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Original Research

Antifungal activity of silver nanoparticles on Fluconazole resistant Dermatophytes identified by $(GACA)_4$ and isolated from primary school children suffering from Tinea Capitis in Ismailia – Egypt

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Abstract: Fungal infections caused by dermatophytes recently became more common. Available antifungal drugs are limited because of emergence of resistant strains due to prophylaxis with them, so there is an urgent need for novel antifungals. This study is aimed to detect the antifungal activity of silver nanoparticles (SNPs) on Fluconazole resistant dermatophytes isolated from primary school children clinically suffering from tinea capitis and attending El-Sheikh Zaid Dermatology Center in Ismailia. The study was done on 112 clinical cases. Examination with potassium hydroxide(KOH) of hair samples was done, followed by routine identification using culturing, macroscopical and microscopical examination and biochemical tests, finally molecular identification using Polymerase Chain Reaction (PCR) with $(GACA)_4$ was done. Fluconazole resistance of these dermatophytes was detected by different methods including agar disc diffusion method and broth microdilution susceptibility testing. Silver nanoparticles susceptibility testing was carried out on these Fluconazole resistant dermatophytes. The Ubiquitin 1 (Ub 1) gene was detected in samples which were Fluconazole resistant but SNPs susceptible. In this study dermatophytes were found only in 70 samples (62.5%). They were belonged to 3 species: *Trichophyton violaceum*, *Microsporum gypseum* and *Microsporum canis*. Fluconazole resistance was found in 58 samples (82.85%). Both *M. canis* and *M. gypseum* were resistant to all used concentrations of SNPs, while *T. violaceum* was susceptible to 50 µg/ml SNPs solution. The Ub1 gene was detected in 1 sample (4.8%). Therefore SNPs can be used for treatment of *T. violaceum*, while they can't be used for treatment of *M. canis* or *M. gypseum*.

Key words: Dermatophytes; $(GACA)_4$; Fluconazole; SNPs; Ub1.

Introduction

Tinea capitis is an infection of the scalp caused by dermatophytes of genera *Microsporum* and *Trichophyton* (1).It is predominant disease in children (2). These dermatophytes are group of fungi invading the keratinized tissues like skin, hair and nails causing dermatophytosis "ringworm infection" (3,4-5,6). Dermatophytes' identification using routine methods such as culturing is time consuming, while molecular identification using $(GACA)_4$ is highly sensitive (7,8). The treatment of the dermatophytes becomes more difficult and their resistance to antifungals like Fluconazole was detected, which might be related to Ubiquitin (Ub) gene (9,10). The purpose of our work is to prove that the SNPs can be used as antifungal agent for the treatment of dermatophytes (11). These nanoparticles are 1-100 nm in size and synthesized by physical, chemical or biological methods (12,13-14).

Materials and Methods

Collection of hair samples

A total of 112 children clinically diagnosed as tinea capitis cases attending El-Sheikh Zaid Dermatology Center during the period from February to September 2013, were involved in this study. Lusterless, dull or short broken hairs were plucked using non-toothed forceps. Epilating is better than cutting because of high amount of spores on the root (1).

Isolation and identification of Dermatophytes

These clinical samples were identified by direct examination with 10% and 20% KOH (15). The identification of isolated dermatophytes was done using macroscopic examination of the colony cultured on Sabouraud Dextrose agar (SDA) containing Chloramphenicol (Oxoid)and Cycloheximide (Bioshop) followed by microscopic examination by Lactophenol Cotton Blue (LPCB) stain (15,16). Further identification was done by biochemical tests including urease test, hair perforation test and rice grain test (17,18).

Molecular identification

DNA extraction

Fungal growth obtained on SDA was washed several times with 0.1 M MgCl2, then ground finely with the liquid nitrogen in sterile mortars. The resulting powder was aliquot into sterile, 1.5-ml microcentrifuge tubes (19). Then the subsequent steps were done as prescribed in the Wizard® Genomic DNA Purification Kit (Promega, USA) till obtaining the extracted DNA.

PCR using (GACA) $_4$

The primer (GACA) $_4$ was used as a single primer for dermatophytes' identification (7,19). The reactions were performed in volumes of 50 µl containing 25µl of Maxima Hot Start PCR Master Mix (2X) (colourless) (THERMO SCIENTIFIC), approximately 150-200 ng of the (GACA) $_4$ (Bio Basic Canada Inc.), approximately 20-30 ng of template DNA and the reaction volume was made up to 50 µl with sterile PCR water. PCR was performed as reported by Faggi *et al.* (7) in a thermal cycler (BIORAD T100 Thermal Cycler) and then PCR products were visualized under UV light. They were photographed using a Gel Documentation System (G: box, SYNGENE, Cambridge, England).

Antifungal susceptibility testing

It was done on 70 isolated dermatophytes using the following methods:

Agar disc diffusion method

It was done as prescribed by Nweze *et al.* and Matnani *et al.* using 9 different antifungal discs including Nystatin (NY) 100 IU, Griseofulvin (AGF) 10 µg, Amphotericin B (AMB) 20 µg, Flucytosine (AFY) 1 µg, Itraconazole (ITC) has 2 concentrations 8 µg and 15 µg, Fluconazole (FLU) has 2 concentrations 25 µg and 100 µg, Ketoconazole (KTC) has 2 concentrations 10 µg and 15 µg, Miconazole (MCL) 10 µg, and Voriconazole (VO) 1 µg. All antifungal agents' discs were purchased from LIOFILCHEM, Italy (20,21).

Broth microdilution susceptibility method

It was performed according to the Clinical and Laboratory Standards Institute (CLSI formally NCCLS) guidelines in the document M38-A2 of filamentous fungi using Fluconazole and Itraconazole from their respective manufacturers (22).

Silver Nanoparticles Susceptibility Testing

The solution of SNPs (Nanotech, Egypt) was prepared by chemical reduction (14). This test was performed on Fluconazole resistant isolates. This resistance was detected by Agar disk diffusion method and Broth microdilution susceptibility testing using CLSI guidelines (20,22). The inoculum suspensions of these isolates were prepared from cultures grown on potato dextrose agar at 28°C. The fungal colonies were covered with 10 ml of distilled water, and then scraping the surface with the tip of a sterile loop. The obtained mixture containing fungal conidia and hyphal fragments was removed by vortex and transferred to sterile tube. The final suspension was 0.5 McFarland concenteration (21,23). Antifungal activity of SNPs was determined by measuring diameter of inhibition zones (11). The Packialakshmi and Musbira Banu's method was used with some modifications including fresh fungal culture was spread on surface of the both (PDA) and Muller Hinton Agar (MHA) plates with the swab, 4 different concentrations of SNPs solutions were used (12.5, 25, 50, and 100) µg/ml either in distilled water or absolute ethanol as a solvent, 4 cups were made in each plate and then put 200 µl of each concentration of SNPs in the corresponding cup, finally incubate at 28º-30º C for 1 week except *T. violaceum* incubated for 2 weeks.

Ubiquitin 1 gene detection

The Ub1 gene detection was done on previously extracted DNA of samples which were SNPs sensitive and Fluconazole resistant as the following: initial denaturation at 94º C for 7 minutes, then 35 cycles, each cycle consisting of denaturation at 94º C for 30 seconds, annealing at 62º C for 30 seconds and extension at 72º C for 30 seconds. Then the final extension was at 72º C for 7 minutes. The Ub1 gene (Macrogen, Korea) used was newly designed from the Blast database of NCBI using the Clustal W2 program. The used primer sequences were 5' GGCACCCTAACATCTACGACAACGGC3' and 5'CTCAGGCTCACTCCCAGTGTCCTG 3'. Reactions were done in volumes of 25 µl containing 100 ng extracted DNA, 0.5 µg of the primer pair, 12.5µl EmeraldAmp® GT PCR master mix (Takara Bio Inc., Japan), then volume completed up to 25µl by sterile PCR water.

Results

Phenotypic identification

Direct microscopical examination of hair specimen with KOH:

The results showed that 100 samples (89.3%) of a total 112 clinical cases were positive using either 10% or 20% KOH. Ectothrix hair invasion was represented by 59 samples and endothrix type was represented by 41 samples as shown in Figure 1.

Dermatophytes were found in 70 samples (62.5%) including *Trichophyton violaceum* (29 samples, 41.4%), *Microsporum gypseum* (27 samples, 38.6%) and *Microsporum canis* (14 samples, 20%) as shown in Figure 2.

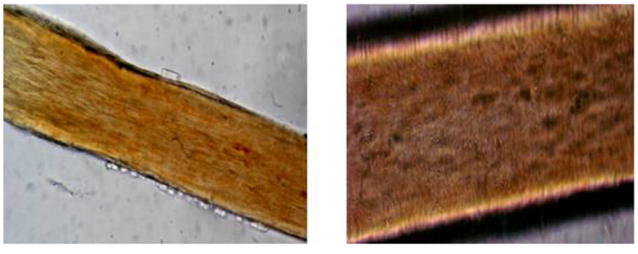
Biochemical test results

The dermatophytes of the species *M. canis* and *M. gypseum* were positive for hair perforation test while *T. violaceum* was negative. Also *M. canis* and *M. gypseum* were urease positive and *T. violaceum* showed negative urease test in 7 days then slow positive thereafter. *M. canis* was rice grain test positive as shown in Figure 4.

Molecular identification by PCR using (GACA) $_4$

PCR using (GACA) $_4$ gave band patterns specific to dermatophyte species or strains. These band patterns appeared were with size ranged from 300 bp. to 2500 bp. as shown in Figure 5 and Table 1.

Antifungal susceptibility testing



(A) (B)

Figure1. Direct microscopical examination with KOH, where (A) showing ectothrix hair invasion and (B) showing endothrix hair invasion.

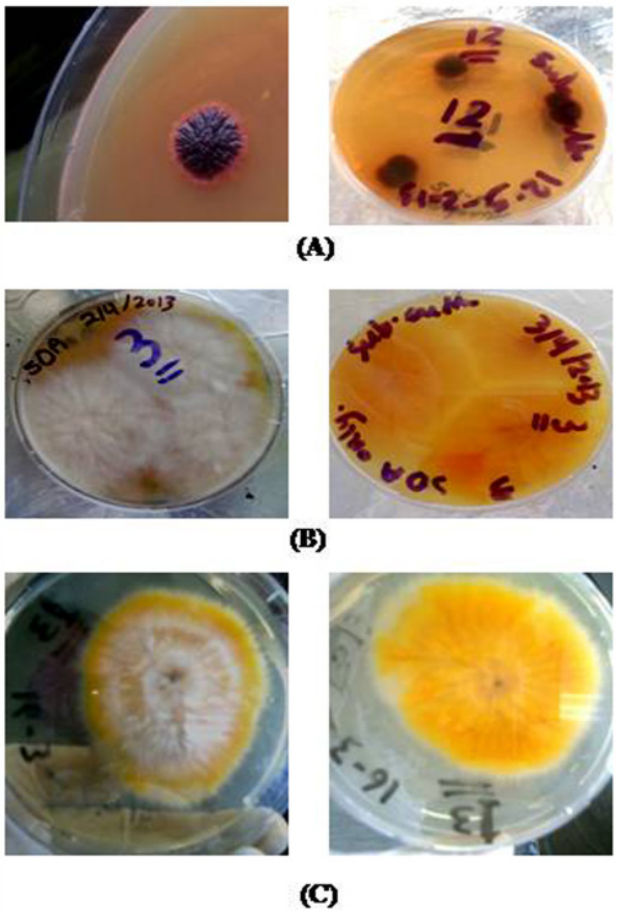


Figure 2. Culture macroscopic examination, where (A) showing surface view and reverse side view of culture macroscopic examination of *T. violaceum*, (B) showing surface view and reverse side view of *M. gypseum* and (C) showing surface view and reverse side view of *M. canis*.

Agar disc diffusion method and Broth microdilution susceptibility method showed the high frequency of Fluconazole resistance in dermatophytes as shown in Tables (2& 3) and Figure 6.

Silver Nanoparticles susceptibility testing results

From 58 Fluconazole resistant dermatophytes, 12 samples (17.14%) of *M. canis* and 25 samples (35.71%) of *M. gypseum* were resistant to antifungal activity of SNPs, while 21 samples (30%) of *T. violaceum* were susceptible to 50 µg/ml concentration of SNPs solution in either absolute ethanol or distilled water as shown in Figure 7.

Ub 1 gene detection

The Ub 1 gene was detected at 441 bp. in 1 sample (4.8%) of total 21 *T. violaceum* Fluconazole resistant, SNPs susceptible samples as shown in Figure 8.

Table 1. Band patterns of *M. canis*, *T. violaceum* and *M. gypseum* that obtained by PCR using $(GACA)_4$.

M. canis	*T. violaceum*	*M. gypseum*
600 bp	600 bp	300 bp
1200 bp	700 bp	400 bp
	1000 bp	600 bp
	1200 bp	700 bp
		1000 bp

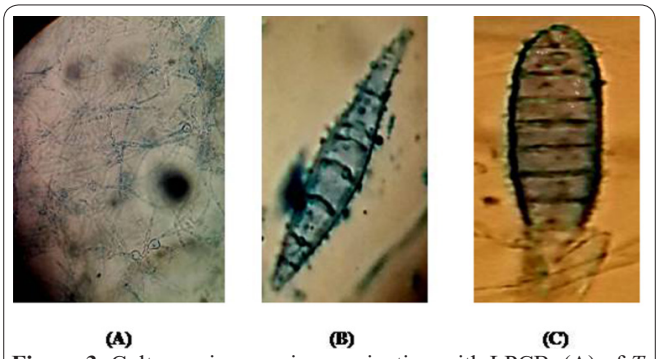


Figure 3. Culture microscopic examination with LPCB, (A) of *T. violaceum*, (B) of *M. canis* and (C) of *M. gypseum.*

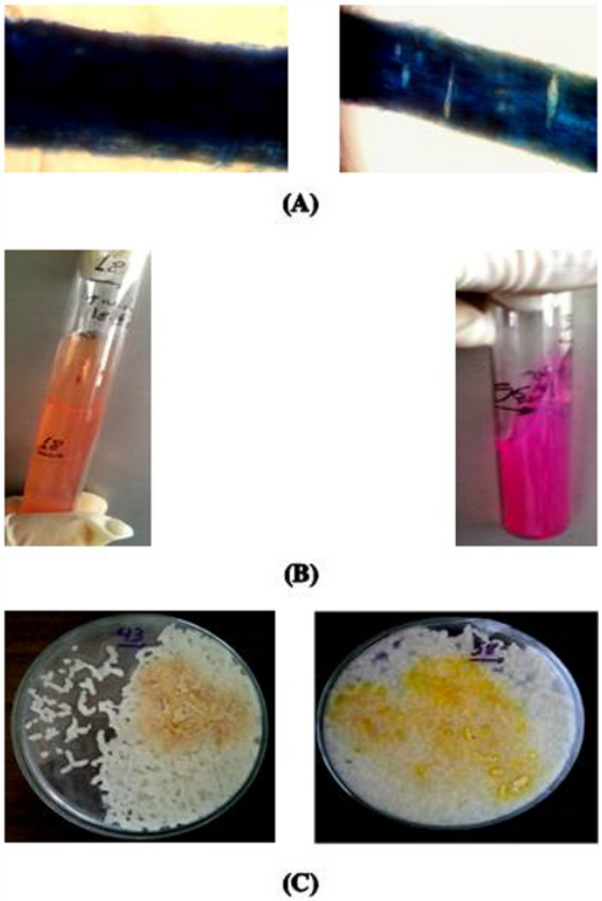


Figure 4. Biochemical tests, (A) showing negative and positive hair perforation test, (B) showing negative and positive urease test and (C) showing negative and positive rice grain test.

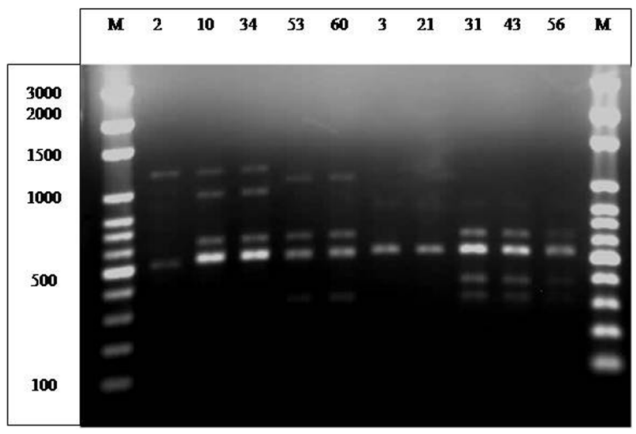


Figure 5. Molecular identification by PCR using $(GACA)_4$. M, molecular weight marker, sample No. 2 (*M. canis)*, samples No. 10, 34, 53 and 60 (*T. violaceum),* samples No. 3, 21, 31, 43 and 56 (*M. gypseum*).

Table 2. Percentage of resistance to antifungal agents.

Antifungalagent	Resistance (%)
Ketoconazole (KCA) 10 µg	0%
Ketoconazole (KCA) 15 µg	0%
Miconazole (MCL) 10 µg	0%
Voriconazole (VO) 1 µg	1.4%
Amphotericin (AMB) 20 µg	8.6%
Nystatin (NY) 100 IU	10%
Griseofulvin (AGF) 10 µg	14.3%
Fluconazole (FLU) 100 µg	47.1%
Fluconazole (FLU) 25 µg	90%
Itraconazole (ITC) 50 µg	94.3%
Itraconazole (ITC) 8 µg	98.6%
Flucytosine (AFY) 1 µg	98.6%

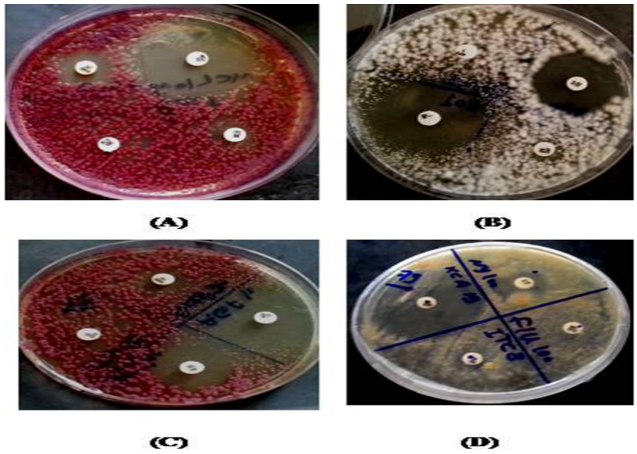


Figure 6. Agar disc diffusion antifungal susceptibility testing, (A) of *T. violaceum* showing sensitivity to Miconazole and Amphotericin and resistance to Itraconazole and Fluconazole, (B) of *M. canis* showing sensitivity to Voriconazole and Nystatin and resistance to Flucytosine and Itraconazole, (C) of *T. violaceum* showing sensitivity to Griseofulvin, Nystatin and Amphotericin and resistance to Fluconazole and (D) of *M. gypsum* showing sensitivity to Ketoconazole and Nystatin and resistance to Fluconazole and Itraconazole.

Discussion

Our study showed that the false positive results obtained in 30 samples (26.8%) by KOH direct examination are similar to that obtained by Singh and Beena (24). This difference might be due to non-viability of fungal elements in some cases. Dermatophytes recently became more common infections as they were found in 62.5% of cases. This finding was in accordance with others (25,26-27) and was dissimilar to Bhatia and Sharma (28). This dissimilarity might be due to difference in climatic conditions and hygiene. From this study, the etiologic agents of tinea capitis were *T. violaceum*, *M. gypseum* and *M. canis*. Agree with studies of Nasser and Mohammed, T. *violaceum* representing the main isolate (41.4%) (25,29).

The molecular identification of dermatophytes using $(GACA)_4$ was performed to remove confusion which occurred due to difference between culture macroscopic and microscopic examination because it was found that it was simple, rapid, accurate and highly sensitive technique as reported by Faggi *et al.,* Miao *et al.,* and Shehata *et al.* (7,8-19). There were slight differences

Table 3. Frequency of resistance to both concentrations used of Fluconazole in the isolated dermatophytes.

Dermatophyte isolates	No. of Fluconazole resistant samples	Percent
T. violaceum	21	30
M. gypseum	25	35.71
M. canis	12	17.14
Total No.	58	82.85

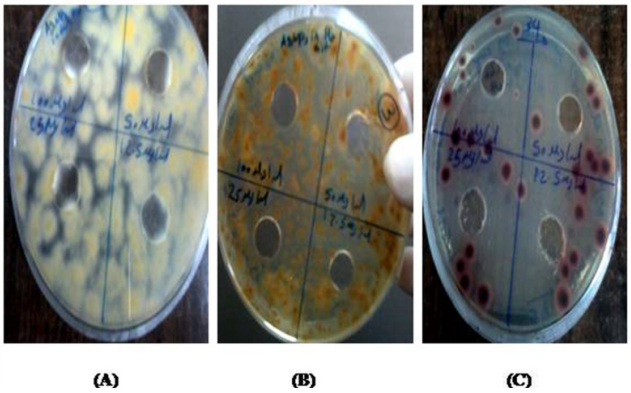


Figure 7. Silver Nanoparticles Susceptibility testing, (A) of *M. canis*, (B) of *M. gypseum* and (C) of *T. violaceum*.

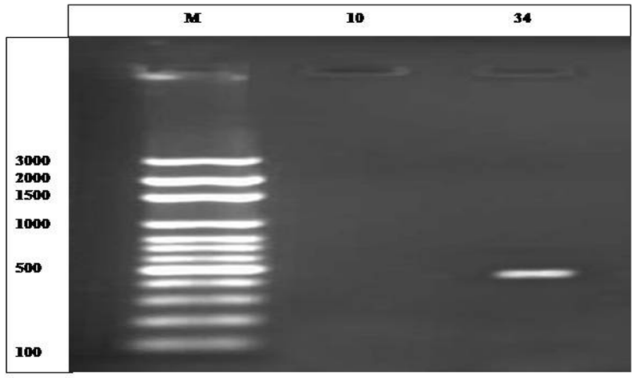


Figure 8. Ub 1 gene detection. M, molecular weight marker, samples No. 10 and 34 (*T. violaceum)*.

in amplicon sizes of brightly colored bands produced of our PCR profiles of *M. canis* and *M. gypseum* and that of Miao *et al.* This difference might be due to band pattern production, difference in DNA concentration or using Maxima Hot Start PCR Master Mix.

Our present study revealed that the best antifungals for treatment were Ketoconazole, Miconazole and Voriconazole as reported byWeitzman and Summerbell, Venugopal and Venugopal and Carrillo-Munoz *et al.*(1,30-31).On the other hand, results in our laboratory showed high resistance of the isolated dermatophytes to Fluconazole, this was as detected by Araujo *et al.*(32).

In the present study, both *M. canis* and *M. gypseum* were resistant to all concentrations used of SNPs solutions, while *T. violaceum* was susceptible to solution of 50 µg/ml concentration. While Packialakshmi and-Musbira Banu found that the 64 µg/ml concentration was able to inhibit growth of dermatophytes (11). This difference could be due to difference in species used, because they used *Trichophyton rubrum*. The study also showed that no difference in results by using either distilled water or ethanol as a solvent dissimilar with Al-Qurashi and Awad who found that ethanol having antifungal activity (33).

Agree with Kano *et al.,* Ub might be related to Fluconazole resistance in dermatophytes because Ub 1 was detected in 1 sample (4.8%) (10). Fluconazole resistance of the other 57 samples (98.3%) might be due to other different biochemical mechanisms (e.g. decrease in drug uptake, structural changes in the target site or increase in drug efflux) which were regulated by genes other than Ub gene as reported by Martinez-Rossi *et al.*(34).

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