

ANTIFUNGAL AND SYNERGISTIC EFFECTS OF ZNO NANOPARTICLES AGAINST *T.VERrucosum* CAUSED RINGWORM IN COWS

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ABSTRACT

The current study was aimed to determined the main causes of ringworm in cows and antifungal and synergistic effects of ZnO nanoparticles. For this purpose 50 skin scrapes were collected from cows infected with ringworm, culture media, staining and genetic methods used for diagnosis. MIC and MFC for antifungal and ZnO were determined. The result showed that *Trichophyton spp* was isolated in rate of 76%. The isolation rate of *T.verrucosum*, *T. mentagrophytes* and *T. rubrum* were 68.4%, 21.0% and 10.5% respectively. MIC of Nystatin, fluocytosin, ZnO, Nystatin+ ZnO and Fluocytosin + ZnO were 200,150,200,150 and 100 µg/ml respectively. in conclusion, that *T.verrucosum* is main caused of Ringworm and ZnO has antifungal and synergistic effects.

Keywords: Trichophyton, synergism treatment, tinea

خلف

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التأثير المضاد للفطريات والتأزري لجسيمات الزنك النانوية ضد الشَّعْرَوِيَّة التُّوْلُوِيَّة المسببة للقوباء الحلقية في الأبقار

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المستخلص

هدفت الدراسة الحالية إلى تحديد الأسباب الرئيسية للقوباء الحلقية في الأبقار والتأثيرات المضادة للفطريات والتأزري للجسيمات النانوية ZnO. ولهذا الغرض جمعت 50 كشط جلدي من أبقار مصابة بالسعفة، استخدمت الاوساط الزرعية والطرق الوراثية في التشخيص. تم تحديد التركيز المثبط الأدنى والتركيز القاتل الأدنى لمضادات الفطريات و جسيمات الزنك النانوية. أظهرت نتائج عزل جنس جنس الشَّعْرَوِيَّات عزل بنسبة 76%. اظهرت الدراسة ان نسبة عزل الشَّعْرَوِيَّة التُّوْلُوِيَّة، الشَّعْرَوِيَّة الدُّقَانِيَّة، الشَّعْرَوِيَّة الحَمْرَاء كانت 68.4%، 21.0%، 10.5% على التوالي. كانت التركيز المثبط الأدنى للنستاتين، الفلوكسيستين، اجسام الزنك النانوية، النستاتين+اجسام الزنك النانوية، الفلوكسيستين+ اجسام الزنك النانوية 200، 150، 200، 150، 100 نانو غرام/مل على التوالي يمكن أن نستنتج أن *T.verrucosum* هو السبب الرئيسي للسعفة وأن ZnO له تأثيرات مضادة للفطريات وتأزريّة.

الكلمات المفتاحية: جنس الشَّعْرَوِيَّات، العلاجات التأزريّة، سعفة الجلد

INTRODUCTION

Ringworm is infectious disease caused by Dermatophytes, that infected Keratinized epithelial cells of skin, hair and nail, there are three genera of Dermatophytes can caused ringworm which are Trichophyton, Microsporum and Epidermophyton (32). these fungi can caused sever inflammatory response, the pathological lesion appear as circular lesion about 3cm in diameter, lose of hair and scales formation(15). trichophyton is genera characterized by formed of cottony, powdery, waxy and granular colony, in color of red, white, yellow, orange, violet, brown, pink and purple. it have small conidia (micro conidia) many in number, transparent, with a thin wallm while large conidia are rarely appear. these genera apple to use of protein, peptide and amino acid as carbone sources and have many enzyme like protease, lipase, keratinase, alkaline phosphatase, esterase and etc (8,16). Antifungal is the substance have ability to kill of inhibition of fungi growth, there are many antifungal group like polyenes (amphotericin b, nystatin), azoles (ketoconazole.. triazoles and fluconazole), allylamines (terbinafine, and butenafine), echinocandins (caspofungin and anidulafungin), fluorinated (pyrimidine and flucytosine) and tetrahydrofuran (derivativesm, sordarins and azasordarins) (18,33). Because of broad and miss use of antifungal also toxic and side effect of these agent, the search of alternative therapeutic substances began, nanoparticles is one of these alternative therapeutic substances. ZnO is nanoparticles have antifungal activity, present of reactive oxygen may be responsible on their activity, and caused damage on cytoplasmic membranes also ZnO caused inhibition mycelium growth, cytoplasmic content liquefaction and cell wall damage of the fungus (12).

MATERIALS AND METHODS

Samples: 50 skin scrapes collected from cows in age 1-4 year suffering from hair loss and pathological lesion suspected of ringworm. Direct examination of skin scrapes: conducted by use of KOH and examined microscopically (under 40 x lens.) to detect the hyphae and/ or spore of fungi *Trichophyton spp.* isolation and identification : the skin scrapes cultivation on sabourauds' dextrose agar (Oxoid - England)

with Choramphenical and cycloheximide according to (10). and incubated at (28C° for 1- 4 weeks. then examined to shows colony observation (color, consistency and topography), colony reverse (color, significant pigment) and microscopic morphology by lactophenol cotton blue stain to shows spore and hyphae.

PCR test for confirming *T.verrucosum* diagnosis

DNA extraction: conducted by taken of part of *T.verrucosum* colony and according to (13).

Primers : specific *T.verrucosum* primers which are

-ITS1 (5'-TCCGTAGGTG AACCTGCGG-3)

-ITS4 (5'-TCCTCCGCTTATT GATATGC-3')

PCR mixture are final volumes of 50 µl consist from 25 µl master mix, 0.3 µl from each primers, 3 µl of DNA templates and 21.4 µl of DNase free water. While thymocyclar program consisted of initial denaturation at: 94°C for 3 minutes, followed by 35 cycles of 30 seconds at 94°C, 1 minute at 60°C, and 30 seconds at 72°C and a final extension at 72°C for 10 minutes. PCR products were separated in 1.5% agarose gel, stained with ethidium bromide : according to (13,25).

Determination of MIC and MFC of antifungal alone and with nanoparticles against *T.verrucosum* conducted on Sabourauds' Dextrose broth

- Antifungal (Nystatin and fluocytosin): prepared on concentration of 100-150-200-250µg/ml

- ZnO nanoparticles: prepared on concentration of 100-150-200-250 µg/ml

- MIC : the lowest concentration of antifungal and/or nanoparticles that caused growth inhibition of *T.verrucosum* (clear tube)

- MFC: the lowest concentration of nan antifungal and/or nanoparticles that caused growth inhibition of *T.verrucosum* (clear and septic tube when sub culturing on agar

RESULTS AND DISCUSSION

Result of *Trichophyton spp* isolation: Out of 50 cows skin infected sample 38 *Trichophyton spp.* isolated in rate of 76%. This result agreed with study of (4,14,19,22). The negative sample of isolation and identification for Trichophyton due to other fungi such as Microsporum and epidermophyton (6,9) also other systemic

fungi such as aspergillus, penicillium and candida may be caused skin infection or predisposing to skin infection (23,28). In the current study three type of Trichophyton were isolated which are *T.verrucosum*, *T. mentagrophytes* and *T. rubrum*, the main character of these type as the following:

***T.verrucosum*:** after 7-10 days of incubation at 25°C on SDA, colonies appeared as yellow to brown colonies contained radial depressions, the center is slightly high than edge (Figure. 1) back of plate appeared yellow to orange. When staining by cotton lactophenol cotton blue showed chains of chlamydial spores

***T. mentagrophytes*:** after 7-10 days of incubation at 25°C on SDA, colonies appeared as cottony texture, white and rise in the center with thin radial edges, while the back of the plate was yellowish brown (Figure.2). When staining by cotton lactophenol cotton blue showed spherical microconidia.

***T. rubrum*:** after 7-10 days of incubation at 25°C on SDA, colonies appeared as fluffy wool smooth in texture, white-creamy while the back of the plate was brown(Figure.3) When staining by cotton lactophenol cotton blue showed large elongated conidia like tear with divided long hyphae.



Figure 1. *T.verrucosum* colony on SDA



Figure 2. *T. mentagrophytes* colony on SDA



Figure 3. *T. rubrum* on SDA

Results of PCR test for confirming of *T. verrucosum* diagnosis: by using of specific

primers, all *T. verrucosum* colony gave positive results on PCR test (figure 4)

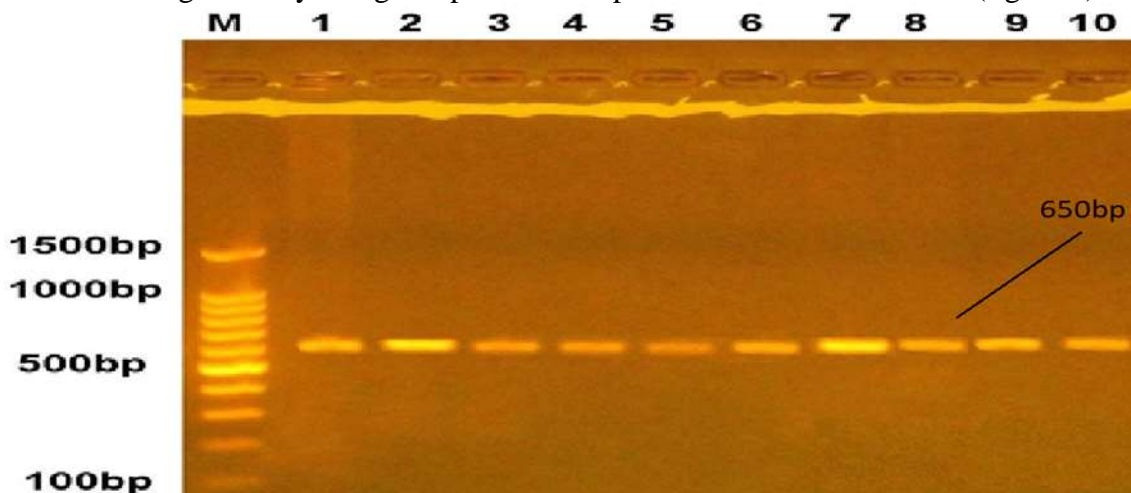


Figure 4. Positive result of PCR test for *T. verrucosum* , M: 100bp DNA marker lens (1-10) positive sample which gave band in size 650 bp

From Table (1) showed that isolation rate of *T. verrucosum*, *T. mentagrophytes* and *T. rubrum* were 68.4%, 21.0% and 10.5%

respectively The isolation of these type is agreed with study of (1,17,22).

Table 1. Species of Trichophyton isolated in the current study

Genus and types of fungi	No of isolate	Isolation rate
<i>T. verrucosum</i>	26	68.4%
<i>T. mentagrophytes</i>	8	21.0%
<i>T. rubrum</i>	4	10.5%
Total	38	100%

Results of MIC and MFC : from Table (2) showed that MIC of Nystatin, fluocytosin, ZnO, Nystatin+ ZnO and Fluocytosin + ZnO were 200,150,200,150 and 100 µg/ml respectively while MFC of Nystatin,

fluocytosin, ZnO, Nystatin+ ZnO and Fluocytosin+ ZnO were more than 250,200,250,200 and 150 µg/ml respectively and showed clear synergistic effects of nanoparticles with antifungal

Table 2. MIC and MFC of antifungal alone and/ or with ZnO nanoparticles

Antifungal	Concentration µg/ml			
	100	150	200	250
Nystatin	Growth	Growth	Clear / no septic (MIC)	Clear / no septic
Fluocytosin	Growth	Clear / no septic (MIC)	Clear / septic (MFC)	Clear / septic (MFC)
ZnO	Growth	Growth	Clear / no septic (MIC)	Clear / septic (MFC)
Nystatin+ZnO (1:1)	Growth	Clear / no septic (MIC)	Clear / septic (MFC)	Clear / septic (MFC)
Fluocytosin+ZnO (1:1)	Clear / no septic (MIC)	Clear / septic (MFC)	Clear / septic (MFC)	Clear / septic (MFC)

Results in Table (2) show that MIC for fluocytosin and Nystatin were 150 and 200 µg/ml respectively this result disagreed with result of (3). Whom recorded MIC (25-100 µg/ml). That's may be due to difference in sources of isolate. In compare between MIC for fluocytosin and Nystatin showed that the MIC for Nystatin more that MIC for fluocytosin, that's agreed with results

(5,20,30). The current study showed clear effect on *T. verrucosum* as antifungal or synergistic to antifungal, this results agreed with results of (7,21,24,27). ZnO consider as safe chemical material and have antimicrobial effects and used as cosmetic or pharmaceutical ointments, also ZnO my be act as antifungal by physical and chemical pathway, the physical pathway by their effect on cell wall,

and alteration on proteins functioning, also ZnO interaction with biological system which lead to interference with ions and electrons transport (2,29). The chemical action of ZnO by inducing of oxidative stress propitiated by the reactive oxygen species (ROS) that may be generated by hydrogen peroxide (H₂O₂) hydroxyl radicals (OH) and superoxide anions(O₂⁻). Also ZnO able to penetration of biological barrier and release of toxic metallic substance which lead to damage on DNA (11,31).

In conclusion: *Trichophyton* is main Dermatophytes genus caused Ringworm in cow, and *T.verrucosum* is the dominant species, ZnO has antifungal and synergistic effect with other antifungal drags

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