

ORIGINAL ARTICLE

In-vitro Evaluation of Different Commercial Antimycotics and Disinfectants against *Trichophyton verrucosum* Isolated from Beef Farm in Beni Suef, Egypt

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Abstract

Dermatophytosis is a fungal disease that affects both animals and humans. The improper use of antimycotics and disinfectants led to an acquired resistance of dermatophytes to most of the commonly used antimycotic agents. The aim of the present study was to estimate the prevalence of dermatophytosis in a calf-beef farm and to evaluate the in-vitro efficacy of six antifungal agents and three disinfectants. Out of the 120 examined calves, 42 (35%) showed ringworm lesions. *Trichophyton verrucosum* was isolated from a total of 45 samples including 42 skin scrapping from infected animals and 3 hair samples from farm workers followed by molecular identification using PCR. Antimicrobial sensitivity profile was performed by agar-based disk diffusion method using six antifungal agents. Three disinfectants with different concentrations were tested against 45 strains of *T. verrucosum* isolates using broth macro-dilution at different contact times (20 sec, 30min, 1h and 24h). Animal isolates were sensitive to Fluconazole (100%), meanwhile human strains were sensitive to Itraconazole (66.7%) ($P < 0.001$). Recovered isolates were sensitive to iodine (7%) at contact time 1h and 24h (47 and 69 %, respectively) at $P < 0.001$, and to Virkon S (1%) after 24 h contact time (55%) at $P < 0.001$. Results prove the antimycotic action of Fluconazole and Itraconazole on *Trichophyton verrucosum* and highlights the significant role of increasing the contact time on decreasing the resistance pattern of dermatophytes to Virkon S (1%) and Iodine (7%) that give a promising results when used to control the infections in the surrounding environment.

Keywords

Dermatophytosis, Fluconazole, Itraconazole, PCR, *Trichophyton verrucosum*

1. Introduction

Ringworm or as known dermatophytosis is a contagious fungal disease that affects both animals and humans by damaging primarily the superficial layer of the skin, hair, nails and claws (Alaa et al., 2010). Dermatophytes are Gram positive, strict aerobic, non-motile filamentous fungi that belong to one of three genera *Epidermophyton* (*E*), *Microsporum* (*M*) or *Trichophyton* (*T*) (Dave et al., 2014; Weitzman and Summerbell, 1995). The infection with such pathogen causes a significant morbidity and huge economic losses due to its direct effect on the quality of skin, hide and fur of infected animals, the resistance to most used antifungal drugs, and the reduction of the weight in butchered animals, but in the same time mortalities are scarce (Radostits et al., 2007; Yuan et al., 2009).

Humans could be affected by anthropophilic, zoophilic as well as geophilic dermatophytes (Simpanya, 2000). The infection with zoophilic dermatophytes is considered the severest type as it produces more inflammatory reactions (Degreef, 2008). Generally, signs in immune-compromised individuals are more serious and aggressive including folliculitis, kerion formation, alopecia, abscess, and cellulitis. It is known that cutaneous mycoses affect up to 20-25% of the world's population (Pal, 2017). Dermatophytosis occurs either in epidemic form or as sporadic cases (Dalis et al., 2014; Pal, 2017). The infection could be transmitted through direct contact with infected animals or indirectly through contaminated fomites (Pal and Dave, 2006). The disease is more prevalent in animals housed in poor closed confinement and in worm humid climates that favor the survival of spores

also it is more likely to occur in young animals (under one year of age) due to their ill developed immune system and alkalinity of their skin (Pal and Dave, 2006).

Diseased animals and contaminated environments consider the main source and reservoir of infection among susceptible animals (Murray et al., 2005). Infected animals can disseminate huge numbers of infective fungal spores in the surrounding environment that adhere to hairs or skin scabs. Infective fungal spores can survive in contaminated environments up to 18 months due to their relative high resistance to ultraviolet rays, desiccation and chemical disinfectants (Euzeby, 1992).

Due to Scarcity of information about the prevalence of dermatophytosis in beef farms and the increased resistance to wide range of the commonly used antifungal drugs, this study was performed to determine the prevalence of dermatophytosis in the farm under the study and to evaluate the efficacy of different antifungal drugs as well as disinfectants that are commonly used in the field to control it.

2. Materials and Methods

2.1. Study Area and Animals

This study was performed in a private beef farm of 120 calves at Beni Suef (coordinates 28° 54' 22.9" N 30° 56' 18.7" E), Egypt from November 2020 to February 2021. Calves were kept overcrowded. The hygienic condition prevailed during the period of the study was fair. During the study, forty two out of 120 calves showing skin lesions were carefully examined. The farm workers (n=3) who were in close contact with the animals showed inflamed rounded scaled lesions in their fore arms. Diseased animals and humans were subjected to sample collection.

Animal samples gathered during the study were approved by International Animal Care and Use Committee (IACUC), Ref. No: IORG 0001092), Beni-Suef University, Egypt while human samples were authorized by Institutional Review Board (IRB), Ref. No: IORG 0001101), Beni-Suef University, Egypt.

2.2. Samples Collection

2.2.1. Animal Samples

A total of 42 Skin scrapings were gathered from periphery of the lesion with a sterile scalpel blade after aseptically cleaned with 70% alcohol. Hairs were taken by removing the dull fragmented hairs from the margin of the lesion using sterile tweezers (Cheesbrough, 1992). All samples were individually sited in clean plastic cups then transported to Laboratory of Animal Hygiene and Zoonoses Department, Faculty of Veterinary Medicine, Beni-Suef University for different dermatologic investigations.

2.2.2. Human Samples

Skin scales and crusts were collected from the three workers by scraping through the inflamed lesion's margin by the blunt

edge of a sterile surgical blade. While hair specimens were obtained by using epilating forceps to pluck along the base of the hair shaft. Each sample was carefully identified, labeled and sent to the laboratory where the fungal examination was initiated.

2.3. Mycological Examination

2.3.1. Wet Mount Preparation for Fungal Examination

Skin scrapings of crusts, scabs, loosely attached scales and hair plugs were strictly gathered, placed on a clean slide, gently mixed with equal volumes of 10% potassium hydroxide (KOH) and 40% Dimethyle sulphoxide (DMSO), then covered with a cover slide, heated gently, and left for at least 30 minutes to one hour. The prepared slides were thoroughly inspected using both low power (10X) and high power (40X) magnification for detection of hyphae and/or arthroconidia. The existence of spores on the surface of the shaft of infected hairs showing the mosaic arrangement (ectothrix infection) while the invasion of hyphal fragments and arthroconidia in the internal hair structure is named endothrix infection (Shalaby et al., 2016).

2.3.2. Isolation and Identification of Dermatophytes

The collected hair scales and crusts are cultivated onto the surface of Sabouraud's dextrose agar (SDA, Oxoid, UK) supplemented with; chloramphenicol (500mg/L), cycloheximide (400mg/L), thiamine (10mg/L) and inositol (50mg/L). Cultures were incubated aerobically at 25-30°C. Plates were noticed daily for fungal growth up to 30 days. Positive cultures were examined using both macroscopically (color of the surface and reverse, texture and topography) and microscopically using of lactophenol-cotton blue stain (type of conidia appeared either in the form of small unicellular microconidia or larger septate macroconidia) for species identification (Halley and Standard, 1973). In case of growth, an individual colony was picked up and the mold was examined by means of both low power (10X) and high power (40X) magnifications. In the absence of any growth after the fourth week, the sample was considered negative (Chermette et al., 2008).

2.4. Molecular Identification of Dermatophytes

2.4.1. DNA Extraction

Fungal colony (0.5–1.0cm in diameter) was cut from the agar plate with a scalpel, transferred to a mortar and ground in liquid nitrogen. The mycelial powder was transferred to an Eppendorf tube. DNA extraction was performed using QIAamp DNeasy Plant Mini kit (Qiagen, Germany, GmbH) based on the kit's instructions.

2.4.2. Polymerase Chain Reaction for Dermatophytes

PCR runs were performed in a total volume of 25µl/reaction containing 12.5µl of PCR master mix (Takara, Japan). 1µl of

each primer of 20pmol concentration, 5.5µl of water, and 5µl of DNA template. Data of the primer's sequences are listed in **Table (1)**. The reaction was performed in an applied biosystem 2720 thermal cycler. The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15µl

of the products was loaded in each gel slot. A gene ruler 100bp DNA Ladder (Fermentas, Thermofisher, Germany) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software of DigiDoc-It Imaging System.

Table 1. Primers sequences, target gene, amplicon size and cycling conditions for conventional PCR.

Target gene	Primers sequences	Amplified segment (bp)	Primary denaturation	Amplification (35 cycles)			Final extension	Reference
				Secondary denaturation	Annealing	Extension		
<i>T. verrucosum</i>	CCGCGCTCT CCCC GATTCACGGAATTCTGCAA TTCAC	220	94°C 5 min	94°C 30 sec	55°C 30 sec	72°C 30 sec	72°C 7 min	Ohst et al., (2016)

2.5. Evaluation of Germicidal Efficacy of Tested Antimicrobial Agents

2.5.1. Antifungal Susceptibility Testing

Antifungal susceptibility testing was performed by agar-based disk diffusion method using six antifungal agents: Fluconazole (FLC, 25µg), Itraconazole (IT, 10ug), Nystatin (NS, 100U), Amphotericin B (AP, 100U), Fusidic acid (FC, 10ug), Vericonazole (VRC, 1ug) (Oxoid, UK) against 45 strain of *T. verrucosum*. Mycelium and conidia growth were picked up from SDA surface by sterile forceps and suspended in a tube containing 1ml distilled water; the mixture was left sediment for 30min. Swabs dipped into the inoculum suspensions were streaked evenly over the surface of SDA (Oxoid, UK) plates. The antifungal disks were impregnated into the agar plates then incubated at 25°C for 5-10 days. When growth took place, the zone of inhibition around the disks was measured and recorded. Criteria of susceptibility and resistance of antifungal agents were measured and interpreted according to **Pakshir et al., (2009)**.

2.5.2. Disinfectant Sensitivity Testing

Germicidal power of three commercially disinfectants; Virkon S (potassium per-oxymonosulfate 50%, NaCl 3%, UBM, Egypt) at conc. 0.5 and 1%, Iodine (6th October 3rd Industrial Area, Egypt) at conc. 5 and 7% and TH4 (Didecyldimethyl Ammonium Chloride, Dioctyldimethyl Dimethyl Ammonium Chloride, Octyl Decyl Dimethyl Ammonium Chloride and Glutraldehyde, SOGeval) at conc. 1: 200 and 1: 400 were tested against 45 strains of *T. verrucosum* isolates using broth macro-dilution at different contact times (20sec, 30min, 1h and 24h) according to **Li et al., (2008)**.

Statistical Analysis

Data obtained were recorded and the frequency of *T. verrucosum* in the collected samples as well the germicidal efficacy of tested antifungal agents and disinfectants were calculated using non-parametric tests (Chi-Square Test) using SPSS (Inc. version 22.0, Chicago, IL, USA).

3. Results and Discussion

Dermatophytes is considered one of the public health concerns that affect all domestic animals and humans worldwide (**Abd-Elmegeed et al., 2015**). *T. verrucosum* is the predominant zoophilic dermatophyte causal species of dermatophytosis in cattle and can occasionally spread to humans through direct contact with livestock or infected fomites (**Papini et al., 2009**).

On clinical examination, 35.0% (42/120) of calves showed heavy gray, white crusty circular lesions of 1-5cm in diameter (**Table, 2**). These lesions are commonly found around the eyes, ears and neck (**Fig. 1A**). Noticeably the farm workers were free of infection at the start of the study then later and as the lesion started to appear on the animals, it was obvious on the forearm of them (**Fig. 1B**). Forty-five samples out of one hundred and twenty-three (36.6%) were significantly positive for isolation of dermatophytes and PCR (**Fig. 2**) at $X^2 = 132.382$ $P > 0.001$. Where all the three samples obtained from farm's workers were positive for microbial isolation and PCR (100%), meanwhile 42 animals' samples were positive (35%). It was noticed that worker was completely recovered after their treatment with iodine bandages and topical antifungal spray containing Itraconazole as active principle.

Table 2. Prevalence of *T. verrucosum* in the examined animal and human samples.

Samples	Total number	No. positive	Percentage (%)
Animal	120	42	35
Human	3	3	100
Total	123	45	36.6
<i>P-value</i> $X^2 = 132.382$		$P < 0.001$	



Fig. 1. Affected calf showed typical circumscribed crusty lesions in head region (A) erythematous circular scaly lesions with advancing edges in the arm area of the infected farm workers in the current study (B).

The high prevalence rates of infection in the examined farm might be attributed to climatic condition, poor hygienic measures, and faulty housing system where calves housed in close contact to each other for long periods without sun light exposure. Winter season that is characterized by cold temperatures and high humidity rates play a role in the maintenance and existence of spores in the surrounding environment and the soil (Radostits et al., 1997). Moreover, Ringworm caused by *T. verrucosum* is characterized by rapidly spreading among susceptible animals. The prevalence rate (35%) observed in this study is consistent with other reports from Egypt, 30% (Abd-Elmegeed et al., 2015), 12% (EL-Ashmawy et al., 2015) and 12.5% (Mousa and Abdeen, 2018). On the other hand, Aboueisha and El-Mahallawy (2013) denoted higher detection rates than the current study (61.9%). In Nigeria, 13% prevalence of ringworm was reported by Dalis et al., (2019). However, low prevalence rate (3.75%) was documented in Afghanistan by Langar et al., (2020). Variation in the prevalence of the disease among countries is possibly attributed to climatic conditions, housing system, animal breed and production.

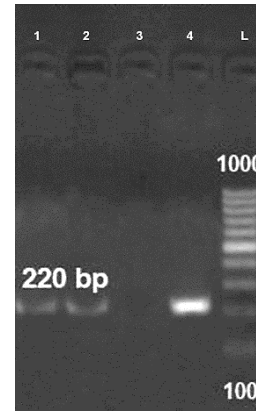


Fig. 2. Agarose gel electrophoresis for PCR products of *T. verrucosum*. Lane 1: Animal samples, Lane 2: Human samples, Lane 3: control negative, Lane 4: control positive, L: 100bp DNA ladder.

Concerning the *in-vitro* sensitivity of human and animals' isolates recovered from the farm under the study to most used antifungal drugs was illustrated in Fig. (3) and Table (3). It was clear that both human and animal traits showed multi-drug resistance where animal isolates ($n=42$) were resistant to Fusidic acid, Nystatin, Amphotericin B, (85.7, 78.6, 71.5 and 59.5% respectively) and they were intermediately resistant to Voriconazole (50%) at $P > 0.001$. On the other hand, they were sensitive (100%) to Fluconazole $P > 0.001$. Meanwhile human isolates were resistant to Voriconazole, Amphotericin B and Fluconazole (100, 66.7 and 66.7% respectively) but they showed equal degree of resistance to Fusidic acid, and Nystatin (33.3%), and they showed sensitivity to Itraconazole (66.7%) at $P > 0.001$.

Table 3. *In-vitro* antifungal sensitivity pattern of the isolated *T. verrucosum* from animals and humans.

Species	Antifungal discs																		p. value
	Fusidic acid (FC, 10ug)			Itraconazole (IT, 10ug)			Nystatin (NS, 100U)			Voriconazole (VRC, 1ug)			Amphotericin B (AP, 100U)			Fluconazole (FLC, 25ug)			
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	
Animal (n=42)	2	4	36	1	30	11	3	6	33	7	21	14	7	10	25	42	-	-	0.001
	4.76	9.5	85.7	2.4	71.5	26.2	7.1	14.3	78.6	16.7	50	33.3	16.7	23.8	59.5	100	0	0	
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	
Human (n=3)	1	-	2	2	-	1	-	-	3	-	-	3	-	1	2	1	-	2	0.5
	33.3	0	66.7	66.7	0	33.3	0	0	100	0	0	100	0	33.3	66.6	33.3	0	66.6	
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	

S= sensitive, I= intermediate, R= resistant

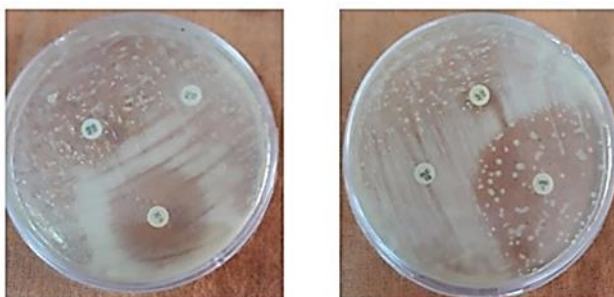


Fig. 3. Antifungal susceptibility test of *T. verrucosum*

The higher rate of fungal resistance might be contributed to low level of hygiene due to incomplete disinfection and/or inappropriate use of disinfectants as well due to overuse or

abuse of antifungal agents. Like our finding, Khatri et al., (2017) reported that six out of nine isolates from patients were resistant to Fluconazole (66.67%), but reciprocally two (16.6%) were intermediate to Itraconazole. Conversely to our findings, Lagowski et al., (2020) reported that human isolates of dermatophytosis were resistant to Itraconazole but cattle isolates were resistant to Itraconazole, Clotrimazole and ketoconazole which is nearly the same as our findings. On the other hand, Pakshir et al., (2009) reported that most dermatophytes spp. showed resistance to Fluconazole which might be due to the culturing media (SDA) that contains substance (triazole) that interfere with active principle of the drug.

The results illustrated in **Table (4)** showed significant resistance to all of the used disinfectants in different concentrations at contact time 20sec at $P > 0.001$ in addition to a significant resistance to all of the used disinfectants after contact time 30min. at $P > 0.001$, meanwhile the tested isolates showed decreasing the resistance pattern with the increase of contact time in case of iodine disinfectant where they were sensitive to iodine (7%) after 1h contact time (47%) that increase to (69%) after 24h of contact time at $P > 0.001$. regarding Virkon S, the tested isolates showed similar

pattern to that of iodine where they showed decreasing resistance with the increase of contact time, as they were sensitive to Virkon S (1%) after 24 h contact time (55%) ($P > 0.001$). The obtained results came in accordance with that of **Marchetti et al., (2006)** who reported that Virkon S treated hairbrushes have stopped the mycotic growth (87.14%) within 10min contact time. The complete recovery of worker after their treatment with iodine bandages and topical antifungal spray containing Itraconazole as active principle strengthen our results.

Table 4. *In-vitro* biocidal efficacy of tested disinfectants against the isolated *T. verrucosum* traits.

Tested disinfectant	Sensitivity pattern (%) of <i>T. verrucosum</i> isolates (n=45) at different exposure times												
		20sec			30min			1h			24h		
		S	I	R	S	I	R	S	I	R	S	I	R
Iodine	5%	5	8	87	7	9	84	10	11	79	15	15	70
	7%	13	8	79	25	15	60	47	22	31	69	11	20
TH	1:200	7	5	88	11	10	79	18	15	67	25	20	55
	1:400	11	6	83	19	10	71	23	15	62	30	19	51
Virokin S	0%	9	3	88	15	5	80	29	15	56	35	25	40
	1%	15	7	78	22	10	68	30	20	50	55	15	30
<i>P value</i>		0.001			0.001			0.001			0.001		

Incontrovertible, *T. verrucosum* in the current study exhibited a high degree of multi- drug resistance to both antifungal agents and disinfectant that might be related to its arthrocoidia which is considered the most resistant *Trichophyton conidia* (**Rippon, 1988**). Also, it might be attributed to the miss use and/or abuse of those agents. The miss uses and /or the abuse of antifungal drugs as well as disinfectants in the animals' surrounding environment might eventually end up with increased levels of microbial resistance to both antifungal agents and disinfectant that will cause economic losses concerning the costs of the wasted agents and failed treatments of animals

4. Conclusion

From the present study we concluded that *Trichophyton verrucosum* is prevalent in the studied areas in Egypt and has an occupational hazard. Although both human and animal isolates showed multi-drug resistance, the *in-vitro* sensitivity of animals and human isolates to Fluconazole and Itraconazole, respectively was observed. The study also highlights the significant role of increasing the contact time on decreasing the resistance pattern of dermatophyte to Virkon S (1%) followed by Iodine (7%) that give a promising results when used to control the infections in the surrounding environment.

5. Authors Contributions

All authors contributed equally to study design methodology, interpretation of results and preparing of the manuscript.

6. Conflict of Interest

The authors declare no conflict of interest.

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