies. To 1 m*l* of a mixed suspension of *C. neoformans* (5×10^2 cells/0.5m*l*) and *C. albicans* (5×10^2 cells/0.5m*l*), 20 μl of the coated beads was added and IMS performed as described in the IMS method. The respective colony numbers for *C. neoformans* or *C. albicans* were determined by characteristic coloration of each fungus on a CHROMagar (Kanto Kagaku Co., Tokyo) plate.

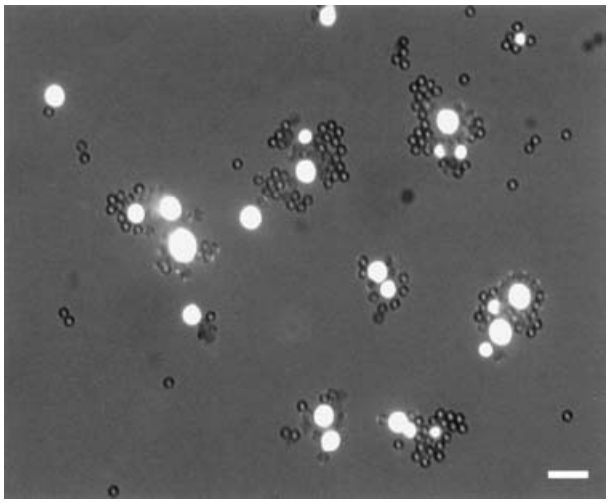

Fig. 1. Microscopic observation of *C. neoformans* cells surrounded by immunomagnetic beads. Bar indicates 10 μm.

Application of IMS method for selective separation of *C. neoformans* from environmental samples:

Eight samples of soil or pigeon droppings were collected from various sites in Chiba Prefecture (Table 1), and about 500 mg of soil or pigeon droppings was mixed into 5 m*l* of 0.8% saline and agitated for 8 to 10 minutes. After standing for a few minutes, the suspension was filtered through sterilized gauze. Twenty microliters of beads was added to the resultant filtrate and gently agitated for 1 hr at 4℃. After magnetic isolation of the beads, 2 to 3 m*l* of PBS-BSA was added and the beads were washed three times, then resuspended in 500 μl of PBS-BSA. One hundred microliter aliquots were spread onto 5 PDA plates containing chloramphenicol (100 μg/m*l*, final concentration). After incubation at 30℃ for 72 to 96 hr, fungal colonies were selected.

Confirmation of fungal species identification as *C. neoformans*:

Fungal DNAs were extracted by a modified method of Poonwan *et al.*[12], Aoki *et al.*[13] and Tamura *et al.*[14]. The PCR primer pair of CN4 and CN5, which was originally prepared by Mitchell *et al.*[15] and is specific for this fungus, was used to identify species of *C. neoformans*. Amplification reactions were performed in a volume of 30 μl of distilled water containing 1 μl of primer (20 pM), 1 μl of genomic DNA (1 μg/m*l*) and one PCR bead (Ready-to-Go PCR bead, Pharmacia Biotech, Tokyo). The PCR was performed by the method of Mitchell *et al.*[15]. All reaction products were characterized by electrophoresis on 1.5% agarose gels in 1 × TBE buffer at 80 V for 90 min and then stained in 0.5 μg/m*l* of ethidium bromide solution.

Table 2. Recovery of *C. neoformans* by IMS beads coated with *C. neoformans* polyclonal antibody

Cell number [a]	Number of colonies without IMS (mean ± SD) [b]	Number of colonies with IMS (mean ± SD)	Recovery rate (%) [c]
10^3	840.5 ± 23.5	707.0 ± 7.0	84.1
10^2	93.0 ± 1.0	67.5 ± 4.5	72.6
10^1	8.5 ± 2.5	4.0 ± 1.0	47.1
NC [d]	0	0	0

a) Cell numbers: *C. neoformans* cells were adjusted to the designated numbers and were incubated with and without IMS beads.
b) SD: Standard deviation.
c) Recovery rate: number of colonies obtained with IMS/number of colonies obtained without IMS (%)
d) NC: Negative control.

Crypto Check (Iatron Laboratories) was used to determine the serotype of *C. neoformans*[16].

Determination of fungal species by PCR using ITS sequence information:

For amplification of the ITS1 and ITS2 regions the following PCR primer pair was used: ITS-5: 5'-GGAAGTAAAAGTCGTAACAAGG-3', and ITS-4: 5'-TCCTCCGCTTATTGATATGC-3'. Amplification reactions were performed by the same methods used by Tamura *et al.*[14]. The PCR products were purified with a PCR product presequencing kit (USB corp., Cleveland, Ohio, USA). The DNA sequences were determined with an automatic sequence analyzer (ABI PRISM™ 310, PE Applied Biosystems, Tokyo) using a dye terminator cycle sequencing kit (PE Applied Biosystems). Sequences of the ITS regions were compared by performing a sequence database search using BLAST, and sequence similarity values were calculated manually.

Results and Discussion

Binding of the magnetic beads to *C. neoformans* cells was confirmed microscopically (Fig. 1). The number of beads around the fungal cells varied from 1 to 10 per cell. To determine the binding ability of the beads to *C. neoformans* cells, a serially diluted solution of fungal cells was added to the beads and the recovery rates were determined. As shown in Table 2, the rates of recovery were more than 47% in this modeled spiking experiment, and this was confirmed by duplicate experiments.

The usefulness of the magnetic beads for the specific isolation of *C. neoformans* was determined using a mixture of fungal cells of *C. neoformans* and *C. albicans*. When the beads were added to the mixture of both fungal cells, the recovered cells were mainly *C. neoformans* (more than 97%), as shown in Fig. 2A and 2B. This data clearly

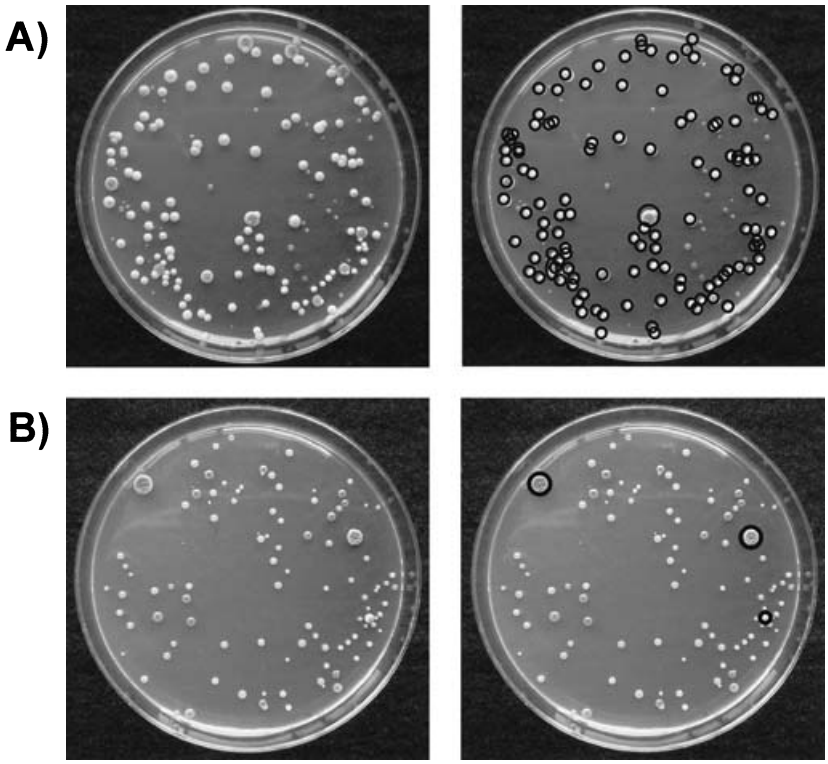

Fig. 2. Selective separation of *C. neoformans* from the mixture of *C. neoformans* and *C. albicans* cells by IMS techniques. Each fungal cell was separated by color selection of purple-white colony of *C. neoformans* from green colored colony of *C. albicans* on CHROMagar. Colonies of *C. albicans* were surrounded by a circle (right plate). A): Control plate with the mixed cells of *C. neoformans* and *C. albicans*, B): *C. neoformans* colonies with small numbers of *C. albicans* by the IMS treatment.

shows that the beads selectively bind to *C. neoformans*, even in the presence of other fungi.

Since in our present experiment the specificity of the magnetic beads to *C. neoformans* cells was confirmed, this IMS technique was applied to environmental samples, i.e. soils and pigeon droppings. At first, 20 μl of the beads was used per ml of the filtered solution in accordance with the original operation described by Enroth and Engstrand[6]. But the beads agglutinated in the test tube and did not work well; therefore, we used 20 μl of beads per tube. With this technique, 8 samples, including 5 pigeon droppings and 3 soils collected from different sites in Chiba Prefecture, were tested for the presence of *C. neoformans*. As shown in Table 1, we isolated *C. neoformans* cells from 3 of the 8 samples tested (2 pigeon droppings and 1 soil).

These results show that this technique is useful for the selective isolation of *C. neoformans* cells from various environments. We reported previously that the number of these cells in soils or pigeon droppings was too small to be detected by original plate culture methods[1, 17-19], even using various selective media or a single PCR method, and that a nested PCR system is necessary to detect the fungus[20]. The nested PCR method indicated that 18.2% of pigeon droppings contain *C. neoformans* DNA.

We also reported that fresh pigeon droppings obtained from a zoo had no detectable level of *C. neoformans*[20], suggesting that the droppings were contaminated by the fungus via nearby soils and other environments after their excretion. Therefore, we selected old pigeon droppings in the present experiments, but these did not always contain *C. neoformans* cells. Throughout the present studies, we found that soil samples obtained from near pigeon aviaries contained a higher amount of fungal cells than did pigeon droppings (data not shown). These facts may suggest that contaminated soils with avian excreta are more important reservoirs of *C. neoformans*, and that such soil particles may be an important source of human infection.

During our isolation studies of *C. neoformans* from soils and pigeon droppings, about 100 colonies were observed on PDA plates. The fungal cells that showed yeast-like colonies (about 60% among 100 colonies/plate) were isolated from 5 PDA plates. These cultures were inoculated on CHROMagar plates, and all of the cultures

Table 3. Recovery of *C. neoformans* serotype B strain by IMS beads coated with *C. neoformans* serotype B specific purified polyclonal antibody [a)]

Cell [b)] number	Number of colonies without IMS (mean ± SD) [c)]	Number of colonies with IMS (mean ± SD)	Recovery [d)] rate (%)
10^3	1192.5 ± 449.5	1125.0 ± 455.0	94.3
NC [e)]	0	0	0

a) Factor serum No.5 (Crypto Check) was used for this experiment.
b) Cell numbers: *C. neoformans* cells (serotype B) were adjusted to the designated number.
c) SD: Standard deviation.
d) Recovery rate: number of colonies obtained with IMS/number of colonies obtained without IMS (%)
e) NC: Negative control.

produced faint purple colonies. Since these characteristics like faint purple coloring were similar to those of *C. neoformans*, we first believed all of them to be *C. neoformans*. However, PCR using CN primers failed to detect the specific 136 bp of amplified band in some of these colonies: approximately 50% of these isolates were identified as *C. neoformans* by PCR and ITS sequence analysis, and these were reconfirmed by Crypto Check as serotype A *C. neoformans*. In subsequent PCR identification studies using the ITS region sequence analysis we found that some of the remaining fungal isolate colonies with faint purple coloring belonged to a bacidiomycetous fungus, *Filobasidium uniguttulatum* (accession number submitted to DDBJ is AB097079). In addition to these fungi, another filamentous fungus, which was easily differentiated from *C. neoformans*, was also isolated by this technique, although the number of colonies found was small. Since we coated the beads in this experiment with a polyclonal rabbit antibody that is supposed to be specific for *C. neoformans*, isolation of this *C. neoformans*-related fungus or other non-identified filamentous fungus may be due to its being immunologically related to *C. neoformans*. Further experiments using a purified polyclonal antibody for *C. neoformans* were therefore conducted. As shown in Table 3, when *C. neoformans* serotype B serum (purified factor serum No. 5 from Crypto Check) was used, the beads were found to be highly effective for the isolation of serotype B strain and the recovery rate was more than 94 %. When the mixed cells of *C. neoformans* serotypes B and A were used, this specificity was also confirmed. The results suggest that the IMS specific for serotype B strains can isolate this serotype more selectively from the mixture of serotypes A and B, with a recovery rate of 80%. Therefore, introduction of specific anti-serotypes C, D and A/D is also expected to lead to the specific separation of each serotype from various environmental sources.

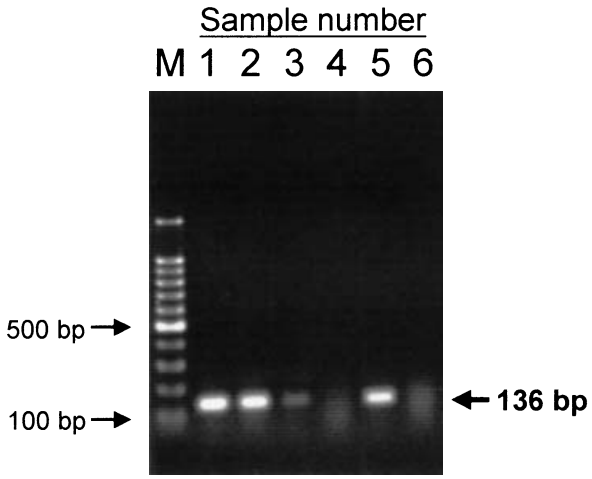

Fig. 3. Fungal species confirmation of *C. neoformans* isolated in the present experiments via PCR. Only *C. neoformans* cells showed amplification of PCR band at 136 bp. Sample number shows the colony number obtained by random selection from PDA plate. Sample numbers 1, 2, 3 and 5 indicated positive samples, the others negative.

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