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Molecular Detection of Putative Virulence Factors (Fungalysin and Subtilisin) in *Aspergillus tamarii* Isolated from Human Skin

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Abstracts

The genus *Aspergillus* is widely distributed containing a number of species that are opportunistic pathogens for both humans and animals. Some of the species of this genus were identified based on morphology. However, the molecular detection of the pathogens is necessary. The detection of the virulence factors of *Aspergillus tamarii* associated with the human skin was performed in this study. *Aspergillus tamarii* isolated from skin infections were examined using morphological and molecular criteria. The phylogenetic relationship of isolates was determined using sequences from the ITS gene. Fungalysin (*MEP3*) and subtilisin (*SUB1*) genes were also detected in the isolates using Polymerase Chain Reaction. Out of ten morphologically identified *Aspergillus tamarii* seven were identified to be *Aspergillus tamarii* using molecular methods. All the molecularly identified *A. tamarii* had fungalysin (*MEP3*) gene while the subtilisin (*SUB1*) gene was detected in six out of seven isolates. The nucleic acid sequencing offers an additional tool for a better diagnoses and understanding of the phylogenetic relationships within genus or species of pathogenic microbes.

Keywords: Aspergillosis, *Aspergillus tamari*, Cutaneous infections, Fungalysin, Subtilisin

1. Introduction

Not until the recent time, aspergilloses have not been reported to be rare opportunistic fungal infections. The *Aspergillus* species have been implicated in both localized and invasive infections of man with a higher occurrence especially among immunocompromised patients (Mustafa *et al.,* 1992; Walmsley *et al.,* 1993; Klein and Tebbets, 2007). Cutaneous aspergillosis has been reported to be more frequent among burns victims, neonates and children. Individuals with cancer, bone marrow and solid organ transplant recipients have also been reported to be disposed to cutaneous aspergillosis (Martin *et al.,* 2003). Surgical wounds (Brock, 2009), traumatic inoculation (Gupta *et al.,* 2001; Latge and Calderone, 2002), or exposure to high spore counts in occupations such as farming (Chakrabarti, 2005) have been identified as pre-disposing factors for healthy individuals to contract cutaneous aspergillosis. Cutaneous aspergillosis may produce pustules or lesions with purulent discharge which generally are common among neonates (Granstein *et al.,* 2005). *Aspergillus fumigatus, A. flavus,* and *A. niger* are the prominent members of the genus that have been implicated in most cases of aspergillosis (John and Shadomy, 1987; Grossman *et al.,* 2005). Non-*flavus* and –*fumigatus Aspergillus* rarely cause cutaneous lesion.

Some dermatophytes have been reported to produce fungalysin (an endometalloprotease) which has been recognised as keratin-induced proteases that expresses during fungus-keratinocyte interaction (Brouta *et al.,* 2001; Tarabees *et al.,* 2015). Fungalysin has been reported to have pronounced collagenolytic, elastinolytic and keratinolytic activities that contribute to the virulence of the secreting fungi (Jousson *et al.,* 2004). *Aspergillus* spp. produce and secrete various hydrolytic enzymes including subtilisin. Subtilisin is an extracellular-located enzyme that is also connected with the cell wall. It generally contributes to the fungal virulence and facilitates tissue colonization. There is a considerable correlation between their activity and the severity of infections (Alp and Arikan, 2008). Subtilisin has the ability to degrade protein (Hogan *et al*., 1996), and specifically attacks elastin, collagen, fibrin and fibrinogen in the tissue of the host (Moutaouakil *et al*., 1993; Tomee and Kauffman, 2000).

There is dearth of information on the association of *A. tamarii* with skin infections and the virulence factors of the fungus isolated samples from the study area. Therefore, this study was conducted to investigate the association of *A. tamarii* with human skin infections, and to detect the

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presence of fungalysin and subtilisin, two putative virulence factors in the organism.

2. Material and Methods

2.1. Sample Collection, Macroscopic Examination and Cultural Identification of the Isolates

Scrapings or swabs were collected from children [(n=23) aged 5-14 years] with different skin infections, and were plated on Saboraud Dextrose Agar supplemented with chloramphenicol. They were incubated for five days at 25 ^{o}C. The fungal identification was carried out based on microscopic examination (growth characteristics, pigment formation). A wet preparation of each colony was prepared using lactophenol cotton blue solution on a clean grease-free slide as described by Raper and Fennell (1965). Fungal spore and hypha were picked on the slide, lactophenol blue was added, and a cover slip was used. The samples were examined under the microscope by X10 and X40 objective lenses.

2.2. Molecular Identification

The identities of the isolates were confirmed further using polymerase chain reaction. The DNA extraction was carried out on the isolates using the Zymo Fungal/Bacteria DNA extraction kit according to the manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a nanodrop (ND 1000) Spectrophotometer (Thermo Scientific, USA). All the samples showed a DNA yield between 5ng - 25ng, and the extracted DNA was optimally pure showing A_{260}/A_{280} between 1.60-1.80.

Polymerase chain reaction was carried out to amplify the ITS gene of the fungus using the primer pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG) and ITS-4 (5'-TCCTCCGCTTATTGATATGC). The PCR reaction was carried out as described earlier.

Eppendorf Vapo protect thermal cycler (Nexus Series) with an initial denaturation of 95 °C for fifteen minutes followed by 35 amplification cycles of thirty seconds at 95 °C; one minute at 58 °C and 1.5 minutes at 72 °C was used for the thermal cycling. The final extension step of ten minutes at 72 °C was followed. The amplification product was separated on a 1.5 % agarose gel, and electrophoresis was carried out at 80 V for one hour and thirty minutes. The DNA bands were visualized by ethidium bromide staining. One hundred base pair DNA ladder was used as DNA molecular weight standard.

Purified PCR products were sequenced with Exo sap, and were sent to Epoch Life science (USA) for Sanger sequencing. The consensus sequences obtained from both primers were edited and subjected to BLAST searches to assign tentative identity and phylogenetic inference.

2.3. Determination of the Presence of Pathogenic Factors Using PCR

DNA extraction was carried out from the isolates using the Zymo Fungal/Bacteria DNA extraction kit according to the manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a NANODROP (ND 1000) Spectrophotometer (Thermo Scientific, USA). All the samples showed a DNA yield between 5 ng – 25 ng, and the extracted DNA was optimally pure showing A_{260}/A_{280} between 1.60-1.80.

2.4. PCR Amplification of Fungalysin (MEP3) and Subtilisin (SUB1) Genes

Polymerase chain reaction was carried out to amplify the *MEP3* gene and *SUB1*of the fungi using the primer pair *MEP*3 forward 5' GCCATGTCCTTCTCCAAG 3' reverse 5' AGACCACGCTTAGCAAAG 3' and *SUB1* forward 5' ATCCTGTCTATGCCTCATG 3', reverse 5' AATCGAAGTCGAAGTTATC 3' as reported by Lemsaddek *et al.* (2010) and Alp and Arikan (2008) respectively. The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl reaction containing 1X Blend Master mix bufferBuffer (Solis Biodyne), 1.5mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pMol of each primer (BIOMERS, Germany), 2 unit of Hot FIREPol*DNA* polymerase (Solis Biodyne); However, additional Taq DNA polymerase was incorporated into the reaction mixture to reach 2.5 final units. Proof reading enzyme, 2 ng of the extracted DNA, and sterile distilled water was used to complete the reaction mixture.

Thermal cycling was conducted in an Eppendorf Vapo protect thermal cycler (Nexus Series) as described earlier. The amplification product was separated on a 1.5 % agarose gel, and electrophoresis was carried out at 80 V for one hour. After electrophoresis, DNA bands were visualized by ethidium bromide staining. 100 bp DNA ladder was used as DNA molecular weight standard.

3. Results

After culturing on Glucose Peptone Agar (supplemented with chloramphenicol to inhibit the growth of any bacteria from the culture), only ten out of the twenty-three samples were positive for *Aspergillus* species. The Isolates, that produce abundant conidia heads in dull yellowish green shades, becoming metallic bronze at maturity with diameters between 6.0 and 7.0 cm in eight days when cultured at 37 ^{o}C, were suspected to be *A. tamarii.*

The genomic DNA extracted from all the ten fungi isolates was identified by PCR analysis. Seven were identified to be *A. tamarii* and others were one *A. nomius,* one *A. aculeatus* and one *Pichia kudriavzevii.* Only seven that were positive for *A. tamarii* were chosen for this study.

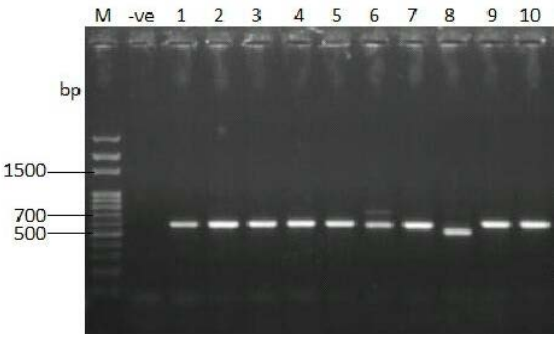

Figure 1. Gel electrophoresis of PCR amplification of *ITS* gene, Lane M is a 1500 bp ladder, Lane -ve is the negative control lane while Lanes 1 to 10 are morphologically identified *Aspergillus tamarii* isolated from human skin

Table 1. Sequence spacing significant alignment of the isolates isolated from skin infection

Isolates	Max score	Total score	Query cover (%)	Identity (%)	Accession number	Identification
A	11024	1145	96	99	KC874831.1	*Aspergillus tamarii*
B	1011	1011	75	99	JQ257030.1	*Aspergillus tamarii*
C	1061	1061	63	96	KR905619.1	*Aspergillus nomius*
D	1022	1363	85	99	NR135325.1	*Aspergillus tamarii*
E	1016	1400	87	99	MR135325.1	*Aspergillus tamarii*
F	1016	1158	95	99	JQ257030.1	*Aspergillus tamarii*
G	952	1376	61	97	KJ706301.1	*Pichia kudriavzevii*
H	1271	2086	94	95	KP965728.1	*Aspergillus aculeatus*
I	1014	1193	97	99	KP784375.1	*Aspergillus tamarii*
J	1016	1016	54	99	JQ257030.1	*Aspergillus tamarii*

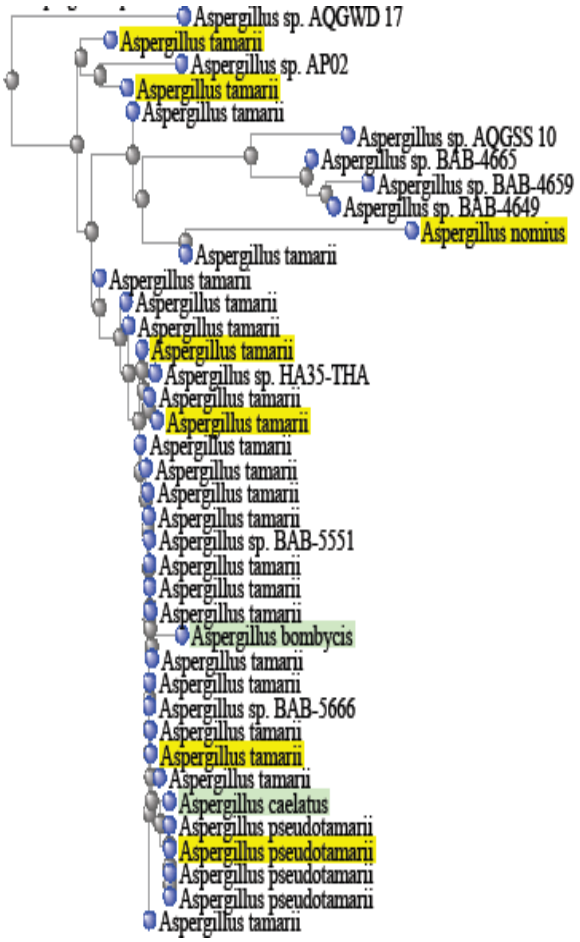

Figure 2. Phylogenetic tree of *Aspergillus tamarii* constructed from aligned combined DNA sequences of the ITS gene. Sequences obtained during this study are presented in yellow.

3.1. Determination of Some Pathogenic Factors from the Isolates

The seven selected *Aspergillus tamarii* isolates were tested for the presence of two virulence factors, and they are *MEP3* and *SUB1* gene. For the polymerase chain reaction, all the seven isolates showed amplification using keratin, elastin and collagen (*MEP3*) gene with amplification band of 850 bp and fungilisin and subtilisin (*SUB1*) gene primers with amplification band 1250 bp (Figure 2 and 3).

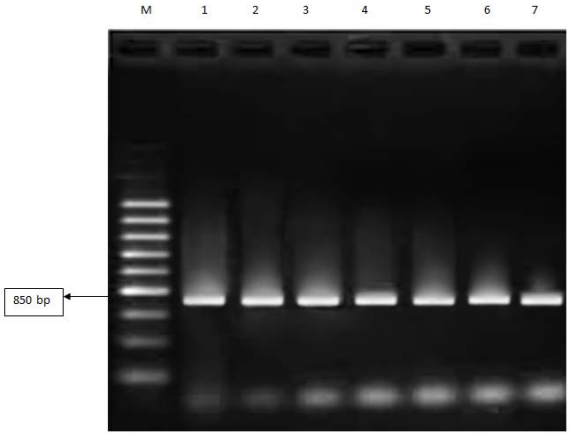

Figure 3. Gel electrophoresis of PCR amplification of *MEP3* gene, Lane M is a 1500bp ladder. Lanes 1 to 7 are *Aspergillus tamarii* isolated from human skin

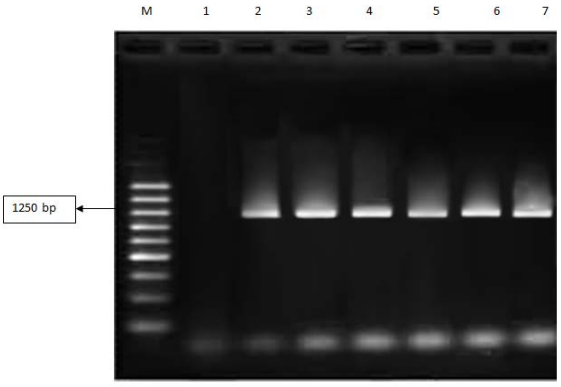

Figure 4. Gel electrophoresis of PCR amplification of *SUB1* gene. Lane M is a 1500 bp ladder. Lanes 1 to 7 are *Aspergillus tamarii* isolated from human skin

4. Discussion

Out of the twenty three samples collected only ten were identified as *Aspergillus tamarii* based on their growth characteristics, pigment formation, and gross colony morphology identification features. Further molecular identification, PCR showed that only seven out of the isolates were *Aspergillus tamarii*, one of each was *Aspergillus nomius*, *Aspergillus aculeatus* and *Pichia kudriavzeuii*. This further proved the specificity and reliability of molecular identification over the conventional methods of fungi identification (Tomee and Kauffman, 2000). The infection caused by the organisms maybe as a result of low immunity of the children or malnutrition. Constant contact with the soil, the reservoir of *Aspergillus* spp. may also increase the chances for the infection by the

organisms among children (Casadevall, 2007; Ganaie *et al.*, 2010).

The presence of the fungalysin (*MEP3* gene) and subtilisin (*SUB1* gene) in *Aspergillus tamarii* is an indication of the degree of pathogenicity of the organism as reported by Tomee and Kauffman (2000). All the isolated *Aspergillus tamarii* harbored *MEP3* gene, and six out of the seven strains screened has the *SUB1* gene. These putative factors have already been characterized and shown to be responsible for the elastinolytic, keratinolytic and collaginolytic activities of the fungus (Tomee and Kauffman, 2000; Brouta *et al.*, 2001; Baldo *et al.*, 2010). Keratin protects the epithelial cells from damage or stress (Gupta and Ramnani, 2006), while collagen assists the connective or fibrous tissues (Chen *et al.*, 2009). The elastase production by the organism assists it to degrade the connective tissue that assists the tissues in the body to snap back to their original shape after being stretched or contracted (Almine *et al.*, 2010).

The *MEP3* genes codes for keratinolytic activity which will allow the fungus to degrade one of the most important constituents of the skin, nails and hair. *Aspergillus tamarii* have been reported to infect the eyelids and are responsible for onychomycosis (Lise *et al.*, 2004). *Aspergillus tamarii* have been isolated from the skin for the first time in Ekiti State of Nigeria. The presence of *MEP* and *SUB* genes in *A. tamarii* indicated that it is a dermatophytes to watch out for in the clinical practice.

References

Almine JF, Bax DV, Mithieux SM, Nivison-Smith L, Rnjak J, Waterhouse A, Wise SG and Weiss AS. 2010. Elastin-based materials. *Chem Soc Rev*, **39:** 3371-3379.

Alp S, Arikan S. 2008. Investigation of extracellular elastase, acid proteinase and phospholipase activities as putative virulence factors in clinical isolates of *Aspergillus* species. *J Basic Microbiol.* **48:** 331–337.

Baldo A, Tabart J, Vermout S, Marty A, Collard A, Losson B and Mignon B. 2010. Secreted subtilisins of *Microsporum canis* are aspergillosis in a leukemic child. *Arch Dermatol*. **114:** 78–80.

Brock M. 2009. Fungal metabolism in host niches. *Cur Opin Microbiol*. **12**: 371-376.

Brouta F, Descamps F, Fett T, Losson B, Gerday C and Mignon B. 2001. Purification and characterization of a 43.5 kDa keratinolytic metalloprotease from *Microsporum canis*. *Med Mycol*. **39**:269-275.

Brouta F, Descamps F, Monod M, Vermout S, Losson B and Mignon B. 2002. Secreted metalloprotease gene family of *Microsporum canis*. *Infect Immun*. 70: 5676-5683.

Casadevall A. 2007. Determinants of virulence in the pathogenic fungi. *Fungal Biol Rev* **21**: 130–132

Chakrabarti A. 2005. Microbiology of systemic fungal infection. *J Postgradrad Med*. **51(Suppl1)**: S16–S20

Chen Z, Shin MH, Moon YJ, Lee SR, Kim YK, Seo JE, Kim JE, Kim KH and Chung JH. 2009. Modulation of elastin exon 26A mRNA and protein expression in human skin *in vivo*. *Exper Dermatol.* **18**: 378-386.

Ganaie MA, Sood S, Rizvi G and Khan TA. 2010. Isolation and identification of keratinophilic fungi from different soil samples in Jhansi city (India). *Plant Pathol J*. **9:** 194-197.

Granstein RD, First LR and Sober AJ. 2005. Primary cutaneous aspergillosis in a premature neonate. *British J Dermatol.* **103:** 681–684.

Grossman ME, Fithian EC, Behrens C, Bissinger J, Fracaro M and Neu HC. 2005. Primary cutaneous aspergillosis in six leukemic children. *J Amer Acad Dermatol*. **12:** 313–318.

Gupta M, Weinberger B. and Whitley-Williams PN. 2001. Cutaneous aspergillosis in a neonate. *The Pediatr Infect Dis J*. **15:** 464–465.

Gupta R and Ramnani P. 2006. Microbial keratinase and their prospective applications. *Appl Microbiol Biotechnol*. **70 (1)**: 21-33.

Hogan LH, Klein BS and Levitz SM. 1996. Virulence factors of medically important fungi. *Clin Microbiol Rev.* **9**: 469–488.

John PU and Shadomy HJ. 1987. Deep fungal infections. In: Fitzpatrick TB, Eisen AZ, Wolff K. (Ed) , **Dermatology in General Medicine.** 3rd Ed. New York: McGraw-Hill; p. 2266-8.

Jousson O, Lechenne B, Bontems O, Capoccia S, Mignon B, Barblan J and Quadroni M. 2004. Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. *Microbiol*. **150**: 301-310.

Klein BS and Tebbets B. 2007. Dimorphism and virulence in fungi. *Curr Opin Microbiol*. **10:** 314–319

Latge JP and Calderone R. 2002 Host–microbe interactions: fungi Invasive human fungal opportunistic infections. *Curr Opin Microbiol.* 5: 355–358

Lise K, Jorgen S and Aksel O. 2004. Onychomycosis due to *Aspergillus tamarii* in a 3-year-old Boy. Department of Clinical Microbiology, Herning Sygehus, DK-7400 Herning and The Skin Clinic, Herning, Denmark.

Martin GS, Mnnino DM, Eaton S and Moss M. 2003. The epidemiology of sepsis in the United States from 1979 through 2000. *The New Engl J Med.* **348:** 546–1554.

Moutaouakil M, Monod M, Prévost MC, Bouchara JP, Paris S and Latge JP. 1993. Identification of the 33-kDa alkaline protease of *Aspergillus fumigatus in vitro* and *in vivo*. *J Med Microbiol*. **39**: 393–399.

Mustafa MM and McCracken GH (1992). Perinatal bacterial diseases. *In*: Feigin RD, Cherry JD, eds. **Textbook of Pediatric Infectious Diseases**. 3rd edition Philadelphia: Saunders: 891-924.

Raper BK and Fennell DI. 1965. **The Genus *Aspergillus***, The Williams and Wilkins, Baltimore, Md, USA.

Tarabees R, Sabry M and Abdeen E. 2015. Incidence of fungalysins virulence genes (*MEP*1-5) in dermatophytes isolated form infected cases in Egypt. *Assiut Vet Med J.* **61**: (144): 56-64.

Tomee JF and Kauffman HF. 2000. Putative virulence factors of *Aspergillus fumigatus. Clin Exper Aller*. **30:** 476–484

Walmsley S, Devi S, King S, Schneider R, Richardson S and Ford-Jones L. 1993. Invasive *Aspergillus* infections in a pediatric hospital: a ten-year review. *The Pediatr Infect Dis J*. **12**: 673-82.