Benha Veterinary Medical Journal 37 (2019) 161-164

Official Journal Issued by Faculty of Veterinary Medicine

Benha Veterinary Medical Journal

Journal homepage: https://bvmj.journals.ekb.eg/

Since 1990

Original Paper

First record of *Cryptococcus cerealis* from camel meat lesions in Egypt

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ARTICLE INFO

Keywords

Camel

Cryptococcus cerealis

Meat

PCR

Received ××/××/2019
Accepted ××/××/2019
Available On-Line ××/××/2020

ABSTRACT

Camels were formerly considered resistant to most of the diseases commonly affecting livestock. Recently, camels were found to be susceptible to a large number of pathogenic agents. The aim of this work was to identify the causative agent of meat lesion encountered in one humped male camels' carcass at abattoir in Matrouh governorate, Egypt. Out of 50 carcass of slaughtered male camel, 12 meat lesions were macroscopically found. Blackening and necrotic lesions were exposed to routine mycological examination. This revealed the presence of Cryptococcus in one lesion. Cryptococcus infection is an opportunistic infection that occurs primarily among patients who are immunosuppressed or getting access through accidental penetration of skin barriers. The isolated fungi were tested by PCR amplification of the ITS region and sequencing of 28S ribosomal RNA gene. The alignment of the fungal isolates 28S region sequences revealed 94% identity with Cryptococcus cerealis in the gene bank library. For the best of our knowledge, this is the first isolation of *C. cerealis* from camel meat lesion in Egypt.

1. INTRODUCTION

Camels contribute hugely to human survival in many parts of African, Asian, and Arabian deserts. They have been used for transportation and as a source of food for long time. Nowadays, they are so important in many parts of the arid world as sustainable livestock species (Burger, 2016). Camels have adapted to the harsh arid environments inhabit allowing them to maximize the digestion of low-quality feeds to a greater extent than ruminants (Manefield and Tinson, 1997). There is a lack of research in camel nutrition and data is often extrapolated from ruminants (Ellard, 2000). Temperatures can vary between less than 10 °C and more than 30 °C in Matrouh province, dependent on the climate conditions.

Cryptococci are present in the nature in soil, bird dropping (especially pigeon) and trees. Opportunistic fungi such as cryptococcosis, have a preferred habitat independent from the living host and cause infection after accidentally penetration of intact skin barriers, or when immunologic defects or other debilitating conditions exist in the host (Casadevall and Pirofski, 2000). *Cryptococcus* has been isolated from a variety of environments, including soil, water or stored agricultural products (Barnett et al., 2000; Olstorpe et al., 2010).

The present study was planned to identify the mycological cause of lesion (blackening area) encountered in meat of slaughtered camel in Matrouh governorate, Egypt.

2. MATERIAL AND METHODS

2.1. Sampling:

Meat lesions were taken out from 50 slaughtered one humped male camel (4-9 years old) in the period from December 2015 to June 2016. These samples were collected from main abattoirs in Matrouh governorate, Egypt, where camel rearing is common. Samples were collected directly after slaughtering during early morning. From these slaughtered camels, twelve meats with macroscopic lesions were obtained. Then, these meats were aseptically sampled and transferred to Mycology Research Lab, Desert Research Center, Egypt, for mycological and molecular investigations.

2.2. Isolation strategy:

The fungi were isolated by the direct plating method according to Dhingra and Sinclair (1995) on Sabouraud dextrose agar medium plates (oxoid) with Tetracycline 0.5 gm/L. The plates were then incubated at 28±2 °C for 5-7 days (Roberts, 1986) and every single fungal colony was purified, identified and preserved by transferring to a pure slant containing malt extract agar medium and hence stored at 4 °C.

2.*2.1. Culture and morphological identification*:

Isolates were identified based on routine cultural characteristics and morphological characteristics according to McClenny (2005)

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2.3. Electron Microscope:
Specimens were fixed in 2.5% (v/v) Glutaraldehyde for 20 min. The fixed specimens were dehydrated through a series of increasing concentrations of ethanol, ending in a 100% dehydrating liquid of the highest possible purity. Typically, these are steps of 10, 20, 30, 50, 70, 90, 95, 100% at 10 minutes for each, with 3 changes at 100%. Acetone was used as the intermediate fluid because it is miscible with carbon dioxide. Critical point drying (CPD) was used to prevent collapse in Scanning Electron Microscope (SEM). The samples were sputter-coated with gold using an Emitech K550X coating unit. Then the specimens were examined using scanning electron microscope.

2.4. Molecular identification:
2.4.1. Fungal DNA extraction was carried out by Quick-DNA™ Fungal/Bacterial Microprep Kit (Zymo research #D6007). Then the ITS region was amplified by PCR using ITS1 and ITS4 in the fungal isolates. PCR was done by using Maxima Hot Start PCR Master Mix (Thermo K1051). Briefly 1 µl of forward and reverse ITS primer (20 µM), 5 µl of fungal DNA, 25 µl of PCR Master Mix (2X) were added and completed to 50 µl of nuclease free double distilled water. The mixtures were vortexed and spin down and put in the thermal cycler for initial denaturation at 95 °C for 10 min this was followed by 35 cycles each consisted of denaturation at 95 °C for 30 sec., annealing at 57 °C for 1 min, extension at 72 °C for 1.5 min and final extension at 72 °C for 10 min.

2.4.2. Horizontal gel electrophoresis
PCR-amplified products were separated by agarose gel electrophoresis using a horizontal submarine gel system (Agagel Medi, Biometra) as well as agarose (Gibco BRL Life Technologies) at a concentration of 2% (w/v). Electrophoresis was conducted in 0.5× TBE buffer at 10 v/cm for various times, depending on the size of the gel unit, DNA size marker was used as standard (GeneRuler 100 bp plus DNA ladder; Fermentas Biotech. Inc.). The Run was continued for 1 hr, then agarose gel with DNA bands were stained with ethidium bromide (10 mg/ml), visualized and photographed under UV light according to Weising et al. (1995).

2.4.3. Purification of PCR product
The PCR product underwent cleaning up using Gene JET™ PCR Purification Kit (Thermo K0701) according to the manufacture guidance.

2.4.4. Sequencing of ITS gene
The sequencing of the PCR product was made on GATC German Company using ABI 3730xl DNA sequencer by ITS1 and ITS4 primers in addition to 28S ribosomal RNA gene partial sequences. The DNA sequence was determined by automated DNA sequencing method. The automated DNA sequencing reactions were performed using Big Dye terminator ready sequencing kit. The reaction was conducted in a total volume of 20 µl, containing 8 µl of terminator ready reaction mix, 1 µg of DNA, and 3.2 pmole of forward primer. The cycle sequencing program was 96 °C for 10 sec, 50 °C for 5 sec, and 60 °C for 4 min, repeated for 25 cycles with rapid thermal ramping. The nucleotide sequence was determined automatically by the electrophoresis of the cycle sequencing reaction product on ABI 3730xl DNA sequences. The data were provided as fluorimetric scans from which the sequence was assembled using the sequence analysis software.

2.4.5 Alignment analysis
The obtained nucleotide sequences of ITS of isolate in addition to 28S ribosomal RNA gene partial sequences were analyzed using GenBank database (Altschul *et al*., 1997) using BLAST program available on the National Center of Biotechnology Information website and where compared to the sequences on library in order to assess the gene similarities.

3. RESULTS

3.1. Macroscopic characteristic of the isolated fungi on Sabouraud Dextrose Agar (SDA) media showed streak like structure at 25 °C (Fig. 1).

Fig. 1 Macroscopic characteristics of Cryptococcus examined by naked eye. Fungal sample was isolated from meat lesion as described in Material and Methods showing streak like structure.

3.2. Microscopic character: Direct microscopic examination of fungi isolates stained with India ink showed cells being globose to subglobose and occur singly or in pairs encapsulated yeast (Fig. 2).

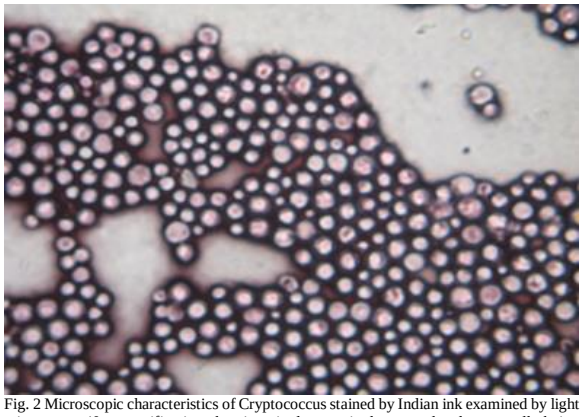

Fig. 2 Microscopic characteristics of Cryptococcus stained by Indian ink examined by light microscope 40x magnification showing single or paired encapsulated yeast cells being globose to subglobose

3.3. Scanning Electron Microscope (SEM) examination of 1 µm magnified ×9.000 and 2 µm magnified ×8500 showed the characteristics of basidiomycetous yeast genus with the lack of basidiocarps. The Hyphae and pseudohyphae were usually absent, sexual reproduction were not observed with

the presence of budding cells giving the characteristics of *Cryptococcus* species (Fig. 3).

3.4. Molecular identification by ITS

The alignment of a 501 bp region clarified that the specific gene was resemble to *Cryptococcus cerealis* by 94% which support the morphological identification (Passoth et al., 2015) (Fig. 4-6).

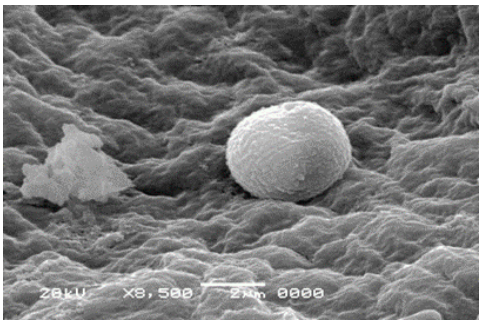

Fig. 3 *Cryptococcus cerealis*, 2um x8500. The basidiomycetes lack basidiocarps. The Hyphae and pseudohyphae are usually absent, sexual reproduction is not observed, budding cells are present.

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1   GAGACTCGTG CCGTGTACTC TGCTCACGGC GCTATGTCTT TATATCCATA GCCGCTGTGC
61  TCTTGATGCA TGTTCTTGAT ACGTACGATA GAAGCGGAGG TCCATAGTCA TCCCCTTACA
121 CTAAACAATA ATGTATCAAA TGTAGTCTTA TTATAACATA ATAAAACTTT CTTCAACGGA
181 TCTCTTGGCT CTCGCATCGA TGAAGAACGC AGCGAAATGC GATAAGTAAT GTGAATTGAC
241 TGAATTCAGT GAATCATCCA ATCTTTGAAC GCACCTTGC GCTCCTTGGTA TTCCGAGGAG
301 ACATGCCTGT TTGAGTGTCA TGAAAACCCT CAACCTTATA TTGGTTATTT GACCTTTCTT
361 TGGCTTGGAT TTGGACGTTT GCCGATGTCA AGTCAGCGCG TCGTAAAAGT AATAACTGGA
421 TCAGTGTCGT GAGATGGTTT GACGTGGTGT AATAGGTAGG GCGGCAGTGA GGGCCTCGGA
481 TGGGCGTGGT GCGCGAAGGA
```

Fig. 4 Sequence of Cryptococcus isolates using ITS primer set 1 and 4 (501 bp)

Description	Max score	Total score	Query cover	E value	Ident	Accession
Naganishia friedmanni CBS 7160 ITS region; from TYPE material	540	540	98%	3e-154	92%	NR_111047.1
Naganishia cerealis CBS 10505 ITS region; from TYPE material	496	496	85%	6e-141	94%	NR_111371.1
Naganishia randhawae CBS 10160 ITS region; from TYPE material	496	496	85%	6e-141	94%	NR_111118.1
Naganishia antarctica CBS 7687 ITS region; from TYPE material	483	483	85%	4e-137	93%	NR_111049.1
Naganishia bhutanensis ATCC 22461 ITS region; from TYPE material	477	477	85%	2e-135	93%	NR_077082.1
Naganishia uzbekistanensis CBS 8683 ITS region; from TYPE material	459	459	85%	8e-130	92%	NR_073219.1
Cryptococcus consortionis JCM 9425 ITS region; from TYPE material	453	453	85%	4e-128	92%	NR_077112.1
Naganishia vishniacii CBS 7110 ITS region; from TYPE material	453	453	85%	4e-128	92%	NR_111045.1
Naganishia adeliensis CBS 8351 ITS region; from TYPE material	448	448	85%	2e-126	91%	NR_111050.1
Naganishia albidosimilis JCM 8843 ITS region; from TYPE material	442	442	85%	8e-125	91%	NR_077113.1
Naganishia albida CBS 142 ITS region; from TYPE material	442	442	85%	8e-125	91%	NR_111046.1
Naganishia diffluens CBS 160 ITS region; from TYPE material	442	442	85%	8e-125	91%	NR_111051.1
Naganishia liquefaciens CBS 968 ITS region; from TYPE material	442	442	85%	8e-125	91%	NR_073220.1
Filobasidium oeirense CBS 8681 ITS region; from TYPE material	363	363	85%	6e-101	87%	NR_077106.1
Filobasidium magnum CBS 140 ITS region; from TYPE material	353	353	85%	4e-98	86%	NR_130655.1
Filobasidium floriforme CBS 6241 ITS region; from TYPE material	348	348	85%	2e-96	86%	NR_119429.1

Naganishia friedmannii CBS 7160 ITS region; from TYPE material

Fig. 5 Phylogenic analysis result showing the fungal isolates specific gene with 94% identity to Cryptococcus cerealis gene sequences present in the national library

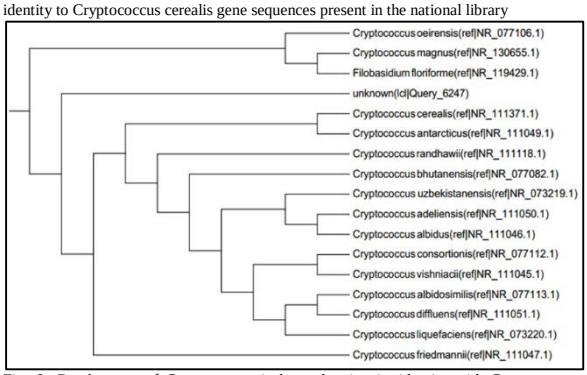

Fig. 6 Dendrogram of Cryptococcus isolates showing its identity with Cryptococcus cerealis present in the public library

4. DISCUSSION

Opportunistic fungi such as *Cryptococcus* may cause infection after the skin barrier of animal was accidentally penetrated or when animal was immunocompromised (Casadevall and Pirofski, 2000). In this study, we isolated *Cryptococcus cerealis* from meat lesion of camel carcass. This finding may indicate that the tested carcass was from immunocompromised animal. Fungal infection could be increased in animals exposed to long term antibacterial treatment and immunosuppression (Pfaller et al., 2006). However, at risk for fungal infection include also normal individual as cases of invasive aspergillosis have been reported in normal hosts after extensive environmental exposure to *Aspergillus* spores in the form of tree-bark chippings (Butler et al., 2013), or after inhalation of a great deal of dust in a mushroom factory (Shimaoka et al., 2006). Considering the desert nature of the sampling area and the continuous windy environment beside the severe cold condition that camel are exposed in winter in the North of Egypt in the current study may explain the chance of getting fungal infection in some weak camel that are not sheltered all the time.

The isolated fungus from this lesion was identified by SEM examination as one of *Cryptococcus* species. Further investigation of the isolates using phylogenetic analysis based on transcribed spacer (ITS1 and ITS4) showed that, the strain represents a single species in the genus Naganishia that is distinct from other species. The identity to *Cryptococcus cerealis* was 94%. These data collectively indicate that the meat lesion encountered in the camel carcass is caused by *Cryptococcus cerealis*. Indeed, camels in Saudi Arabia were found to be infected with *Cryptococcus gattii* with the appearance of pathological changes including gelatinous and granulomatous of which the granulomatous lesion consisted of histiocytes, giant cells and lymphocytes (Ramadan *et al*., 1989). Meanwhile, other animal as lama were reported to be infected with *Cryptococcus gattii* while the animal suffered mainly from meningitis (Bildfell et al.,2002). Internal transcribed spacer (ITS) region sequences analysis has been proved to be validated for fungal spp. identification (deHoog and Horré 2002). It also was chosen as the official barcode for fungi identification by a consortium of mycologists (Schoch et al., 2012). The identification by ITS region sequences analysis of the isolate being *Cryptococcus cerealis* with 94% identity confirm without any doubt that this isolate was *Cryptococcus cerealis.* Thus, these results may indicate that this species can infect camel and produce lesion that can affect carcasses of camel especially those are under severe harsh environmental condition that has effect on the immune status of the animal.

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