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Prevalence of Yeasts in Human, Animals and Soil Sample at El-Fayoum Governorate in Egypt

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Abstract: A survey was done for the most common yeast isolates from different clinical sources from human and animals in addition to environment samples from soil in El-Fayoum Governorate. The total of tested samples was 802 clinical and environmental samples. They consisted of fifty human vaginal swabs, seventy one human throat swabs, three hundred fifty seven animal vaginal swabs, one hundred seventy nine animal nasal swabs and one hundred forty five soil samples. The yeast isolates were representatives for only 3 species *Candida albicans* (110 isolates), *Cryptococcous neoformans* (20 isolates) and *Rhodotorula rubra* (66 isolates), on the basis of the conventional mycological identification tests. The results were confirmed by molecular - identification for the representative isolates of *Candida albicans* and *Cryptococcous neoformans* recovered from different sources.

Key words: Prevalence · *C. albicans* · *C. neoformans* · Animal · Human · Soil · PCR

INTRODUCTION

Fungal infections, particularly yeast infections, represent the most wide spread and prevalent mycotic diseases of man and animals [1]. The incidence and prevalence of serious mycoses continue to be a public health problem. Despite aggressive treatment with new or more established licensed antifungal agents, these infections are important causes of morbidity and mortality, especially in immunocompromised patients [2].

C. neoformans causes a lethal meningoencephalitis in immunocompromised patients and has become the most common cause of meningitis due to AIDS- related infections in Africa [3]. On the other hand, *C. albicans* is considered a commensal organism of humans colonizing the oral cavity, gastrointestinal and reproductive tracts. However, when host defenses are compromised, *C. albicans* can transform into a tissue invasive pathogen [4]. Yeast strains are identified according to morphological cultural, physiological characteristics and biochemical tests [5]. Recently, molecular biology techniques are increasingly used in the identification of yeasts, particularly *C. albicans* [6- 8].

The accuracy of the PCR was found to be 70.85%, where PCR proved to be a rapid diagnostic technique for detection of pan fungal genome directly from clinical specimens [9]. The sensitive and specific nested PCR assay as well as the rapid and quantitative Light Cycler PCR assay might be useful for the diagnosis and monitoring of human crytococcal infections [10].

The aim of this work was to study the prevalence of *C. albicans* and *C. neoformans* in the vaginal mucosa and animal (cattle, buffaloes, sheep and goats) as well as the soil in their vicinity in El-Fayoum Governorate by conventional and PCR identification techniques.

MATERIALS AND METHODS

Collection and Cultivation of Samples

Human and Animal Samples: A total of 657 swab samples was collected from different pathological and healthy cases including 121 from human (50 vaginal and 71 throat) and 536 from animal (357 vaginal and, 179 nasal), in addition to 145 soil samples collected from farms, pastures and houses of cattle, buffaloes, sheep and goats as well as from the floor of veterinary clinics and from the canopies of eucalyptus trees. All the samples were taken

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by sterile swabs in sterile saline and transferred directly to the laboratory and inoculated into sterile brain-heart-infusion broth, incubated at 37°C for 6-18 h, then streaked onto SDA plates with chloramphenicol and incubated at 37°C for 2-5 days.

Soil Samples: Soil samples were collected in sterile plastic bags and the samples were prepared by mixing about 3-5 g of each sample into sterile test tubes containing 15-25 ml sterile normal physiological saline solution containing 2 mg streptomycin and 500 I.U. penicillin / ml and closed with sterile rubber stoppers. The tubes were shaken vigorously by vortex, then allowed to stand for about 15 min. The supernatant of each prepared sample was taken and streaked onto plates of SDA with chloramphenicol and incubated at 37°C for 2-5 days.

Isolation and Conventional Identification of Yeast Isolates: Pure yeast isolates were subjected to different mycological conventional identification methods including morphological and differential biochemical identification tests [11].

Extraction of Genomic DNA from both *C. albicans* and *C. neoformans*: The genomic DNA was extracted from the prepared samples according to Sambrook [12].

The concentration of the resulting DNA was then evaluated. Recovery yield should be between 10 and 20 ng with purity satisfactory for PCR reaction.

Detection of *C. albicans* DNA by PCR

***Candida albicans* Specific Primers Set:** SAP123: forward primer (5′-CTG ATT TAT GGG TTC CTG AT-3′) chosen for specific amplification of SAP1, SAP2 and SAP3 gene and the used reverse primer was SAP3 (5′- CAT GTC CCT TGT GAA GTA GT- 3′) (Fermentas, AB. Gene) (MWG,oligosynthesis-Germany). The expected size of the amplified fragments from *C. albicans* SAP3 gene was 172 bp [13].

The PCR reaction was performed in 25 µl mixture containing Tris-HCL [20 mM] [PH 8.4], KCL [50 mM] and $MgCl_2$ [2 mM], ach at a concentration of 0.4 µM, 0.2 mM of each dNTPs and 2U Taq DNA polymerase. Concentration was 3 µl of each template. The cycling conditions of denaturing - annealing - synthesizing cycle for amplification with a DNA thermal cycler were as follows; one cycle of initial denaturation 94°C for 5 min, followed by 35 cycles of denaturation 94°C for 1 min, annealing 55°C for 1 min, extension 72°C for 2 min and final extension 72°C for 5 min.

Detection of *C. neoformans* DNA by Nested-PCR: *Cryptococcus neoformans* specific primers set: Primer oligonucleotides I and II. (2 external primers for 1st round PCR), 5′ GTT AAA AAG CTC GTA GTT G 3′, 5′ TCC CTA GTC GGC ATA GTT TA 3′. Primer oligonucleotides cryp III and IV. (2 internal primers for 2nd round PCR) 5′ TCC TCA CGG AGT GCA CTG TCT TG 3′, 5′ CAG TTG TTG GTC TTC CGT CAA TCT A 3.′ where the expected size of fragment amplified in all positive samples was 278 bp [13]. 10 µl of template DNA in a final volume of 50 µl was used for the 1st round PCR and 1 µl of the 1st round amplification reaction mixture was used as a template for the 2nd round PCR. The cycling conditions of denaturing - annealing - synthesizing cycle for amplification with a DNA thermal cycler were as follows: denaturation for 5 min at 94°C before 35 cycles of 94°C for 30 sec, 50°C for 30 sec (1st round PCR) and 72°C for 1 min and final extension at 72°C for 5 min. then initial denaturation 5 min at 94°C, 30 cycles of 94°C for 30 sec., 65°C for 30 sec (2nd round for nested PCR) and 72°C for 1 min and final extension at 72°C for 5 min [10]. Ten µl of PCR product were mixed with 2 µl of the orange /Blue 6X loading dye [Promega, Madison, WI.USA]. The whole mixture was delivered into the corresponding well in the agarose gel. Samples were electrophoresed on 1.5% [w/v] agarose gel in 1x TBE buffer containing [0.3 µg/ml] ethidium bromide for about 50-60 min. at 70 voltages in a minigel electrophoresis unit using 1x TBE as electrophoresis buffer [12]. The DNA bands [amplification products] were visualized on UV transilluminator and photographed. The sizes of the amplification products were compared with the used DNA marker (100 bp DNA marker ladder, Promega, Madison, WI.USA) that was loaded with the samples simultaneously.

RESULTS AND DISCUSSION

Yeasts were reported as potential pathogens and caused different diseases conditions in human and animals. particularly after prolonged antibiotic therapy, where the isolation of the same species of yeasts from the samples collected from the apparently healthy cases, does not indicate, as it is usually reported, that these yeasts are part of normal flora [14]. The incidence of infections by yeast pathogens in immunocompromised-patients has increased in the last two decades. High percentage of the examined cases was due to *C. albicans* infection [15]. *C. albicans* is known to cause several infections in man and animals as oral thrush, glossitis, stomatitis, vaginitis, placentitis, bronchial and pulmonary

Table 1: Results of isolation of *C. albicans*, *C. neoformans* and other yeasts from human, animals and soil samples:

		Cryptococcus Neoformans		*Candida albicans*		*Candida. species.*		*Rhodotorula rubra*		Total	
Samples	No. of samples	No.	%	No.	%	No.	%	No.	%	No.	%
Throat swabs Human	71	3	4.20	7.00	9.90	-	-	4	5.60	14	19.70
Nasal swabs Cattle	67	1	1.49	6.00	8.90	4	5.90	11	16.40	22	32.83
Buffalo	29	1	3.45	2.00	6.90	1	3.45	14	48.20	18	62.08
Sheep	39	-	-	8.00	20.50	8	20.50	3	7.69	19	48.70
Goats	44	1	2.20	13.00	29.50	8	18.10	4	9.00	26	59.00
Vaginal swabs Human	50	4	8.00	8.00	16.00	4	8.00	-	-	16	32.00
Cattle	180	4	2.20	35.00	19.40	47	26.10	12	6.60	98	54.40
Buffalo	102	3	2.90	10.00	9.80	24	23.50	10	9.80	47	46.00
Sheep	35	-	-	5.00	14.30	9	25.70	3	8.50	17	48.57
Goats	40	1	2.50	6.00	15.00	8	20.00	2	5.00	17	42.50
Soil	145	2	1.37	10.00	6.89	15	10.30	3	2.00	30	20.68
Total	802	20		110.00		128		66		324	

affections, chronic mucocutaneous candidiasis, nail infections, as well as generalized candidiasis and alimentary tract infection [16, 17].

In the present study, mycological isolation and identification of different yeast species from various human sources were reported (Table 1). The yeast isolation percentage was [32%] in human vaginal samples examined where, the most frequently isolated yeast species was *C. albicans* [16 %]. Other *Candida* species and *C. neoformans* were recovered in [8%]. The yeast isolation percentage was [19.7%] in human throat samples examined, where the most frequently isolated yeast species were *C. albicans* [9.9%], *R. rubra* [5.6 %] and *C. neoformans* [4.2%].

Several authors isolated different yeasts in different percentages, as *C. albicans* strains were isolated from vaginal swabs of asymptomatic normal women in percentage of 7.4 % [18]. Also, *C. albicans* was the most encountered Candida species with an incidence of 63% in candidemic cases [19]. Moreover, different members of *Candida* spp. were recovered from candidemic patients including *C. albicans* [40.4 %], *C. parapsilosis* [22.3%], *C. tropicalis* [16 %] and *C. glabrata* [12.8%] [20]. Therapy with two or more antibiotics, corticosteroid administration, intravascular catheterization for over 24 hours and neutropenia are the most accountable predisposing factors for *C. albicans* infection [21].

Moreover, it was reported that the respiratory tract [63.2%], followed by the urinary tract [10.5%] were the most common sites of infection with *C. albicans, C. parapsilosis, C. tropicalis* and emerging species such as *C. krusei* and *C. guilliermondii* [22, 23]. Cryptococcal pneumomia has been recognized as a distinct clinical disease. Moreover, in experimental animal primary pulmonary cryptococcosis can lead to extra pulmonary dissemination [24].

In the present study, the mycological isolation of different yeast species from various animal sources was positive in 47.8% of animal vaginal samples including [54.4 %] in cattle, [48.57 %] in sheep, [46 %] in buffalo and [42.5 %] in goats. The total yeast isolation percentage was 47.5% in total animal nasal samples including [62.08 %] in buffalo, [59 %] in goats, [48.7 %] in sheep and finally [32.83 %] in cattle.

The most frequently isolated yeast species were *Candida* species other than C. albicans from animal vaginal discharges [24.6%], followed by *C. albicans* [15.6 %], *R. rubra* [7.5 %] and *C. neoformans* [2.24 %], while, the most frequently isolated yeast species from animal nasal discharges were *R. rubra* [17.8 %], *C. albicans* [16.2 %], *Candida* spp. other than *C. albicans* [11.7 %] and *C. neoformans* [1.67 %].

Similar results were reported, where yeasts including *C. neoformans*, *C. albicans*, other *Candida* species, *R. rubra*, *G. candidum* and *T. cutaneum* were isolated from the reproductive tracts through vaginal discharges examined from different animals with reproductive disorders and apparently healthy ones including cattle, buffalo, sheep and goats in a total percentage of 51.8% [25]. It was reported that, significant differences in the species spectrum and distribution were documented between yeasts from dogs, cows and pigs. This is probably due to different environmental conditions and the endogenous origin of the yeast isolates [26].

The high percentages of yeasts isolated from vaginal discharges of human and animals might have a good support from the speculation that the opportunistic yeasts under many stress factors could become potentially pathogenic, that establish a disease condition or may be introduced to vagina on top of the secondary infections as the reproductive tracts of different animals are the major sources of yeasts [27]. On the other hand, it was mentioned that, *Candida* species can be found commensally in the vagina [28].

Additionally, yeasts may be introduced to the vagina as a result of insemination with infected male semen either naturally or artificially. This is applied also to pathogenic yeasts such as *C. neoformans*, which was isolated from neat bull semen [29] and from fresh semen samples from apparently healthy cattle and buffaloes as well as from the frozen semen of both local and foreign bull breeds and from the preputeal sheath wash samples collected from cattle bulls and buffalos [30]. Moreover, yeasts may be introduced through vagina as a result of setting of animals on contaminated soils due to the bad hygienic measures [31].

It is known that, yeasts reach the respiratory tracts of different animal species mainly through inhalation of the contaminated dusts. Also the environmental events such as construction or renovation act as being specific risk factors for invasive fungal diseases as a result of bad ventilation [32] as in case of fungal pneumonias which are most frequently caused by *C. neoformans*, especially in an enlarging immunocompromised population, where both *C. neoformans* and *Cryptococcus bacillisporus* often cause pulmonary mass lesions [33].

On the other hand, several authors recovered *C. neoformans* from goats suffering from severe pulmonary disease [34]. They mentioned that *C. neoformans var. gattii* is associated with Eucalyptus trees so, we might find an explanation from the fact that inhalation of the dust from the canopies of Eucalyptus trees by these goats may cause their infection with pulmonary cryptococcosis. Accordingly, environmental infections play an important role in the epidemiology of cryptococcosis.

In the present work, the isolation percentages of *C. neoformans* from human sources were high, specially that from vaginal discharges samples, where most of the vaginal discharges samples were taken from women rearing pigeon and bird breeders, as pigeons act as a natural carrier of *C. neoformans* in their droppings [35]. The infection is mainly by inhalation of dust mixed with contaminated pulverized droppings [36-38] especially that *C. neoformans* remains viable for two years or more in moist or desiccated pigeon excreta [39]. So, the infection becomes generalized in the body through hematogeneous spread, where infection may reach genital system leading to contaminated vaginal discharges.

The total yeast isolation percentage was 20.68 % including *Candida* species other than *C. albicans* in a percentage of 10.3, *C. albicans* 6.89, *R. rubra* 2 % and *C. neoformans* in a lowest percentage of 1.37.

Cryptococcus neoformans was isolated from soils of Virginia [40] where, the virulent strains of *C. neoformans* were found commonly and abundantly in pigeon manure under roosting sites. This saprophytic source has been recognized in many areas of the world [41] in Copenhagen and Odense, [42], Japan [34], Thuringia [43], Brazil [44] and in Egypt [25, 35]. In India, *C. neoforman* and *C. gatti* are widely spread in decayed wood inside trunk hollows of trees and in soil near the base of various trees [45].

In the present study, it is evident that, *C. neoformans* was isolated in a low percentage from soil samples. This may be due to the fact that *C. neoformans* strains are more susceptible to heat [46] and ultra violet radiation [10], as all soil samples were collected from Fayoum governorate characterized by a high climatic desert temperature.

The identification of *C. neoformans* and *C. albicans* using the conventional morphological and metabolic characteristics required several days after isolation [47] and in many critical and confusing situations, diagnosis needs other accurate, sensitive and rapid techniques. Therefore, molecular biology techniques were suggested for fungal infections diagnosis as the PCR fingerprinting, which can be a beneficial tool for global epidemiological studies [48].

In the present work, PCR of *C. albicans* and *C. neoformans* strains confirmed the identification in 90 % of the isolates. The profile of *C. albicans* isolates recovered from different sources showed an identical genotype of *C. albicans*. Only one isolate did not show the DNA band [band 7], which was recovered from nasal swab of sheep (Fig. 1).

The profile of *C. neoformans* from different sources [1-9] in Fig. 2 showed an identical genotype of *C. neoformans*. Only one isolate, which was isolated from nasal swab of goat [10], did not show the DNA band.

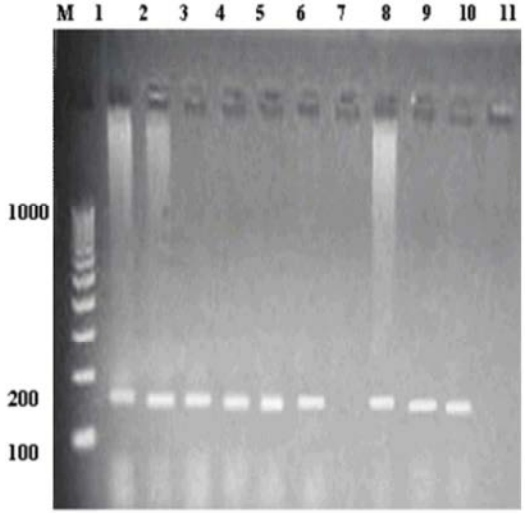

Fig. 1: Detection of *C. albicans*-DNA by using PCR, Lame M, 100 bp melecula weight market. Lanes 1-6 and 8-10, amplification of *C. albicans*-DAN from the isolates. Lame 7, no amplification of *C. albicans*-DNA and Lame 11, negative control

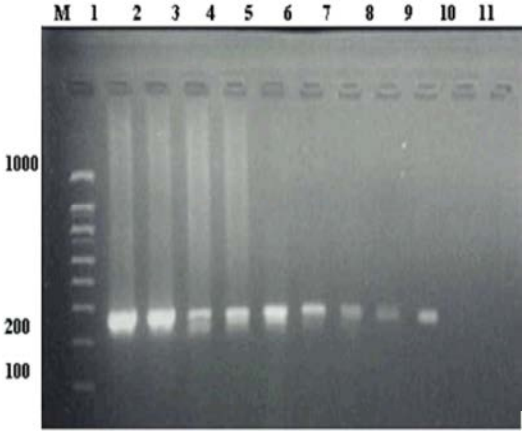

Fig. 2: Detection of *C. neoformons*-DNA by using nested- PCR, Lane M, 100 bp molecular weight marker. Lanes 1-9 amplification of *C. neoformons*-DNA from the isolates recovered from human, buffaloes and cow,s. Lane 10, no amplification of *C. neoformons*-DNA from goat isolatd and Lane 11, negative control

PCR-based assay was used to rapidly identify *Candida* spp. from positive blood culture bottles [49]. This assay used fungus-specific, universal primers for DNA amplification and species-specific probes to identify *C. albicans, C. krusei, C. parapsilosis, C. tropicalis* or *C. glabrata [Torulopsis glabrata]* amplicons. The accuracy of the PCR was found to be 70.85%, where PCR proved to be a rapid diagnostic technique for detection of pan fungal genome directly from clinical specimens. The sensitive and specific nested PCR assay as well as the rapid and quantitative LightCycler PCR assay might be useful for the diagnosis and monitoring of human cryptococcal infections [9].

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