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Microscopic, Molecular, and Pathogenic Characterization of Acremonium: Insights into its Biology, Culture Characteristics, and Clinical Implications

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Abstract **Original Research Article**

Fungal genus *Acremonium sclerotigenum* reported as rare opportunistic pathogenic fungi. *A.sclerotigenum* is a rare clinical isolate that has not been reported previously from Southern Punjab Pakistan. The Goal of study is to determine the actual spectrum of morphological, microscopic and molecular characterization of *A.sclerotigenum*. *Acremonium sclerotigenum* was analyzed for phenotypic diversity on different growth medias, humidity effect on fungal growth by culture characteristics, microscopy and identified at molecular level. Morphological examination revealed hyaline, septate hyphe giving rise to conidiophores with numerous micro conidias. *A.sclerotigenum* showed optimum growth on sabourad dextrose agar and oat meal agar medium *A.sclerotigenum* produce white, smooth cottony colony at 92% humidity with maximum growth. *A.sclerotigenum* strain was analyzed by culture characteristics and further identified by PCR and sequencing of ITS region. Study revealed that sabourad dextrose agar media is an appropriate growth media for *A.sclerotigenum* while optimum temperature for *A.*sclerotigenum culturing ranges from 25°C to 35°C. Study suggested *A.sclerotigenum* has adaptability to physiological factors such as humidity. A detailed research on the factors and aspects involved in pathogenicity caused by *A.sclerotigenum*, is still to be investigate.

Keywords: *Acremonium Sclerotigenum*, Microscopic examination, Relative humidity, Molecular characterization, pathogenic fungi.

1. INTRODUCTION

Acremonium is major polyphyletic genus that includes about 200 species. They are associated to two major *ascomycetous* orders (i.e Hypocreales, sordariales). *Acremonium* are mostly soil saprobes and considered as opportunists pathogens to human and animals. *Acremonium* species are very similar to each other in terms of morphology and can be differentiated only by some subtle differences such as morphological slow growing colonies including thin hyphae, narrow, tapered or long phalides. Many *Acremonium* species also produce conidiophores having verticillate or simple branching. In several clinical reports many etiological agents are investigated as *Acremonium* specie, which made the reports doubtful and is the reason of under investigated clinical incidence of most of the *Acremonium* species (Perdomo H *et al*., 2011).

Acremonium is known to be *cephalosporium* formally and thought to be a cause of skin infection in humans. Mycetoma by fungi caused by many other varieties of fungi but not by *Acremonium* commonly. Dermatophytes and the Fusarium species are most common infectious agents of Onchomycosis followed by *Acremonium* species (Fakharian *et al.*, 2015). The most common anatomic sites for *Acremonium* isolates were the respiratory tract (41.3%), nails (10.7%), and the eye (9.3%) (Perdomo H *et al*., 2011). *Acremonioum*

Sclerotigenum is a type of filamentous fungi those are associated with spoilage of food. It has association with many animals and plant sources, shows capability to grow at high temperature relatively with some general compatibility i.e morphological. Contamination of the foods those are heat disinfected is facilitated by its heat tolerance ability (Summerbell RC *et al*., 2018).

Acremonium related infections occur uncommonly in humans but they commonly affect individuals facing immune competency. Followed by trauma, gastrointestinal track, eyes, lung's colonizing infections, invasive infections include arthritis, osteomyelitis, peritonitis and sinusitis (Fincher RM *et al*., 1991). Fungal superficial infections those are commonly called as tinea infections occur due to dermatophytes, belongs to group of Filamentous Fungi. They have special ability to breakdown keratinized tissues (i.e skin, nails, hair). Dermatophytosis is the mycotic infection which leads to serious consequences related to public health. According to an estimate 20% of human population is affected by dermatophytes worldwide (Pal M *et al*., 2017). Dermatophytosis can affect various anatomical areas of human body which are described as tinea capitis that manifest scalp, tinea unguum nails, tinea cruris groin region, tinea barbae face, tinea pedis which is also called as Athlete's foot, a chronic mycotic infection of feet. In United States tinea pedis is known to be second common disease of skin and it is estimated that 15% of population is affected by it (Al Hassan *et al*., 2004). To cause infection, dermatophytes first attach to the surface of host, adhere to epithelial cells and then get nutrients from them for their survival. Dermatophytes secretes proteases including keratinases and other enzymes to produce infection in their host (Achterman *et al*., 2013).

In the majority of clinical cases, the etiological agent was either not recognised at all or incorrectly identified due to the challenges associated with morphologically distinguishing *Acremonium* isolates and the overall lack of trustworthy sequences in public databases. As a result, it is unknown how frequently *Acremonium* species are actually involved in therapeutic settings. The spectrum of fungal species that can be recognised in a clinical setting has increased recently with the use of molecular technologies. With regard to *Acremonium*, a recent genetic investigation that examined its prevalence in a large collection of clinical isolates from the United States. A. sclerotigenum-A. egyptiacum complex revealed among most commom clinical isolate (Fernandez-Silva *et al*., 2014).

Study is aimed to characterize the *Acremonium sclerotigenum* for physiological, molecular and more realistic morphological identifications.

2. MATERIAL AND METHODS

Sample Collection:

Samples were collected from patients infected with tinea pedis at the Civil hospital Bahawalpur. Before the collection of skin scrapings and nail samples, infection site was cleaned with 70% ethanol. The skin and broken nail scraps were removed from the toe nail, site of infection of tinea pedis.

3. Culture Medium

Sabouraud dextrose agar (SDA) medium was used to culture the samples, supplemented with 1% peptone, 4% dextrose and 1.5% agar in 100mL distilled water containing chloramphenicol (0.05g/ 100mL) to inhibit bacterial growth and cycloheximide (0.04g/ 100mL) which inhibits the growth of saprophytic fungi. From the SDA slant, spores were collected and spore suspension was prepared in autoclaved distilled water. They were then centrifuged at 4000rpm for 2min. Supernatant was then discarded and sterilized water 1mL added to conidial suspension. Conidial suspension 5µL was inoculated from (2×10^6) conidial suspension (Ismail F *et al*., 2021).

3.1 Microscopic identification

Lacto phenol cotton blue (LCB) staining was used for the phenotypic observation of *A. sclerotigenum*. 3µL conidial suspension from 2×10^6 conidial suspension, examined under light microscope stained with 2µL of LCB dye prior to which 1µL ethanol was mounted on slide (Taha M *et al*., 2017). Conidia were examined under the light microscope at 100X magnification.

3.2 Optimum morphology on Different Media

In order to investigate the optimum morphology of *A.sclerotignum* four different media such as SDA (sabourad dextrose agar), PCA (potato carrot agar media) and OMA (oat meal agar), CA (casein agar) were used to grow *A.sclerotignum*. SDA medium is supplemented with 1% peptone, 2% dextrose and 1.5% agar; PCA medium with 20% carrot infusion, 25 % potato infusion and 1.5% agar. OMA medium supplemented with 6% oat meal and 1.25% agar; CA medium composition skim milk 1% and agar 2%. Autoclave media was poured in petri plates to inoculate the spore suspension (containing 1×10^2 spores per mL in water), incubated at 30°C for 3-10 days depending on growth period of fungi (Mishra PK *et al*., 2016).

3.3 Growth of *A.sclerotigenum* at Relative Humidity at different humidity levels

Humidity Stress was determined by using saturated solutions of salts which were prepared to maintain required humidity level; $MgCl_2$ (33% RH), KI (65% RH), NaCl (75% RH), KNO_3 (92% RH). Relative humidity levels maintained by keeping saturated salt solutions in 30°C incubator. 50ml beaker filled with saturated salt solution kept in large plastic box that

contain stack of the inoculated petri dishes (Mishra PK *et al*., 2016).

3.4 Molecular Identification

3.4.1 DNA Extraction

Fungal isolate was grown in the nutrient broth to get enough cell biomass for the DNA extraction. After seven days of growth, broth was filtered and biomass on the filter paper then allowed to dry and grinded into fine powder. Mycelia were allowed to grow in liquid nitrogen. 1mL of DNA extraction buffer (appendix/ table) were added and incubated at 37°C for one hour in water bath.

700 µL of phenol, chloroform 1:1 mixture was added in each eppendorf and centrifuge for 12000rpm for 10 minutes. Supernatant was transferred into another 2.0 mL tube and added 0.7mL tri chloro methane, shaken gently and again centrifuged at 12000rpm for 10 minutes. 400 µL of supernatant was transferred to new 1.5 mL tube and added 240 µL isopropyl alcohol, mixed gently and placed at 4°C for 1-2 hours till liquid became opaque.

Centrifuged and discarded the supernatant to get DNA. 70% ethanol 1mL was added and centrifuged at 7500rpm for 5 minutes, discard the supernatant and this step was repeated for 2 minutes. Tubes were dried at room temperature and DNA pellet was diluted with d.dH2O.

3.4.2 Gel Electrophoresis

For the conformation of DNA extraction, 0.8% gel was prepared. For the preparation of gel 1XTBE buffer was used. At first stock of 5XTBE buffer (Appendix/ table) was prepared and diluted for the process. 0.8g agarose gel dissolved in 1XTBE buffer. This mixture was boiled until solution became clear then 5µL of ethidium bromide was added. Gel was poured into casting tray. After solidification of gel, samples were loaded with loading dye (Bromophenol blue). Gel was run with 1XTBE buffer and seen in UV light to check the presence of DNA.

3.4.3 PCR Amplification of ITS regions

For molecular identification of the isolated starin Hot star PCR was used on the basis of ITS region amplification. For this purpose, universal primers, Forward primer (ITS1 (5'TCCGTAGGTGAACCTGCGG3') and the (Reverse primer: ITS4 (5'TCCTCCGCTTATTGATATGC3') were used. Master mix (Appendix/ table) was prepared. Total volume of each PCR tube was maintained up to 25µL containing 25µL master mix and 1µL of DNA. Initial denaturation temperature was set at 95ºC for 5 minutes then 95ºC for 1 min, annealing temperature at 65 ºC for 30 seconds, elongation temperature at72ºC for 1min, and then final extension step at 72ºC for 10 min. No of cycles were 30. To check result of PCR 2% agarose gel with 1XTBE buffer was prepared. Gel was run on 70 voltages and observed under UV. DNA samples were then sent for sequencing (Ismail F *et al*., 2021).

4. RESULTS

4.1 Microscopic Identification

A.Sclerotigenum was identified on the basis of macro morphology (forward and reverse side colony color) exhibits cottony white to rusty colony and micromorphology (microconidia and macroconidia). Mycelium was observed with numerous microconidia and hyaline macroconidia producing septate hyphae. Hyphae of *A.sclerotigenum* grew fine and smooth. Hyphaes of Acremonium synthesized phalides which were simple. Conidiophores giving rise to microcoidias as shown in Figure 4.1.

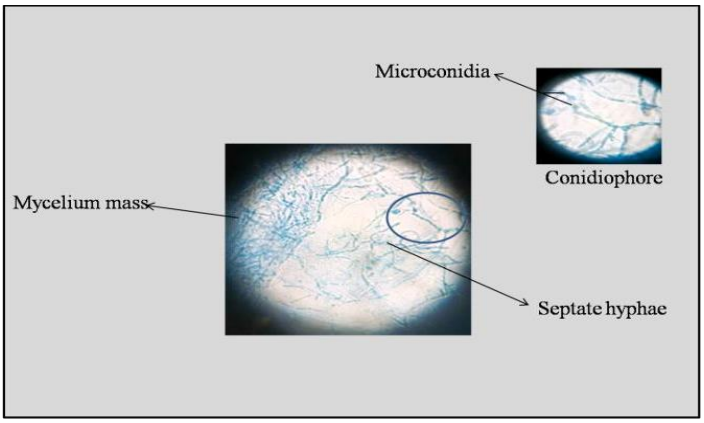

Figure 4.1: Shows the Microscopic image of *Acremonium sclerotigenum* (MK732096.1) producing septate hyphae with numerous conidiophores

4.2 Optimum morphology of *A.sclerotignum* on different Media

In order to investigate the optimum morphology of *A.sclerotignum* on four different media such as SDA (sabourad dextrose agar), PCA (potato carrot agar Media), OMA (oat meal agar) and CA (casein agar) were used to grow *A.sclerotigenum*. Fungal strain are further incubated at 30°C.

A.sclerotigenum on PCA media grown in four to seven days. *A.sclerotigenum* colony on PCA shown, with morphological features as off white cottony appearance as presented in Figure 4.2 and Table 4.1.

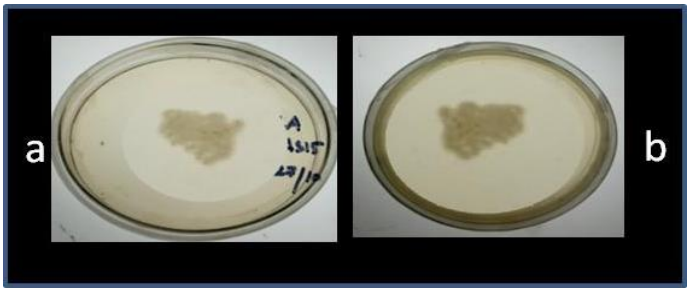

Figure 4.2: Culture morphology of *A.sclerotigenum* on PCA media, a) Front view, b) reverse view

A.sclerotigenum on SDA media developed in five to ten days. *A.sclerotigenum* colony on SDA ; with morphological features as white cottony appearance on both front and orange to rust pigmentation on reverse side as shown in Figure 4.3 and Table 4.1.

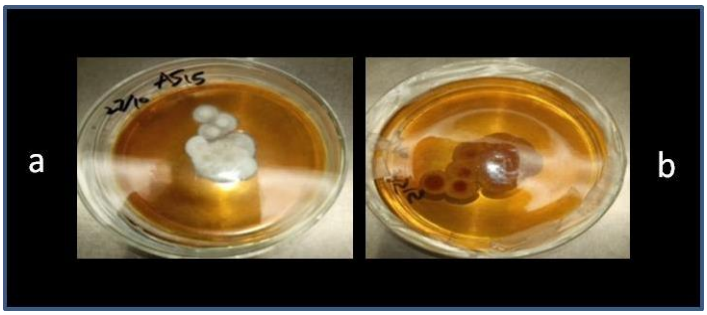

Figure 4.3: Culture morphology of *A.sclerotigenum* on SDA media, a) Front view, b) reverse view

A.sclerotigenum on OMA media developed in three to five days. *A.sclerotigenum* colony on OMA; with morphological features such as white, creamy and moist appearance as shown in figure 4.4 table 4.1.

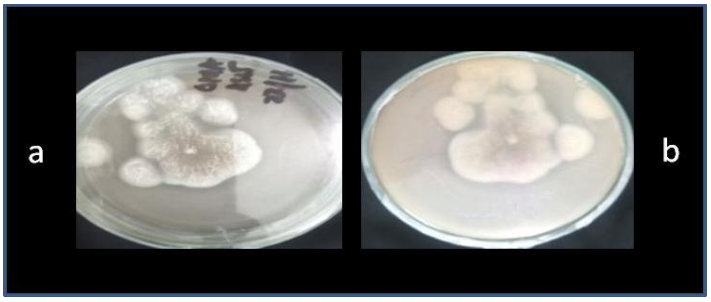

Figure 4.4: Culture morphology of *A.sclerotigenum* on SDA media, a) Front view, b) reverse view

On CA media *A.sclerotigenum* colony appeared in three to six days. *A.sclerotigenum* colony on CA exhibited creamy velvety appearance as presented in figure 4.5 and table 4.1.

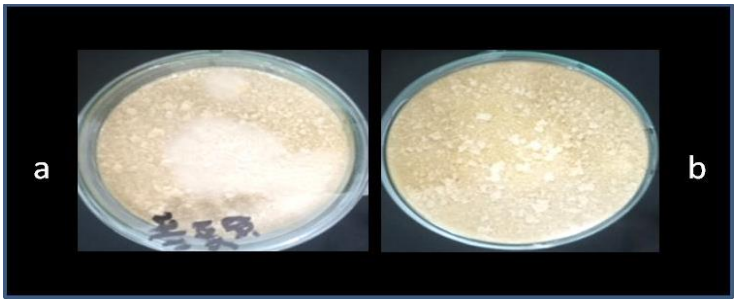

Figure 4.5: Culture morphology of *A.sclerotigenum* on CA media, a) Front view, b) reverse view

Table 4.1: Represents *A.sclerotigenum*'s (MK732096.1) colony appearance, colony pigmentation and growth period on four different medium

Sr. No	Growth Media	Colony Appearance	Front Side	Reverse Side	Days required to grow at 30◦C
1	**Potato Carrot Agar**	Slight Cottony	Off white	Off white	4-7
2	**Sabourad Dextrose Agar**	Cottony	Off White	Rusty pigmented	4-10
3	**Oat meal Agar**	Creamy, moist	White	White	3-5
4	**Casein Agar**	Fluffy, velvety	Creamy	Creamy	3-6

4.3 Growth of A.sclerotigenum at various relative humidity levels

Humidity stress was determined by using saturated solutions of salts which were prepared to maintain required relative humidity level. Growth count was examined at four different levels of humidity. *A.sclerotigenum* exhibited growth at all 4 levels of relative humidity.

A.sclerotigenum showed growth at 33% humidity with colony diameter 8mm. Growth was accompanied by increase in humidity level. At 65% and 75% humidity levels, 1.1 cm and 1.6 cm colony diameter was calculated respectively. Best growth was examined at 92% humidity with colony diameter approximately 2.0 cm and least at 33% humidity as shown in figure 4.6 and table 4.2.

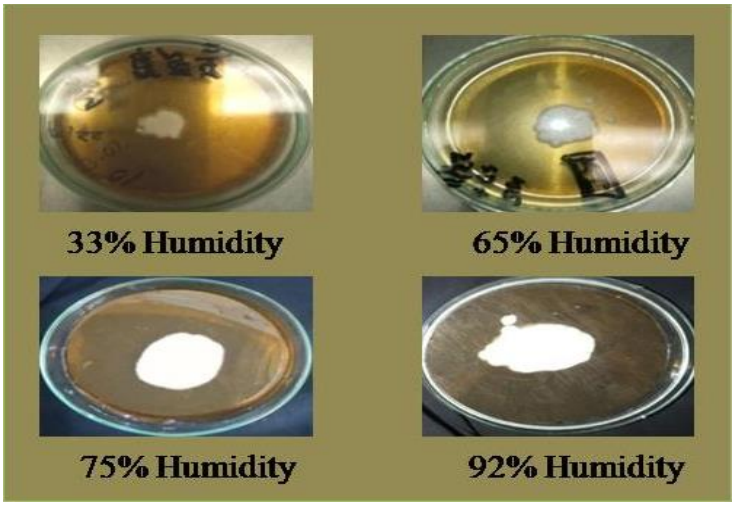

Figure 4.6: Represents the growth of *A.sclerotigenum* on different levels of Relative humidity (RH) provided by saturated solutions of Salts

Table 4.2: Growth of *A.sclerotigenum* at 4 different level of humidity

Sr. No	Salt	Relative Humidity (RH)	Sporulation	Colony Appearance	Diameter of colony
1	**MgCl2**	33%	Moderate	Smooth, thick and cottony	8mm
2	**KI**	65%	Good	Thick, smooth and cottony	1.1 cm
3	**NaCl**	75%	Very Good	Thick, smooth and cottony	1.6 cm
4	**KNO3**	92%	Excellent	Smooth, thick and cottony	2.0 cm

4.4 Molecular Identification

Molecular identification was performed by sequencing the conserved ITS region. Universal primer was used as forward primer ITS1(5'TCCGTAGGTGAACCTGCGG3') and the Reverse primer ITS4(5'TCCTCCGCTTATTGATATGC3'). DNA extraction was performed and confirmed by running 0.8% agarose gel. PCR amplification was done after amplification of ITS regions by using universal primer and confirmed by running 2% agarose gel as shown in Figure 4.7.

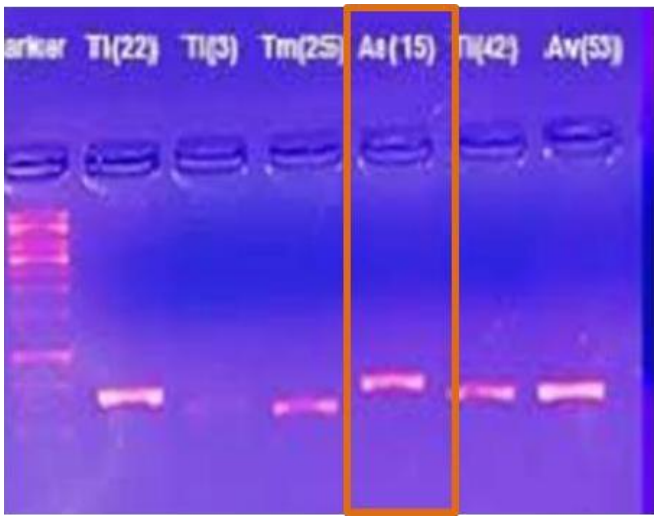

Figure 4.7: DNA confirmation through gel electrophoresis and PCR amplification

Sequencing of ITS Region

PCR products were sequenced by Sanger sequencing method and results were observed through NCBI BLAST. Sequences were aligned by multiple sequence alignment tool (Clustal w) as showed in Table 4.3.

Table 4.3: Sequence Identification by BLAST

Strain no	Clinical type	Identification According to ITS Sequence	Percentage % Identification by BLAST	Accession no
As(15)	**Tinea pedis**	***A.sclerotigenum***	**99.65%**	**MK732096.1**

5. DISCUSSION

Acremonium sclerotigenum are plant and soil saprophytic fungi. The species *A. sclerotigenum* is known to be a common soil fungus, but its presence as endophyte has been previously described. They are opportunistic fungi can act as a causative agent of human superficial infections such as onchomycosis. According to World Health Organisation (WHO) reports from 2005, tinea capitis prevalence in underdeveloped country populations can reach up to 19.7%. There have been reports of high prevalence rates of onychomycosis and tinea pedis in a number of occupational groups, including soldiers (16.4-58%), miners (21-72.9%), and marathon runners (22-31%) (Dogra *et al*., 2019).

Our study based on morphological identification of *A.sclerotigenum* by both microscopically and macromorphologically. Microscopic characteristics of *A.sclerotigenum* comprised of mycelium with septate and simple hyphae giving rise to hyaline phalides. Conidiophores produce enormous single cell micro conidias. As for as macro morphological identification was concerned, *A.sclerotigenum* grown on 4 different growth media such as potato carrot agar (PCA), sabourad dextrose agar (SDA), casein agar (CA) and oat meal agar (OMA). PCA was least effective for growth of *A.sclerotigenum*. CA and SDA exhibited proficient white velvety and cottony colony appearance respectively. OMA and SDA were most efficient with white cottony and white smooth creamy colonies respectively

To investigate the optimum humidity level for the growth of *A.sclerotigenum*. *A.sclerotigenum* was grown on 4 different levels of relative humidity. Phenotypic results revealed that growth of *A.sclerotigenum* increased gradually with an increase in humidity level. *A.sclerotigenum* showed growth at 33% humidity with colony diameter 8mm. Growth was accompanied by increase in humidity level. At 65% and 75% humidity levels, 1.1 cm and 1.6 cm colony diameter was calculated respectively. Best growth was examined at 92% humidity with colony diameter approximately 2.1cm and least at 33% humidity While on 65% and 75% humidity moderate growth was observed. Humidity had an impact on fungal enzyme activities as it is previously reported that enzyme activities increase as humidity increase (Han, B.Z *et al*., 2003).

It is challenging to identify Acremonium species since they are morphologically so similar to one another that the sole criteria for differentiation are small changes. Species identification calls for DNA-based methods (Jangla, S. M., 2024). *A. sclerotigenum* are identified on the basis of DNA sequence data analysis. PCR products were sequenced by Sanger sequencing method and results were observed through NCBI BLAST. *A. sclerotigenum* showed a high level of genetic diversity. Nucleotide sequences of ITS regions of *Acremonium* isolates assayed were deposited in GenBank. Sequences were aligned by multiple sequence alignment tool (Clustal w). Sequence is identified through BLAST showed 99.65 percent similarity already present sequence of GenBank.

6. CONCLUSION

Acremonium Sclerotigenum is non dermatophyte opportunistic fungi cause human superficial skin infections. *Acremonium* is major polyphyletic genus that includes about 200 species. They belong to *ascomycota* group. Morphological characterization revealed that *A.sclerotigenum* have septate hyphae, conidiophores with several microconidias. *A.sclerotigenum* grows with the full fungal characterization on sabourad dextrose agar and oat meal agar medium. *A.sclerotigenum* colonies exhibit white cottony and white creamy appearance respectively. *A.sclerotigenum* showed utmost growth at highest humidity. *A.sclerotigenum* identified at molecular level by sequencing of ITS region. On the basis of morphology, growth rate organization and conidial structure as well as genetical identification it is confirmed as *A.sclerotigenum*. *Acremonium* is an opportunist pathogen that should not be disregarded as a simple contaminant as it can cause all forms of onychomycosis with significant therapeutic implications. Focus should be placed on both prompt care and the fungus's continued expansion. A throughout investigation on the infectious agents involving in virulence caused by *Acremonium* still need to be explored.

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