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Detection of Cryptococcus sp. from clinical and subclinical mastitis in dairy cows and assessment antifungal activity to fluconazole, amphotericin B, and nystatin

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Abstract

Cryptococcus species are increasingly recognized as significant pathogens causing mastitis in dairy cows. The objective of this research was to detect *Cryptococcus spp.* in milk samples collected from cows with subclinical and clinical mastitis. Additionally, assessment antifungal activity to fluconazole, amphotericin B, and nystatin. Milk samples were collected randomly from various locations in Diyala province and a total of 27 samples were obtained. Each sample was cultured on Sabouraud Dextrose Agar (SDA) and the resulting isolates were identified using various laboratory methods such as morphological and microscopic examination. Of the 27 samples, 8 (28.6%) were positive for Cryptococcus species, including Cryptococcus neoformans (5 samples, 62.5%), Cryptococcus gattii (2 samples, 25%), and Cryptococcus laurentii (1 sample, 12.5%), as determined by Cryptococcus differential agar (CDA). Antifungal susceptibility examination was performed using disk diffusion method and all 8 isolates were found to be 100% resistant to all examined medications in this study; fluconazole, amphotericin B, and nystatin.

Keyword:

Cryptococcus, Clinical, subclinical Mastitis, Antifungal activity.

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Introduction:

Mastitis, which is an inflammation of the cow's mammary gland, is a major

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disease affecting dairy cows globally, leading to substantial economic losses in the dairy industry due to decreased milk production, early culling, and increased veterinary costs (1). The disease can manifest in both clinical and subclinical forms, with the latter often is undetected due to the absence of visible symptoms (2).

Cryptococcus species are encapsulated yeasts that are omnipresent in the environment, (3), especially in association with birds and bird droppings (4). *Cryptococcus spp.* have also been reported to cause disease in domestic animals, including mastitis in cattle (5,6). The role of *Cryptococcus species* in bovine mastitis has been underexplored, with most studies focusing on bacterial pathogens (7). *Cryptococcus neoformans* and *Cryptococcus gattii* are the most common species associated with human and animal infections (8). *C. laurentii*, a yeast pathogen, has been identified as a rare and opportunistic species found in bovine mastitic milk samples (9).

These fungal pathogens are usually resistant to common antifungal agents used in veterinary medicine (10). The treatment of *Cryptococcus mastitis* is challenging due to the inherent resistance of these fungi to many antifungal drugs (11). Fluconazole, amphotericin B, and nystatin are among the antifungal agents used to treat *Cryptococcus* infections (11). However, the efficacy of these drugs against *Cryptococcus species* isolated from mastitis cases in dairy cows has not been extensively studied. *In vitro* antifungal sensitivity test is a valuable tool for guiding the choice of antifungal therapy (12). The aim of this study was to test the sensitivity of Cryptococcus species, isolated from clinical and subclinical cases of mastitis in dairy cows, to fluconazole, amphotericin B, and nystatin. Fluconazole is a triazole antifungal agent that inhibits ergosterol synthesis (13). Amphotericin B is a polyene antifungal drug that binds to ergosterol in fungal cell membranes, causing leakage of intracellular components and antifungal activity (14). Nystatin is a polyene antifungal that acts by attaching to sterols in the plasma

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membrane of yeast, thereby inhibiting their growth (15).

Materials and Methods:

Sample collection

In this study, milk samples were collected from 27 cows experiencing clinical and subclinical mastitis in various regions of Diyala. The teats of the cows were cleaned with 70% alcohol swabs and allowed to air dry before collecting the milk samples. The first spurts of milk were discarded, then 2-5 ml of milk from each cow was collected into sterile glass flasks after letting the alcohol dry. This was done to ensure clean and sterile milk samples. Subsequently, the samples were transported under cold chain conditions to the Mycology Laboratory at Diyala University College of Veterinary Medicine.

Isolation and identification of *Cryptococcus spp*.

In order to isolate *Cryptococcus spp.* from the milk samples, the primary growth medium used was Sabouraud dextrose agar supplemented with 0.05 mg/ml chloramphenicol. The incubation of all plates was carried out at 37°C for 72 hours. To identify Cryptococcus species morphologically, suspected colonies grown on Sabouraud dextrose agar (SDA) were mixed with a drop of Indian ink on a glass slide and examined under a microscope. Positive colonies were subcultured onto SDA with chloramphenicol and streaked onto Cryptococcus differential agar (CDA) to differentiate *C. neoformans*, *C. gatti*, and *C. laurentii* based on colony color after 37°C incubation for 36-48 hours. Light blue, dry brown mucoid, and brown dry colonies indicated *C. neoformans*, *C. gatti*, and *C. laurentii*, respectively. Pure colonies were further confirmed via biochemical tests including phenol oxidase and urease assays. The oxidase test involved rubbing colonies on paper soaked with NNN'N'-tetramethyl-p-phenylenediamine and examination for a bluish-purple color. The urease test used a urea agar slant to examine the color change from yellow to pink after inoculation and incubation. Isolates positive on all tests were identified as *Cryptococcus spp*.

Antifungal susceptibility test

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The antifungal susceptibility test was performed using the agar diffusion technique. The inoculum was created from 48-hour plate cultures of Cryptococcus species. These colonies were mixed in 0.9% saline, and the cloudiness was matched to the 0.5 McFarland standard to create a yeast suspension with $1x10^6$ to $5x10^6$ cells/ml. This suspension was applied to a sterile cotton swab, which was then rotated and pressed against the tube's inner wall to remove excess inoculum. Cultures were swabbed evenly across Sabouraud dextrose agar plates in three directions, rotating 60° between swabbing to distribute the fungi. Plates were allowed to dry for 5–15 minutes before placing antifungal disks on the agar surface. Plates were incubated at 37°C for 24 hours, with slower-growing isolates needing up to 48 hours before reading results. This agar diffusion method allowed uniform cryptococcal growth and the diffusion of antifungals across the plate for antifungal susceptibility test. Evenly distribution of inoculum, drying time, and optimized incubation enabled standardized testing conditions to determine antifungal sensitivity. All measurements of inhibition zone diameters from disk diffusion tests were conducted following CLSI standards. The interpretive criteria for determining susceptibility of the Cryptococcus isolates to the antifungal disks were as follows: for fluconazole (10 µg): zone diameter (dz) ≥ 19 mm was susceptible (S), 15 mm ≤ dz < 18 mm was susceptible-dose dependent (S-DD), and dz ≤ 14 mm was resistant (R). For amphotericin B (20 µg): dz ≥ 12 mm was S, 10 mm ≤ dz ≤ 11 mm was S-DD, and dz ≤ 10 mm was R. For nystatin (100 IU): dz ≥ 18 mm was S, 15 mm < dz ≤ 17 mm was S-DD, and dz ≤ 15 mm was R.

Results:

Out of 27 milk sample only 8 samples showed positive growth for Cryptococcus species. This indicates the infection rate 28.6% for Cryptococcus mastitis in the collected milk samples.

Cryptococcus species Isolated from Mastitic Milk Samples

On Sabouraud's Dextrose agar, the isolates displayed typical creamy white colonies. When examined under the

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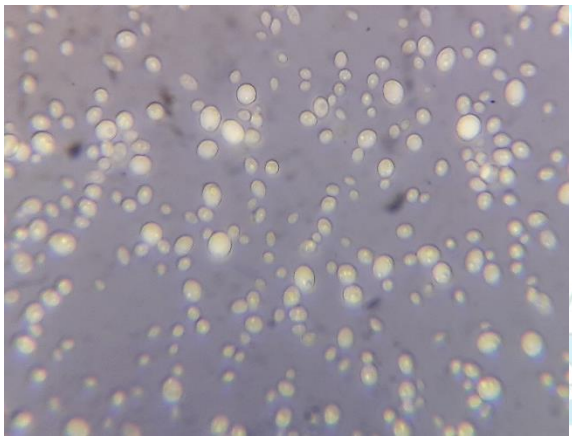
microscope budding yeast cells were observed after Lactophenol cotton blue staining. Additionally, when India ink staining was performed, a bright halo representing the capsular material surrounding the yeast cells was visible (Fig. 1).

Fig. (1): Macroscopic appearance of *Cryptococcus spp.* colonies on Sabouraud Dextrose Agar at 3 days at 37ºC. Microscopic of *Cryptococcus spp.*at 100X show encapsulated budding yeast cells with India ink stain.



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All isolates were found to be positive for urease activity, which is produced in large amounts by C. neoformans. When C. neoformans colonies were rubbed on NNN'N'-tetramethyl-pphenylenediamine dihydrochloride paper, they turned bluish purple quickly, indicating positive results (Fig. 2).

Fig. (2): *C. neoformans* showed (1) urease activity by turning the Christensen's urea slant pink, and (2) oxidase positive as evidenced by the bluish-purple color change on the oxidase test strips.

Cryptococcus differential agar (CDA) was used to differentiate between three *Cryptococcus species*, including *C. neoformans*, *C. gatti*, and *C. laurentii*. After being incubated at 37°C for 7 days, *C. neoformans*, *C. gatti*, and *C. laurentii* exhibited light blue, dry brown mucoid, and brown dry colonies, respectively. The species identified with this method represented three Cryptococcus species, including 5 samples (62.5%) Cryptococcus neoformans , 2 samples (25%) *Cryptococcus gattii*, and 1 sample (12.5%) *Cryptococcus laurentii* (Table 1),(Fig.3).

Table (1): The number of Cryptococcus spp. isolates from Bovine Mastitic Milk Samples

Cryptococcus species	**Isolation No.**	**Percentage %**
Cryptococcus neoformans	5	62.5

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Cryptococcus gattii	2	25
Cryptococcus laurentii	1	12.5

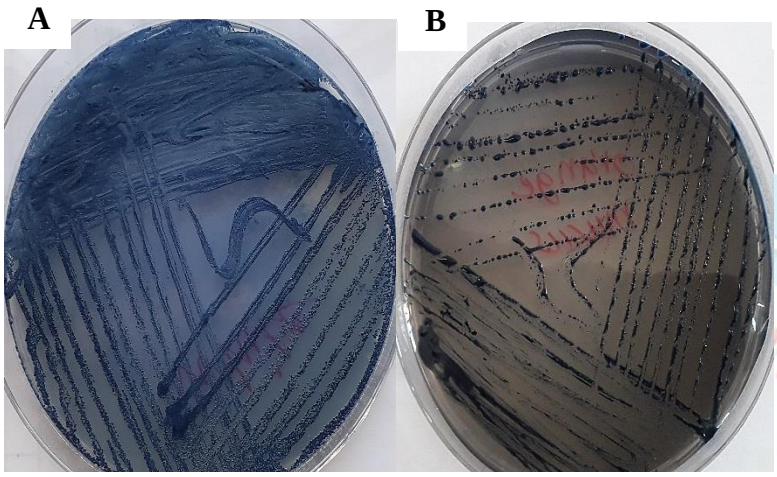

Fig (3): Cryptococcus Differential Agar (CDA) formed (A) light blue dry colonies of *C. neoformans*, (B) dry brown mucoid of *C. gattii*, and (C) brown dry of *C. laurentii* after 7 days of incubation.

Cryptococcus species	**No. /% of isolates**	**Fluconazole (FLU)**			**Nystatin (NY)**			**Amphotericin B (AMP)**		
		S	**SDD**	**R**	**S**	**SDD**	**R**	**S**	**SDD**	**R**
Cryptococcus neoformans	5 62.5%	-	-	5 100%	-	-	5 100%	-	-	5 100%
Cryptococcus gattii	2 25%	-	-	2 100%	-	-	2 100%	-	-	2 100%
Cryptococcus laurentii	1 12.5%	-	-	1 100%	-	-	1 100%	-	-	1 100%
Total	8 100%	-	-	8 100%	-	-	8 100%	-	-	8 100%

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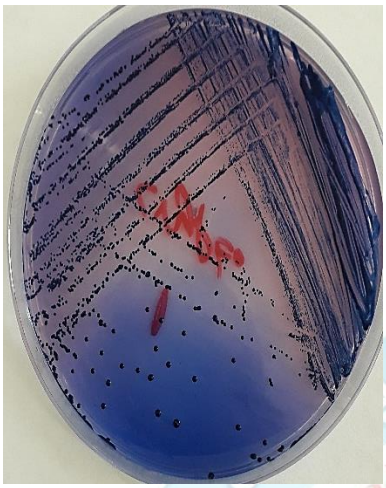

The response of Cryptococcus species to antifungal drugs

The antifungal susceptibility test of Cryptococcus species against Fluconazole, Amphotericin B, and Nystatin was carried out. The findings revealed that 100% of the Cryptococcus isolates tested displayed complete resistance to the antifungal drug Fluconazole. Similarly, all Cryptococcus species isolates exhibited total resistance (100%) to Nystatin. Furthermore, all Cryptococcus species were found to be entirely resistant (100%) to Amphotericin B as shown in (Table 2), (Fig. 4)

Table (2): The antifungal activity of Fluconazole, Nystatin, and Amphotericin B against *Cryptococcus species* was evaluated using the disk diffusion method.

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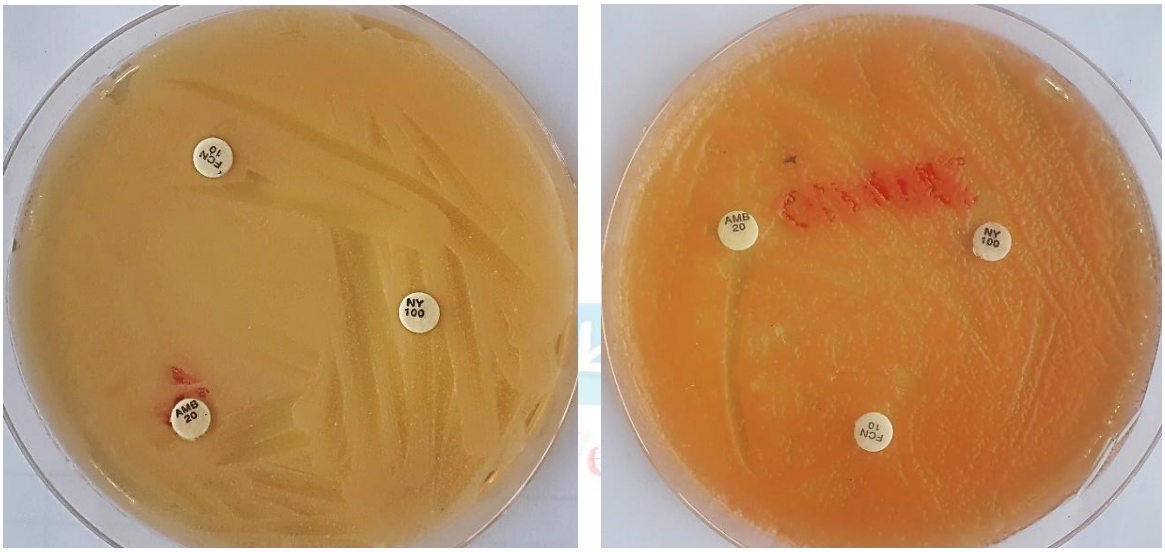

Fig (4): Cryptococcus species showed resistance (no inhibitory zones) to all three antifungal agents tested: FCN, NY, and AMB.

Discussion:

Mastitis, a common issue in the global dairy industry, can be caused by various types of bacteria (16) and can lead to decreased milk production and quality, resulting in economic losses for dairy farmers (17). Cryptococcus species are among the fungi that can be isolated from milk samples obtained from cows with subclinical or clinical mastitis.

The study found that out of 27 samples, 8 (28.6%) tested positive for Cryptococcus species, including Cryptococcus neoformans (5 samples, 62.5%), Cryptococcus gattii (2 samples, 25%), and Cryptococcus laurentii (1 sample, 12.5%). These identifications were made using Cryptococcus differential agar (CDA), a specialized medium designed to differentiate between Cryptococcus species based on their phenotypic characteristics (18).

Several studies have highlighted the role of Cryptococcus species in mastitis. A study investigated the isolation and identification of Cryptococcus species from dairy cows with clinical and subclinical mastitis. An Iraqi study found fungal infections in 80% of 100 mastitis cow's milk

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samples, with Cryptococcus neoformans accounting for 2.5% of these infections (19). In another study in China identified Cryptococcus species as one of the fungal pathogens causing mastitis in cows (20). Similarly, a study in Poland isolated Cryptococcus from milk samples of cows with clinical and subclinical mastitis (21). In Mexico, Cryptococcus laurentii was among the six species of yeasts identified in milk samples from dairy cattle with subclinical mastitis (22).

However, it's important to note that the incidence of mycotic mastitis caused by fungi, including Cryptococcus, is relatively low compared to bacterial mastitis in dairy cows (21).

In this study, the susceptibility of Cryptococcus species to three antifungal medications - including fluconazole, amphotericin B, and nystatin - was tested. Antifungal susceptibility testing was conducted using the disk diffusion method, and it was revealed that all 8 Cryptococcus isolates exhibited complete resistance (100%) to fluconazole, amphotericin B, and nystatin. This high level of resistance is concerning because these antifungal agents are commonly used to treat fungal infections (23). The findings suggest bovine Cryptococcus infections will be extremely difficult to treat with commonly available antifungal drugs.

Several scholarly studies have explored the antifungal sensitivity of various Cryptococcus species. For instance, a study examined in vitro antifungal susceptibility of *Cryptococcus species* isolated from Guangxi province in Southern China. Its findings revealed that all isolates were susceptible to a wide range of antifungal drugs, notably fluconazole and amphotericin B (24). Another study investigated the sensitivity of clinical *Cryptococcus neoformans* isolates from Thailand to five antifungal agents - amphotericin B, 5-flucytosine, fluconazole, itraconazole and ketoconazole. The results showed that all the isolates were susceptible to amphotericin B. However, some isolates displayed resistance towards fluconazole and itraconazole (25). Contrarily, a

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different study recorded a marked resistance in all tested isolates against fluconazole, ketoconazole, and amphotericin B antifungals (26). Finally, a study tested the in vitro antifungal sensitivities of 57 *Cryptococcus gattii* strains against 9 antifungal agents, including fluconazole, amphotericin B and nystatin. The results showed that all the *C. gattii* strains were susceptible to amphotericin B. 98% of strains were susceptible to fluconazole and 96% were susceptible to nystatin (27). There were several factors, including geographic variations, species distinctions (28), and methodological differences (29), along with genetic changes in Cryptococcus populations (30). These factors are likely to contribute to the observed variability in sensitivity profiles across the studies. Tracking this diversity is crucial for comprehending resistance epidemiology.

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