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Diagnosis of *Cryptococcus neoformans* from the milk of goat using Multiplex PCR as diagnostic tool

Shaimaa N. Yassein[1*], Jenan M. Khalaf [2], Hayder M. Samaka [3]

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***Corresponding author:**
Email address:
shaimaa_nabhan@yahoo.com

Abstract
Cryptococcus neoformans (*C. neoformans*) is distributing globally. It is an opportunistic saprophytic fungal pathogen that appears as a dimorphic yeast-like fungus. It causes a wide variety of cryptococcal diseases in immunocompromised man and animals. No previous reports have been recorded the isolation of *Cryptococcus neoformans* from the milk of the mastitic goat in Iraq. Consequently, this study intends to isolate *C. neoformans* from the milk of goats that suffered from clinical and subclinical mastitis in Iraq. Three hundred milk specimens were collected from 163 mastitic and apparently healthy goats. All milk samples were subjected to conventional isolation and characterization tests, moreover, multiplex polymerase chain reaction (mPCR) was used to confirm the diagnosis. The total positive ratio of mycotic mastitis was 41.33% for all tested samples. The total number of yeast isolates was 69 (55.64 %), meanwhile, *C. neoformans* rate was 7.24% (5 out of 69). Multiplex polymerase chain reaction confirmed 5 *C. neoformans* isolates. The results of this study revealed an agreement between the mPCR technique and conventional methods in the diagnosis of *Cryptococcus neoformans*. In conclusion, the results of this study approved the isolation of *Cryptococcus neoformans* from the milk of clinical and subclinical mastitis of goats in Iraq. The authors recommend doing a further future study that includes a large number of goats and from different locations, to investigate the actual situation of *Cryptococcus neoformans* infection in caprine in Iraq using mPCR technique.

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Introduction

One of the most predominant serious problems in the dairy livestock is mastitis. The most common causes of mastitis are bacteria. However, not long ago, there is anincrease in the cases of mycotic mastitis that caused by yeasts or yeast-like fungi (Saleh, 2005). Moreover, *C. neoformans* and *C. albicans* are the more pathogenic types of yeast than other to cause mastitis (Spanamberg *et al.,* 2009 A). Many predisposing factors are

contributing in the development of mycotic mastitis. These factors are chronic diseases, excessive use of antibiotics, corticosteroid, and immunosuppressive drugs. In addition, poor management and hygiene, teat injuries and faulty milking machines have also a role in acceleration the occurrence of mycotic mastitis (Islam *et al.,* 2011). Spanamberg *et al.,* (2008) believed that some mycotic mastitis outbreaks occurred due to ascending infection after incorrect administration of antibiotic preparations during the drying-off period. However, the improper use of antibiotics as feed additives also may increase the complications of fungal infection (Shibat-El-Hamed, 2008). The administration of high doses of antibiotics may result in the decrease in vitamin A, which leads to a defect in the epithelium of mammary gland and changing the natural defence mechanism that represented by the microflora of the mammary glands and facilitated the invasion of molds and yeasts (Şeker, 2010). Environmental molds and yeasts play a significant role in contamination of the teat end of the udder during milking and eventually cause udder infection (Gaudie *et al.,* 2009).

There are scarce publication concerning the epidemiology of cryptococcosis mastitis, although *Cryptococcus neoformans* had been isolated from milk of caprine mastitis case and demonstrated by direct microscopy since 1976 by Pal and Randhawa., (1976).

Direct and indirect methods are used as a conventional tools for the diagnosis of mycotic infections with poor accuracy. Consequently, the researchers look for accurate, more rapid, practical and reliable tests with high sensitivity and specificity for the diagnosis of mycotic infection (Susever and Yeğenoğlu, 2011). Review of literatures revealed scarce reports regarding mycotic mastitis in goats in Iraq. Moreover, the isolation of *C. neoformans* from goat mastitis hasn't reported previously. Therefore, this study intends to investigate the mycotic mastitis caused by *C. neoformans* in goats in Iraq using conventional methods and multiplex PCR technique.

Materials and methods

Samples collection

Three hundred milk samples collected from different areas in Baghdad governorate and the surrounded suburban farms of Abu-Ghraib. Milk samples were collected from totally, 163 doe (goats). Some of these goats were suffered from clinical mastitis while others were apparently health (subclinical mastitis). Ten millilitres of milk collected aseptically in sterile tubes and placed in a cool box and immediately transfer to the laboratory.

Isolation and conventional identification of yeast isolate

Each milk sample centrifuged for 20 minutes at 3000 / RPM. Later on, the sediments were collected and cultured on different media including Sabouraud dextrose (Difco) and Sunflower Seed, brain-heart infusion agars. Duplicate plates were made from each sample and kept at 25 and 37°C for 4 to 6 days.

All yeast isolates were identified by direct microscopic examination using the lacto-phenol-cotton-blue stain and capsular stain by Indian ink. Brownish pigment production on Sunflower Seed Agar for Cryptococcus isolates was observed. Then, several biochemical identification tests were used to diagnose these isolates such as urea

hydrolysis, sugar fermentation, and assimilation tests. In addition, all isolates identified with biochemical Kits (API Candida. The API –yeast-IDENT system used for identification of yeast isolates).

Multiplex polymerase chain reaction (mPCR)

ITS regions is the most important molecular method that has been performed to determine fungi from clinical samples (Barton and Evans, 1999). The mPCR reactions were carried out by using three selected oligonucleotide primers. These primers ITS1 (5-TCCGTAGGTGAACCTGCG-3), ITS3(5-GCATCGATG AAGAACGCAGC-3), and ITS4 (5-TCCTCCGCTTATTGATATGC-3), were used for amplification and targeting the conserved regions of 18S, 5.8S, and 26S rDNA respectively (White *et al.,*1990). The ITS1-ITS4 primer pair was used to amplify the intervening 5.8S rDNA and the adjacent ITS1 and ITS2 regions, while the ITS3-ITS4 primer pair was used to amplify a large portion of the 5.8S rDNA and the adjacent ITS2 region.

Extraction of DNA

Genomic DNA extracted by using DNA-Pure Yeast Genomic Kit (bioWorld, USA) according the manufacturer's instructions. Extracted DNA was precipitated by absolute isopropanol and washed with 70% ethanol and then transferred to a sterile Eppendorf tubes and stored at -20◦C prior to PCR (Samaka *et al.,* 2011). PCR amplification was performed with a volume of 25ul. The Polymerase chain reactions were carried out in a volume of 25µl, containing 5 ul extracted DNA, 12.5µl KAPA2G Robust Hot Start Ready Mix® (KAPA BIOSYSTEM, South Africa) and 1.25µl (10 pmol/µl) for each forward and reverse primers with the remaining volume consisting of distilled water.
The amplification process consisted of an initial denaturation at 94°C for 4 min; 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°Cfor 1 min; and a final extension at 72°C for 5 min. A Techne TC-3000X Thermal Cycler (Bibby Scientific Limited, UK) was used.

Electrophoresis

Gel electrophoresis with 1.5% ethidium bromide - agarose gels was conducted with 1XTBE buffer (0.1 M Tris, 0.09 M boric acid, 1 mM EDTA) at 75 V for 75m. A 100-bp DNA ladder (Promega Corp., USA.). Gene tool analysis software (SynGen, UK) was used to calculate molecular sizes.

Results

Incidence of yeasts species in the milk of goat

Sixty nine (55.64 %) yeast spp isolates were obtained from 300 milk samples that collected from both mastitic and apparently healthy goats. Totally, the rate of mycotic mastitis was 41.33% of all the examined specimens. The numbers of *C. neoformans* were 5 times (7.24%) out of 69 isolates diagnosed by conventional methods.

A multiplex PCR targeting *C. neoformans* was performed directly on the strains that obtained from milk samples to confirm the isolates. The sizes of the 18S, 5.8S, and 26S rRNA genes are essentially identical in most of yeast species and in *Cryptococcus neoformance* (550bp and 372bp) were observed for ITS1-ITS4 and ITS3-ITS4 respectively (Figure 1 and 2). While the lengths of the ITS regions depend on the species, the ITS region is located between the 18S and 26S rRNA genes and is subdivided into the ITS1 region between the 18S and 5.8S rRNA genes, and the ITS2 region, between the 5.8S and 26S rRNA genes.

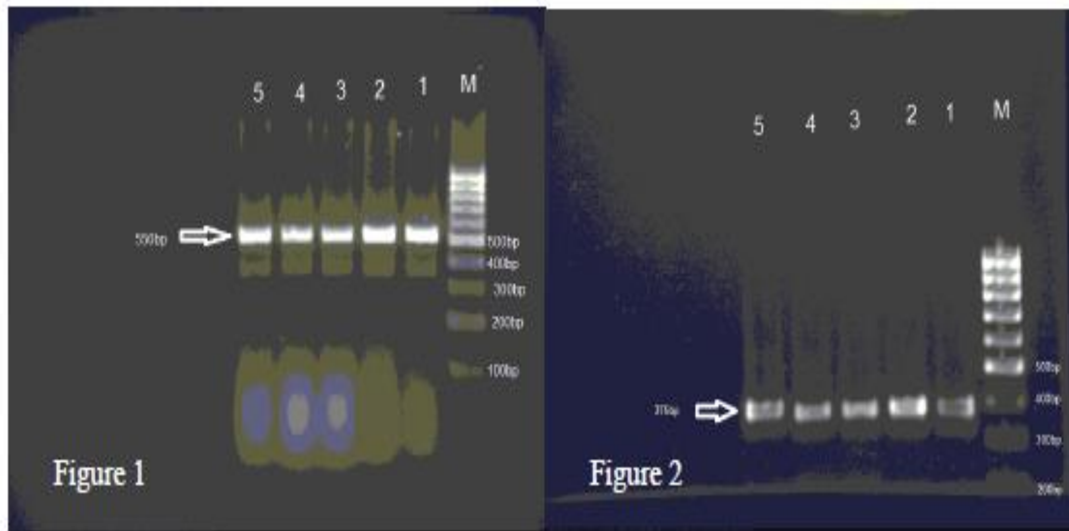

Figure. 1: Shows lane 1, 2, 3 and 4 represent *Cryptococcus neoformans* with 550bp amplicone (ITS1-ITS4); lanes M, 100-bp DNA ladder.
Figure. 2: Shows lane 1, 2, 3 and 4 represent *Cryptococcus neoformans* with 375bp amplicone (ITS3-ITS4); lanes M, 100-bp DNA ladder.

Discussion

Mycotic mastitis could be caused by diverse genera of molds and yeasts. Different molds and yeasts including, *Candida spp*, *Aspergillus spp*, *Cryptococcuss pp*, *Trichosporon spp* and *Penicillium spp* were isolated from mycotic mastitis in cows in Missan Governorate/Iraq (Sahan, 2011).
Bovine Mycotic mastitis caused by *C. neoformans* has been reported by several researcher elsewhere worldwide. In Iraq, *C. neoformans* has also isolated from ovine mastitic milk (Shnawa and Nigam, 1987 and Al-Kubaysi, 2000).In the present study, *C. neoformans* was isolated from milk samples that were collected from both apparently healthy goats and goat with clinical mastitis. There is no previous report regarding the isolation of *C. neoformans* from the milk of the goats in Iraq. This isbelieved to be the first instance of caprine mycotic mastitis in Iraq which *C. neoformans* has been implicated as the etiologic agent that approved by conventional and molecular tools. The results of this study showed that the total isolation rate of *C. neoformans* was 7.24%. This rate considers as a high rate. It might has impact on the public health in Iraq because *C.*

neoformans is one of the important highly infectious mycozoonotic disease. It is well known that *C. neoformans* can easily transmit to human being either directly due to handling the infected animals, or indirectly via consumed not well pasteurized milk without exposed it to the sterility process (Nosnchuk *et al.,* 2000).
The result of this study is compatible with the previous study that was done in Quena Governorate/ Egypt (Abou-Elmagd *et al.,* 2011). Abou-Elmagd *et al.,* (2011) study the prevalence of *Candida albicans* and *Cryptococcus neoformans* in different species of animals, in addition, the isolated yeast were identified using RAPD-PCR. The isolation rate of yeast from milk samples was 19.73% and *C. neoformans* constituted the second predominant yeast detected in all specimens (Abou-Elmagd, *et al.,* 2011). The results of this study is also in agreement with previous study (Hassan *et al.,* 2012). Hassan *et al., (*2012) studied the prevalence of yeast infections in small ruminants. They isolated the *C. albicans* from the mastitic milk with a percentage rates 32% and 24% of mastitis cases of sheep and goat, respectively. Moreover, *C. neoformans* was recognized in 4% of milk of mastitic sheep and 4% of nasal discharge of sheep suffered from respiratory disorders however, *C. neoformans* was not recovered from samples of apparently healthy sheep.
Most types of yeasts own similar morphological and biochemical properties that complicate the conventional diagnostic tools or may give inaccurate results. On the other hand the routine cultivation methods of yeast need long time accompanying with low sensitivity and specificity and the false negative are also possible (Abd El-Razik *et al.,* 2011). All these factors act as obstacle that delay and limit the isolation and diagnosis process of fungus (Sidrim *et al.,* 2010). The controversy regarding the conventional isolation process, encourage researcher to develop new molecular techniques to identify and characterize microorganisms originated from milk (Spanamberg *et al.,* 2009b). Moreover, the advancing in molecular techniques like DNA sequencing can give rapid identification of the biodiversity and ecology of microorganisms isolated from milk and food.
In this study the multiplex polymerase chain reaction (mPCR) was used for accurate diagnosis of *C. neoformans*. The test was done based on restriction-fragment length polymorphism of 5.8S-ITS rDNA region or sequencing of the D1 and D2 domains in the 26S rDNA gene (Rezki, *et al.,* 2013). The purification and identification process was don for each separated colony by sequencing the ITS1-5.8S-ITS2 rDNA region. This manner was performed to eradicate any bias that may have been introduced by selecting colonies based on the morphology (Lavoie *et al.,* 2012). The results of this study revealed identical sizes of the 18S, 5.8S, and 26S rRNA genes that are essentially identical in most of yeast species. The *Cryptococcus neoformance* isolates size band were located in (550bp and 372bp) for ITS1-ITS4 and ITS3-ITS4 respectively. The results of this study is compatible with previous study that used themultiplex PCR as a rapid diagnostic tool for the rapid identification of common and uncommon yeast strains from culture colonies (Fujita *et al.,*2001).
Sidrim *et al.,* (2010) pointed that *C. neoformans* and *C. gattii* can be detected from clinical specimens and cultures by using specific gene sequences. The identification, typing, and the study of population genetics can be done by using these sequences. However, Susever and Yeğenoğlu, (2011) revealed that the rapid diagnosis of *Cryptococcosis* from CSF sample of a patient by detection of *C. neoformans* DNA via PCR can provide the probability for rapid treatment. Meanwhile, Fadda *et al.,* (2010)

found that the Candida zeylanoides was the most common species that isolated from goat's milk and they used an easy method of DNA extraction with a minimal time and low-cost that provided a high quality of DNA for RAPD analysis of 32 isolates of *C. zeylanoides.*

In conclusion, this study investigated and identified *C. neoformans* as the causative agent of caprine mycotic mastitis using conventional and molecular tools. For the author's knowledge, this is believed to be the first report of caprine mastitis in which C. neoformans has been implicated as the etiologic agent in Iraq. The authors recommend to do further future study using the multiplex PCR as a rapid diagnostic tools and including a large number of goats from different locations, to investigate the actual situation of *Cryptococcus neoformans* infection in Iraqi goats.

References

Abd El-Razik KA, Abdelrahman KA, Abd El-Moez SI and Danial EN.(2011). New approach in diagnosis and treatment of Bovine Mycotic Mastitis in Egypt. African Journal of Microbiology Research. 5 (31):5725-5732.

Abou-Elmagd S, Kotb H, Abdalla K and Refai M. (2011). Prevalence of *Candida albicans* and *Cryptococcus neoformans* in Animals from Quena Governorate with Special Reference to RAPD-PCR Patterns. Journal of American Science. 7(12):22-31.

Al-Kubaysi SMA. (2000). Bacterial and mycotic mastitis in ewes in Al-Qaim District-Al- Anbar Province .M.Sc. Thesis-College of Veterinary Medicine-Baghdad University.

Jackson CJ, Barton RC, & Evans EGV. (1999). Species identification and strain differentiation of dermatophyte fungi by analysis of ribosomal-DNA intergenic spacer regions. J. clin. Microbiol.37: 931-936.

Fadda ME , Viale S, Deplano M, Pisano MB, Cosentino S. (2010). Characterization of yeast population and molecular fingerprinting of *Candida zeylanoides* isolated from goat's milk collected in Sardinia. Int J Food Microbiol. 1; 136(3):376-80. doi: 10.1016/j.ijfoodmicro.2009.10.007. Epub 2009 Oct 17.

Fujita S, Senda Y, Nakaguchi S, AND Hashimoto T. (2001). Multiplex PCR Using Internal Transcribed Spacer 1 and 2 Regions for Rapid Detection and Identification of Yeast Strains .Journal of clinical microbiology. 39(10): 3617–3622.

Gaudie CM, Wragg PN, Barber AM (2009). Outbreak of disease due to *Candida krusei* in a small dairy herd in the UK. Veterinary Record, 165: 535-537.

Hassan AA, Manal A Hassan, Rasha MH Sayed El Ahl and Darwish AS. (2012). Prevalence of Yeast Infections in Small in Ruminants with Particular References to their Treatment by some Natural Herbal Extracts. Bulletin of Environment, Pharmacology & Life Sciences. 1(3):12-22. www.bepls.com

Islam MR, Samad MA and Anisur Rahman A KM. (2011). Bacterial Pathogens and risk factors associated with mastitis in Black Bengal goats in Bangladish . Bangl. J. Vet. Med. 9 (2): 155 – 159.

Lavoie K, Touchette M, St-Gelais D, and Labrie S (2012). Characterization of the fungal microflora in raw milk and specialty cheeses of the province of Quebec Dairy Sci. & Technol. 92:455– 468.

Joshua D. Nosanchuk, Shmuel Shoham, Bettina C. Fries, Daniel S. Shapiro, Stuart M. Levitz, and Arturo Casadevall. (2000). Evidence of Zoonotic transmission of *C. neoformans* from a pet cockatoo to an immunocompromised patient. Ann. Intern. Med. 132(3):205-208.

Pal M, Randhawa HS. (1976). Caprine mastitis due to *Cryptococcus neoformans*. Sabouraudia. 14(3):261-3.

Rezki MA, Benbadis L, DeBillerbeck G, Benbayer Z. and François JM. (2013). Isolation and physiological characterization of indigenous yeasts from some Algerian agricultural and dairy products. J. Yeast Fungal Res. Vol. 4(6), pp.75-83.

Sahan HF. (2011). Prevalence of mycotic mastitis in cows in Missan Governorate. M.Sc. Thesis-College of Veterinary Medicine-Baghdad University.

Saleh HAE. (2005). Mycological studies on *C. neoformans* and other yeasts isolated from clinical cases and environment. M.V.Sc. thesis, Department of Microbiology, Faculty of Veterinary Medicine, Cairo University.

Samaka HM, Al-Azzam AH, AL-Naqeeb MM, Al-Hatami AO and Al-Rufai HM. (2011). Genotyping of *Candida albicans* Isolated from Turkeys by 25S Intron Analysis. Kufa Journal for veterinary medical sciences, Special issue for Researches of First Scientific Conference of Faculty of Veterinary Medicine – University of Kufa. 28-29: part 2: 54-62.

Shibat-El-Hamed DMW. (2008). Some studies on poultry Mycosis in Quena Governorate. M.V.Sc. thesis, Department of Poultry diseases, Faculty of Veterinary Medicine, Assuit University

Şeker E (2010). Identification of *Candida* Species isolated from Bovine mastitic milk and their in vitro hemolytic activity in Western Turkey. Mycopathologia 169:303-308.

Shnawa IM and Nigam JM. (1987). A note Cryptococcal mastitis in sheep. Ind.J.Vet.Med.7:175-176.

Sidrim JJC, Costa AKF, Cordeiro RA, Brilhante RSN, Moura FEA, Castelo-Branco DSC, Neto MPA, and Rocha MFG. (2010). Molecular methods for the

diagnosis and characterization of *Cryptococcus*: a review. Canadian Journal of Microbiology. 56(6): 445-458.

Spanamberg A, Sanches EMC, Santurio JM and Ferreiro L. (2009). Mycotic mastitis in ruminants caused by yeasts. Cienc. Rural. 39 (1): 282-290.

Spanamberg A, Ramos JP, Leoncini O, Alves SH and Valente P. (2009). High frequency of potentially pathogenic yeast species in goats raw milk and creamed cheese in Southern Brazil. Acta Scientiae Veterinariae. 37(2):133-141.

Spanamberg A, Wünder Jr EA, Brayer Pereira DI, Argenta J, Cavallini Sanches EM, Valente P, Ferreiro L. (2008). Diversity of yeasts from bovine mastitis in Southern Brazil. Rev. Iberoam Micol., 25: 154-156.

Susever S and Yeğenoğlu Y. (2011). Evaluation of the significance of molecular methods in the diagnosis of invasive fungal infections: comparison with conventional methods. Mikrobiyol Bul. 45(2):325-35.

White TJ, Burns T, Lee S, and Taylor J. (1990). Amplification andsequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322. *In* M.A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (ed.), PCRprotocols. A guide to methods and applications. Academic Press, Inc., San Diego, Calif.