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Prevalence of *Cryptococcus neoformans* in Clinical Specimens

E. DORKO[a], M. KMEŤOVÁ[b], F. DORKO[c], I. BRAČOKOVÁ[a], J. DANKO[d], E. ŠVICKÝ[e], Ľ. TKÁČIKOVÁ[f]

[a]*Department of Physiology,* [b]*Department of Microbiology and* [c]*Department of Anatomy, Faculty of Medicine, Šafárik University, 040 01 Košice, Slovakia*

[d]*Department of Anatomy,* [e]*Department of Pathology and* [f]*Department of Microbiology, University of Veterinary Medicine, 040 01 Košice*

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ABSTRACT. *Cryptococcus neoformans* isolated from various clinical materials in 14 cases, was identified by (*1*) cultivation on Sabouraud glucose agar and CHROMagar *Candida*, (*2*) microscopic examination of Indian-ink-stained preparations and (*3*) determination of biochemical properties (assimilation and fermentation of saccharides, assimilation of KNO_3, production of urease and phenol monooxygenase). *C. neoformans* was determined in five specimens from pædiatric patients in the intensive care unit and in nine specimens from adult patients, most frequently from liquor at meningitis ($n = 3$).

Opportunistic infections induced by capsulated yeast *Cryptococcus neoformans* are a frequent cause of morbidity and mortality particularly in immunosuppressed patients. The disease develops most frequently following inhalation of the causative microorganisms. The *C. neoformans* cells pass *via* blood from alveoli to the target organs, preferentially to the central nervous system where they cause life-threatening meningitis or meningoencephalitis (Arsic *et al.* 1995; Khanna *et al.* 1996; Gomes *et al.* 1997; Dunbar *et al.* 1998). They infect frequently the lungs and less frequently the skin, eyes, liver, bones, joints, muscles, genitals, medulla and others (Klobušický *et al.* 1995; Mitchell and Perfect 1995; Tanaka *et al.* 1996; Kimura *et al.* 1997; Mauri *et al.* 1997; Biswas *et al.* 1998; Drew and Takezawa 1998; Liu 1998; Marcus *et al.* 1998; O'Neill *et al.* 1998; Raftopoulos *et al.* 1998).

Cryptococcosis is the prevalent systemic mycosis in AIDS patients and its clinical manifestations usually appear after the number of $CD4^+$ lymphocytes decreases below 200/μL (Bava *et al.* 1997). Cellular immunity is the essential defense against *C. neoformans* and because of that patients with deficient cellular immunity are infected primarily. The main predisposition factors for the development of cryptococcosis are AIDS, lymphoma, corticosteroid therapy and idiopathic CD4 T lymphopenia (Hogan *et al.* 1996). Despite the world-wide distribution of this disease it occurred only sporadically up to 1980. Typical cases developed in individuals with immune system disorders, such as patients with Hodgkin's disease (Korfel *et al.* 1998). The rapid increase in AIDS pandemic has been associated with increase in *C. neoformans*-induced diseases. The incidence of *C. neoformans* infection in AIDS patients ranges from 3 to 15 % although the incidence in some regions of Africa is higher (Arsic *et al.* 1995; Khanna *et al.* 1996; Bava *et al.* 1997; Molez 1998).

Suspect cryptococcosis cases are detected by direct microscopic examination of the liquor, blood, urine, *etc.* and the proof is obtained by serological examination (latex agglutination, ELISA), PCR, cultivation, and histological biopsy examination (Arsic *et al.* 1995; Mitchell and Perfect 1995; Khanna *et al.* 1996; Tanaka *et al.* 1996; Zerpa *et al.* 1996; Bava *et al.* 1997; Kimura *et al.* 1997; O'Neill *et al.* 1998; Raftopoulos *et al.* 1998).

Here we focus on the incidence of *C. neoformans* in the array of other yeast microorganisms isolated from infectious material.

MATERIAL AND METHODS

The specimens of clinical material originated from patients admitted to the *Faculty Hospital* in Košice. The examined strains were isolated from the liquor, urine and sputum, and swabs taken from the rectum, oral cavity, vagina, conjunctival sac and skin of patients with meningitis, kidney transplant, bronchopneumonia, hydrocephalus, leukopenia, leukæmia, Hodgkin's disease, soor, stomatitis prothetica, vaginitis, keratoconjunctivitis and dermatitis.

Cultivation was carried out on Sabouraud glucose agar (SGA; *Imuna*, Slovakia) and CHROMagar *Candida* (CHaC; *Mast Diagnostica*, England) at 37 °C for 2 d (Odds and Bernaerts 1994; Arsic *et al.* 1995; Khanna *et al.* 1996; Gomes *et al.* 1997). We investigated the ability of yeasts to grow at 37 °C. This property is an auxiliary identification criterion for distinguishing *C. neoformans* from other *Cryptococcus* species.

The yeast isolate was inoculated in parallel to two tubes with agar slants (Sabouraud agar). One of the tubes was incubated at 27 °C, the other one at 37 °C and the growth was checked after 2–4 d; the growth of the yeast both at the higher temperature and at 27 °C indicated positive result (Sandven 1990).

The ability to produce urease was tested on Christensen's agar slants. The respective agar was inoculated with the culture and incubated (27 °C, 5 d). In the case of a positive reaction the color of the medium changed from yellow to pink or to red while in the case of negative reaction the Christensen's agar remained unchanged (Sandven 1990). We investigated also assimilation and fermentation of saccharides, assimilation of KNO_3 and formation of phenol monooxygenase (Otčenášek 1990; Sandven 1990).

The presence of capsule was confirmed in preparations stained with Indian ink. One drop of this ink was applied to a slide and mixed with the material examined (liquor, urine, exudate and others) or with water suspension of the examined strain from the agar medium. The capsulated cells were surrounded by a light unstained zone visible against a dark background (Sandven 1990; Khanna *et al.* 1996; Bava *et al.* 1997; Raftopoulos *et al.* 1998).

The assimilation of saccharides was detected on auxanograms during 1–2 d at 27 °C, and fermentation (7–10 d, 27 °C) on zymograms (Otčenášek 1990).

RESULTS

The *C. neoformans* colonies grown on SGA were 3–7 mm in diameter, white to gray at the beginning of cultivation and creamy, smooth, glossy and mucous later on. They had a convex surface and circular or slightly lobulated but non-fibrous edges. The colonies grown on CHaC were gray or light pink. They grew better at 37 than at 27 °C. They gave positive results in the urease test on the basis of color change of Christensen's medium. The Indian ink stained preparations, prepared either directly from the infectious material or from the cultures grown on SGA, allowed us to observe unstained capsules of varying thickness around the spherical cells. The biochemical properties of *C. neoformans* strains included assimilation of glucose, galactose, sucrose, maltose, raffinose, trehalose, cellobiose and xylose; they failed to assimilate lactose and melibiose; the zymograms showed no fermentation of any of the six saccharides. Formation of phenol monooxygenase was positive (brown color) and assimilation of KNO_3 was negative (Table I).

Table I. Biochemical and morphological characteristics of *C. neoformans*

Assimilation[a]	Glc +, Gal +, Sac +, Mal +, Raf +, Tre +, Cel +, Xyl +, Lac –, Mel –, KNO_3 +
Fermentation[a]	Glc –, Gal –, Sac –, Mal –, Raf –, Lac –
Growth on SGA	colony white, gray, cream, smooth, glossy, mucoid; surface convex; margin spherical or lobulated but non filamentous
on CHaC	colony gray, pale pink
at 37 °C	better than at 27 °C
Production of urease and phenol monooxygenase[a]	+
Microscopy	cells spherical with capsule

[a]Result positive +, negative –; Glc – glucose, Gal – galactose, Sac – sucrose, Mal – maltose, Lac – lactose, Raf – raffinose, Tre – trehalose, Mel – melibiose, Cel – cellobiose, Xyl – xylose.

From among the 336 non-*albicans Candida* species *C. neoformans* was isolated in only 14 cases, from 9 adult patients and 5 premature babies admitted to a neonatal intensive care unit. With neonates we detected 1 strain in each a skin swab, liquor and oral cavity, and 2 strains from the rectum for the following diagnoses: unspecified skin infection with erythematous rash, meningitis, a pædiatric patient subjected to intubation and exhibiting symptoms of oral candidosis, hydrocephalus and an immunodeficient patient with decreased number of leukocytes. Only one of the pædiatric patients was a girl.

The adult patients were 22–71 years old (44.2 on the average); the age of females (n = 4) ranged from 22 to 45 years (34.5 on average) while that of males (n = 5) was in the range of 28–71 years (52.4 on the average). In the group of females *C. neoformans* was isolated successfully from the liquor of a woman with meningitis, from a vaginal swab of a patient with a primary diagnosis as diabetes mellitus, complaining of

a burning sensation and itching in the area of outer genitals, from the urine of a kidney transplant patient in association with immunosuppressive therapy and from the conjunctival sac at keratoconjunctivitis. In the male population one strain was proved with each meningitis, stomatitis prothetica in the oldest patient (71 years) with total dental prosthesis, Hodgkin's disease, chronic leukæmia and bronchopneumonia with lung tumor from the liquor (1), oral cavity swab (3) and sputum (1) (Table II).

The results indicate that the incidence of cryptococcosis was higher in males (9; 64 %) than in females (5; 36 %) and occurred more frequently in adults than in the pædiatric patients.

DISCUSSION

Cryptococcosis prevails currently in immunodeficient patients, particularly in those positive for HIV (Mitchell and Perfect 1995; Khanna *et al.* 1996; Bava *et al.* 1997; Molez 1998). The low prevalence of *C. neoformans* may by related to the relatively low incidence of AIDS in Slovakia in comparison with the developed and developing countries. *C. neoformans* was isolated most frequently from meningitis patients which is in agreement with the studies of many authors and is also related to the affinity of *C. neoformans* for the central nervous system (Arsic *et al.* 1995; Khanna *et al.* 1996; Zerpa *et al.* 1996; Gomes *et al.* 1997; Dunbar *et al.* 1998; Molez 1998).

Table II. Incidence of *C. neoformans* in clinical material in relation to diagnosis

Diagnosis	Material	Number
Bronchopneumonia	sputum	1
Dermatosis	skin	1
Hydrocephalus	rectum	1
Keratoconjunctivitis	conjunctival sac	1
Leukæmia	oral cavity	1
Leukopenia	rectum	1
Meningitis	liquor	3
Hodgkin's disease	oral cavity	1
Soor	oral cavity	1
Stomatitis prosthetica	oral cavity	1
Transplantation of kidney	urine	1
Vaginitis	vagina	1
Total	–	14

The highest number of positive *C. neoformans* isolates was from the oral cavity. One of the positive oral swabs was obtained from a premature baby with introduced plastic endotracheal cannula. Yeast organisms are known for their ability to adhere to plastics (cannula, catheter and similar) and induce infections in individuals with hyperalimentation, endotracheal intubation and similar, common in neonatal intensive care unit (Dorko *et al.* 1999). *C. neoformans* was also isolated from an older patient with stomatitis prosthetica. Resin prostheses are a reservoir of yeasts, particularly in people with inadequate oral cavity hygiene habits despite the antimicrobial action of factors present in saliva (secretory IgA, lysozyme, peroxidase system, lactoferrin) (Budtz-Jörgensen *et al* 1996; Dorko and Ďurovičová 1999). Korfel *et al.* (1998) pointed to the low incidence of cryptococcosis in patients with Hodgkin's disease and risk factors such as lymphopenia and preceding aggressive therapy. In contrast to our results, they isolated *C. neoformans* from the liquor and blood (Korfel *et al.* 1998).

In the case of the two premature babies admitted to neonatal intensive care unit we isolated *C. neoformans* from the rectum swabs with hydrocephalus and leucopenia diagnosis. Both babies were immunodeficient and thus they provided proof of the prevalence of cryptococcosis in immunocompromised patients reported by a number of authors.

One *C. neoformans* strain was isolated from skin and vaginal swabs, from urine, conjunctival sac and sputum; it occurs rarely in females suffering from diseases of sexual organs. Other yeast species predominate in such cases as was presented in our previous study (Dorko *et al.* 1999, 2000) and in the paper by Klobušický *et al.* (1995). One of the possible risk factors predisposing to candida vulvovaginitis is diabetes mellitus diagnosed also in our patient. Cryptococcosis occurs frequently following the transplantation of organs to immunosuppressed patients. We detected it in a patient with kidney transplant in agreement with the results of Arsic *et al.* (1995). *Cryptococcus* eye infections are rare in healthy individuals and occur frequently in immunocompromised patients. They are recurrent and resistant to antifungal therapy (Biswas *et al.* 1998). Our female patient with keratoconjunctivitis showed no immunodeficiency symptoms. On the basis of a large number of papers one can state that pneumonias are most frequent cryptococcal infections and that the infection can spread from lungs to other tissues and organs through the blood. Clinical diagnosis of pulmonary cryptococcosis in patients without clinical symptoms is difficult and is based on biopsy examination of pulmonary tissue, detection of antigens in the serum and the use of polymerase chain reaction (Tanaka *et al.* 1996; Kimura *et al.* 1997). Our results differ considerably from those of other authors who

recorded a high prevalence of this microorganism in patients with pulmonary diseases. We detected *C. neoformans* only in one patient with bronchopneumonia in association with a lung tumor.

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