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In vitro Utility of Zinc oxide Nanoparticles and Antifungal Drugs for the Treatment of Mycotic Mastitis in Dairy Cows in Egypt

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Abstract

Bovine mastitis is an inflammation of mammary gland parenchyma in cows. It is caused by multiple pathogens including bacteria and fungi. Mycotic mastitis is a secondary disease following improper frequent use of antibiotics or an unhygienic environment. The treatment of rising cases of mycotic mastitis is still controversial because of the rapid resistance acquired by the traditional use of antifungal drugs. The present study aimed to investigate the use of zinc oxide nanoparticles (ZnO-NPs), alternative therapy for traditional antifungal drugs, to combat fungal isolates from mastitic cows by examining the in vitro antifungal activity of ZnO-NPs. One hundred milk samples were aseptically collected from cows suffering from clinical mastitis in a governorate in Egypt. The fungal isolates were identified by their colony morphology and microscopical examination and subsequent underwent determining the MIC of traditional antifungal drugs and ZnO-NPs using the disc diffusion methods. Our results showed that 70% of milk samples were positive for mycotic mastitis with 30% yeasts, 15% *Mold*s, and 25% yeast and *Mold*s. *Candida* was the most common yeast species isolated. *Rhodotorula*, *C. guilliermondii*, *C. parapsilosis*, and *C. albicans* showed entire resistant (100%) to all traditional antifungal drugs whereas, the same isolates, except *C. albicans*, were susceptible to ZnO-NPs. *Mold* spp. were susceptible to ZnO-NPs and only Itraconazole, and Clotrimazole. ZnO-NPs are highly effective and promising inexpensive antimicrobial agent for the treatment of bovine mycotic mastitis.

KEYWORDS
Mycotic mastitis, Cow, ZnO-NPs, Antifungal.

INTRODUCTION

Dairy manufacturing is currently targeted on the genetic enhancement of dairy cattle to increase milk yield to be nearly twice that of the nineteenth century (Whitelaw *et al.*, 2016). This dairy zone reports nowadays represents 15% of the total farming production. Selecting of high milking cow to incorporate into the livestock industry is mainly associated with the incidence of mastitis (Bradley, 2002; Reshi *et al.*, 2015).

Bovine mastitis is the most predominant disease affecting milk herds worldwide. It is a complex disease caused by a wide range of infectious agents which triggers large economic losses and harm to the livestock industry in the form of a reduction in milk yield and increasing the costs of treatment and culling (Costa *et al.*, 1998; Zaragoza *et al.*, 2011). Bacteria are usually considered the primary infectious cause of mastitis while fungi represent the secondary etiological agents. The clinical signs of mastitis are usually clear, and the disease can be distinguished through external symptoms such as swelling, heat, redness, hardness, or pain of the udder (Dworecka-Kaszak *et al.*, 2012). The extensive and uncontrolled usage of antibiotics for the treatment of mastitis is one of the main causes of increasing the prevalence of mycotic mastitis among dairy cows (Ilhan *et al.*, 2016; Krömker and Leimbach, 2017).

Fungal mastitis is usually caused secondary to environmental contaminants related to poor hygienic conditions of the animal and surroundings, teat injuries, and inefficient use of milking machines which enhance and accelerate the entry of infectious agents (Sheena and Sigler, 1995; Spanamberg *et al.*, 2018). The fungal bovine mastitis has been reported by several species of yeast or yeast-like microorganisms. *Cryptococcus neoformans* and *Candida* albicans are the most common causes, but other species have also been associated with fungal bovine mastitis (Staroniewicz *et al.*, 2007; Dworecka-Kaszak *et al.*, 2012). The occurrence of mastitis due to yeast is usually low in dairy herds, but it has drastically increased during the last few years. Yeast intramammary contaminations have been reported to be responsible for 10% of all reported cases seen in the veterinary field (Dworecka-Kaszak *et al.*, 2012; Du *et al.*, 2018; Kitila *et al.*, 2021).

To date in the clinical practice in Egypt, bovine mastitis is treated with antibiotics and antifungal drugs regardless of the diagnosis of the causative agents. According to WHO, the frequent use of antibiotics is a major public health issue especially due to the emergence of an antibiotic-resistant microbe and the

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presence of antibiotic residue in milk (Wahab *et al*., 2010; Gomes and Henriques, 2016). Therefore, an urgent need to develop a new, safe and specific drug for mycotic mastitis.

Nanotechnology was applied in a variety of fields like medicine, food, and agriculture. It was used to produce nano-sized particles that have different physical, optical, and chemical properties from their bulk (Nile *et al*., 2020). Zinc oxide nanoparticles (ZnO-NPs) are extensively used multifunctional semiconductive materials for complicated biosensing purposes using their unique physical and chemical properties (Aslan *et al*., 2008; El-Naggar *et al*., 2017). ZnO-NPs have shown a selective treatment for bacteria in several reports (Reddy *et al*., 2007; Hozyen *et al*., 2019). Although ZnO-NPs possess antibacterial, antifungal, and anti-inflammatory properties, the studies on this treatment for mycotic mastitis are scarce.

We hypothesize that the potential of antifungal activity against mycotic mastitis-causing pathogens could be achieved through using of ZnO-NPs. Therefore, this study aimed to clarify, in vitro, the utility of the use of ZnO-NPs as an antifungal drug against certain fungi isolated from mastitis cows in Egypt.

MATERIALS AND METHODS

Animals and sampling

The animals were handled during the whole experiment according to the regulations approved by the Ethical and Animal Care Committee of the animal health research institute, Damanhour branch, Beheira, Egypt. Animals, from three herds in Beheira governorate in Egypt, were inspected for clinical mastitis that was identified through macroscopical observation of the udder, redness, and swelling of the udder, reduction of milk yield, and presence of clots in the milk.

One hundred milk samples were collected from the affected cows with clinical mastitis. Briefly, the udder teats were disinfected with 70% ethyl alcohol before the collection of samples. Ten ml of milk was milked into sterilized tubes and kept in an icebox during transportation to the laboratory. The mycological inspection was carried out within 2 h after sample collection.

Culture and identification of pathogens

The procedure for isolation and identification of pathogens was performed according to previous reports (Pfaller *et al*., 1996; Prescott, 2003; Fitzgerald *et al*., 2004). Briefly, the milk samples were centrifuged (9000 rpm, 10 min), and the precipitate was resuspended in sterile water and cultured on Saboraud Dextrose Agar (40 g/l dextrose, 10 g/l peptones, and 20 g/l agar) supplemented with chloramphenicol 0.05 g/l, then incubated at 37°C and 25°C and examined daily for one week. The plates that showed no representative growth for fungi were considered negative. The identification of fungi belonging to the genus depended on colony features (pigmentation, shape, texture, color). The colonies with the same morphology were assumed to belong to the same genus or species. Negative control was included for the examined samples. The isolated colonies were analyzed under a microscope for confirmation of fungal characteristics. The fungal isolates were mounted using the lactophenol cotton blue stain solution examined microscopically for the presence of spores and vegetative bodies according to a previous study (Shamly *et al*., 2014).

Isolations of yeast were done on common agar lab media under aerobic conditions and at 30°C. Colonies were firstly selected based on colony morphology, and Gram stain microscopical examination (blastoconidia and chlamydospores) according to Qvirist *et al*. (2016). A germ tube test was performed for the differentiation between *Candida* species in which a very light suspension of yeast-like organisms in 0.5-1.0 ml of sterile rabbit serum was used. Incubation occurred at 37°C for no longer than 3 h. One drop of yeast- serum mixture was placed on a slide slip and was examined microscopically for germ tube production.

Antifungal sensitivity testing for isolated pathogens

Two main groups of antifungals were used in the clinical setting to treat fungal infections, polyenes group represented by Amphotericin B (AP), Nystatin (NS), and Azoles group represented by Itraconazole (IT), Fluconazole (FCA), Voriconazole (VRC), Clotrimazole (CC), and Metronidazole (MTZ). The antifungal sensitivity test was conducted using the disc diffusion test. The in vitro sensitivity of the isolates to antimicrobials was determined according to standards of the National Committee for clinical laboratory (NCCLS2002). The isolated fungi were subcultured on sabouroud dextrose agar and incubated at 37°C for yeast and 25°C for *Mold*. A loop full of pure culture from each isolate was mixed well with 9 ml of sodium chloride solution then spread over the surface of sabouroud dextrose agar plates then suctioned excess fluid. Antifungal discs were spread on the surface of the inoculated plate. Plates were incubated for 5 days at 37°C for yeast and 25°C for Mold. The diameter of the inhibition zone of each disc was measured (mm) and judged.

ZnO-NPs preparation and characterization

The metal oxide nanoparticles of ZnO-NPs were prepared by nanotech Co. in Egypt. The ZnO-NPs were white in color with a powdery appearance. The size and shape of ZnO-NPs were determined using a transmission electron microscope (TEM- JEOL JEM-2100 high resolution) at an accelerating voltage of 200 kV. The size was recorded as an average of 30.0±5.0 nm and the shape was spherical like shape. Solubility was determined to be dispersed in water and suspended in ethanol/methanol.

ZnO-NPs antifungal activity

The anti-fungal activity of ZnO-NPs was determined using the disc diffusion method. The antimicrobial activity was checked against the fungal isolates of *A. flavus*, *A. niger*, *C. guilliermondii*, *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. famata*, and *Rhodotorula*. The minimum inhibitory concentration (MIC) for each isolate was determined. Fungal spores were harvested after 7 days of culturing on sabouroud dextrose agar slant. The culture was washed with 10 mL normal saline in 2% Tween 80 with the aid of glass beads to help in the dispersion of the spores. Using the dilution method, the spore suspensions were standardized to 10^5 spores/mL. One mL of each standardized spore suspension (10^5 spores/mL) was evenly spread on the surface of the sabouroud dextrose agar plates. After that, the plate was divided into three parts each having different ZnO-NPs concentrations of (5, 10, and 15 µg/ml) each concentration was dissolved in deionized distilled water, the surface was impregnated with discs covered with 100 µL from each concentration used. Further, the plates were incubated at 37°C for 5 days and the growth inhibition was examined by the formation of the inhibitory zone. The diameters of the inhibition zones were measured in millimeters (mm). The MIC of ZnO-NPs was determined. The inhibitory concentration was defined as the lowest concentration that inhibited the growth.

RESULTS

The incidence of mycotic mastitis isolated from affected cows was recorded. The number of yeast samples was greater than that of Mold, 30, and 15 samples respectively. Twenty-five samples were recorded for both yeast and Molds. Out of 100 samples, 30 samples were negative for mycotic mastitis. The presence of fungi in the samples was identified by colony morphology and microscopical examination. The color of the colony appeared yellow to green for *A. flavus*, dark blackish for *A. niger*, orange colony for *Rhodotorula* and white to cream for *Candida* species. The microscopical appearance showed budding yeast-like cells for *Candida* species. The incidence of different Mold and yeast isolated was present in Table 1. *A. flavus* and niger were predominant for Mold infection, and the *Rhodotorula* spp. was predominant for yeast infection.

Table 1. Incidence of different types of fungi isolated from milk samples of the affected cows.

Fungi spp.	No. of +ve samples	Percent (%)
No. of samples	70	100
Mold		
A. flavus	30	42.8
A. niger	20	28.5
Yeast		
Rhodotorula	40	57.1
C. albicans	10	14.2
C. famata	15	21.4
C. tropicalis	12	17.1
C. parapsilosis	10	14.2
C. guilliermondii	7	10

The activity of antifungal drugs sensitivity against different fungal isolates from milk samples of mastitic cows is summarized in Table 2. *Rhodotorula*, *C. guilliermondii*, *C. parapsilosis*, and *C. albicans* showed entire resistance (100%) to all antifungal drugs used. Whereas *C. tropicalis* and *C. famata* showed a susceptibility property to all antifungal drugs examined except for MTZ5 drug. The *Mold* spp. (*A. flavus* and *A. niger*) was expressed sensitivity only for IT10 and CC10 drugs. The representative images for the description of MIC of the isolated *C. tropicalis*, and *C. parapsilosis* were shown in Fig. 1.

The average diameter of inhibition zones for ZnO-NPs was summarized in Table 3. The presence of cleared inhibition zone is indicative of the antifungal effect of ZnO-NPs with designated concentration against isolated fungi. The three concentrations of ZnO-NPs were effective against entire *Mold* spp. (*A. flavus*, and *A. niger*), and the *Yeast* spp. of *Rhodotorula*, *C. tropicalis*, *C. guilliermondii*, and *C. parapsilosis*. Complete resistance by *C. albicans* was noticed for the three concentrations. While the high concentration of ZnO-NPs (15/ µg/ml) is solely effective against *C. famata*. In all susceptible fungi, the high concentration of ZnO-NPs (15 µg/ml) showed the largest inhibition zone among the other two concentrations (5 and 10 µg/ml) with a clear zone of 30 mm for *C. parapsilosis*. The representative images for the MIC of ZnO-NPs against isolated fungi were presented in Fig. 2.

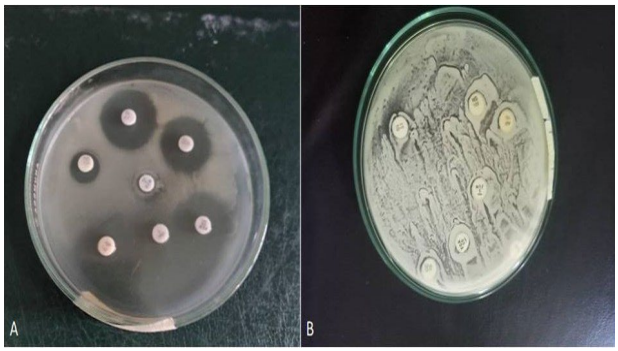

Fig. 1. Representative images for the antifungal activity against (A) *C. tropicalis* (sensitivity) and (B) *C. parapsilosis* (resistance) isolated from cow with clinical mastitis using the disc diffusion method.

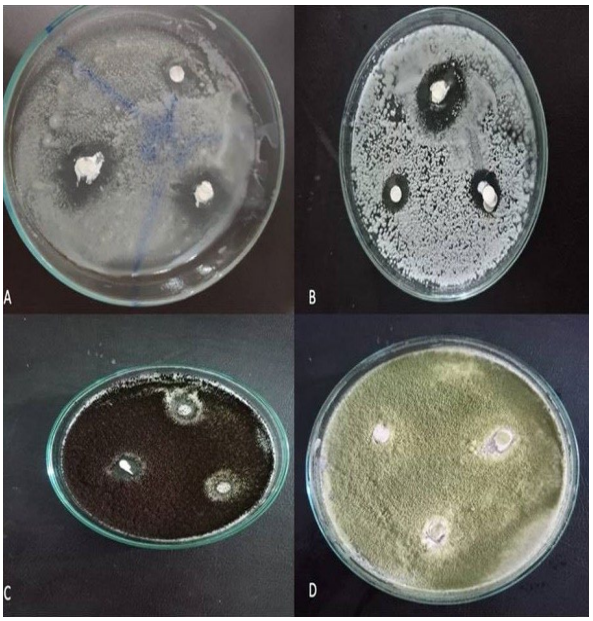

Fig. 2. Representative images for the disc diffusion method for the three concentrations (5, 10, and 15 µg/ml) of ZnO-NPs activity against *C. guilliermondii* (A), *C. parapsilosis* (B), *A. niger* (C), and *A. flavus* (D)

Table 2. Minimal Inhibitory Concentration (MIC) of antifungal drugs against different isolated fungi from mastitic cows.

Species	IT10 (mm)	CC10 (mm)	NS100 (mm)	FCA25 (mm)	AP100 (mm)	VRC1 (mm)	MTZ5 (mm)
A. flavus	27	23	R	R	R	R	R
A. niger	23	17	R	R	R	R	R
Rhodotorula	R	R	R	R	R	R	R
C. albicans	R	R	R	R	R	R	R
C. famata	15	18	13	22	9	11	R
C. tropicalis	16	17	10	13	26	9	R
C. parapsilosis	R	R	R	R	R	R	R
C. guilliermondii	R	R	R	R	R	R	R

IT 10, Itraconazole; CC 10, Clotrimazole; NS 100, Nystatin; FCA 25, Fluconazole; Ap100, Amphotericin; VRC1, Voriconazole; B, MTZ5, Metronidazole; R, Resistant

Table 3. Minimal Inhibitory Concentration (MIC) of ZnO-NPs against different isolated fungi from mastitic cows.

Fungal spp.	ZnO-NPs		
	5 µg/ml	10 µg/ml	15 µg/ml
A. flavus	10 mm	12 mm	16 mm
A. niger	12 mm	13 mm	15 mm
Rhodotorula	8 mm	10 mm	15 mm
C. albicans	R	R	R
C. famata	R	R	11 mm
C. tropicalis	8 mm	9 mm	10 mm
C. parapsilosis	10 mm	15 mm	30 mm
C. guiliermondii	13 mm	19 mm	20 mm

Zn-O-NPs, Zinc oxide nanoparticles; R; resistant

DISCUSSION

The huge mass of bovine mastitis cases is caused by bacteria, but recently there have been a rising number of studies about cases of mycotic etiology (Spanamberg *et al*., 2008). The routine treatment of mastitis with antibiotic drugs without determining the suitable therapy for the causative agent is a big issue in Egypt. The WHO reported that improper frequent use of antibiotics has a public health concern because of the antibiotic residue in milk and increasing the rate of antibiotic resistance (Gomes and Henriques, 2016). Most unresponsive cases from antibiotic treatment were classified as mycotic cases. The traditional antifungal drugs have been used vigorously in the clinical field which raises the resistance property of most fungal species. This study aimed to study the in vitro utility usage of an alternative promising drug of ZnO-NPs against isolated fungi from a cow suffering from mastitis in Egypt.

The incidence of mycotic mastitis has seen an increase in the number of cases documented (Abd El-Razik *et al*., 2011; Ilhan *et al*., 2016). In the current study, 70% of milk samples collected were positive for the presence of fungal pathogens. The sharp increase in the cases reported might be attributed to inadequate milking hygiene, contamination of intramammary syringes, or frequent use of antibiotics as prophylaxis in a dry period. The prevalence of mycotic mastitis was performed in many countries and found to range from 1 to 25 %. The results of our manuscript showed to be higher than the old records (Farid *et al*., 1975; Abd El-Halim, 1979; Awad *et al*., 1980; El-Kholy and Hosein, 1990) in Egypt which reported incidences of 1.1%, 3.6%, 6.13%, and 20% explaining the sharp increase of mycotic cases in Egypt because of the above-mentioned causes. The yeast isolation, especially *Candida* shows a potential increase over that of Mold which is in agreement with the previous studies carried out in Egypt and is usually associated with unhygienic intramammary treatment (Gaudie *et al*., 2009).

In this study, the antifungal susceptibility pattern was targeted. Our results revealed that the traditional antifungal drugs used in the clinical veterinary field in Egypt acquired resistance against several fungi isolated including 100% resistant against *Rhodotorula*, *C. guilliermondii*, *C. parapsilosis*, and *C. albicans* followed by *Molds* (*A. flavus* and *A. niger*) resistance. Those pathogens counted the higher percent of fungi isolated from mastitic cows which may explain the acquired antifungal resistance. Our results confirm the result of the previous study performed by Sonmez and Erbas (2017). It is highly recommended that the traditional antifungal drugs should be decreased in the use for the treatment of mycotic mastitis in affected cows in Egypt. Stimulatingly, the fungal pathogens isolated from cows that had a record free from treatment with antimycotic drugs, indicating that these fungal isolates showed conservational selective pressures represented in the frequent use of azole antifungals drugs in the agricultural field (Rocha *et al*., 2016). Consequently, a distinctive consideration should be paid to animal products for checking the antifungal vulnerability of fungi especially *Candida* spp., meanwhile it may act as reservoirs of strains producing human disease, especially for those immunocompromised persons.

Nanoparticles have seen incorporated into several therapeutic pathways for diseases treatments in animals and humans (Patra *et al*., 2018; Youssef *et al*., 2019). Metal oxide nanoparticles have emerged as promising antibacterial materials (Kadiyala *et al*., 2018). ZnO-NPs are among the most common oxides that have received significant interest worldwide in biological applications and have been regarded as safe materials for animals and humans (Sirelkhatim *et al*., 2015; Islam *et al*., 2022). In the current study, the disc diffusion method was used to determine the zone of inhibition for ZnO-NPs against designated fungi according to CLSI guidelines. All kinds of isolated fungi were susceptible to ZnO-NPs at different concentrations except for *C. albicans*, and *C. famata* with special regard to the higher concentration of ZnO-NPs (15 µg/ml) that showed the larger MIC for all susceptible fungi indicating that high effectiveness of ZnO-NPs for treatment of mycotic mastitis in dairy cows. *C. albicans* is considered the top resistant pathogen in most previous literature (Elad *et al*., 1995; Dworecka-Kaszak *et al*., 2012; Zhou *et al*., 2013). Special attention should be paid to Egyptian dairy farms to *C. albicans* which showed 100% resistance to ZnO-NPs and traditional antifungal drugs.

According to our knowledge, this is the first study to reveal the MIC of ZnO-NPs against fungi isolates from contagious mastitis milk samples. Therefore, several studies are required to investigate the in vivo use of ZnO-NPs for the treatment of mycotic mastitis in the dairy cow as well as describe the relation between nanosized particles and the efficacy of ZnO-NPs. Elucidation of the pathophysiological mechanism of ZnO-NPs to destroy the fungal isolates should be targeted in other studies.

CONCLUSION

This proof-of-concept study proved that mycotic mastitis is a potential disease affecting dairy herds, particularly after prolonged exposure to adverse environmental conditions and the number of cases is increasing every passing year. The lower concentration of ZnO-NPs has a high killing activity agaianst the fungal isolates from mastitis cows. The ZnO-NPs could be a useful substitute for the resistance acquired by traditional antifungal drugs.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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