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Short Communication

Anticandidal Effects of Zinc Oxide Nanoparticles on Fluconazole-Resistant *Candida* Isolates Causing Diarrhea in Calves, *in vitro*

Fozouni, L[1 *], Tahaei, M[1]

1. Department of Microbiology, Gorgan Branch, Islamic Azad University, Gorgan, Iran

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Corresponding Author: lili_kia@yahoo.com

Abstract

Gastrointestinal disease in calves may pose a significant threat to the livestock industry. Due to the increased rate of resistance to antifungal drugs and the side effects of these drugs, it is essential to find suitable alternatives, such as nanoparticles, with favorable antifungal effects and few side effects. This study aimed to determine the frequency of yeast causing diarrhea in calves and evaluate zinc oxide nanoparticles' antifungal effects on fluconazole-resistant isolates. Fecal samples from 94 calves (age: < three months old) with clinical signs of diarrhea were examined by standard microbiological and biochemical methods. The broth microdilution method evaluated the susceptibility of fungi to fluconazole and the antimicrobial activity of zinc oxide nanoparticles on drug-resistant isolates. *Candida albicans* (41.63%) were calves' predominant cause of diarrhea. In addition, 51.2% of the *C. albicans* isolates were resistant to fluconazole. All fluconazole-resistant isolates were eliminated when treated with 119 µg/ml of zinc oxide nanoparticles. The prevalence of diarrhea is relatively high in calves. Considering the predominance of drug-resistant *Candida* and the favorable in vitro effects of zinc oxide nanoparticles against these isolates, it is recommended to investigate the effects of zinc oxide nanoparticles on these isolates *in vivo*.

Keywords: Antifungal Effect**,** zinc oxide nanoparticles, Calve, drug-resistant, Enteropathogenic Candida

1. Introduction

Candidiasis is one of the most common fungal diseases in warm-blooded animals and humans, which has spread significantly in recent years. The causative agent of the disease is the opportunistic fungus from the genus *Candida*, which is part of the normal microflora in the gastrointestinal tract, mucosa, and skin of animals and humans. When the host resistance is reduced locally or systemically, primary or secondary, due to predisposing factors, this agent can cause diseases in various areas of the body as superficial infections (e.g., oral, vaginal, and cutaneous candidiasis) or as deep-seated infections (e.g., candidemia and pulmonary, gastrointestinal, and urinary tract candidiasis) (1). Although *Candida albicans* is the main pathogenic species, non-albicans species such as *Candida tropicalis*, *Candida glabrata*, *Candida parapsilosis*, *Candida guilliermondii*, *Candida krusei*, and *Candida famata* are also important. Although often isolated from air, soil, water, plants, and animal and human feces, *C. famata* (also known as *Debaryomyceshansenii* and *Torulopsis candida*) is recognized as a rare etiologic agent of diseases in humans and animals (2, 3). In animals, *C. famata* has been isolated from the breast tissue of cows with mastitis, ruminants' vaginal discharge, the mouth of pigs with vitamin A deficiency, horses with arthritis, and fecal samples of dogs with diarrhea.

Hydrocarbon-rich diets, strong antibiotics, and metabolic diseases predispose factors for cattle candidiasis. Candidiasis in cattle often affects the gastrointestinal tract, causes thrush lesions in the mouth, esophagus, and foregut, and causes enteritis and diarrhea when affecting the intestines. The disease is often seen in calves receiving long-term antibiotic therapy. Clinical signs depend on the location of the lesions, so in the case of pre-gastric and intestinal involvement, watery diarrhea followed by anorexia, dehydration, weakness, and severe lethargy are seen, which may ultimately progress to paralysis and death (4).

Various antifungal drugs, including azoles, are used to treat these infections. Fluconazole is an oral azole that inhibits ergosterol biosynthesis in fungi. Studies have indicated resistance of *Candida* species to antifungal drugs (5). In addition, due to the side effects of drugs and the risk of drug interactions, the use of alternative antimicrobial compounds, including nanoparticles, with less toxicity and side effects, has recently received much attention. Zinc oxide (ZnO) nanoparticles (NPs) are of great importance due to their diverse industrial and medical applications. An essential characteristic of ZnONPsis their antimicrobial properties at normal pH and in the absence of light. This NP has selective toxicity and can be considered a potential alternative to some antibiotics (6). This study aimed to determine the frequency of enteropathogenic *Candida* strains isolated from calves with diarrhea and to compare the antifungal effects of fluconazole and ZnONPs on the isolates obtained from livestock farms.

2. Materials and Methods

This study was performed on a herd of calves (n=231) aged less than three months in livestock farms around Gorgan (northern Iran) in 2020. After examining vital signs such as respiratory rate, heart rate, and a number of normal rumen contractions, fecal samples from 94 calves with diarrhea were taken and sent to the microbiology laboratory. After ensuring a lack of viral contamination, 1 g of each fecal sample was cultured in 9 ml of peptone water and incubated for 24 hours at 37 °C. The cultivated colonies were sub-cultured on blood agar, MacConkey agar,eosin-methylene blue agar, and in selenite F broth and examined by microbiological and differential tests in order to determine bacterial genus. To isolate fungal species, the samples were cultured on sabouraud dextrose agar with chloramphenicol (Merck, Germany). After 48 hours of incubation at 35 °C, yeast-like fungi were identified based on conventional biochemical techniques using VITEK 2 system (Biomerieux India Private Limited) with YST-ID (7).

2.1. Determination of Drug Susceptibility

After preparing suspensions from 48-hour cultures of *Candida*isolatesand confirmation of 530 nm wavelength and 77-75% light transmission in a spectrophotometer, a primary suspension containing 10^6cfu/ml yeast cells was diluted with 1640 RPMI medium (Sigma, USA) (1:100) to obtain the final concentration of 10^3cfu/ml. Drug susceptibility testing was performed using the broth microdilution method according to the Clinical & Laboratory Standards Institute(CLSI-M27) guideline (8). After preparing the fluconazole solution (Gibco, Germany) in water, dilutions (0.06-512 µg/ml) were made and poured into wells of a 96-well plate containing RPMI 1640 medium (without bicarbonate and with pH indicator and glutamines) and MOPS buffer (Sigma, USA). After inoculating yeast suspensions (1×10^3cfu/ml) into the wells, the microplate was incubated at 35°C for 48 hours. To determine the minimum fungicidal concentration (MFC), 100 µl from the content of each well with a concentration higher than the minimum inhibitory concentration (MIC), together with 100 µl of the positive control (the wells with yeast suspension and RPMI), were separately inoculated on sabouraud dextrose agar and were incubated for 48 hours at 35°C.

2.2. Determination of Antimicrobial Properties of ZnONPs

To prepare the NPs, 5 g of zinc acetate powder (Merck, Germany) was mixed with 50 ml of deionized distilled water at 80 °C. The mixture was then placed at

100±5 °C for 12 consecutive hours and finally at 300±20 °C for 24 hours to form NP crystals. Morphology and size of the formed ZnONPs were examined by transmission electron microscopy using the Leo 906 microscope (KV model, Zeiss, Germany) with an acceleration voltage of 120 kV (Figure 1). In order to determine the crystallographic structure of ZnONPs, X-ray crystallography was performed using an XRD device with CuKa radiation (Olympus, Japan) in the range of 0 to 110 degrees.

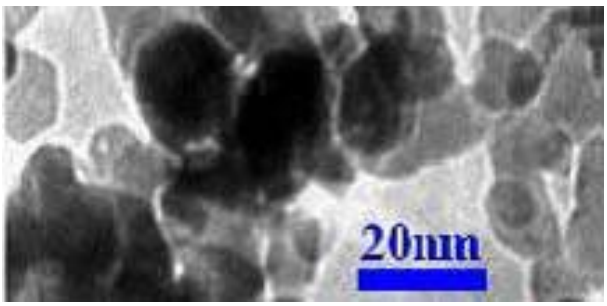

Figure 1. Transmission Electron Microscopy

To determine the antimicrobial properties, serial dilutions (1.85-238 µg/ml) were prepared by mixing ZnO powder with distilled water. After centrifugation at 3800 rpm, the precipitate was dissolved in distilled water. Absorption spectra in the 700-600 nm range were detected using spectroscopy to ensure binding. Next, 10 µl of the colloidal ZnONPs (diameter: 20 nm) solution and 200 µl of sabouraud dextrose broth were separately added to wells of a 96-well plate. After inoculating the yeast suspension (10^3cfu/ml) and 48 hours of incubation, 10 µl were taken from each well and inoculated onto sabouraud dextrose agar with chloramphenicol. Finally, the number of colonies (cfu) was determined.

To determine the minimum fungicidal concentration (MFC), 10 µl were collected from wells where no fungal growth was observed and poured onto sabouraud dextrose agar. The lowest concentration at which no growth was observed was determined as the MFC. To confirm the antifungal effects of ZnONPs, the isolates were treated with sub-inhibitory concentrations of the NPs.

2.3. Statistical Analysis

Data were analyzed with the GraphPad Prism statistical software using Chi-Squared (χ^2) test. A *p*-value of less than 0.05 was considered statistically significant.

3. Results and Discussion

In the clinical picture of the studied calves, involvement of oral and gastrointestinal mucosa in the form of enteritis and diarrhea was observed so that among 231 calves with the same pattern of vital signs and diet, 94 (40.7%) calves had watery diarrhea. Examination of the fecal samples of infected animals did not reveal any viral contamination. Examination of the grown yeasts indicated that diarrhea was predominantly caused by *C. albicans*in in 41 cases (43.61%) and *C. famata* in 8 cases (51.8%). The remaining positive cultures contained bacterial contamination, so 14 samples (14.9%) were infected with *Salmonella*, and 31 samples (32.98%) were infected with *Escherichia coli*.

According to the CLSI guidelines (8), 21(51.2%) *C. albicans* isolates and 3 (37.5%) *C. famata* isolates had MIC values of ≥64 µg/ml and were therefore considered resistant to fluconazole. The results indicated that ZnONPs had inhibitory effects on fluconazole-resistant *Candida* isolates in a dose-dependent manner. At a 119 µg/ml concentration, the ZnONPs inhibited the growth of 97% of fluconazole-resistant *C. albicans* isolates. There was a significant difference between the MIC of fluconazole, which inhibited the growth of 50% of *C. albicans* isolates (MIC50=7.43µg/ml), and the MIC of ZnONPs, which inhibited the growth of 90% of *C. albicans* isolates (MIC90=29.75µg/ml) (*P*=0.01). The results showed that all fluconazole-resistant isolates were eliminated in the presence of 119 µg/ml of ZnONPs (Table 1). As shown in table 2 and figure 2, after treatment of *Candida* isolates with ZnONPs, the absorbance at 570 nm reduced significantly (*P*=0.02) compared to the control group (untreated isolates).

Table 1. Comparison of MIC and MFC of ZnO NPs and fluconazole against *Candida* isolates

Inhibitory agent		*C. albicans* (n=41)	*C. famata* (n=8)	χ^2	*P*-value
ZnO NP	Concentration range (µg/ml)	1.85-238	1.85-238	4.65	0.001
	MIC50*	14.86	7.43		
	MIC90**	59.5	29.75		
	MFC***	119	119		
Fluconazole	Concentration range (µg/ml)	0.06-512	0.06-512	7.33	0.3
	MIC50*	16	8		
	MIC90**	256	128		
	MFC***	512	256		

* Minimum inhibitory concentration that inhibited the growth of 50% of fungi compared to the negative control group.
** Minimum inhibitory concentration that inhibited the growth of 90% of fungi compared to the negative control group.
*** The minimum fungicidal concentration compared to the negative control group.

Table 2. Adsorption response of fluconazole-resistant *C. famata* and *C. albicans* isolates at 570 nm

Fluconazole-resistant *Candida* isolates	0.0	0.1	0.2	0.3	0.4	0.5	0.6
No. 1	-	-	-	N	-	P	-
No. 2	-	-	N	-	-	P	-
No. 3	-	-	-	-	N	-	P
No. 4,32	-	-	-	N	-	P	
No. 5	N	-	-	-	P	-	-
No. 6	-	-	N	P	-	-	-
No. 7,31	-	-	-	-	N	-	P
No. 8	-	-	N	-	-	P	-
No. 9	-	-	-	N	-	P	-
No. 10	-	-	N	-	P	-	-
No. 11	-	-	-	N	-	-	P
No. 12	-	-	N	-	-	P	-
No. 13	-	-	N	-	P	-	-
No. 14,33	-	-	N	-	P	-	-
No. 15	N	-	-	-	P	-	-
No. 16	-	-	-	N	-	P	-
No. 17	-	-	N	-	-	-	-P
No. 18	-	-	N	-	-	P	-
No. 19	-	-	-	-	N	-	P
No. 20,34	-	-	-	-	N	-	P
No. 21	N	-	-	-	-	P	-
No. 22	-	-	N	-	-	P	-
No. 23	-	-	N	-	P	-	-
No. 24	-	-	N	-	-	-	P
No. 25	-	-	N	-	-	P	-
No. 26	-	-	N	-	-	-	P
No. 27	-	N	-	-	-	-	-
No. 28	-	N	-	-	-	P	-
No. 29	-	N	-	-	-	P	-
No. 30	-	N	-	-	-	-	P

P:Pre-treatment
N: Next treatment

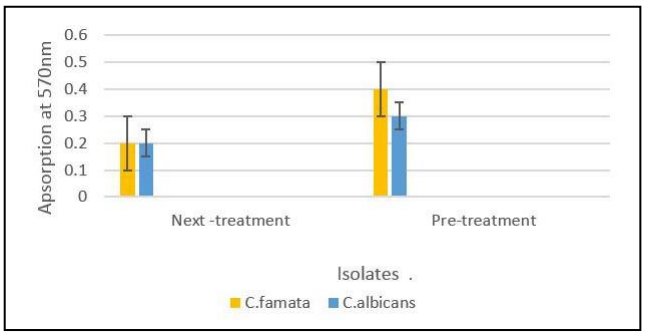

Figure 2. Mean absorption of *Candida* strains before and after treatment with ZnO nanoparticles

Diarrhea is one of the most important causes of death in livestock. In addition to bacteria, viruses, and protozoa, fungi can cause diarrhea in these animals (3). In this study, the prevalence of diarrhea in calves was 40.7%, of which 52.1% was confirmed as candidal diarrhea. Previous studies in Brazil (9), Hungary (10), and the Netherlands (11) reported the prevalence of diarrhea at 40%, 4%, and 2.6%, respectively. The difference in the prevalence rates might be due to the diversity of the geographical area under study and the microbial agents involved, including viruses, bacteria, and fungi.

In the present study, *C. albicans* was calves' predominant causative agent of gastrointestinal diarrhea. As part of the gastrointestinal flora of humans and animals, *C. albicans* is often considered the main causative agent of gastrointestinal infections; however, numerous studies have reported non-albicans species such as *C. glabrata*, *C. guilliermondii*, *C. krusei*, and *C. tropicalis* as the causative agents for diarrhea and severe enteritis (12, 13).

In 2015, *C. famata* was reported for the first time as the predominant cause of gastrointestinal infection in a dairy herd in Iran (14). Epidemiological studies have shown that severe fungal infections are generally caused by drug-resistant fungal species, particularly fluconazole-resistant (15). In the present study, 24 *C. albicans* isolates (49%) were resistant to fluconazole.

The role of ZnONPs in inhibiting drug-resistant microorganisms has long been proven (16). However, the antifungal effects of these NPs have been less exploited. In a study on solar photocatalytic disinfection of a group of microbial aqueous suspensions, ZnO could effectively inhibit the growth of fungi (6).

In the present study, increasing the concentration of ZnONPs to 119 µg/ml significantly increased the antifungal effects. Similarly, previous studies demonstrated that ZnONPs exerts antifungal effects in a dose-dependent manner (17, 18). Researchers reported that ZnO NPs significantly inhibit biofilm formation in *C. tropicalis* at very low concentrations (19). In line with our findings, a study reported that the antibacterial effect of ZnONPs depends on their size and shape, so a smaller-sized NP exerts greater antifungal activity (20). In conclusion, the prevalence of yeast diarrhea in calves is relatively high. Given their favorable antifungal properties, ZnONPs can be used as a suitable option for the treatment of gastrointestinal infections in calves caused by drug-resistant *Candida* strains.

Authors' Contribution

Study concept and design: L. F.
Acquisition of data: M. T.
Analysis and interpretation of data: L. F.
Drafting of the manuscript: L. F.
Critical revision of the manuscript for important intellectual content: L. F.
Statistical analysis: L. F.
Administrative, technical, and material support: M. T.

Ethics

The study protocol were approved by the ethics committee of the Islamic Azad University, Gorgan, Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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