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
Ringworm is a fungal zoonotic infectious disease caused by different species of dermatophytes which affects the animal productivity and performance. To estimate the prevalence of dermatophytosis in beef calf farm and to assess the in-vitro efficacy of six antifungal agents besides three disinfectants with different concentrations against Trichophyton verrucosum isolated from both calves and attendants using broth macro-dilution at different contact times (20 sec, 30min, 1h and 24h) .Skin scrapings and hair samples were collected from both beef calves and attendants Trichophyton verrucosum were isolated from skin scrapings and hair samples. The collected samples were examined by direct microscope, culture and molecularly for isolation and identification of the fungi. Results showed that the prevalence of fungal infection in 35% of calves and 100%. Fluconazole (100%) and Itraconazole (66.7%) showed the highest fungicidal activity (P<0.001) against dermatophytes isolated from calves and human, respectively. Moreover, Iodine (7%) and Virkon S (1%) were significantly active against isolates (P<0.001) at contact time of 1h and 24hr, respectively. In the present study we concluded that ringworm is an infectious fungal diseases that affect the beef calves especially young ages and it has an occupational hazard. Fluconazole and Itraconazole have promising antifungal effect against Trichophyton verrucosum. Moreover, Virkon S (1%) could be applied to control the dermatophytosis in calves’ 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 (Degreer, 2008). Generally, the 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, 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 (Euzehy, 1992). Therefore, environmental contaminations consider a possible source of infection to other animals and to humans that require urgent effective environmental control and not only treating infected animals or humans (Moriello, 2004).

Due to Scarcity of information about the prevalence of dermatophytosis in beef farms and the increased resistance to wide range of antifungal drugs commonly used to control it, this study aimed 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

This study was performed in a private beef farm in Beni-Suef, Egypt during the period from November 2020 till February 2021. The farm consisted of 120 angus calves their ages ranged from 6 to 12 months housed in five groups (n=24) according to age and body weight. Animals housed in confined separate houses where they fed drink and kept during the whole day and were served by three attendants.

The hygienic condition prevailed during the period of study was fair where it was obvious that animals were too many for the allowed space. The investigators visited the farm on a weekly basis at the beginning of the week. During the study some calves (n=42) aged between 6 and 9 months showed signs of focal lesions in their head region. Animals were carefully examined, and samples were obtained from lesion's margins by scalpel blade and were subjected for different mycological processing investigations. In the other hand, the farm workers who were in close contact with the animals showed inflamed rounded scaled lesions in their fore arms.

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. All collected samples were prepared for direct microscopic examination, isolation and characterization using PCR. Antimicrobial sensitivity profile was done on six antifungal disks using agar disc diffusion method, and three disinfectants with different concentrations.

2.1. Calves' Samples

Out of 120 examined animals, 42 of them showed focal lesions in the head area and with characteristic round shape (Fig. 1A). Skin scrapings were gathered by from the periphery's 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. Human Samples

The three workers cared for the examined animals showed typical rounded erythematous lesion in their arms, the lesions were covered by scales (Fig. 1B). Skin scales and crusts were collected 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.

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

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 sulfoxide (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 (500 mg/L), cycloheximide (400 mg/L), thiamine (10 mg/L) and inositol (50 mg/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 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. Data of the primer’s sequences are listed in Table 1 (Ohst et al., 2016).

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

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primers sequences</th>
<th>Amplified segment (bp)</th>
<th>Primary denaturation</th>
<th>Secondary denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. verrucosum</td>
<td>CCGCGCTCT CCC</td>
<td>220</td>
<td>94°C 5 min</td>
<td>94°C 30 sec</td>
<td>55°C 30 sec</td>
<td>72°C 30 sec</td>
<td>72°C 7 min</td>
<td>Ohst et al.,(2016)</td>
</tr>
</tbody>
</table>

2.3.3. Molecular Identification of Dermatophytes

2.3.3.1. 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. Data of the primer’s sequences are listed in Table (1) (Ohst et al., 2016).

2.3.3.2. 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). 1 µl of each primer of 20pmol concentration, 5.5µl of water, and 5µl of DNA template. Data of the primer’s sequences are listed in Table1. The reaction was performed in an applied biosysystem 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 ruer 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 (Fig. 2).

2.3.4. Evaluation of Germicidal Efficacy of Tested Antimicrobial Agents

2.3.4.1. 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 (Fig. 3). 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 30 minutes. 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 to 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).

Fig. 2. Agarose gel electrophoresis for PCR products of T. verrucosum using the specific gene amplified 220bp. Lane (L): 100bp Ladder “Marker”, Lane :1, animal sample, 2, human sample, the examined samples, Lane Pos: Positive control, Lane Neg: Negative control.

Fig. 3. In-vitro antifungal susceptibility testing of T. verrucosum.
2.3.4.2. Disinfectant Sensitivity Testing
Germicidal power of three commercially disinfectants proven their efficacy; Virkon S (potassium peroxymonosulfate 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 (Didecylidimethyl Ammonium Chloride, Dioctyldimethyl Dimethyl Ammonium Chloride, Octyl Decyl Dimethyl Ammonium Chloride and Gluteraldehyde, SOGeval) at conc. 1: 200 and 1: 400 were tested against 45 strains of T. verrucosum isolated from animals and human samples 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, causing highly inflammatory skin and hair dermatophytoses (Papini et al., 2009).

On clinical examination of the prevalence of T. verrucosum in the examined samples of both animals and humans (Table, 2) it was revealed that 35% (42/120) of calves showed heavy gray, white crusty circular lesions of 1-5 cm in diameter. These lesions are commonly found around the eyes, ears and neck (Fig. 1A). Forty-five samples out of one hundred and twenty-three were significantly positive for isolation of dermatophytes and PCR (36.6%) at X2= 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%). 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 forearms of them (Fig. 1B). It was noticed that worker was completely recovered after their treatment with iodine bandages and topical antifungal spray containing Itraconazole as active principle.

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 (Radosits 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.

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

<table>
<thead>
<tr>
<th>Samples</th>
<th>Total number</th>
<th>No. positive</th>
<th>Percentage (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal</td>
<td>120</td>
<td>42</td>
<td>35</td>
</tr>
<tr>
<td>Human</td>
<td>3</td>
<td>2</td>
<td>66.6</td>
</tr>
<tr>
<td>Total</td>
<td>123</td>
<td>44</td>
<td>35.8</td>
</tr>
</tbody>
</table>

P-value X²= 132.382 P<0.001

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 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 immediately 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.

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 dermatophytes 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 complete 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 30 min. 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. In contrast to our results Matloob et al., (2019) found that 3% hydrogen peroxide was efficient enough to kill isolated dermatophytes where the main active principle of Virkon S is potassium oxy-per-oxymonosulfate which is an oxidizing agent. To some extent the results of Gupta et al., (2001) where he reported that chlorine (1%) which is an oxidizing agent showed an efficient result in controlling T. mentagrophytes. Similarly, also Gupta et al., (2001) reported quaternary ammonium salts exhibited low level antifungal activity.

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 arthrocodia 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.

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

<table>
<thead>
<tr>
<th>Antifungal discs</th>
<th>Fusidic acid (FC,10ug)</th>
<th>Itraconazole (IT, 10ug)</th>
<th>Nystatin (NS,100U)</th>
<th>Voriconazole (VRC, 1ug)</th>
<th>Amphotericin B (AP,100U)</th>
<th>Fluconazole (FLC, 25ug)</th>
<th>p. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>I</td>
<td>R</td>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>36</td>
<td>1</td>
<td>30</td>
<td>11</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>4.76</td>
<td>9.5</td>
<td>85.7</td>
<td>2.4</td>
<td>71.5</td>
<td>26.2</td>
<td>7.1</td>
<td>14.3</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>0.001</td>
<td></td>
<td></td>
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</table>

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

<table>
<thead>
<tr>
<th>Tested disinfect</th>
<th>Sensitivity pattern (%) of T. verrucosum isolates (n=45) at different exposure times</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20sec</td>
</tr>
<tr>
<td>S</td>
<td>I</td>
</tr>
<tr>
<td>Iodine</td>
<td>5%</td>
</tr>
<tr>
<td>TH</td>
<td>1:200</td>
</tr>
<tr>
<td>Virkon S</td>
<td>1%</td>
</tr>
<tr>
<td>P value</td>
<td>0.001</td>
</tr>
</tbody>
</table>

4. Conclusion

Controlling dermatophytes in animals can prevent some cases of zoonotic dermatophytosis in humans. Infected animals should be treated, and the premises and fomites should be cleaned and disinfected. Contact with infected animals should be limited, and gloves and protective clothing should be used if these animals are handled. 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 of contact time on decreasing the resistance pattern of dermatophyte to Virkon S (1%) followed by Iodine (7%) that gives a promising result 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.

7. References


