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Experimental Pathogenicity and Molecular Characterization of an Environmental Isolate of *Chrysosporium zonatum* Al-Musallam and Tan (Family: Onygonaceae, Order: Onygonales)

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ABSTRACT

A total of 15 keratinophilic fungi including 8 *Chrysosporium* species were isolated from the rural environment of Ismailia, Egypt, using the hair bait technique. Mainly, *C. zonatum* was isolated from soils collected from fields, animal cages, besides cows and buffaloes hairs and was identified macroscopically, microscopically and using the molecular technique by determining the DNA sequences of ITS1-5.8S-ITS2 region sequences. Its pathogenic potentiality was studied using white mice as an experimental animal model. *C. zonatum* proved potentially pathogenic to infected mice causing symptoms of severe respiratory distress ending with death. It was recovered from lungs of all infected animals. Most of the infected mice showed distinct histopathological changes in their lungs, mainly granuloma formation. They showed the presence of fungal spores in necrotic lung tissue, while the presence of fungal spores and slight necrosis of liver and kidney tissues was occasionally evident.

Key Words: *Chrysosporium zonatum;* ITS1-5.8S-ITS2; Pathogenicity; Keratinophilic fungi

INTRODUCTION

Members of the genus *Chrysosporium* are ubiquitous saprophytes and are predominantly recovered from soil, decaying wood, animal pastures and chicken yards using the conventional hair baiting technique. Many of them are keratinolytic involved in the breakdown of shed keratinized residues. (Carmichael, 1962; Pandey *et al*., 1990; Moubasher *et al*., 1990; Abdel-Hafez & El-Sharouny, 1990; Vissienon, 1999). Many of the *Chrysosporium* species are encountered in the diagnostic mycology laboratory as contaminants of cutaneous and respiratory specimens. The pathogenicity of the *Chrysosporium* species to humans and animals is still uncertain. However, their conidia were able to remain viable for weeks in organs of experimentally infected animals (Abdullah & Hassan, 1995). There are only few reports of human deep infection caused by *Chrysosporium*. Osteomylitis is an immunocompetent host (Stillwell *et al*., 1984), endorcarditis is a prosthetic aortic valve (Toshniwal *et al*., 1986), Sinusitis (Levy *et al*., 1991), disseminated infections invading brain, lungs, sinuses, livers and kidney is a bone marrow recipient (Warwick *et al*., 1992; Morrison *et al*., 1993). However, most of these reports were difficult to evaluate and have been related to the genus level only (Roilides *et al*., 1999).

Chrysosporium zonatum is poorly known thermo-tolerant keratinolytic species, having a wide distribution. It was first isolated in Kuwait using the hair bait technique from horse dung (Al-Musallam & Tan, 1989). Subsequently it was recovered from India, Southern Europe, Italy, Greece, United States, Japan and Middle East (Sigler *et al*., 1998). Mating test had proven that *C. zonatum* is the anamorph of the heterothallic ascomycete, *Unicinocarpus orissi* (Synonym: *Psecudoarchniotus orissi*) family Onygonaceae (Sigler *et al*., 1998).

Recently, *C. zonatum* was isolated from the sputum and affected bone of a patient with chronic granulomatous disease who developed a lobar pneumonia and tibia osteomyelitis (Roilides *et al*., 1999). This investigation was conducted with the aim of studying the prevalence of *C. zonatum* in the highly populated rural areas of Ismailia, Egypt and assessing its pathogenicity using white mice as an experimental animal model.

MATERIALS AND METHODS

Collection of samples and isolation of keratinolytic fungi. Soil samples were collected from animal cages and bird enclosures (usually found inside or in close connection with farmer houses) and fields especially on the border of small water streams. Feathers were collected from domestic birds including chicken, ducks, geese and pigeons. Hairs were separately colleted from cows, buffaloes, goats and donkeys in clean sterilized plastic bags, transferred to the laboratory. Samples were prepared by sieving soil samples from straw and cutting bird feathers and animals hair into suitable

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pieces and processed for isolation of keratinophilic fungi using the hair bait technique as follow:

Soil samples. Three plates (oven sterilized) were half filled with soil samples, pieces of child hair (steam sterilized) was sprinkled over the surface of the soil after being partially moistened with sterilized water.

Bird feathers and animal hairs. Each sample was sprinkled over the surface of three plates half filled with steam sterilized soil for three successive times. All plates were wrapped with papers and incubated at 28°C for a period of two month and examined periodically for the growth of keratinolytic fungi around the sprinkled hairs and feathers. Samples of hairs and feathers showing fungal growth were aseptically picked up and sub-cultured on SDA plates with cycloheximide (0.5 gm L^{-1}), incubated at 28°C till complete fungal growth. The samples were examined microscopically, identified according to their macro- and micromorphological characteristics following the manuals proposed by (Carmichael, 1962; Van Oorschot, 1980; De Hoog & Guarro, 1995).

Molecular Typing Techniques

Extraction of DNA. All fungal strains were grown on Sabouraud's dextrose agar. A small amount of mycelium was suspended in 200 µL of TE buffer (100 mm Tris-Hcl, pH 8.0, 1 mm EDTA) in an Eppendorf tube (1.5 mL). DNA extraction was carried out according to the procedure described by Sandhu *et al*. (1995). A 250 µL of GPT reagent (6 m guanidine thiocyanate dissolved in 50 mM Tris [pH 8.3]) and 700 µL of phenol-buffered in Tris (pH8.0) were added to a washed fungal inoculum in a screw-cap tube and boiled for 15 min. A 250 µL of chloroform-isoamyl alcohol was added and the aqueous phase was separated by centrifugation at 14,000 × g, mixed with an equal amount of 100% isopropanol and 1/10 volume of 3 M ammonium acetate and placed at 20°C for 1 h. Samples were centrifuged at 14,000 x g for 20 min and the pellet was washed with ice-cold 70% ethanol, dried and re-suspended in sterile TE-buffer at a concentration of 5 µg m^{-1}.

Oligonucleotides. The oligonucleotide primers used for amplification and sequencing of the ITS regions were those described by White *et al*. (1990). ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) were purchased from Pharmacia Biotech CO., LTD (Tokyo, Japan).

PCR and DNA sequencing of ITS1-5.8S-ITS2 region rRNA of fungal strains. Amplification reactions were performed in 25 µL of distilled water containing 2.5 µL of each primer (20 pm), 2.5 µL of genomic DNA (5 µg mL^{-1}) and one PCR bead. PCR was performed using the initial denaturation at 94°C for 4 min, followed by 35 cycles at 94°C for 2 min, 55°C for 2 min and 72°C for 2 min and a final extension at 72°C for 10 min. The PCR reaction products sequenced directly using a big Dye terminator reagent kit including *Taq* polymerase according to the manufacturer protocols (Model 310 automated DNA sequencer, Perkin-Elmer/Applied Biosystems, Japan).

Phylogenetic analysis. DNA sequences were aligned with Clustal W (Version 1.83) (Thompson *et al*., 1994) and the alignment was visually corrected. The phylogenetic tree was constructed by the neighbor-joining method that was applied to DNA distance matrices calculated according to Kimura two-parameter model (Saito & Nei, 1987). The confidence values of branches were determined by a bootstrap analysis (Felsenstein, 1981).

Enzymatic activity of the tested fungi. *C. zonatum* and *C. indicum* (as the most common and a control species for comparison) were inoculated onto agar plates of specialized media, incubated at 28°C for 2 to 4 weeks as follow:

(a) Keratinase. SDA slants enriched with keratin powder as a sole nitrogen source and monitored for fungal growth, (Singh, 1997).

(b) Gelatinase. Beef extract, peptone & gelatin medium, gelatin hydrolysis was indicated by failure of immediate solidification in a refrigerator (Hazen *et al*., 1970).

(c) Urease. A 0.5 mL of 2% filter sterilized urea solution was added to SDA medium with phenol red indicator. The development of deep pink color confirmed the urease production (Difco Manual, 1984).

(d) Protease. Filter sterilized bovine serum albumen (BSA) was added to a basal medium (agar 4.5%, yeast carbon base 2.1%) at a final concentration of 0.08%, then bromophenol blue was added. The development of clarification zone confirmed the protease production (Jil-Dostal *et al.*, 2003).

(e) Lipase. One ml of sterilized Tween 20 was added per 100 mL of medium)containing 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2$ & agar 20 gm L^{-1}). Precipitate formation around colonies indicated lipase production (Sierra, 1957).

Growth at 28 and 37°C. Two sets of SDA slants were inoculated with both the fungi, incubated at 28 and 37°C, respectively for 2 weeks to determine their temperature tolerance.

Animal inoculation studies. *C. zonatum* and *C. indicum* (as a control species for comparison) were subcultured on SDA plates with chloramphenicol till profuse growth. Then, the spore suspension was prepared for each fungus by scrabing fungal growth onto screw cap tube containing 10 mL of sterilized phosphate buffered saline, the concentration was adjusted to approximately 106 spores mL^{-1} using haemocytometer.

Three groups of white albino mice (7 mice each) weighing 25-30 g each were used. The first group was intraperitoneally injected with 0.5 mL of C. *zonatum* spore suspension. The second group was injected with that of *C. indicum* and the third was injected with 0.5 mL of sterilized phosphate buffered saline and kept as control. All mice were kept in their cages with continuous supply of food and water for 8 weeks, monitored for the development of clinical symptoms and mortalities. Died or severely ill mice were sacrificed by cervical dislocation, dissected, their lungs, livers, kidneys were excised. Some parts of these organs were subcultured on SDA with chloramphenical. Other parts were fixed in 10% formal saline routinely processed

for histopathological sections, stained with Hematoxylin and Eosin and Gomari methane amine silver stain (GMS), examined and evaluated.

RESULTS

A total of 15 keratinophilic species belonging to eight genera, out of which eight *Chrysosporium* specie were isolated from the rural environment of Ismailia. *C. indicum* was the most prevalent species, where it was recovered from all collected samples. *C. zonatum* was recovered from agricultural soils collected either directly from fields or after being periodically transferred to animal cages and from hairs collected from abdomen and tails of cows and buffaloes (Table I).

Macro- and micro-morphology of C. *zonatum.* Colonies were growing on potato dextrose agar (PDA) at 28°C within 7 days (with slightly faster growth at 37°C). They were flat powdery, initially white to yellow, turning buff with un-colored reverse. Conidia were club shaped borne at the end of short slightly curved conidiophores or occasionally sessile.

Molecular typing. *C. zonatum* strain yielded a unique PCR amplification. The sequence of ITS1-5.8S-ITS2 rDNA region were 516 bp. NCBI GenBank was assessed to identify the isolated species by using obtained ITS data. The ITS data of the isolated strain of *C. zonatum* were identical to the ITS data of *C. zonatum* (Gen Bank Accession Number AJ390393). Pairwise alignment data showed more than 99% identities (Fig. 1), which confirmed the identification of the *C. zonatum* strain.

Animal Inoculation Model

Clinical symptoms and mortality. Mice infected with *C. zonatum* showed lack of appetite and weight loss, being sluggish in their cages with symptoms of severe respiratory distress ending with death. Starting from the day 7, all mice were dead by the day 27. Mice infected with *C. indicum* were quiet in their cages at the first day of infection, they returned their activity on the 2nd day, and remained clinically normal till the end of the experiment.

Fungal recovery from internal organs. *C. zonatum* was recovered from lungs of all infected animals and from livers and kidneys of only two mice, while *C. indicum* was cleared completely and did not recover from any of these organs.

Fungal proliferation and related histopathologcial changes. Mice infected with *C. zonatum* showed distinct histopathological changes in their lungs, mainly granuloma formation and presence of fungal spores in necrotic lung tissue in most of the infected mice. The presence of fungal spores and slight necrosis of liver and kidney tissues was found in two mice only. Mice infected with *C. indicum* showed no histopathological changes in their lungs, liver and kidneys, and were similar to the control group (Fig. 2).

DISCUSSION

Keratinophilic fungi display potentially pathogenic characters for human beings and animals. Thus they could be considered as bioindicators of environmental pollution with human and animal feaces, and pose a risk of infectious diseases arising from such contamination (Hunter, 1997). Therefore, the environmental study of these fungi is of hygienic and epidemiological importance especially in highly populated areas, where keratin remnants and animal manures are continuously delivered (Ulfig, 2000; (Filipello-Marchisio *et al*., 1991; Soon, 1991; Kornillowicz, 1993). Hair is a nutrient for this group of fungi and the hair bait technique is the more efficient qualitative method for their detection (Ajello, 1956).

Pathogenicity is defined as an expression of two component system, the host-parasite relationship, which emphasizes the host as an environment for the fungus. Therefore, the fungus should have the capacity to use the host environment as growth medium and to overcome the defense mechanism of the host (Odds, 2002). The fungal infectious particles are firstly attacked by peritoneal macrophages resulting in engulfing, ingestion and efficient killing, followed by action of peripheral blood macrophages if disseminated and finally, pulmonary macrophages. These defenses may be counterbalanced by fungal virulence factors including enzyme production. Conidia, which escape and overcome early host defense, may germinate (Kappe *et*

Table I. Frequency of occurrence of keratinophilic species isolated from rural areas in Ismailia governorate

	Soil			Animal hairs			Bird Feather		
	F.	A.C	B.E	C & B	D	G	Ch.	D	Pig.
Chrysosporium indicum (Randhawa & Sandhu) Garg	+	+	+	+	+	+	+	+	+
C. zonatum Al- Musallam & Tan	+	+	-	+	-	-	-	-	-
C. tropicum Carmichael	+	+	-	+	-	-	-	-	-
C. keratinophilum De Frey ex Carmichael	+	+	+	+	-	-	+	-	-
C. xerophilum Pitt	+	-	-	-	-	-	-	-	-
C. merdarium (Link ex Grev) Carmichael	+	+	-	+	-	-	-	-	-
C. pseudomerdarium van Oorschot	+	+	+	+	-	-	-	-	-
C. queenslandicum Apinis & Rees	+	+	-	-	-	-	+	+	-
Acremonium falciforme (Carrion) W. Gams	+	+	-	-	-	-	-	-	-
Scopulariopsis brevicaulis (Sacc.) Bain	+	+	+	-	-	-	-	-	-
Geotrichum candidum (Diddens & lodder) V. Arx	+	+	-	-	-	-	-	-	-
Myceliophthora vellerea (Sacc & Speg) van Oorschot	+	+	-	-	-	-	-	-	-
Cephalosporuim acremonium corda	+	+	-	-	-	-	-	-	-
Paecilomyces lilicans (Thom) Samson	+	+	+	-	-	-	-	-	-
Aphanoascus terreus (Randhawa & Sandhu) Apinis	+	-	-	-	-	-	-	-	-

F: fields, A.C: animal cages, B.E: bird enclosures, C&B: cows & buffaloes, D: donkeys, G: goats,Ch: chicken, D: ducks, Pig: pigeons

Table II. Temperature tolerance and enzymatic activity of *C. zonatum* and *C. indicum*

	Growth at		Enzymatic activity				
	28 °C	37 °C	Keratinase	Gelatinase	Urease	Protease	Lipase
C. zonatum	+	+	+	+	+	+	+
C. indicum	+	-	+	+	-	+	-

Fig. 1. Interspecific alignments of the 5.8S rDNA and the flanking internal transcribed spacers (ITS1 & ITS2) of *Chrysosporium zonatum* AJ390393 and isolated strain

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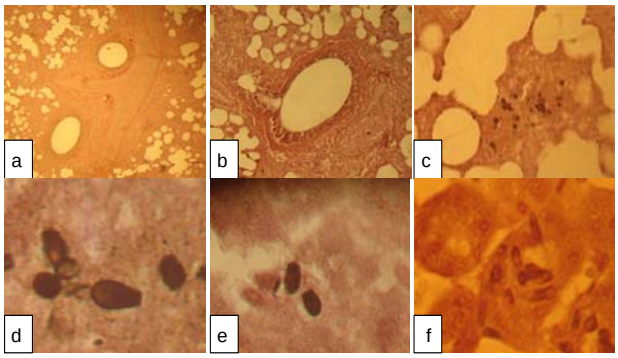
C.zonatum      ATGTGTTTCGGAGC
Isolated strain ATGTGTTTCGGAGC
**************
C.zonatum
CTGGCACACCCATCTCTACTTGGGATGGGTGTGGCAGCGCCCCCACCGTGTTT
ACTGAA
Isolated strain
CTGGCACACCCTCTCTACTTGGGATGGGTGTGGCAGCGCCCCCACACGTGTTT
ACTGAA
********************** ********************************
C.zonatum
CTTTGTTGCCTTGGTAGGCCTGCCGTTCTGGCTGCCAGGGGCGCCTAACCGTG
CCCTCGG
Isolated strain
CTTTGTTGCCTTGGTAGGCCTGCCGTTCTGGCTGCCAGGGGCGCCTAACCGTG
CCCTCGG
************************************************************
C.zonatum
CGTGTGCCTACCAGTGGAGCATTTGAACTCAAGAGAAGATTGAATGTCTGAG
CATTTATG
Isolated strain
CGTGTGCCTACCAGTGGAGCATTTGAACTCAAGAGAAGATTGAATGTCTGAG
CATTTATG
************************************************************
C.zonatum
CAAATTATACAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG
AACGCAG
Isolated strain
CAAATTATACAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAG
AACGCAG
************************************************************
C.zonatum
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTT
TGAACGCA
Isolated strain
CGAAATGCGATAAGTAATGTGAATTGCAGAATTCCGTGAATCATCGAATCTT
TGAACGCA
************************************************************
C.zonatum
CATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTGCAAA
CCCCTCA
Isolated strain
CATTGCGCCCTCTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTGCAAA
CCCCTC
ACCCTCA
```

al., 1992). There is evidence that pathogenic fungi secrete various lytic enzymes, such as proteases and lipases, which may play part in invading and parasitizing the host tissue. These enzymes help fungi in a number of ways; they enhance survival in tissues by chemically or physically altering the immediate environment or act directly by digesting host proteins, lipids, thus providing a source of energy (Ogawa *et al*., 1992). Variations in enzymatic potential of a fungus may be responsible for differences in the pathogenic effect of various strains, however, it must be realized that the production of these enzymes *in vitro* is not necessarily parallel with its ability to degrade host tissues *in vivo*, as a great variety of fungi could secret various enzymes *in vitro* with no concrete evidence of their pathogenic role (Odds, 2002).

It should also be noted that, the capacity of

Fig. 2. Histopathological sections of mice infected with *C. zonatum* (a) granuloma formation in lung tissue (b) enlarged granuloma in lung tissue (c) conidia in necrotic lung tissue (d, e) necrotic liver tissue with conidia (f) conidia in necrotic kidney tissue



microorganisms to produce lytic enzymes is usually estimated under controlled conditions of laboratory cultures. It is therefore quiet likely that some strains may be able to produce varying amounts of enzymes under growth conditions available inside the host tissues, while others lacking such pathogenic behavior, might be due to other factors such as its inability to tolerate the high temperature of the host. Therefore, the ability to grow at or near temperature of the host is an important factor in the pathogenicity of a microorganism (Hogan *et al*., 1996).

Presumably, *C. zonatum* has ability to interfere with the defense mechanism of the host-immune system, the ability of *in vivo* thermoadaptation in the host, secreting digesting enzyme *in vivo* providing essential nutrient and subsequent proliferation in these tissues. Contrarily, *C. indicum* lacks such mechanism of host-environment adaptation and being completely killed and cleared off by the immune system. The failure of peritoneal macrophages to eliminate *C. zonatum* conidia, leading to its dissemination from peritoneal cavity of the infected mice and proliferating mostly in their lungs, with particular dissemination to other organs (liver & kidney), coincides and confirms previous reports concerning its recovery from specimens of clinical cases. *C. zonatum* has been recovered from percutaneous aspirate and bronchoalveolar lavage fluid of a Greek patient with lung chronic granulomatous disease, supposing that it was responsible for the disease and been acquired through the exposure to air borne conidia during the patient's outdoor activities in his countryside farm (Roilides *et al*., 1999). Also, it was isolated from respiratory specimens of two Japanese patients with suspected prior tuberculosis, which did not respond to bacterial antibiotics. One of the patients died and the other recovered following amphotercin B. therapy (Hayashi *et al*., 2002). Our findings along with previous clinical reports might constitute a clear integrated picture aiding in studying and understanding the pathogenic role of this fungus, also, suggesting its respiratory tropism.

Most of moulds, primary pathogens or opportunistic, occur in nature as soil saprobes from where they are

released into the air loaded with dust particles. Fungal infections noted in susceptible hosts are actually acquired from their overloaded surrounding environment either by over inhalation, lodging of active fungal propagules on skin abrasion or accumulation in accidental trauma especially under conditions of illness (Mishra *et al*., 1992; Weitzman & Summerbel, 1995). Accordingly, farmers inhabiting such highly populated rural areas are permanently exposed to this fungus (especially those with various underlying diseases affecting their immune system defense mechanism), should be aware to this real threat of pulmonary infection, which might be misdiagnosed, subjected to mistaken therapy and resulting in subsequent dramatic complications.

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