Prevalence of *Candida albicans* and *Cryptococcus neoformans* in Animals from Quena Governorate with Special Reference to RAPD-PCR Patterns

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Abstract: The present study aimed to isolate different yeast types, particularly *Candida albicans* and *Cryptococcus neoformans* from different animals in Quena Governorate (Upper Egypt). For this study, 4527 samples were collected from buffaloes, cattle, sheep and chickens to be examined mycologically. Different yeast strains were recovered from 535 out of 4527 (11.81 %) animal samples. The percentage of yeast strains recovered from chickens (319 out of 1283; 24.86 %) was higher than that recovered from all other examined animals (216 out of 3244; 6.65 %). The examination revealed that the isolation percentage of *Candida* species (6.44 %) was higher than that of *C. neoformans* (2.54 %). The percentage of positive samples for *Candida albicans* was higher in chicken (12.15 %) than that obtained from other examined animals (3.2 %). Also, the number of positive samples for *C. neoformans* was obtained in a higher percentage in chickens (6.31 %) than that of all other examined animals (1.04 %). RAPD-PCR fingerprinting developed by OPA-18 primer showed two distinctive bands for all *C. albicans* strains recovered from different animal samples. The primer OPE-18 indicated the highest polymorphism for all fungal strains. R2 primer revealed identical RAPD-PCR patterns for all *C. neoformans* isolates recovered from buffalos, chickens and sheep.

[Shimaa Abou-Elmagd, Hosam Kotb, Khaled Abdalla and Mohamed Refai **Prevalence of *Candida albicans* and *Cryptococcus neoformans* in Animals from Quena Governorate with Special Reference to RAPD-PCR Patterns**] Journal of American Science 2011; 7(12): 20-31]. (ISSN: 1545-1003). http://www.americanscience.org.

Key words: *Candida albicans*, *Cryptococcus neoformans*, Animals, Chickens, RAPD-PCR

1. Introduction

Yeasts are found on a wide variety of substances such as soil, plants, water, nectar of flowers, fruits, trees and exudates of animals. They cause diseases in both man and animals such as thrush, disseminated candidosis, cryptococcosis, mastitis, etc. (Asfour *et al*., 2009). The excessive use of antibiotics, corticosteroids, immunosuppressive drugs as well as chronic diseases are the major contributing factors in increasing the incidence of diseases caused by yeasts (Jand *et al*., 2003; Das and Josef, 2005). The intestinal tract provides an important reservoir for many nosocomial pathogens, including *Candida* species and some bacterial species. Disruption of normal barriers, such as gastric acidity and endogenous microflora of the colon, facilitates the overgrowth of pathogens (Donskey, 2004). Refai (1998) reported that *C. albicans* and other *Candida* species can cause gastro intestinal candidosis in animals. The reproductive tracts of different animals are the major reservoir of yeasts such as *C. albicans* and *C. neoformans* (Chengappa *et al*., 1984; Kotb, 1990; El-Naggar *et al*., 1999). The fungal infections can occur in cervicovaginal cavity of Holstein dairy cows with or without reproductive diseases (Garoussi *et al*., 2007). Mastitis is one of the most serious problems in the dairy cattle farms. The great majority of cases are caused by bacteria, but recently there have been an increasing number of reports about cases caused by yeasts or yeast-like organisms (Saleh, 2005). However, *C. neoformans* and *C. albicans* are the most common pathogenic organisms of bovine mastitis. The incidence of mastitis due to yeasts is usually low in dairy herds, but sometimes it can occur in epizootic proportions. Teat injuries may predispose to the establishment of a yeast infection (Gonzalez, 2001; Jand *et al*., 2003; Spanamberg *et al*., 2009). In recent years, the growing economic value of poultry has led to the increase of research of poultry diseases. The fungal diseases of poultry have become problematic as bacterial and viral diseases (Darwish, 1989). The main goal of this study was to investigate the incidence of yeast infection, particularly *C. albicans* and *C. neoformans* in animals from Quena Governorate and characterize

the recovered fungal isolates at the DNA molecular level.

2. Materials and Methods

2.1. Media

All used media in this study were prepared according to Larone (2002).

2.2. Samples

A total of 4527 samples, either from animal products (feces, milk and blood) or rectal, nasal, vaginal, ear and conjunctival swabs, were collected from buffaloes, cattle, sheep and chicken. All samples were brought to the laboratory under complete aseptic conditions.

2.3. Preparation and cultivation of samples

Milk samples were collected aseptically in sterile screw capped bottles from mastitic or apparently healthy cattle, buffaloes and sheep. After centrifugation at 2000 rpm, the pellets were streaked on Sabouraud's dextrose agar (SDA) plates supplemented with chloramphenicol.

Vaginal, throat, nasal, ear and conjunctival swabs were taken by sterile bacteriological 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 supplemented with chloramphenicol.

Faecal samples were collected from cows, buffaloes and sheep then placed directly in sterile plastic bags. The samples were prepared by mixing about 3-5 g of each sample into sterile test tube 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 samples was taken and streaked onto SDA plates supplemented with chloramphenicol.

Chicken viscera were grossly examined for any macroscopical changes. Any organ with visible inflammatory lesions was taken to be cultured after being touched by flamed spatula then sliced with sterile scalpel. The incised tissue was printed with gentle squeezing on the surface of culture media (SDA) then streaked with the platinum loop onto SDA plates supplemented with chloramphenicol. All plates were incubated at 37°C for 2-5 days.

2.4. Isolation and conventional identification of yeast isolates

All samples were processed and cultivated on different media including SDA, brain-heart infusion agar, rice agar, urea hydrolysis, Myc. 10/20 (Dye medium) and Guizotia abyssinica-creatinine agar. The pure yeast isolates were subjected to different mycological conventional identification methods including morphological identification by direct microscopic examination using the slide mount technique, growth on rice agar medium, germ tube test, melanin pigment production for *Cryptococcus* isolates and capsular stain by Indian ink. In addition, the yeast isolates were examined by differential biochemical identification tests such as sugar fermentation tests (glucose, galactose, sucrose, maltose and lactose sugar media), sugar assimilation tests, nitrate reduction and urease tests.

2.5. DNA extraction

DNA was extracted using Wizard Genomic DNA purification kit (Promega, Germany) according to the manufacturer's instructions.

2.6. RAPD-PCR analysis of Candida species

The RAPD-PCR reactions were performed using five selected oligonucleotide primers as indicated in Table 1. OP primers were purchased from Operon Company (Germany). Each reaction mixture contained 3 µl of DNA template (20 ng/µl), 2.5 µl of 10× Taq buffer, 2.5 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs mix, 2 µl of 10 pmol primer (Bioanalysis Centrosud, Italy), 0.4 µl of *Taq* DNA polymerase (5 U/µl) and distilled water up to 25 µl final volume.

Table 1. Primers used for RAPD-PCR analysis of *Candida*

primer	Sequence	Reference
-CDU	5`-GCGATCCCCA-3`	(Sullivan *et a*l., 1995)
-The core sequence of the phage M13	5`-GAGGGTGGCGGTTCT-3`	(Bautista-Munoz *et al.*, 2003)
-OPA10	5`- GTGATCGCAG -3`	
-OPA18	5`- AGCTGACCGT -3`	
-OPE18	5`- GGACTGCAGA -3`	

2.7. RAPD-PCR analysis of Cryptococcus neoformans

The RAPD-PCR reactions were performed using three selected oligonucleotide primers as shown in Table 2. Three µl of 10 ng/µl genomic DNA were used for each RAPD-PCR reaction in a final volume of 25 µl containing 20 pmol of each primer, 2.5 µl 10× buffer, 200 µM dNTPs and 2.5 units *Taq* polymerase (Fermentas, Germany).

Table 2. Primers used for RAPD-PCR analysis of *Cryptococcus neoformans*

primer	Sequence	Reference
R2	5`- ATTGCGTCCA -3`	(Goodwin and Annis, 1991).
OPA1	5`- CAGGCCCTTC -3`	
OPA4	5`- GTGACATGCC -3`	

PCR reactions were carried out in DNA Thermal cycler 9600 (Applied Biosystems, USA) for *Candida* and *Cryptococcus* samples at an initial 5 min denaturation step at 94°C followed by 35 cycles of denaturation step at 94°C for 40 second, annealing step at 35°C for 90 seconds and extension step at 72°C for 2 min. After the 35 cycles, there was an additional extension step at 72°C for 7 min. A negative control in which DNA was replaced by sterile distilled water was also included. Aliquots of 10 µl of amplified products were analyzed by electrophoresis on 1.5 % agarose containing 0.5 µg/ml ethidium bromide at 80 V for 90 min and PCR products were detected by UV transilluminator.

3. Results

3.1. Incidence of yeasts in animals (buffaloes, cattle and sheep)

As shown in Table 3, the highest number of animal samples from which yeasts were recovered was the faecal samples (25.56 %), followed by milk samples (19.73 %), conjunctival swabs (3.42 %), rectal swabs (2.96 %) and vaginal swabs (2.3 %). The blood samples (0.06 %) were the lowest samples that were contaminated with yeasts.

Table 3. Incidence of yeasts in animal sample

Type of samples	No.of samples	No.of positive samples for yeasts	(%)
Conjunctival swab	146	5	03.42
Ear swab	125	2	01.60
Nasal swab	270	8	02.96
Vaginal swab	217	5	02.30
Rectal swab	135	4	02.96
Blood samples	1500	1	00.06
Milk	456	90	19.73
Faeces	395	101	25.56
Total	3244	216	06.65

3.2. Incidence of yeasts in viscera and cloacal swabs of chickens

As illustrated in Table 4, the highest number of samples that were positive for yeasts was from cloacal swabs (39.15 %), followed by bursa of fabricious and proventriculus (37.5 %), brain, liver and intestine (23.68 %), kidney (22.8 %), crop (21.05 %), spleen (14.91 %), lung (14.03 %) and finally trachea (8.77 %).

3.3 Incidence of yeasts species isolated from animals and chickens

Table 5 shows that *C. albicans* was the most common yeast species in all samples which was detected in 260 out of 535 yeast isolates (48.59 %). The incidence of *C. albicans* was almost the same in both chickens (48.9 %) and animals (48.15 %). *Cryptococcus neoformans* was the next common yeast identified in all samples (21.49 %), chickens (25.39 %) and animals (15.74 %).

Table 4. Incidence of yeast in chicken samples

Type of samples	No.of samples	No.of positive samples for yeasts	(%)
Brain	114	27	23.68
Trachea	114	10	08.77
Lung	114	16	14.03
Crop	114	24	21.05
Liver	114	27	23.68
Spleen	114	17	14.91
Kidney	114	26	22.80
Intestine	114	27	23.68
Proventriculus	8	3	37.50
Bursa	8	3	37.50
Cloacal swab	355	139	39.15
Total	1283	319	24.86

Table 5. Incidence of different yeast species in animals and chickens

	Animal		Chicken		Total	
	No. of isolates	%	No. of isolates	%	No. of isolates	%
C. albicans	104	48.14	156	48.90	260	48.59
C. neoformans	34	15.74	81	25.39	115	21.49
Other *Candida*	7	3.24	25	7.84	32	5.98
G. candidum	17	7.87	2	0.63	19	3.55
Mixed yeast	54	25.00	55	17.24	109	20.37
Total	216	100.00	319	100.00	535	100.00

The 33 isolates recovered from milk were identified as *C. albicans* (15 isolates); *C. neoformans* (6 isolates); *G. candidum* (1 isolate) in addition to 11 mixed yeast cultures. *C. albicans* was isolated from all organs of chickens except the proventriculus. The highest rate of isolation was from the cloacae (20.28 %), followed by brain (13.15 %), intestine (12.28 %), lung (10.52 %), kidneys (10.52 %), liver (8.77 %), spleen (7.01 %) and crop (6.14 %). The percentage from the bursa is misleading. The 81 *C. neoformans* isolates were recovered also from all organs except the bursa. The highest rate of isolation was from the cloacae, brains, livers and kidneys.

3.4. RAPD-PCR of Candida strains

Fig. 1 shows the gel electrophoresis image for the RAPD-PCR products of 4 isolates of *Candida albicans*, Fig. 1.A shows RAPD-PCR result using OPA-18 primer. The number of bands varied between 5 in cattle milk strain and 11 in chicken pink strain, while the chicken white strain showed 8 bands and the previously identified strain 10 bands. Two bands of 600 bp and 2000 bp were found in all strains which can be considered as specific bands for *C. albicans*, representing 12.5 % of the bands. The rest of the bands were variable among all the strains, giving the percentage of polymorphism of 87.5 %. As shown in Fig. 1.B, 4 *Candida albicans* isolates were tested using OPE-18 primer. The number of bands was lowest (3 bands) in white chicken strain and cattle milk strain, the previously identified *C. albicans* strain showed 6 bands while the pink chicken strain had the highest (7 bands) number of bands. It is clear that none of the bands was common to all strains, i.e. the polymorphism among these strains was 100 %. However, it was interesting that the chicken strains irrespective of the color had 2 common bands of 600 and 700 bp. On the other hand, the previously identified strain had two unique bands of 300 and 360 bp.

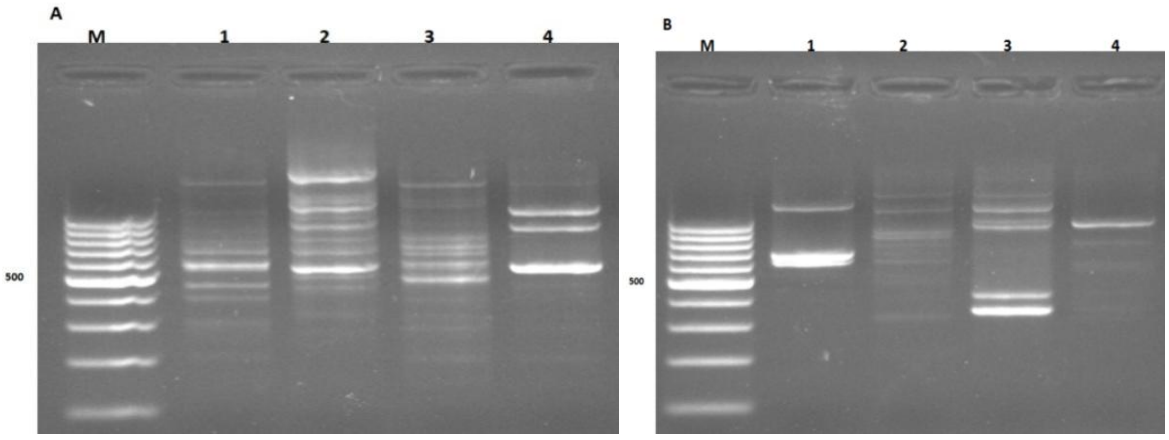

Fig. 1. RAPD-PCR products for *Candida albicans*. A: RAPD-PCR using OPA-18 primer. B: RAPD-PCR using OPE-18 primer. M: 100 bp marker, Lane 1: chicken strain (white), Lane 2: chicken strain (pink), Lane 3: *C. albicans* strain (white) and Lane 4: cattle milk strain (white)

3.5. RAPD-PCR of C. neoformans strains

The gel electrophoresis for RAPD-PCR products of *C. neoformans* strains using R2 primer is demonstrated in Fig. 2. Both pink chicken strains had the lowest number of bands (3-5), while all other strains, namely chicken, sheep and buffalo creamy strains, as well as the previously identified *C. neoformans* strain had 6 bands, each.

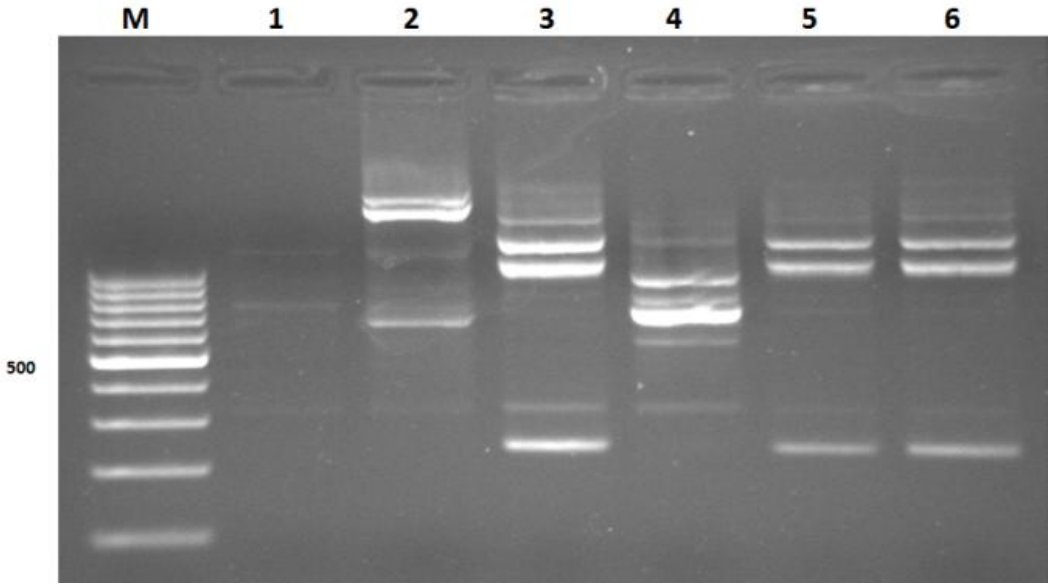

Fig. 2. RAPD-PCR products using R2 primer for *C. neoformans* strains. M: 1 kb marker, Lane 1: chicken strain (pink at 4°C), Lane 2: chicken strain (constant pink), Lane 3: chicken strain (creamy) Lane 4: *C. neoformans* strain, Lane 5: sheep strain (creamy), and Lane 6: buffalo strain (creamy).

3.6. Degree of similarity and dendrogram of C. albicans strains

As shown in Table 6, the similarity among all strains was low. The highest similarity (60 %) was only among the 2 white and pink chicken strains. The similarity between the chicken strains and the previously identified strain was between 25 and 50 %, while it was zero % between cattle milk strain

and an old white *C. albicans* strain. The use of OPE-18 primer confirmed the similarity between chicken white and pink strains (60 %). On the other hand, the cattle milk strain showed the lowest similarity with the old white strain. The results of dendrogram patterns of *C. albicans* showed variable clustering On the other hand, the OPE-18 primer yielded 2 clusters. The first cluster contained the cattle milk strain and the white chicken strain, while the second cluster contained the previously identified strain and the pink chicken strain.

systems when the two different primers were used. The OPA-18 primer gave 3 clusters (Fig. 3), a cluster comprising the cattle milk strain and pink chicken strain, a nearby cluster of the previously identified strain and far-distant cluster of the chicken white strain.

Table 6. Degree of similarity among *C. albicans* strains using OPA-18 primer

	Degree of similarity with OPA-18			
	Chicken strain (white)	Chicken strain (pink)	*C. albicans* (white)	Cattle milk (white)
Chicken strain (white)	100	60	50	40
Chicken strain (pink)	60	100	25	33.3
C. albicans (old, white)	50	25	100	0
Cattle milk (white)	40	33.3	0	100

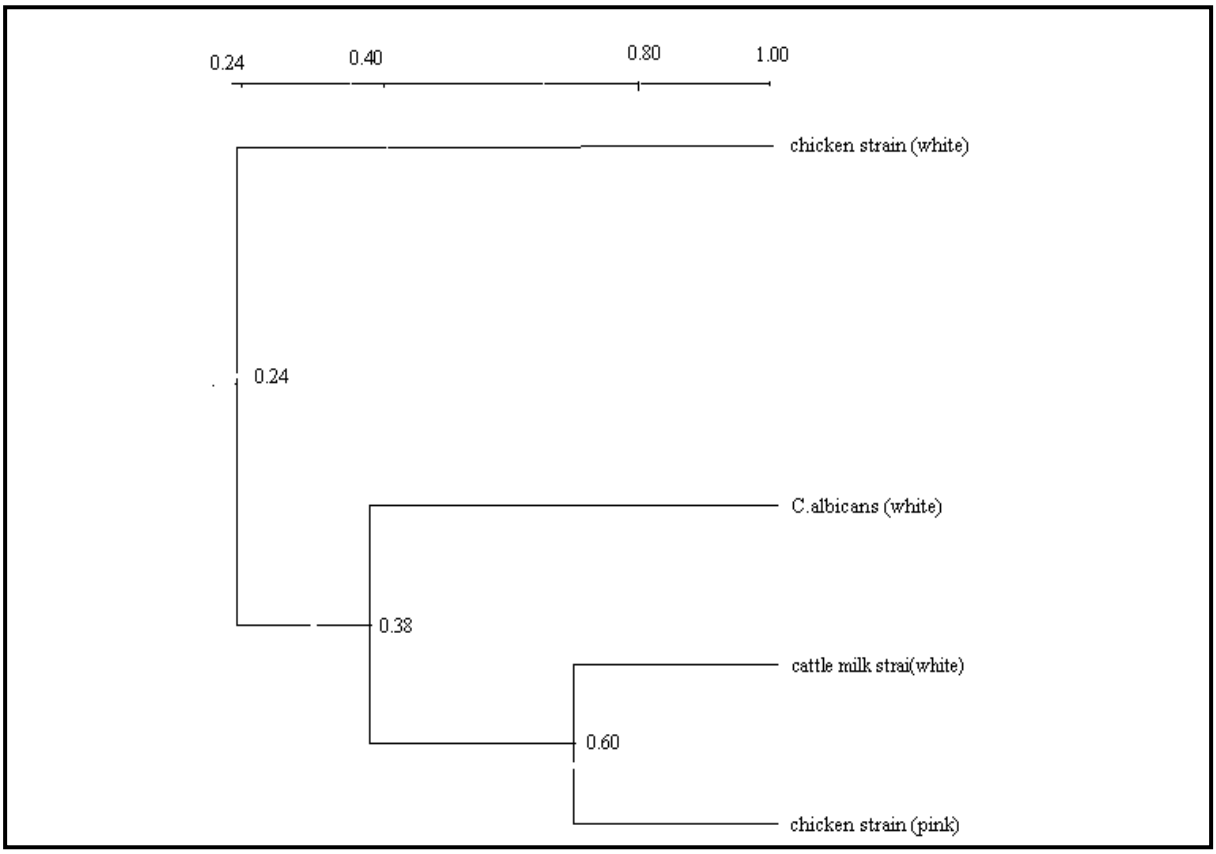

Fig. 3. Dendrogram pattern of primer OPA-18

3.7. Degree of similarity and dendrogram of C. neoformans strains

As illustrated in Table 7, the buffalo, chicken and sheep strains were 100 % similar to each other, while the similarity between these strains and the remaining strains ranged between 50-60 %. The dendrogram (Fig. 4) showed the classification of the *C. neoformans* strains from various sources into 3 clusters. One cluster represented by the pink strain, one by the previously identified strain and the largest cluster contained the buffalo, sheep and chicken creamy strains.

Table 7. Degree of similarity among *Cryptococcus neoformans strains* isolated from chicken and various animal species using R2 primer

	Bu	Chick	Sh	3	2	Cr.
Bu	100	100	100	60	50	50
Chick	100	100	100	60	50	50
Sh	100	100	100	60	50	50
3	60	60	60	100	50	66.7
2	50	50	50	50	100	60
Cr.	50	50	50	66.7	60	100

Bu: Buffalo strain, chick.: chicken strain, sh.: sheep strain, 3: constant pink strain, 2: pink at 4°C, and Cr.: *C. neoformans* strain.

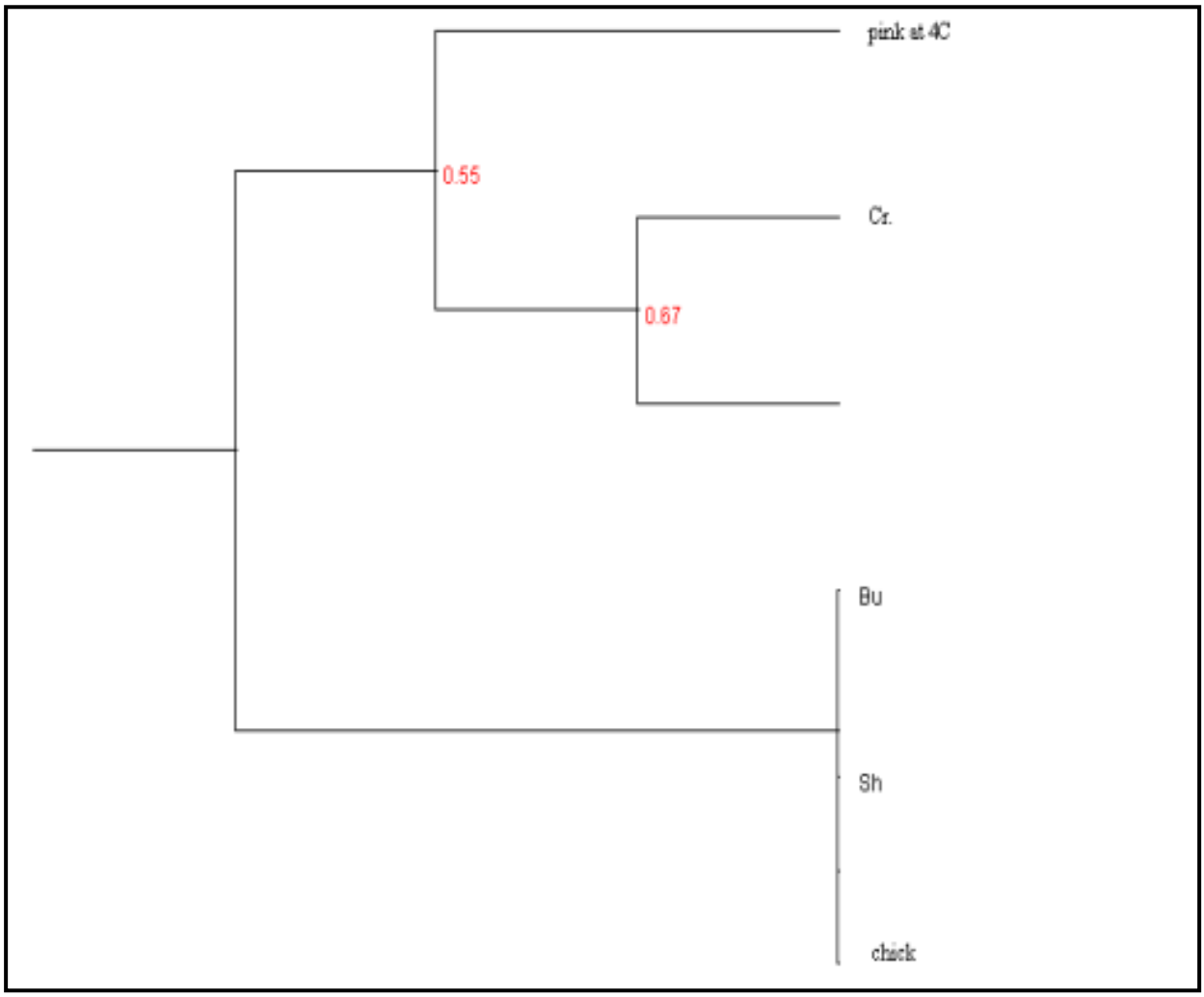

Fig. 4. Dendrogram pattern of primer R2

4. Discussion

In this study, 535 samples out of 4527 were positive for yeasts (11.81 %). This percentage may be attributed to that yeast infections are among the infrequent common fungal infections in animals and birds. The percentage of yeasts isolated was higher in chicken (24.86 %) than that isolated from different animal species (6.65 %). This can be expected due to the wide use of antibiotic preparations in the treatment of many diseases as well as the extensive use of antibiotics as feed additives, which enhance mycotic complications (Shibat-El-Hamed, 2008). Also this may be attributed to the bad hygienic measures in the farms from which samples were collected. Moreover *C. neoformans* is capable of multiplying to large numbers in faeces of pigeons and other birds, where it can remain viable for months (Junis and Schrauwen, 2003). It is clear in the present work that, out of 101(25.56 %) yeast isolates recovered from 395 faecal samples examined from all the studied animal species, *Candida* species were recovered 54 times (53.46 %) of which, 50 isolates were *C. albicans* (49.50 %), while the other *Candida* species (data not shown) were recovered 4 times (3.96 %). *C. neoformans* was obtained 19 times (18.81 %), *G. candidum* was obtained 4 times (3.96 %) and the mixed yeasts were obtained 24 times (23.76 %). It is known that, the digestive tract of different animals is one of the major sources of yeasts (Chengappa *et al.,* 1984). Some yeast species may be considered as commensals of the digestive tract and consequently their isolation from the intestinal contents or faecal samples has no clinical significance (Elad *et al.,* 1998). Moreover, *C. albicans* was considered to be normally present in faeces of different animals (Chengappa *et al.,* 1984). This is substantiated by the results obtained in the present study, where *C. albicans* was isolated from the faecal samples of apparently healthy cases in high percentage (49.50 %). The incidences of cases with positive mycotic findings in Egyptian cattle, buffalo, sheep and goat at various conditions of normal reproduction were 10 %, 0 %, 25 % and 50 % for these animal species, respectively (Osman and Abou Gabal, 1977). Yeasts were recovered, in the present study, from the reproductive tract of apparently healthy buffaloes, cattle and sheep in percentages of (3.33 %), (4.54 %) and (1.81 %) respectively. This finding might have a good support from the speculation that the opportunistic yeasts under many stress factors could become potential pathogenic that establish a disease condition or may be introduced to the vagina on top of the secondary infections. The incidence of yeasts in mastitic and apparently normal milk was (19.73 %). Out of 90 yeast isolates recovered from the milk samples examined from all the studied animal species, 39 isolates were *C. albicans* (43.33 %) , while *C. neoformans* was recovered 10 times (11.11 %). Ten milk samples were positive out of 31 samples from cattle showing clear udder inflammation and milk changes in a percentage of (32.25 %). The high rate of isolation in mastitic cattle milk resembles that reported by other authors (Hoffmann *et al*., 1968). The isolation of *C. albicans* from cattle milk samples was also reported by Abdel-Halim (1979); Nicklas *et al.* (1980); Moretti *et al.* (1998). Moreover, *C. albicans* was the most frequently isolated yeast in the present study especially from the mastitic cases which did not respond to the prolonged antibiotic therapy, where such result is in agreement with the work of Natalia and Hastiona (1985). *C. albicans* was isolated from chicken's samples in a percentage of (12.15 %). Pennycott *et al.* (2003); Kedar and Esmeraldo (2009) have reported the isolation of *C. albicans* from chickens and turkeys. Additionally in the present study, *C. neoformans* was isolated from chicken samples in a percentage of (6.31 %) as it was reported by Irokanulo *et al.* (1997); Mahmoud (1999); Singh and Dash (2008). Recent data indicated that, molecular techniques based on PCR and RAPD-PCR have been used as tool for diagnosis of several fungal species (Senses-Ergul *et al*., 2006; and Noumi *et al*., 2009). In the present study, the use of RAPD-PCR for *C. albicans* strains isolated from different sources using OPA-18 primer indicated the presence of 2 distinctive bands in all strains tested, which means that these bands are specific for *C. albicans* and may be used for diagnosis. This conclusion may be substantiated by the finding of Bautista-Munoz *et al.* (2003), who reported that RAPD-PCR patterns enabled the direct identification of common opportunistic pathogenic *Candida* species, including *C. albicans*. On the other hand, the use of RAPD-PCR with OPE-18 primer on the same *C. albicans* strains did not show any specific bands for this yeast. This is contrary to the results reported by Baires-Varguez *et al*. (2007), who mentioned that the RAPD-PCR patterns obtained with OPE-18 primer for identification of clinical isolates were consistent, specific and sensitive for the identification of *Candida glabrate*, *C. guilliermondii*, *C. tropicalis* and *C. albicans*. The failure to obtain specific pattern for *C. albicans* in our work, raises the question of reliability of this primer in the diagnosis of *C. albicans*. Perhaps more isolates may be needed to test this primer to reach a definite conclusion. Nevertheless, the high polymorphism of stains obtained in the present study may be useful in the epidemiological studies of *C. albicans* infection in tracing the source of infection. In the present study OPE-18 primer confirmed the similarity between

chicken white and pink strain. On the other hand, the dendrogram indicated the clustering of the white chicken strain and cattle milk strain in one cluster, while a second cluster contained the pink strain with the standard strain isolated also from milk. These results speculate the transmission of *Candida* species from cattle to chicken and vice versa. The results of RAPD-PCR for *C. neoformans* were of particular interest, as the buffalo, chicken and sheep strains were 100 % similar to each other. This was confirmed by the dendrogram, where the three strains were found in one cluster. This indicate that it is the same strain circulating among buffalo, sheep and chicken and most probably, the bird droppings and soil are the source of infection for all of them (Refai *et al.*, 1983; Kotb, 1990). RAPD-PCR was used by many authors to differentiate the serotypes of *C. neoformans* (Bockhout and Belkum, 1997; Passo *et al*., 1997; Nakamura *et al*., 2000). The use of R2 primer could be used for detecting the polymorphism among *C. neoformans* isolates.

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