Diplomarbeit

Identification and characterization of heat shock protein 60 and 70 of common environmental fungi of medical interest

eingereicht von

Helmut Josef Friedrich Salzer

Mat.Nr.: 0213219

zur Erlangung des akademischen Grades

Doktor der gesamten Heilkunde

(Dr. med. univ.)

an der

Medizinischen Universität Graz

ausgeführt am

Institut für Hygiene, Mikrobiologie und Umweltmedizin

unter der Anleitung von

Priv. Doz. Mag. Dr. Walter BUZINA

Graz, am 31.05.2008 Unterschrift:

Eidesstattliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst habe, andere als die angegebenen Quellen nicht verwendet habe und die den benutzten Quellen wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

Graz, am 13.06.2008 *Unterschrift:*

Acknowledgment

This diploma thesis describes a study which proceeded at the Institute of Hygiene, Microbiology and Environmental Medicine at the Medical University of Graz. I am grateful for all the support I have received during the whole last year working in the laboratory at the Department of Mycology and writing up this diploma thesis. The scientific work with the superior objective to obtain entirely new knowledge which may help for a better understanding in the field of medicine is a completely new approach of thinking for a medical student.

First of all I want to thank my supervising tutor Walter Buzina for giving me that golden opportunity to work in such an awesome team as I have found it at the Department of Mycology. As the head of the department he understands to originate a fantastic working atmosphere and so he inspired me to engage myself with the interesting topic of fungi related diseases.
In the same intense way I would like to thank Reinhard B. Raggam who introduced me to the precisely procedure of laboratory work and for the personal and amicable scientific sessions.
It was also a big pleasure to work with Bettina Heiling and I`m really thankful for her great patience.
Furthermore I want to thank the whole team of the Department of Mycology, their competence and support is part of the success of this diploma thesis.

In particular I want to take the chance to thank my incredible family for supporting me the whole six years in any imaginable way. THANKS a lot!

Index

1. Abbreviations

ABPA	Allergic bronchopulmonary aspergillosis
AFS	Allergic fungal sinusitis
AMV	Avian Myeloblastosis Virus
BLAST	Basic local alignment search tool
$CdCl_2$	Cadmiumchloride
cDNA	Complementary desoxyribonucleic acid
CRS	Chronic rhinosinusitis
CT	Computed tomography
DNA	Desoxyribonucleic acid
EDN	Eosinophil-derived neurotoxin
EFRS	Eosinophilic fungal rhinosinusitis
ER	Endoplasmic reticulum
HSP/ hsp	Heat shock protein
kDa	Kilodalton
MGPs	Magnetic Glass Particles
NCBI	National Center for Biotechnology Information
NHIS	National Health Interview Survey
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
Sp	Stress protein

2. Zusammenfassung

HINTERGRUND: Hitzeschockproteine (Hsps) oder Chaperone sind oligomere, hoch konservierte Proteine, welche an den fundamentalen Funktionen der Zellphysiologie beteiligt sind und konstitutiv als auch auf Antwort auf zahlreiche Stresssituationen exprimiert werden.

METHODE: Um Hsp60 und Hsp70 in neun unterschiedlichen allergenen Pilzen nachzuweisen, zu charakterisieren und eine mögliche hitzeinduzierte Antwort zu provozieren, wurden sie kultiviert und unter verschieden Temperaturbedingungen inkubiert. Vollautomatische RNA Isolation und anschließende cDNA Synthese waren gefolgt von quantitativer real time RT-PCR und abschließender Sequenzierung. Die pilzspezifischen Amplifikationsprodukte wurden via Schmelzkurvenanalyse und Gelelektrophorese verifiziert. Für die Auswertung der Relativen Quantifizierung wurde die vergleichende SYBR Green/Δ C_t Methode gewählt und mittels der C_t Werte konnte gegen das β-Tubulin, welches konstitutiv exprimierte wird, normalisiert werden.

ERGEBNISSE: Sowohl Hsp60 als auch Hsp70 wurden einheitlich in allen untersuchten Pilzen identifiziert. Eine temperaturabhängige Expression von Hsp60 mRNA war bei *A. fumigatus, A. terreus, P. chrysogenum, T. mentagrophytes* und *S. apiospermum* induzierbar, während bei *C. cladosporioides* kein signifikanter Hitzeschock beobachtet wurde. Eine durch erhöhte Temperaturgradienten induzierte Expressionssteigerung wurde bei *A. fumigatus, A. terreus, C. cladosporioides* und *T. mentagrophytes* festgestellt, indessen zeigte *P. chrysogenum* und *S. apiospermum* keine signifikante Stressantwort. A. fumigatus zeigte als einziger Pilz in dieser Studie eine 2,1-fache Zunahme der Hsp70 mRNA Expression auf eine Exposition mit 8 µM $CdCl_2$ bei 25°C. Die Sequenzierung der generierten cDNA ergab im Vergleich mit bereits publizierten pilzlichen Hsp60 und Hsp70 eine hohe Gleichartigkeit.

SCHLUSSFOLGERUNG: Mit dieser Studie konnte gezeigt werden, dass unterschiedliche Temperaturgradienten zu einer gesteigerten Expression von Hsp60 und Hsp70, welche ubiquitär in den untersuchten Pilzen nachgewiesen wurden, führen.

3. Abstract

INTRODUCTION: Heat shock proteins (Hsps) or chaperones are oligomeric proteins which fulfill fundamental functions for the cellular homeostasis and are constitutively and stress induced expressed. They may adopt an important role in inflammatory and immunomodulating processes and hence play an essential role in immunogenic fungi related diseases.

METHODS: To identify and characterize Hsp60 mRNA and Hsp70 mRNA, nine diverse immunogenic fungi were initially cultured and incubated at different temperatures to induce a possible heat-shock response. Fully automated RNA extraction and cDNA synthesis was followed by quantitative real time RT-PCR and sequencing of the amplification product. The specific primers were designed to perform a LightCycler© protocol for Hsp60 and Hsp70 of the entire fungi with two tailored cycling conditions. Fungi specific real time RT-PCR amplification products were verified using melting curve analysis. For the relative quantification of specific hsp60/hsp70 mRNA expression, the comparative SYBR Green/Δ C_t method was used and normalized against the housekeeping gene β–tubulin.

RESULTS: Both, Hsp60 and Hsp70 were consistently detected in all analyzed fungi. Temperature dependent expression of Hsp60 was inducible in *A. fumigatus, A. terreus, P. chrysogenum, T. mentagrophytes* and *S. apiospermum*, wherein *C. cladosporioides* no significant heat-shock response was observed. A temperature dependent increase of Hsp70 expression was found in *A. fumigatus, A. terreus, C. cladosporioides* and *T. mentagrophytes,* but *P. chrysogenum* and *S. apiospermum* showed no stress induced response. In the present study, *A. fumigatus* was the only fungus that exhibited a 2.1 fold up regulation of the Hsp70 expression when incubated with cadmium. Sequencing of the obtained cDNA showed a high similarity in comparison to published sequences of other fungal Hsp60 and Hsp70.

CONCLUSION: The enhancement of fungi specific Hsp60 mRNA and Hsp70 mRNA duo to different temperature gradients revealed a strong stress induced response in common environmental fungi. Hence, the strong difference in expression levels in comparison to the diverse temperature gradients and the heavy metal exposure of $CdCl_2$ shows the ubiquitous importance of the heat shock proteins in this context.

4. Introduction

“That which drug fails to cure, the scalpel can cure. That which the scalpel fails to cure, heat can cure. If the heat cannot cure, it must be determined to be incurable.”

Hippocrates

4.1 Heat shock proteins

In 1962 Ritossa Ferruccio discovered a novel puffing pattern in the polytene chromosomes of *Drosophila busckii* and *Drosophilia melanogaster*. After the incidentally increase of temperature he observed that hyperthermia at the cell level is reflected by the pattern of active gene loci in the larval salivary gland chromosomes. The puffing patterns encoded for a unique nature of proteins and due to the heat response it has commonly been entitled as “heat shock proteins” (hsp`s). In addition, the puffs were also detectable after exposure to chemical stressors (Ritossa 1962; Tissieres 1974). Further investigations showed that the heat dependent puffing pattern appeared within minutes after increasing temperature (Berendes 1965; Ashburner 1970).

Since the first observations of the stress inducible puffs by Ritossa and the subsequent confirmation of the gene products by Tissieres et al., hsp`s have been observed in all studied organisms from prokaryotic to eukaryotic species so far (Matthews 1998, Gunther and Walter 1994). Heat shock proteins are oligomeric proteins and due to their high similarity of the nucleotide sequences is it assumed that they are evolutionary highly conserved proteins. They are found in numerous intracellular compartments, including the nucleus, endoplasmic reticulum (ER), cytoplasm and mitochondria. But they are not only restricted to the intracellular region, thus mammalian molecular chaperones have also been verified on the surface of cells, have been secreted into the extracellular milieu and have been found in extracellular fluids like blood, synovial fluid or bronchoalveolar secretions.

While incipiently identified as an up-regulated response to thermal stress, heat shock proteins are triggered by reams of diverse endogenous and exogenous stressors. Those stressors can be biotic or abiotic factors and lead to an increase in hsp synthesis. Those demanding situations include environmental, pathological and physiological stimuli like radiation, heavy metal, different toxins like ethanol and arsenic, increased temperature, hypoxia, starvation, infections, malignancies, growth factors or cell differentiation (Lindquist 1988, Jäättelä 1999, Pfister 2005). For that reason the term stress proteins (sp) and stress response may be more representative.

The classification of the stress proteins is based on their molecular weight which ranges from about 7 to 110 kDa (e.g. -60, -70 kDa Hsps) and their related function (e.g. chaperones; proteins with catalytic activity et cetera). Regarding to the Cold Spring Harbor Meeting 1996 the family name of the heat shock proteins is written with capitals, for example HSP60. Members of a family are commonly marked as hsp60 (Hightower 1996).

HSPs aren´t only a response to various conditions of stress, constitutively expressed, they fulfill an essential function in the physiological cellular homoeostasis. Due to the fact that heat shock proteins exist without increasing their expression as a response to stress we differentiate the molecular chaperones from the stress induced heat shock proteins. Chaperones assure the intracellular transport and the proper folding of newly synthesized proteins, which prevent misfolding and aggregation of proteins in the cell. They recognize and interact with native and denatured proteins and help these to refold to their active state or dispose them to lysosomes for degradation (Welch 1992, Welch 1993, Hartl 1996, Hartl and Hayer-Hartl 2002). Hence, ubiquitous heat shock proteins or chaperones play an essential and necessary role for cell survival.

More recently, it has also been shown that hsps`s play an important role in numerous different medical fields like infections, autoimmune diseases, prion diseases or multifactorial indispositions like arthritis or atheriosclerosis (Ranford 2002, Benagiano 2005). Several of diverse proteins which respond to a number of stress situations have been defined so far. The major stress proteins are listed in Table 1.

Table 1

Major eukaryotic stress proteins (Offermanns S. Molecular Pharmacology, Springer).

Family	Members	Prokaryotic homologues	Funktional turnover	Comments
HSP100	Hsp104, Hsp100	C1pA, C1pß	Protein turnover	Have ATPase activity
HSP90	Hsp90, Grp94	C62.5	Maintenance of proteins such as steroid receptors in an inactive form until appropriate	Drosophilia and yeast proteins known as hsp83
HSP70	Grp78, Hsp 70, Hsc70, Hsx70	dnaK	Protein folding and unfolding, assembly of multi- protein complexes	Hsx70 only in primates
HSP60	Hsp60	groEL, Mycobacterial 65kDa antigen	Protein folding and unfolding, organelle translocation	Major antigen of many bacteria and parasites which infect man
HSP56	Hsp56	None	Protein folding, associated with hsp90 and hsp70 in steroid receptor complex	Have peptidyl prolyl isomerase activity, target of immune-suppressive drugs
HSP47	Hsp47	None	Protein folding of collagen and possibly other proteins	Has homology to protease inhibitors
HSP32	Hsp32		Cleaves heme to yield carbon monoxide and the protective anti- oxidant molecule biliverdin	Also known as heme oxygenase- 1
HSP27	Hsp27, Hsp26 etc.	Mycobacterial 18 kDa antigen	Protein folding, actin binding proteins	Very variable in size (12- 40 kDa) and number in different organisms
Ubiquitin	Ubiquitin	None	Protein degradation	Also found conjugated to histone H2A in the nucleus

Several families of Hsp`s have been identified so far. The class of the 70-kDa HSP family is considered as one of the potent immunogenic protein families. Hsp70 has a great impact in the field of medicine and enhances systemic murine candidiasis (Bromuro 1998). The two major members of the family are the stress inducible Hsp70 with a molecular mass of 72 kDa and the constitutively expressed Hsc70 with approximately 73 kDa (Lindquist and Craig 1988). The arrangement of the amino acid sequence of both Hsps exhibits a similarity of 85% (Gunther and Walter 1994). Hsc70 is just slightly inducible, while Hsp70 is significantly inducible to various stressors. The comparison of the sequences of Hsp70s demonstrated a high conservation and they are evident in all major cellular compartments of eukaryotic organisms. The “typical” Hsp70 is found in the cytoplasm, but they are also verified in the ER, the mitochondria and from plastids (Boorstein 1994). The translated Hsp70 consists of 641 amino acids and comprises two-domain structures. A carboxy-terminal domain that binds short regions of polypeptides and a conserved amino-terminal ATPase domain. The HSP 70 family is involved in a variety of processes, including protein folding and assembly of newly synthesized oligomeric protein structures, the transport of protein structures across membranes, the refolding of misfolded proteins to prevent aggregation and the regulated activation of signal of transduction proteins (Bukau and Horwich 1998, Jensen and Johnson 1999, Gehrmann 2003).

Heat shock protein 60 is an essential protein for the cellular homeostasis and hence part of the chaperones. It is typically located within the mitochondrial membrane, but it`s also be found as a cytosolic chaperone. It provides the correct folding of newly synthesized proteins or helps misfolded ones into their properly folded structure. Briefly, a complex of Hsp10 and Hsp60, which was simply entitled as a “giant breathing machine” by Richardson et al. engulfs misfolded proteins and affords the right re-folding before their release (Figure 1) (Richardson 1998).

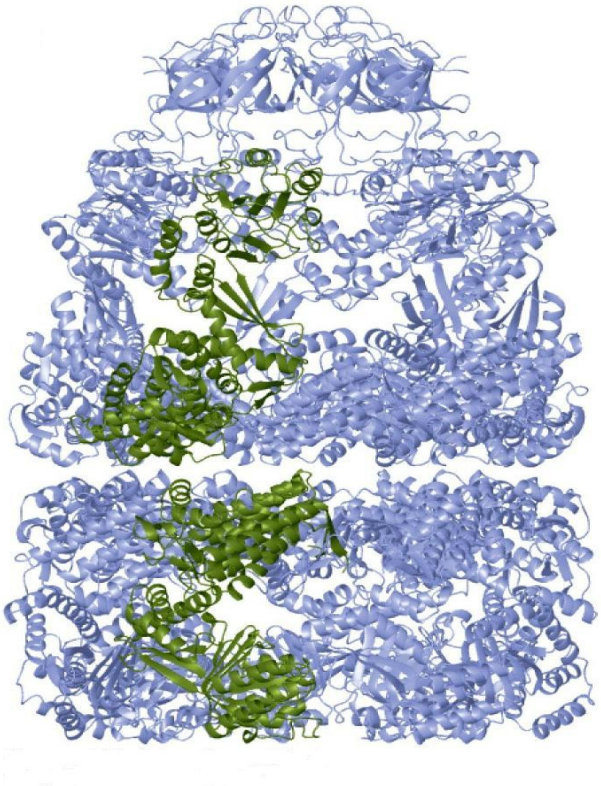


Figure 1: Hsp60/ Hsp10 complex
(sandwalk.blogspot.com/2007_01_28_archive.html)

Experimental models and clinical trials showed that heat shock proteins induce T cell regulation of chronic inflammation, promote production of anti-inflammatory cytokines and prevent inflammatory damage (Hauet-Broere 2006). Arterial endothelial cells express their self 60 kDa Hsp which may become a target for autoreactive T-cells and cross-reactive T-cells to microbial 60 kDa Hsp via mechanisms of molecular mimicry (Benagiano 2005).

A Study by Bromuro et al. showed that 70-kDa recombinant hsp of Candida *albicans* is highly immunogenic and enhances systemic murine candidiasis (Bromuro 1998). Fungal Hsp`s get identified as antigens by the human immune system in mycotic infections (Raska 2004). The identification of specific epitopes of different pro- and eukaryotic Hsp60 species showed different binding sites on primary macrophages (Habich 2005). Hsp60 proteins isolated from different pathogenic fungi were successfully used for a protective vaccination in different infection-models. Vaccination trials with recombinant Hsp60 from *Histoplasma capsulatum* induced an immune response in mice that protected against a sub-lethal dose of the fungus (Gomez 1995).

4.2 The Kingdom of Fungi

Fungi have a worldwide distribution and represent a unique entity within living organisms. They are eukaryotic and heterotrophic (Greek: *heterone* = (an)-other; *trophe*= nutrition) organisms that lack chlorophyll. The vast majority of fungi depend on oxygen, just a few are anaerogenic. They depend on external nutrition from organic materials and therefore exist as saprophytes, parasites or symbionts of animals and plants. Nutrition takes place through secretion of enzymes and the absorption of solute.

Table 2

Differences between fungi and bacteria;

Fungi	***Bacteria***
Eukaryotic	Prokaryotic
Nucleus	Nucleoid
More than one chromosome	One pseudo- chromosome
Steroids in plasma membrane	No steroids

Fungi have a wide range of functions. As mycorrhiza they fulfill an indispensable symbiontic relationship with the roots of plants. On the other hand fungi are the only saprophytes, which abolish lignin, a polysaccharide which is integrated in the cell walls of plants (Fackler 2006). We enlist the assistance of fungi for producing and refinement of foods like the yeast species of the genus *Saccharomyces* for fermentation of beer and wine.

In the field of medicine fungi play an important role as causing organisms of infectious diseases. As opportunists they cause a wide range of different symptoms and manifestations in immunocompromised patients and can develop a severe progression leading to death. There are existing different types of fungi that bring severe infections on immunocompetent people like dimorphic fungi. In 1835 Robert Remak discovered the transmissibility of the fungus *Trichophyton schoenleinii.* Generally a disease of the scalp, but it can occur on any region of the skin. It was the first discovered fungal infection and is commonly entitled as favus.

But fungi are not only present as pathogens, they also play an important role for producing drugs and vaccines e.g. antibiotics, statins, cyclosporine or hepatitis-B-surface-antigen (Hof 2005).

Table 3

Differences between fungi, animals and plants;

	Fungi	*Animals*	*Plants*
Cell wall	Chitin, Glucan	---	Cellulose
Steroids	Ergosterol	Cholesterol	Stigmasterol
Energy	Glycogen	Glycogen	Starch
Nutrition	Heterotrophic	Heterotrophic	Autotrophic

4.2.1 Classification

There are a number of recommended classifications for fungi and each of them has it`s certain eligibility (Alexopoulos 1966, Ainsworth 1973, Vanbreuseghem 1978). Fungi can be divided into myxomycota (= slime fungi) and eumycota (= true fungi). The division of eumycetae after Ainsworth has proved to be uncomplicated (Table 4).

Table 4

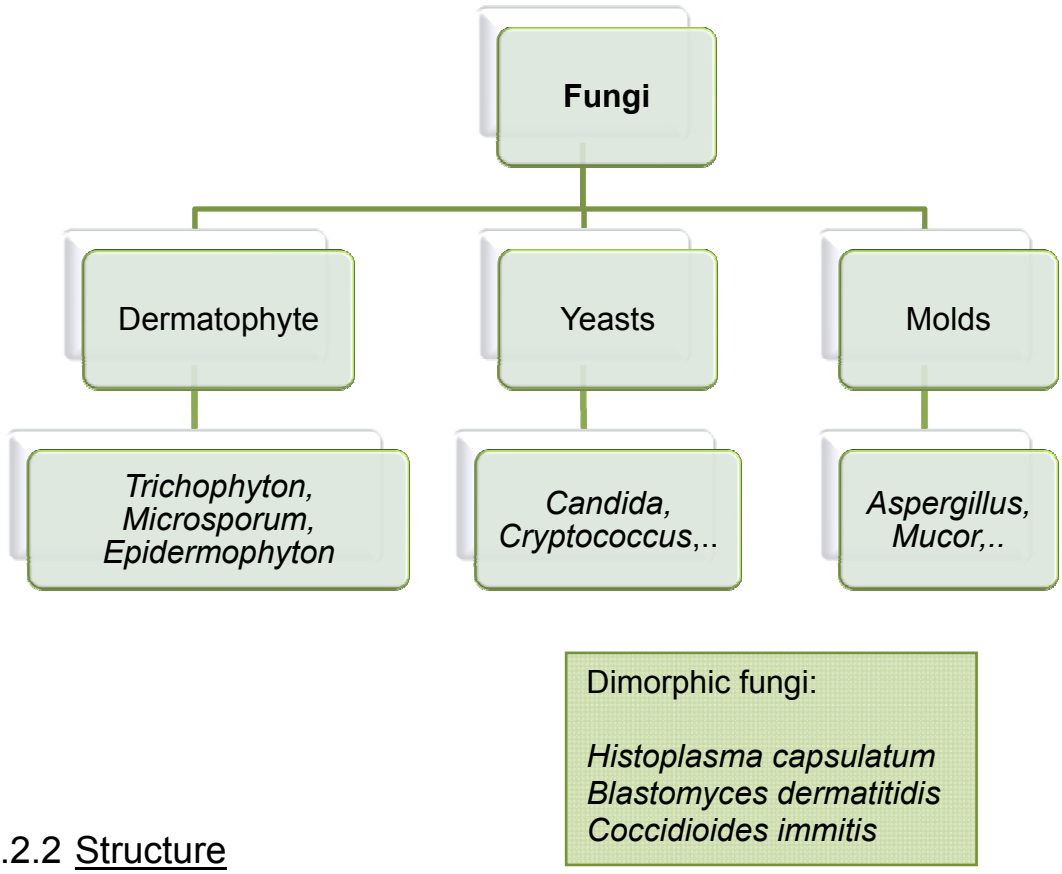
Classification of eumycota (Ainsworth 1973)

Fungi perfecti	**Fungi imperfecti**
Mastigomycotina	
Zygomycotina	
Ascomycotina *	Deuteromycotina *
Basidiomycotina *	

**…… Higher fungi*

The way of reproduction is of vital importance for the classification in the field of botany. Fungi that produce sexual spores are called Fungi perfecti whereas fungi that form asexual spores are entitled as Fungi imperfecti.
In the field of medicine the most common and practicable classification of fungi is the arrangement into three (four) groups (Figure 2).

Figure 2



4.2.2 Structure

The cell wall of fungi consists of a derivative of glucose named chitin, a long chain-polymer of an N-acetylglucosamine, and the polysaccharides glucan and mannan. Depending on the growth fungi can be divided into filamentous fungi (hyphae, mycel) that are multicellular and yeasts (Figure 3).
Most fungi are composed of a mycelium (synonym thallus), a variable dense ball of long, branching cells, which grow filamentous and are called hyphae. Depending on their form of appearance it`s possible to distinguish different types of hyphae.

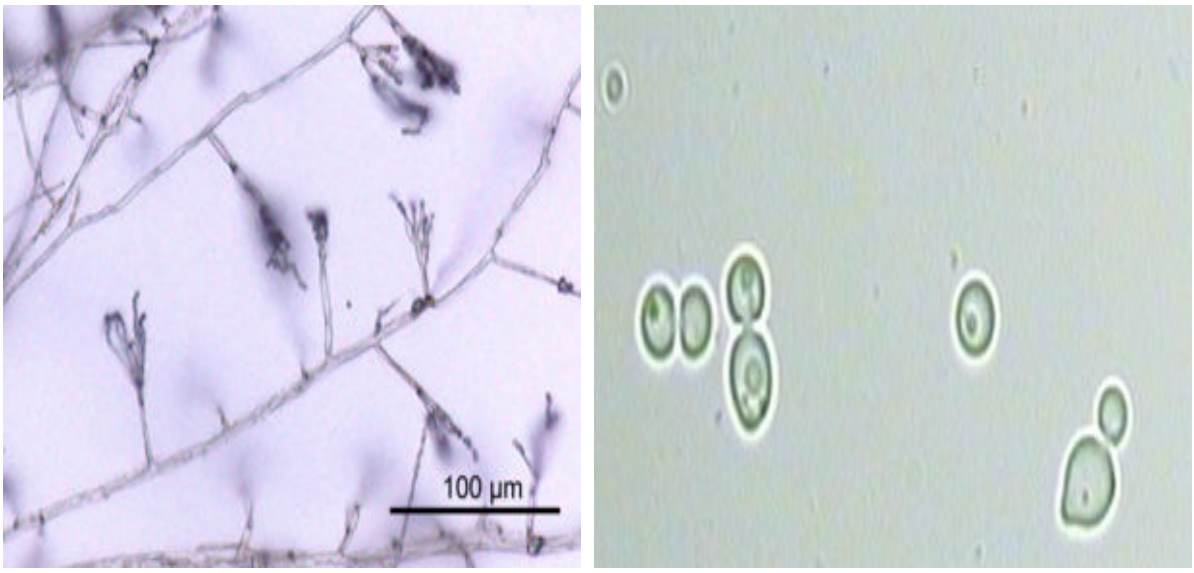


Figure 3: a) Hyphae (*Penicillium spp*.) vs. **b)** yeast (*C. albicans*)

Two major categories are established. Hyphae that are divided into compartments/ cells by septa are called septate. Each cell contains one or more nuclei. Ascomycota, basidiomycota and deuteromycota are not divided by internal septa they get appointed as aseptate. Zygomycota have pseudohyphae which are singular, elongated, connected cells. Hyphae abstract necessary substances from the environment to provide the centrifugal, equably or radial growth of the mycelium (i.e. younger parts in the center, dead fractions peripheral). The fact whether hyphae or yeast cells are formed is important for the classification of fungi. Fungi of such a mycelium type are called eumyzetae and consist of two parts. The vegetative part of the fungus (= mycelium) which is organized by hyphae and the reproduction part that arise from the vegetative part. The reproduction of fungi is very complex and is carried out by asexual and sexual reproduction via spores. Depending on the environment fungi are also able to reproduce both asexual and sexual. Fungi which propagate via vegetative spores are part of the asexual reproduction and it allows a faster spread as the sexual reproduction via meiosis.

Spores are resistant to numerous environmental conditions and are part of the life-cycle of fungi. Fungi have been found to exist under nearly all environmental conditions indoor and outdoor. The measurement of the total airborne fungal spore concentration in office buildings at the United States ranged from < 24 to 1000

spores/ m^3 and did not vary significantly between winter and summer. Buildings with natural ventilation exhibited higher spore concentrations (MacIntosh 2006).

A study by Guine et al. investigated the outdoor environmental levels of *Aspergillus spp.* spores across the province of Madrid (Spain). The air load of *Aspergillus* spores appeared almost constant throughout the year with the highest spore levels in autumn. More *Aspergillus spp.* isolates were found in the urban environment than in the rural environment (P= 0.11). The concentration in the air has been strongly influenced by atmospheric parameters such as temperature, wind speed or humidity (Gunine 2006).

Similar data were shown by Buzina et al. A total of 619 samples of fungal isolates from patients with chronic rhinosinusitis and healthy individuals were collected for a period of 28 month. 81 different fungi could be identified within the nasal mucos. Most prevalent findings contained *Penicillium, Aspergillus, Cladosporium, Alternaria* and *Aurobasidium*. Most fungi were found almost constant throughout the year, only *Alternaria alternate, Cladosporium cladosporioides* und *Aureobasidium pullulans* showed a significant higher occurrence in late summer and early autumn.

4.2.3 Medical interest

There have been more than 100.000 different fungal species found so far. A few hundred perform as opportunists and can cause life threatening events with a high mortality rate in immuncompromised persons as systemic fungal infections (Nucci 2005, Ruhnke 2006). But there are also fungi which are able to induce a severe fungal infection in healthy immunocompetent individuals like the group of dimorphic fungi. The clinical relevance of fungi is the possibility to provoke allergy, intoxications or infections (= mycosis).

4.2.3.1 Intoxications

The cells of a fungus accumulate different secondary metabolites after the growth period. In form of spores the fungus cell is able to store them or to dispense the metabolites. Therefore absorption is possible via ingestion (or aerogen). Because of the toxicity for humans and animals these metabolites are called mycotoxins. This low-molecular substances can cause acute or chronic Intoxications. Some of them have teratogenic, immunosuppressive or carcinogenic effects. The most important mycotoxins are listed in table 5.

Table 5

Toxin	Fungus	Sources	Consequences
Ergotamin	*Claviceps purpurea*	Cereals	Vascular damage
Ethyl alcohol	*Saccharomyces cerevisiae*	Beer, wine	Neurotoxic, hepatotoxic
Aflatoxin B	*Aspergillus flavus*	Nuts	Carcinogenic, immunosuppressive
Ochratoxin	*Aspergillus ochraceus*	Cereals, coffee	Hepatotoxic, nephrotoxic
Trichotecene	*Fusarium spp.*	Corn, wheat	Neurotoxic, teratogenic
Zearalone	*Fusarium spp.*	Corn	Carcinogenic, estrogenic effect, immunotoxic
T2	*Fusarium spp.*	Corn	Immunotoxic
Patulin	*Penicillium spp.*	Fruits	Mutagenic, neurotoxic
Gliotoxin	*Aspergillus spp.*	Corn	Cytotoxic, immunosuppressiv

4.2.3.2 Infections

About 150 species of fungi are able to colonize and/ or infect humans. Pathogen fungi just have mean aggressive virulence factors and often serve as opportunist. Never the less they can cause local or even severe systemic mycosis

in immunocompromised patients with high mortality rates. In contrast to obligate pathogenic fungi which belong in the majority of cases to dimorphic fungi.

4.2.3.3 Immunresponse - Allergy

Numerous fungi that are found under different environmental conditions or even certain edible mushrooms contain antigens which are able to activate the immune system. Immunogenic reactions include a number of different diseases like allergic rhinitis, allergic fungal sinusitis, eosinophilic fungal rhinosinusitis, allergic bronchopulmonary aspergillosis, hypersensitivity pneumonitis, atopic dermatitis and others (Aukrust 1985, Tariq 1996, Vailes 2001). A re-exposure with the antigen can then cause an allergic reaction like asthma bronchiale. Especially the antigens of molds are likely the origin of allergies, maybe more previous than pollen (Pulimood 2007). Because of cross- reaction it`s difficult to identify a define fungus as the proper trigger. In contrast to pollen, fungi provoke not only hypersensitivity reactions of IgE-mediated type I allergy, but also cause type II, III and IV allergies (after Coombs and Gell). Threshold concentrations for triggering allergic symptoms are estimated to be 100 spores/ m^3 for *Alternaria species* and 3000 spores/ m^3 for *Cladosporium species* (Graveson 1979).

Several reports have demonstrated increased acute asthma episodes occurring during thunderstorms and that sensitization to molds is a risk factor for severe and fatal asthma (O`Hollaren 1991, Codina and Lockey 1998, Dales 2003, Guy 2007).

A study by Pulimood et al. showed that an increase of fungal spores as aeroallergens significantly increases the number of asthma admissions in hospitals. During the period of interest for this study, grass pollen levels were decreasing and measurements were far below the peaks seen a few weeks earlier. The results of this case-control study demonstrated that *Alternaria alternata* sensitivity is a compiling predictor of epidemic asthma in patients with seasonal asthma. Morbidity and mortality was significantly higher in those patients. It`s not only an important factor during epidemic thunderstorm-related asthma, but also a more severe phenotype of asthma was reported (Pulimood 2007).

Animal experiments demonstrated an intense immune response to mold spores. Two intraperitoneal injections before intranasal challenge of *Alternaria alternate* and *Cladosporium herbarum* spores into BALB/c mice induced specific IgM and IgG1 antibodies and elevated Immunglobulin E (IgE) serum levels. An increase of Th2 cytokine like IL-4, IL-5 and IL-13 in comparison to nonsensitized mice has been reported. The mice developed an allergic lung inflammation and hyperreactivity (Havaux 2005). Further investigations showed that also a chronic intranasal administration of mold spores to unsensitized mice led to allergic pulmonary diseases and lung remodeling (Denis 2007).

Eosinophilic granulocytes are important mediators of allergic responses and are implicated in antigen presentation to T-cells (Shi 2004). Environmental fungi are able to induce activation and degranulation of human eosinophils. *Alternaria alternata* and *Penicillium notatum* increased intracellular calcium concentration, expression of CD63 and CD11b and induced the production IL-8 and the exocytosis of eosinophil-derived neurotoxin (EDN) in eosinophils from normal individuals. *A. alternata* didn`t activate neutrophils. Size exclusion chromatography showed that the stimulating agent for degranulation could be a protein with the size of about 60 kDa which was highly heat labile compared with no heat treatment of extract ($p < 0.01$) (Inoue 2005).

4.2.3.4 Fungi of medical interest

Table 6

Candida albicans	Aspergillus fumigatus	Aspergillus terreus	Cladosporium cladosporioides
Vaginal candidiasis	Inhalations mycosis → colonization + invasion → allergic reaction	Allergic or invasive bronchopulmonary aspergillosis	Pulmonary infections
Mucocutaneous candidiasis → Osteomyelitis	Systemic aspergillosis	Nosocomial infections	Cutaneous infections
Peritonitis (via catheters, lesions or perforations)	Pulmonary aspergillosis	Cutaneous infections	Keratitis
Shunt infections	Disseminated infections	Ophthalmic infections	Dental granulome
Systemic candidiasis	Traumatic mycosis, wound-contaminations	Pulmonary infections	Phaeohyphomycosis
Pericarditis (via thoracic surgery)	Colonization of non-pulmonary cavities (cave: invasive)	Disseminated infections	Subcutaneous cyst
Prosthetic valve endocarditis	Fungal sinusitis (fungal balls)	Keratitis	Sinusitis?
Empyemas	Cerebral aspergillosis	Arthritis	Mixed disseminated infections
Disseminated infections (in neutropenia, haematologic malignancies, antimicrobial therapy, chemotherapy, abdominal surgery, severe burns)	Nosocomial infections (in immuncompromised patients)	Spondylodiscitis, Otitis	
Candida pneumonia			
Endophthalmitis, Otitis			
Oral candidiasis			

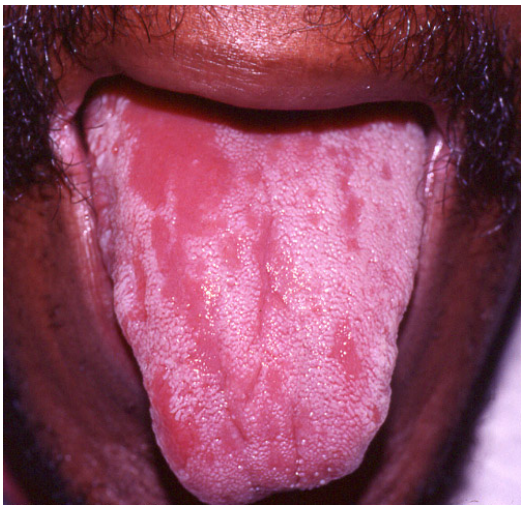


Figure 2a: Oral erythematous (atrophic) candidiasis in a HIV patient. (depts.washington.edu/.../oral/case1/fig5d.html)

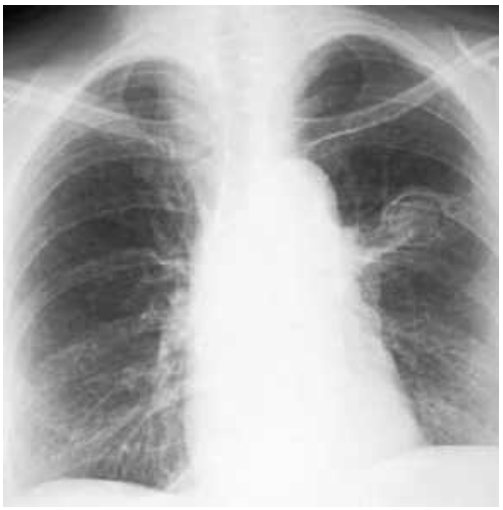


Figure 2b: Pulmonary aspergilloma with a homogeneous round lesion on the left side within a cavity and the typical annular accumulation of air.

Table 7

Scedosporium apiospermum	Saccharomyces cerevisiae	Penicillium chrysogenum	Trichophyton mentagrophytes
Involved in arthritis, otitis	Superficial mycosis	Penicilliosis	Tinea pedis, Tinea cruris
Cutaneous infections	Infections in AIDS patients	Acute dissiminated penicilliosis (in AIDS patinents)	
Ophthalmic infections	Deep fungemia in transplant patients		
Opportunist (in immun↓, leukemic, transplant patients)	Sepsis in leukemic patients		
Disseminated infections, systemic mycosis			
Allergic reactions			
Sinusitis			
Pneumonia			

4.2.3.5 Chronic Rhinosinusitis

Chronic rhinosinusitis (CRS) has a great impact on the medical care policy. The health statistics from the National Health Interview Service (NHIS) for the civilian non-institutionalized adult population in the United States (USA) in 2005 showed a prevalence of 13 % of adults with sinusitis. The percentage of adults with sinusitis was higher in the south than in any other region in the United States. Women were more likely than men to have been told they had sinusitis (Pleis 2005). Hence, it`s not surprising that the economic burden of sinusitis is significant. A study by Ray et al. estimated the health care expenditures attributable to sinusitis in 1996 with $5.8 billion in the United States (Ray 1996). It is assumed that the situation in Europe is similar. Due to the fact that sinusitis mostly involves the nasal cavity and that there are no significant differences with the rhinosinusitis the term rhinosinusitis is preferred. CRS is defined as a chronic inflammatory disease of the mucosa in the nasal cavity and the paranasal sinuses which lasts more than three month with persistent symptoms and is associated with the development of morphological variations of the mucosa like a chronic inflammatory swelling and the formation of polyps as an end stage (Kaliner 1997, Lanza and Kennedy 1997). The diagnosis of chronic rhinosinusitis is still based on the symptoms and signs of the patient. The conformation with objective tests like computed tomography (CT) or nasal endoscopy remains controversial. The etiopathology of this common chronic disease is still insufficiently established. CRS seems to be a complex and multifactorial illness. To enable the clinical diagnosis of CRS Lanza and Kennedy divided the symptoms and signs into two categories (major and minor symptoms). For the clinical diagnosis the patient has to suffer from two or more major symptoms or the combination of a major and two minor symptoms (Table 8) (Lanza and Kennedy 1997).

Table 8

Major and minor symptoms for the clinical diagnosis of chronic rhinosinusitis of adults (Lanza and Kennedy).

Major symptom	*Minor symptom*
Facial pain/ pressure pain	Headache
Feeling of pressure	Fever
Constricted nasal breathing	Halitosis
Running nose/ postnasal drip (purulent, colored)	Faintness
Hyposmia/ anosmia	Cough
Purulent secretion during examination	Toothache
Fever (just with acute exacerbation)	Earache/ feeling of pressure

Already in 1983 Katzenstein et al. described a newly recognized form of chronic sinusitis. The clinical and pathological features of seven cases showed that most patients were young adults with a history of asthma, chronic nasal polyps, a positive surgical anamneses and the recurrent sinusitis was common. Resected mucus from the sinuses presented histological findings of eosinophils, Charcot-Leyden-crystals and *Aspergillus* hyphae. Because of the preference of the sinuses, the histological findings in the mucous and the analogy with the allergic bronchopulmonary aspergillosis (ABPA) they termed it allergic aspergillus sinusitis (Katzenstein 1983). A few years later a case report by Robinson et al. demonstrated that also other fungi have to be considered as pathogens (Robinson 1989). From there on it was termed allergic fungal sinusitis (AFS). In 1994 Bent and Kuhn proposed five criteria for the diagnosis of AFS which also included the aspect of an atopy (Typ-1-hypersensitivity) against fungi. The conclusion was based on the anamneses, positive skin- and serological tests of 16 patients (Bent and Kuhn 1994). After intensive computer-based literature research (99 reviewed case reports from 1978-1993) and their own findings of investigation DeShazo and Swain confuted the aspect of the atopy and established new diagnostic criteria for AFS without atopy (Table 9). The analyses of the case reports also showed the findings of numerous different species of fungi isolated from patients with

previously reported AFS and that young adults are more commonly affected at the time of diagnosis (DeShazo and Swain 1995).

Table 9

Criteria for the diagnosis of AFS in the study of DeShazo and Swain.
1. Sinusitis of one or more paranasal sinus on x-ray film.
2. Identification of allergic mucin by rhinoscopy or at the time of sinus surgery or subsequently on histopathologic evaluation of material from the sinus.
3. Demonstration of fungal elements in nasal discharge or in material obtained at the time of surgery by stain or culture.
4. Absence of diabetes, previous or subsequent immunodeficiency disease, and treatment with immunosuppressive drugs.
5. Absence of invasive fungal disease at the time of diagnosis or subsequently.'

In 1999 Ponikau et al. determined the incidence of AFS in patients with chronic rhinosinusitis, reevaluated the current criteria for diagnosing AFS and described novel methods for collecting and culturing fungi from nasal mucus. The results of this prospective study showed a great success of the new methods for a proper evidence of fungi from nasal mucus. Fungal cultures of nasal secretion were positive in 202 (96%) of 210 consecutive CRS patients. Based on the histopathologic findings and culture results AFS was diagnosed in 93% of 101 consecutive surgical cases with CRS. The clinical and histopathologic criteria of AFS were detectable in 93% of the patients, but the majority of AFS patients showed no immunoglobulin E-mediated hypersensitivity to fungal allergens. In accordance with the findings the research group advocated the novel term “eosionophilic fungal rhinosinusitis” (EFRS) (Ponikau 1999). This substantial change for collecting and culturing fungi from nasal mucus were also verified in an open prospective study by Braun et al. at the university hospital in Graz, Austria. With the introduction of the novel methods for fungal detection in culture and histology as proposed by Mayo Clinic researchers the data in our hospital altered essential. The positive detection of fungal cultures of the mucus of CRS patients developed from 7% in the past up to 87% at the present. The histological findings in 37 surgical CRS patients showed in 94,6 % eosinophilic clusters and in 75,5%

were fungal elements detected within the mucus. Overall 89,2% of the surgically treated CRS patients confirmed the criteria for the diagnosis of AFS postulated by DeShazo and Swain. In the collective of 238 CRS patients and 23 healthy test persons 88 different genera grew. The healthy control group yielded in 91,3% a positive fungal culture and this demonstrated that positive fungal cultures from nasal secretion has to be considered as normal findings (Braun 2003).

5. Materials and methods

With this project we had the intention to obtain new scientific knowledge about the heat shock proteins 60 and 70 of common environmental fungi to make a contribution to a better understanding.

The investigations contained:

1. Relative quantification of hsp60 and 70 specific mRNA expression of selected human pathogenic fungi and its dependency of different cultivation temperatures and exposure to cadmiumchloride ($CdCl_2$) by real-time RT-PCR.
2. Sequencing of the real-time RT-PCR amplification products and comparison of the nucleotide sequences.
3. Translation of the nucleotide sequences into corresponding amino acid sequences and a homology analyses (Internet Database) (Data in progress).

In context of the preliminary work of Buzina et al. concerning the characterization and temperature-dependent quantification of heat shock protein 60 of the immunogenic fungus *Alternaria alternata* and the growing medical interest regarding stress proteins, the stress response in immunogenic fungi were analysed (Buzina 2008).

5.1. Cultivation of nine immunogenic fungi, incubation under different temperatures and co-stimulation with 8 µM cadmiumchloride (CdCl2) concentration

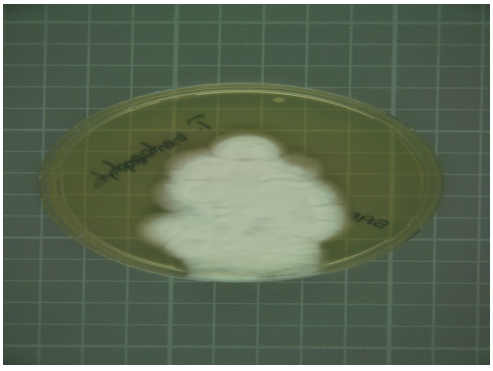
A strain of each appropriate fungus was cultured on two separate Sabouraud agar plates in an incubator set at room temperature. For *Alternaria alternata* we

preferred to use carrot-potato agar as an appropriate medium for enhanced sporulation. The analysis contained molds, yeasts and dermatophyte.

1) *Alternaria alternata*[1] (CBS 109803),
2) *Aspergillus fumigates* (ATCC 204305),
3) *Aspergillus terreus* (NEQAS 262),
4) *Cladosporium cladosporioides* (Clinical isolate, culture collection of Walter Buzina [022.99]),
5) *Penicillium chrysogenum* (environmental isolate, culture collection of Walter Buzina [053.99]),
6) *Scedosporium apiospermum (Pseudallescheria boydii)* (CBS 118233),
7) *Candida albicans*[2] (ATCC 90028),
8) *Saccharomyces cerevisiae*[2] (ATCC 9763),
9) *Trichophyton mentagrophytes* (DSMZ 4870)

Buzina et al. Med Mycol 2008[1].
C. albicans and S. cerevisiae weren`t evaluated at the time of completion of this diploma thesis[2].

After conidiation the plates were flooded with 2 ml Sabouraud bouillon (SAB). The adherent conidia were loosend under the use of an applicator to obtain suspensions. The conidia containing suspensions were transferred into 500 ml Erlenmeyer flasks prefilled with 300 ml liquid SAB medium and incubated on a shaker (100 rpm) for 72 h at room temperature. For the yeasts a suspension with 2 ml natriumchlorid (NaCl) corresponding to a McFarland 1 standard were prepared and continued under the same procedure.



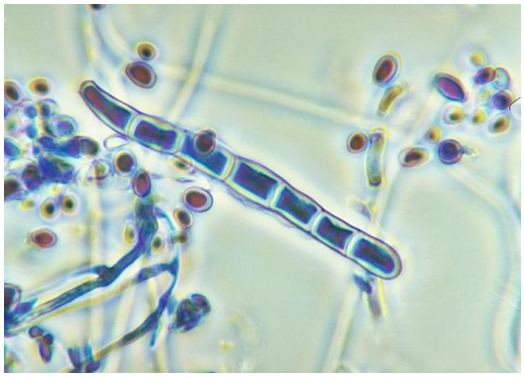


Figure 2.1a: Cultivation of *T. mentagrophytes* on Sabouraud agar plates.
Figure 2.1b: Microscopic view (300 x) of typical conidia of *Trichophyton rubrum* (http://de.wikipedia.org/wiki/Trichophyton).

Depending on the habit, 3 to 4 of the formed fungi balls in SAB or 2 ml of fungus suspension were filled in 2 ml microfuge tubes to provoke a temperature dependent response. The assigned sample tubes were incubated for 3 h at 25°C, 30°C, 35°C and 40°C, respectively each of another assigned sample tube was added with 8 µM $CdCl_2$ at 25°C to simulate a heavy metal exposure. So we investigated 8 different conditions of each fungus. After 3 h of incubation the tubes were centrifuged for 10 min at 13200 rpm. Supernatant was discarded and samples were washed once with isotonic phosphate buffered saline (PBS) and centrifuged for another 10 min at 10000 rpm. PBS was discarded and samples were snap-frozen and stored at -70°C if not processed immediately.

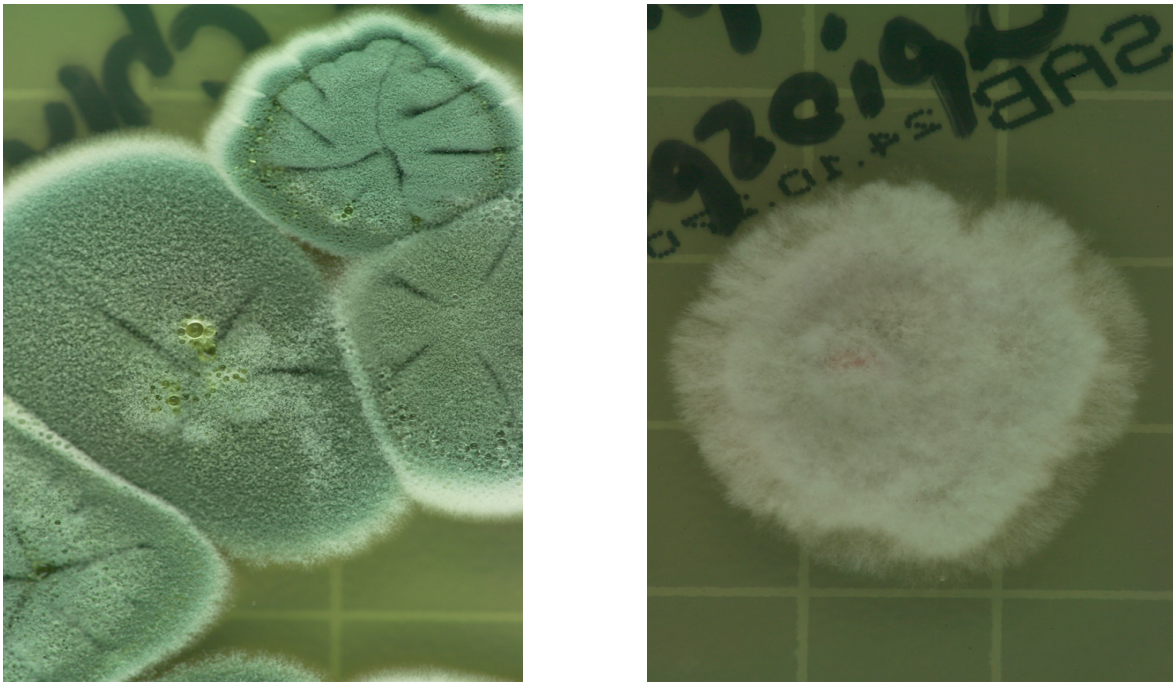


Figure 2.2: a) Cultivation of *Penicillium chrysogenum* and **b)** *Scedosporium apiospermum (Pseudallescheria boydii)* on Sabouraud agar plates.

5.2. Homogenization of the samples via MagNA Lyser

For the homogenization of the solid sample material of the snap-frozen fungi the specimens were transferred into the MagNa Lyser Green Beads Tubes (Roche Applied Science, Mannheim, Germany). The MagNa Lyser Green Beads consists of 2 ml screw tubes prefilled with 1.4 mm ceramic beads. 500 µl of lysis buffer in

each tube which were constantly set on ice for an optimal homogenization were added. According to the manufacturer`s instructions the MagNa Lyser Green Beads filled with fungi specimens and lysis buffer were loaded in the MagNa Lyser Instrument rotor (Roche Applied Science). As specified in the product description the cell disruption process during a run with the MagNa Lyser Instrument is caused by the fast moving, oscillating reciprocal motion of the rotor which leads to the collision of ceramic beads with the sample in the sample tubes. Appropriate for our sample material the disruption parameters (speed, time) were optimized and repeated the cycle up to three times with 6000 rpm for 30 seconds. Between each cycle the tubes were set on ice for two minutes. After the homogenization of the fungi the samples were centrifuged to pellet the cell debris and transferred 350 µl of the lysate supernatant into the 2.0 ml sample tubes for nucleic acid preparations.

Figure 2.2: MagNa Lyser Instrument© (Roche)

5.3. Fully automated RNA extraction on the MagNA Pure Compact Instrument

For the total RNA-isolation the MagNa Pure Compact Instrument was used (Roche Applied Science) together with the MagNa Pure Compact RNA isolation Kit (Roche Applied Science) according to the manufacturer`s instructions. The instrument carries out RNA purification automatically using the “RNA tissue” protocol. 20 µl of the DNase solution was transferred onto the bottom of the sample tube and selected the volume of the elution buffer to 50 µl. The RNA isolation procedure is based on the MagNA Pure Magnetic Glass Particle Technology. As described from Roche Applied Science the sample homogenate is

lysed by incubation with the lysis/ binding buffer containing a chaotropic salt and proteinase K, which destroys remaining proteins including nucleases. Magnetic Glass Particles (MGPs) are added and nucleic acids are immobilized on the MGPs surfaces. Genomic DNA is degraded by incubation with DNase. This substantially reduces the DNA content of the sample. Unbound substances (*e.g.,* proteins, cell debris, PCR inhibitors etc.) are removed by several washing steps. Purified total RNA is eluted from the MGPs.
The obtained RNA was measured and quantified photometrically ($A_{260/280}$ wavelength ratio).

5.4. cDNA synthesis using the 1st strand cDNA synthesis kit for RT-PCR

For the 1st strand synthesis of single-stranded cDNA as the starting reaction for RT-PCR the first strand cDNA synthesis kit for RT-PCR [AMV] were applied (Roche Diagnostics) according to the instruction of the manufacturer. 2 µg of the quantified RNA were evaporated in the SpeedVac system for drying down each RNA sample. Afterwards 16.4 µl sterile water was added for dissolution. After approximately 30 minutes of residence time half of the solution that equates 8.2 µl was transferred into a new tube. The samples were snap-frozen and stored at -70°C as an aliquot.
The residual 8.2 µl RNA sample was used for the cDNA synthesis. Preparation of the master mix contained 2 µl reaction buffer, 4 µl of 25mM MgCl, 2 µl of random primer p(dN)6, 1 µl of Rnase inhibitor and 0,8 µl AMV (=Avian Myeloblastosis Virus) reverse transcriptase for each sample. The master mix was subsequently aliquoted into RNA sample tubes and briefly vortexed. Afterwards the reaction was incubated at 25°C for 10 minutes and then at 42°C for 60 minutes. After the primer annealing to the RNA template and the reverse transcription of the RNA in the template cDNA, the AMV reverse transcriptase was denatured by incubating the reaction mix at 99°C for 5 minutes. After the last step, samples were put on ice for another 5 minutes before they were stored at –20°C if not processed immediately.

5.4.1. Fungus specific primer alignment for hsp60 and hsp70

For this study, the same Hsp60 primers were applied as in the preliminary study of Buzina et al. about the characterization and temperature- dependent quantification of heat shock protein 60 of the immunogenic fungus *Alternaria alternate* (Buzina 2008). The sequences were obtained from the National Center for Biotechnology Information (NCBI) nucleotide GenBank (Tab.1) (http://www.ncbi.nlm.nih.gov./sites/entrez?db =Nucleotide) from the following fungi:

Tabelle 1

Fungi	**GenBank accession numbers**
Ajellomyces capsulatus	L11390
Aspergillus nidulans	XM_658601
Candida albicans	XM_707984
Chaetomium globosum	XM_00122816
Saccharomyces cerevisiae	M33301
Schizosaccharomyces pombe	D50609
Trichophyton mentagrophytes	AF199024

Table 2

Primers for RT-PCR and Sequencing		
Primer	**Sequence**	**Used for**
hsp60_761	5'-GCNGGNTGYAACCCNATGGA-3'	RT-PCR, Sequencing
hsp60_1100	5'-CTCTCCGAGAAGAAGATCTC-3'	Sequencing
hsp60_1406	5'-ACCATYACYAAGGARGACAC-3'	Sequencing
hsp60_1119r	5'-GAGATCTTCTTCTCGGAGAG-3'	Sequencing
hsp60_1425r	5'-GTGTCYTCCTTRGTRATGGT-3'	Sequencing
hsp60_1577r	5'-RTATRACRGCAANACCNCC-3'	RT-PCR, Sequencing

* Degenerated primers were named after the position of S.cerevisiae M33301 hsp60

The sequences were aligned (Tab.2) using ClustalW free software under http://www.ebi.ac.uk/Tools/clustalw/ as described by Kopecek et al. (1999).

After extensive research of the literature Hsp70 primers were aligned out of 6 fungi genome sequences. For Hsp70 the sequences were obtained from NCBI nucleotide GenBank from the following fungi (Tab.3): (http://www.ncbi.nlm.nih.gov./sites/entrez?db=Nucleotide)

Table 3

Fungi	**GenBank accession numbers**
Saccharomyces cerevisiae	X12926
Cladosporium herbarum	X81860
Trichophyton rubrum	AF052391
Aspergillus oryza	AB030231
Candida albicans	X97723
Penicillium marneffei	AY960135

The sequences were aligned using ClustalW free software under http://www.ebi.ac.uk/Tools/clustalw/ as described by Kopecek et al. (1999).
Primer sequences of the housekeeping gene beta-tubulin are listed in Table 4.
Segments with the highest similarity were verified for their suitability as PCR primers using the program Primer Designer 2.0 (Scientific & Educational Software, Durham, USA). The degenerated primers were named after the position of *Saccharomyces cerevisiae* X12926 Hsp70 (Tab.5) and commercially produced (Invitrogen, Germany).

Table 4

Beta-tubulin primers for RT-PCR and Sequencing		
Primer	**Sequence**	**Used for**
bt2a	5'-GGTAACCAAATCGGTGCTGCTTTC-3'	RT-PCR, Sequencing
bt2b	5'-ACCCTCAGTGTAGTGACCCTTGGC-3'	RT-PCR, Sequencing

Table 5

Primers for RT-PCR and Sequencing		
Primer	**Sequence**	**Used for**
Hsp70_646f_sacch	5`-TTGCCAACGAWCAAGGTAAC-3`	RT-PCR + Sequencing
Hsp70_1181r_sacch	5`-GAAACATCGAAAGTACCACCACC-3`	RT-PCR + Sequencing

5.5. Fungus specific hsp60 and hsp70 mRNA real-time RT-PCR

The real-time PCR was performed by the LightCycler 2.0 PCR Instrument (Roche Diagnostics) using fungus specific hsp60/hsp70 primers and the LightCycler SYBR Green DNA Fast Start Kit (Roche Applied Science. Fluorescence of the fluorescent dye SYBR Green was measured in channel 1 that equates 530 nm. The different primers were designed to perform the Hsp60`s and Hsp70`s for all fungi with two tailored cycling programs in order to acquire a reduced processing time and a faster turn-around. A significant amplification product was committed with a fluorescence value beyond of 2. The facility to accomplish a melting curve analysis after expired PCR discriminates unspecific PCR products like the formation of primer dimers.

Figure 2.3a: LightCycler© 2.0 Instrument

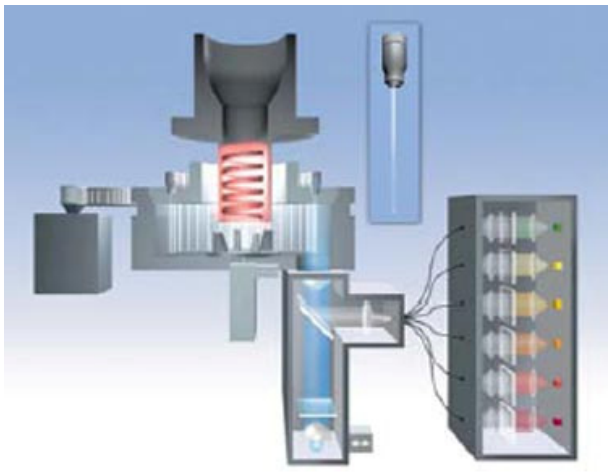


Figure 2.3b: Schematic cross section of the LightCycler© 2.0 Instrument

The PCR reactions contained 18 µl of master mix and 2 µl of template cDNA to yielding in a PCR reaction mix of total 20 µl.

For the hsp60 master mix 13.2 µl H_2O, 2.4 µl $MgCl_2$, 0.2 µl forward primer 50 µM, 0.2 µl reverse primer 50 µM and 2 µl DNA master to receive a master mix with 4 mmol/L $MgCl_2$ were combined. The master mix for hsp70 contained 3 mmol/L $MgCl_2$ (Tab.5).

Table 5

Master Mix hsp60/ 70 light cycler RT-PCR		
	Vol/ sample **hsp60**	Vol/ sample **hsp70**
H_2O	13.2 µl	13.8 µl
$MgCl_2$	2,4 µl	1.8 µl
Primer f 50 µM	0.2 µl	0.2 µl
Primer r 50 µM	0.2 µl	0.2 µl
cDNA Master	2 µl	2 µl
Master Mix	**18 µl**	
cDNA template	2 µl	
PCR Reaction Mix	**20 µl**	

After preparation of the master mix the required number of LightCycler© capillaries were placed in the pre-cooled centrifuge adapter. The master mix was carefully sampled by pipetting up and down, before 18 µl were prefilled into each capillary followed by adding 2 µl of template cDNA. The capillaries were transferred into the sample carousel of the LightCycler© Instrument and centrifuged using the LightCycler© carousel centrifuge.

5.5.1. Hsp60 real-time RT-PCR cycling protocol

Cycling conditions for hsp60 included one cycle of 95°C for 7 minutes followed by 60 cycles consisting of denaturation for 3 seconds at 95°C, annealing for 10 seconds at 62 °C and secondary target temperature at 57°C, and elongation for 30 seconds at 72°C. After the last cycle the capillaries were cooled for 2 seconds at 40°C, before the melting curve was started. To obtain the melting curve, the temperature was slowly raised (0.1°C/ sec) from 40°C to a target temperature of 95°C.

5.5.2. Hsp70 real-time RT-PCR cycling protocol

A different cycling protocol was used for hsp70. Cycling conditions included one cycle of 95°C for 7 minutes followed by 60 cycles consisting of denaturation for 3 seconds at 95°C, annealing for 10 seconds at 65°C secondary target temperature at 60°C, and elongation for 30 seconds at 72°C. As described before, the melting curve program continued with the same settings.
In summery the light cycling RT-PCR procedure of hsp70 differed from hsp60 in a lower concentration of $MgCl_2$ and in higher annealing temperatures.
A negative control was added to every LightCycler© run with the samples, which contained PCR-grade water instead of the template cDNA.

For the relative quantification of the specific gene expression level the comparative SYBR Green/Δ C_t method was used. The calculation of the crossing points (CP) was automatically done by the LC software. The analysis of data generated during amplification and melting curve were carried out using the LC-software 4.1 from Roche Applied Science. The results were normalized using ΔC_t

values obtained from the β-tubulins RNA amplifications products included in the same runs (Guilemette 2004).

5.6. Purification and sequencing of real-time RT-PCR amplification products

20 µl of the real-time RT-PCR products, originated from the amplification reaction were purified by the High Pure PCR Purification Kit from Roche Diagnostics. The High Pure PCR Product Purification method was performed according to the manufacturer`s instructions.

Cycle sequencing was performed by using 1 µl of purified RT-PCR product employing the BigDye 3.1 Kit (Applied Biosystems, Foster City, USA). After reaction the product was purified with an ethanol based extraction, dissolved in 15 µl HiDi and analyzed using the 3130 Automated Capillary Sequencer (Applied Biosystems).

6. Results

The expression of Hsp60 and Hsp70 in cultured fungi containing *A. fumigatus, A. terreus, C. cladosporioides, P. chrysogenum, T. mentagrophytes and S. apiospermum (P. boydii)* were examined by quantitative real time reverse transcriptase polymerase chain reaction (real time RT-PCR) [*C. albicans* and *S. cerevisiae* weren`t evaluated at the time of finishing this diploma thesis]. In all of the investigated fungal specimens both Hsp60 and Hsp70 mRNA were consistently detected. Additionally, specific β-tubulin mRNA was detected in all of the investigated specimens too. The fungus specific real time RT-PCR amplification products (Figure 1) were verified using melting curve analysis (Figure 2) and gel electrophoresis (Figure 3). For relative quantification the fungus specific Hsp60, alternatively Hsp70 and the corresponding β-tubulin incubated at 25°C were paired as target and reference calibrator and set to 1.00. The results of the expression of Hsp60 in *A. alternata* are expressed in the preliminary work of Buzina et al. (Buzina 2008).

Figure 1

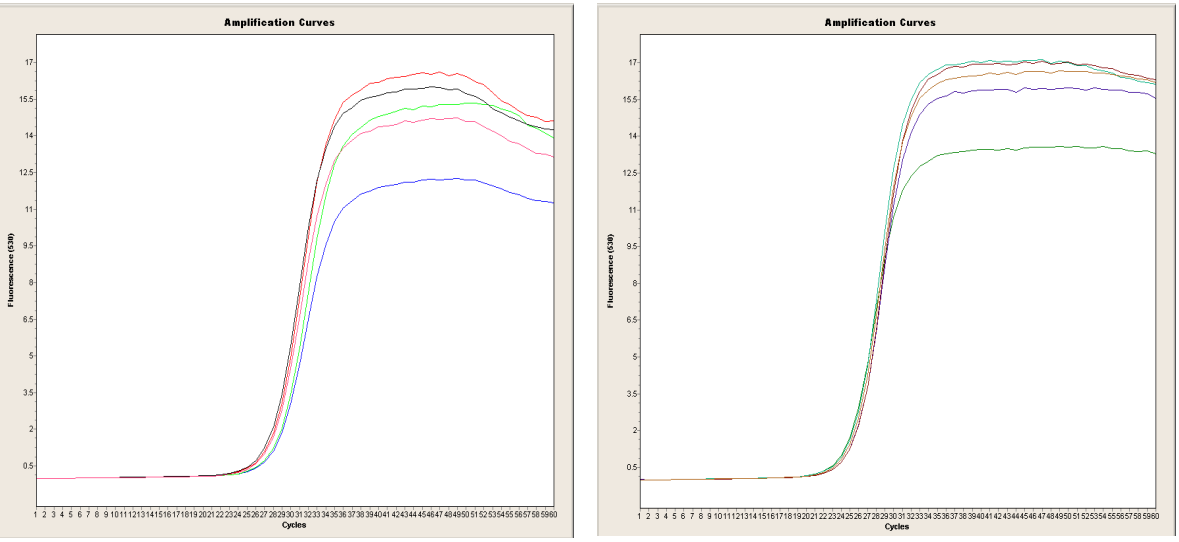


Figure 1: Fluorescence versus cycle number plots of the *A. fumigatus* specimens incubated at different temperatures (25°C, 30°C, 35°C, 40°C) and under incubation with 8 µM $CdCl_2$ showing the five curves of the **a)** hsp60-specific amplification products and the **b)** corresponding β-tubulin specific amplification products.

Figure 2

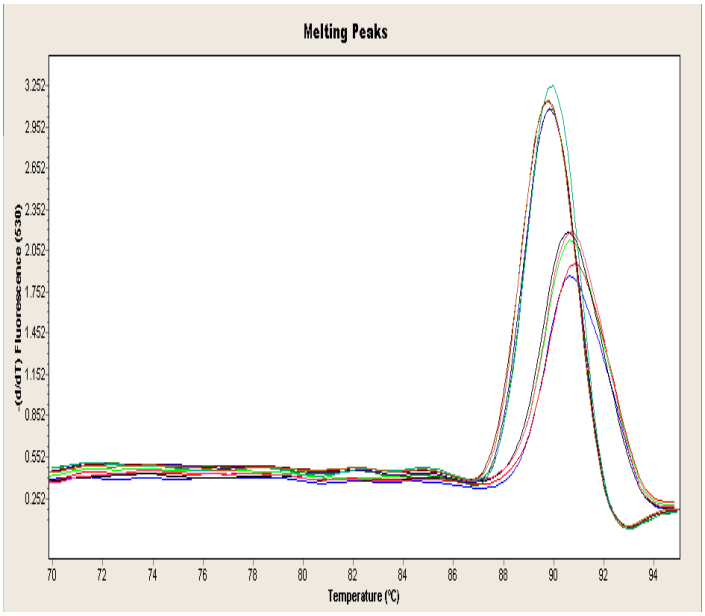


Figure 2: Derivative melting curve of the fungus *Penicillium chrysogenum* of Hsp60 specific- and β-tubulin specific products with the melting temperature (T_m) for each product in real time.

T_m Hsp60 mRNA: 89.7°C
T_m β-tubulin: 90.6°C

Figure 3

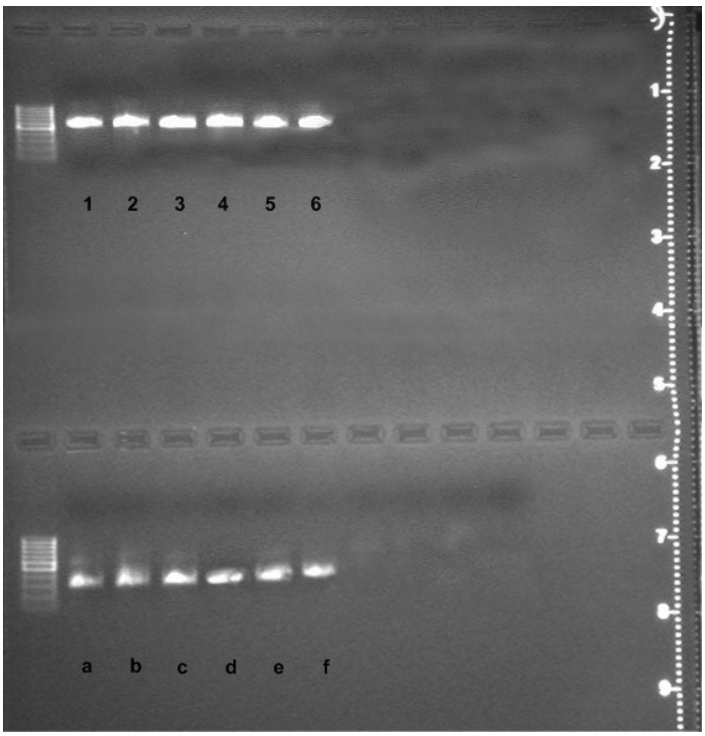


Figure 3: Determination and verification of the specific Hsp60 mRNA and the corresponding specific β-tubulin fragment-length for each fungus using gel electrophoresis.

Hsp60 mRNA	β-tubulin
1) *A. fumigatus*	**a)** *A. fumigatus*
2) *A. terreus*	**b)** *A. terreus*
3) *C. cladosporioides*	**c)** *C. cladosporioides*
4) *P. chrysogenum*	**d)** *P. chrysogenum*
5) *T. mentagrophytes*	**e)** *T. mentagrophytes*
6) *S. apiospermum*	**f)** *S. apiospermum*

A temperature dependent expression of specific Hsp60 was detected in five fungi including *A. fumigatus* (Tab.1), *A. terreus* (Tab.2), *P. chrysogenum* (Tab.4), *T. mentagrophytes* (Tab.5) and *S. apiospermum* (Tab.6) as the normalization against the housekeeping gene β-tubulin has shown. While in *C. cladosporioides* (Tab.3) no significant heat response was observed and the expression of Hsp60 mRNA showed even lower levels than specimens incubated at 25°C (Figure 4). In all of the investigated fungi the expression of Hsp60 mRNA was not inducible by stimulating with 8 µM $CdCl_2$ at 25°C.

A. fumigatus incubated at 35°C and 40°C showed an induction of Hsp60 mRNA in comparison to 25°C. The highest value was observed within an incubation temperature of 35°C with a 1.4 fold up-regulation. After cDNA sequencing the comparison with published sequences of other fungal Hsp60 confirmed a similarity of 99% with *A. fumigatus* Hsp60 (Basic local alignment search tool, BLAST).

A. terreus incubated at 40°C enhanced the Hsp60 expression to a 6.7 fold up-regulation in comparison to 25°C. Stimulation at 35°C resulted in a 2.8 fold up-regulation and showed a similarity score of 98 % with *A. terreus* when sequences were compared with other fungal Hsp60.

A 1.9 fold up-regulation was observed in *P. chrysogenum* incubated at 35°C and sequencing of the obtained cDNA yielded the highest similarity data in comparison to other published sequences within the mitochondrial protein Hsp60 of *Neosartorya fischeri* XM_001260424 (91%) and the putative mitochondrial protein Hsp60 of *Aspergillus clavata* Hsp60 XM_001267849 (90%). In the online data base (BLAST) was no specific *P. chrysogenum* Hsp60 sequence listed so far.

T. mentagrophytes incubated at 40°C showed a 4.4 fold up-regulation of Hsp60 expression in comparison to 25°C and comparison of the obtained cDNA sequences were confirmed with the fungal Hsp60 sequence of *T. mentagrophytes* with a similarity of 99%. A heat dependent response was also observed in *S. apiospermum* incubated at 35°C with a 2.7 fold up-regulation compared to the reference of 25°C. The alignment with published sequences of other fungal Hsp60 revealed the highest similarity with Hsp60 of *Neurospora crassa* XM_951407 (91%) and *A. alternata* Hsp60 EU285274 (90%).

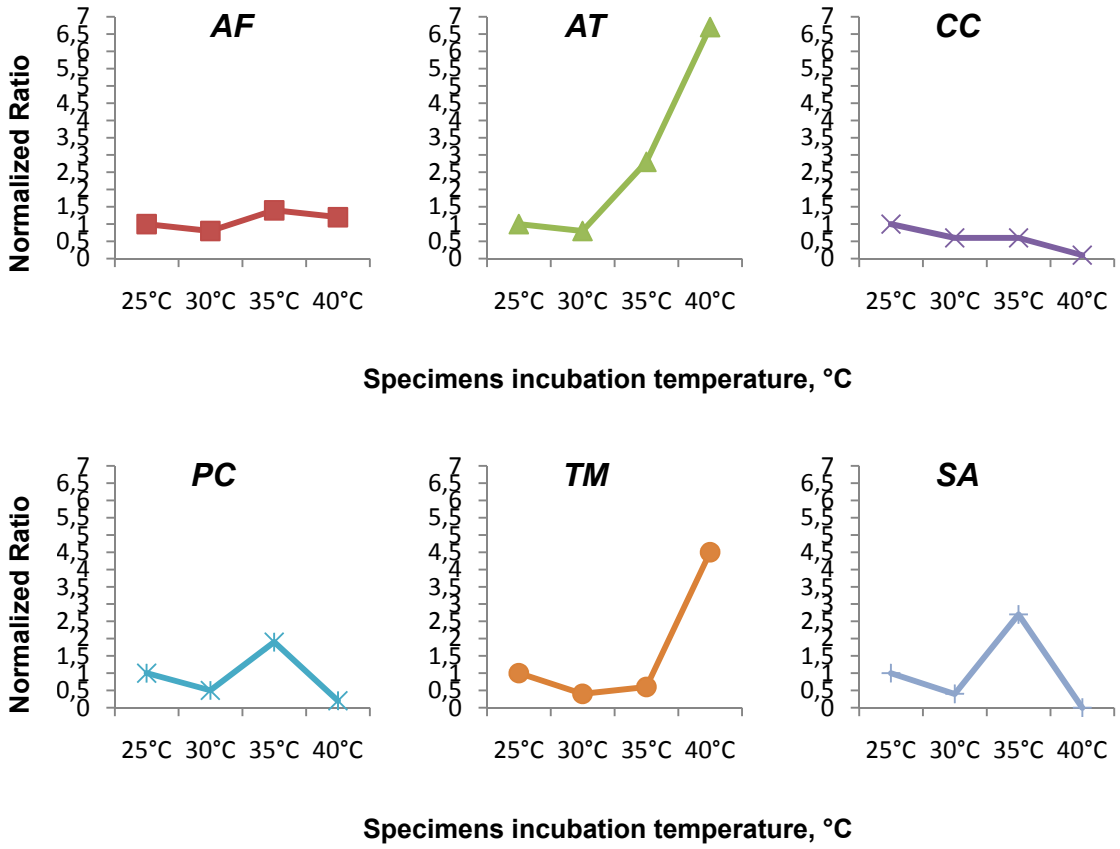


Figure 4: Expression of Hsp60 mRNA of *A.fumigatus* (*AF), A.terreus (AT), C.cladosporioides (CC), P.chrysogenum (PC), T.mentagrophytes (TM),* and *S.apiospermum (SA)* incubated at different temperatures (25°C, 30°C, 35°C, 40°C). For relative quantification Hsp60 and β-tubulin of the appropriate fungal specimens incubated at 25°C was paired as target and reference calibrator.

The expression of Hsp70 mRNA was verified in a temperature dependent fashion for *A. fumigatus* (Tab.8), *A. terreus* (Tab.9), *C. cladosporioides* (Tab.10), *T. mentagrophytes* (Tab.12), when normalized against the housekeeping gene β-tubulin which was detected in all of the six fungi (Figure 5).

P. chrysogenum and *S. apiospermum* exhibited no heat dependent response. The expression levels were even lower in comparison to an incubation temperature of 25°C. The comparison of the 70 kDa Hsp sequence of *P. chrysogenum* with online published sequences of other fungal Hsp70 showed the highest similarity with the Hsp70 of *A. clavatus* XM_001269420 (85%), *Neurospora crassa* U10443 (83%) and *Nicotiana tabacum* AY372069 (83%). The

comparative data for *S. apiospermum* showed the highest similarity with *Hypocrea jecorina* Hsp70 AY281746 (86%) and *Nicotiana tabacum* heat shock protein 70 AY372070 (86%).

A. fumigatus incubated with 8 µM $CdCl_2$ at 25°C was the only specimens that showed an increased expression of Hsp70 mRNA with a 2.2 fold-up regulation in comparison to 25°C without $CdCl_2$ stimulation. All other fungi incubated with 8 µM $CdCl_2$ at 25°C showed no stress induced response to $CdCl_2$ at 25°C.

In *A. fumigatus* all of the observed incubation temperatures showed an induction of the Hsp70 expression level. The consistently enhancement of the expression levels lead to a 2.6 fold up-regulation as the highest induction in *A. fumigatus* incubated at 35°C in comparison to 25°C. The stimulation with higher temperatures showed a decrease in the expression levels, but still a 2.3 fold up-regulation was observed. The cDNA were sequenced and the comparison to published sequences of other fungal Hsp70 showed the highest similarity with *A. fumigatus* Hsp70 XM_744501 (99%) and *Neosartorya fischeri* Hsp70 XM_001260091 (95%). In all *A. terreus* specimens an increase of the Hsp70 expression was observed with the highest induction in *A. terreus* incubated at 40°C with a 12.6 fold up-regulation. The comparison of the sequence data confirmed a similarity with the published sequence of *A. terreus* Hsp70 XM_001209480 with 98%, whereas the similarity with other published fungal Hsp70 sequences like *A. niger, T. rubrum* or *T. verrucosum* were less than 92%.
Also *C. cladosporioides* showed the highest enhancement when incubated at 40°C. The comparison of the 70 kDa Hsp sequences showed a similarity with with the putative Hsp70 of C. herbarum X81860 (92%), Gibberella zeae XM_3810114 (85%) and A. terreus XM_001209480 (84%).
The highest expression level of Hsp70 mRNA was inducible with a 13.4 fold up-regulation in *T. mentagrophytes* incubated at 25°C in comparison to 8 µM $CdCl_2$ at 25°C which showed no stress inducible response.

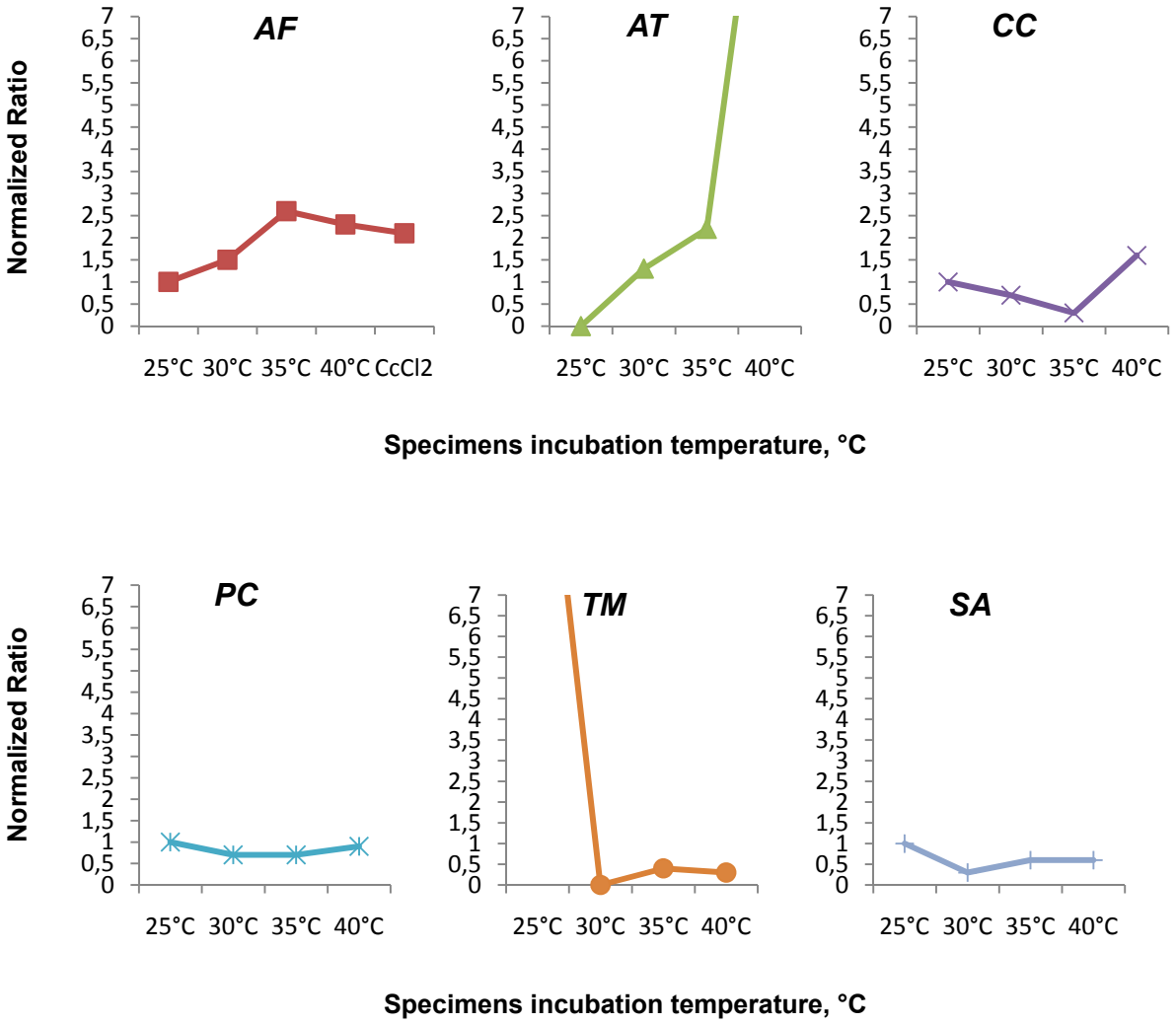


Figure 5: Expression of Hsp70 mRNA of *A.fumigatus (AF), A.terreus (AT), C.cladosporioides (CC), P.chrysogenum (PC), T.mentagrophytes (TM), and S.apiospermum (SA)* incubated at different temperatures (25°C, 30°C, 35°C, 40°C – for *AF* incubation with 8 μM $CdCl_2$ is added). For relative quantification Hsp70 and β-tubulin of the appropriate fungal specimens incubated at 25°C was paired as target and reference calibrator. Under certain LC-software conditions *TM,* incubated at 25°C with $CdCl_2$ was set as target and reference calibrator.

Table 1

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp60* Calibrator	25°C	28.81	0.04	1.00
	Reference β-tubulin Calibrator	25°C	24.02		
Result Set 2	Target *hsp60*	30°C	29.93	0.03	0.78
	Reference β-tubulin	30°C	24.79		
Result Set 3	Target *hsp60*	35°C	29.26	0.05	1.38
	Reference β-tubulin	35°C	24.94		
Result Set 4	Target *hsp60*	40°C	29.06	0.04	1.20
	Reference β-tubulin	40°C	24.54		
Result Set 5	Target *hsp60*	8 μM $CdCl_2$	29.93	0.03	0.71
	Reference β-tubulin	8 μM $CdCl_2$	24.66		

Relative quantification of Aspergillus fumigates *hsp60* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 2

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp60* Calibrator	25°C	28.92	0.02	1.00
	Reference β-tubulin Calibrator	25°C	23.25		
Result Set 2	Target *hsp60*	30°C	29.46	0.02	0.81
	Reference β-tubulin	30°C	23.49		
Result Set 3	Target *hsp60*	35°C	28.33	0.05	2.78
	Reference β-tubulin	35°C	24.14		
Result Set 4	Target *hsp60*	40°C	26.58	0.13	6.71
	Reference β-tubulin	40°C	23.65		
Result Set 5	Target *hsp60*	8 μM $CdCl_2$	30.63	0.01	0.57
	Reference β-tubulin	8 μM $CdCl_2$	24.15		

Relative quantification of Aspergillus terreus *hsp60* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 3

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp60* Calibrator	25°C	27.28	10.13	1.00
	Reference β-tubulin Calibrator	25°C	30.62		
Result Set 2	Target *hsp60*	30°C	28.99	5.81	0.57
	Reference β-tubulin	30°C	31.53		
Result Set 3	Target *hsp60*	35°C	28.69	5.88	0.58
	Reference β-tubulin	35°C	31.24		
Result Set 4	Target *hsp60*	40°C	27.28	0.56	0.06
	Reference β-tubulin	40°C	26.45		
Result Set 5	Target *hsp60*	8 µM $CdCl_2$	29.56	3.74	0.37
	Reference β-tubulin	8 µM $CdCl_2$	31.47		

Relative quantification of Cladosporium cladosporioides *hsp60* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 4

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp60* Calibrator	25°C	23.97	0.13	1.00
	Reference β-tubulin Calibrator	25°C	21.06		
Result Set 2	Target *hsp60*	30°C	26.62	0.07	0.54
	Reference β-tubulin	30°C	22.81		
Result Set 3	Target *hsp60*	35°C	24.67	0.25	1.86
	Reference β-tubulin	35°C	22.64		
Result Set 4	Target *hsp60*	40°C	27.03	0.03	0.19
	Reference β-tubulin	40°C	21.73		
Result Set 5	Target *hsp60*	8 µM $CdCl_2$	26.43	0.08	0.63
	Reference β-tubulin	8 µM $CdCl_2$	22.84		

Relative quantification of Penicillium chrysogenum *hsp60* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 5

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp60* Calibrator	25°C	32.45	1.74E-4	1.00
	Reference β-tubulin Calibrator	25°C	19.96		
Result Set 2	Target *hsp60*	30°C	32.94	6.36E-5	0.36
	Reference β-tubulin	30°C	19.00		
Result Set 3	Target *hsp60*	35°C	31.81	1.10E-4	0.63
	Reference β-tubulin	35°C	18.66		
Result Set 4	Target *hsp60*	40°C	31.65	7.79E-4	4.46
	Reference β-tubulin	40°C	21.32		
Result Set 5	Target *hsp60*	8 µM $CdCl_2$	32.95	5.89E-5	0.34
	Reference β-tubulin	8 µM $CdCl_2$	18.90		

Relative quantification of Trichophyton mentagrophytes *hsp60* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 6

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp60* Calibrator	25°C	28.01	0.04	1.00
	Reference β-tubulin Calibrator	25°C	23.27		
Result Set 2	Target *hsp60*	30°C	27.74	0.02	0.42
	Reference β-tubulin	30°C	21.75		
Result Set 3	Target *hsp60*	35°C	25.93	0.10	2.74
	Reference β-tubulin	35°C	22.64		
Result Set 4	Target *hsp60*	40°C	30.22	2.38E-3	0.06
	Reference β-tubulin	40°C	21.50		
Result Set 5	Target *hsp60*	8 µM $CdCl_2$	30.00	2.95E-3	0.08
	Reference β-tubulin	8 µM $CdCl_2$	21.59		

Relative quantification of Scedosporium apiospermum (Pseudallescheria boydii) *hsp60* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 7

	Paired Samples Types	Incubation Temp °C	Aspergillus fumigatus	Aspergillus terreus	Cladosporium cladospor.	Penicillium chrysogenum	Scedosporium apiospermum	Trichophyton mentagrophytes
Result Set 1	Target *hsp70* Calibr. Reference β-tubulin Calibrator	25°C 25°C	1.00	1.00	1.00	1.00	1.00	1.00
Result Set 2	Target *hsp70* Reference β-tubulin	30°C 30°C	0.78	0.81	0.57	0.54	0.42	0.36
Result Set 3	Target *hsp70* Reference β-tubulin	35°C 35°C	1.38	2.78	0.58	1.86	2.74	0.63
Result Set 4	Target *hsp70* Reference β-tubulin	40°C 40°C	1.20	6.71	0.06	0.19	0.06	4.46
Result Set 5	Target *hsp70* Reference β-tubulin	8 µM $CdCl_2$ 8 µM $CdCl_2$	0.71	0.57	0.37	0.63	0.08	0.34

Compendium of the Relative quantification values of A.fumigatus, A.terreus, C.cladosporioides, P.chrysogenum, S.apiospermum and T.mentagrophytes *hsp60* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all

Table 8

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp70* Calibrator	25°C	27.31	0.08	1.00
	Reference β-tubulin Calibrator	25°C	23.62		
Result Set 2	Target *hsp70*	30°C	27.58	0.12	1.52
	Reference β-tubulin	30°C	24.48		
Result Set 3	Target *hsp70*	35°C	26.93	0.20	2.57
	Reference β-tubulin	35°C	24.60		
Result Set 4	Target *hsp70*	40°C	26.66	0.18	2.28
	Reference β-tubulin	40°C	24.15		
Result Set 5	Target *hsp70*	8 µM $CdCl_2$	26.85	0.17	2.15
	Reference β-tubulin	8 µM $CdCl_2$	24.26		

Relative quantification of Aspergillus fumigates *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 9

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp70* Calibrator	25°C	27.45	0.04	1.00
	Reference β-tubulin Calibrator	25°C	22.96		
Result Set 2	Target *hsp70*	30°C	27.22	0.06	1.30
	Reference β-tubulin	30°C	23.11		
Result Set 3	Target *hsp70*	35°C	26.96	0.10	2.22
	Reference β-tubulin	35°C	23.62		
Result Set 4	Target *hsp70*	40°C	24.00	0.56	12.60
	Reference β-tubulin	40°C	23.16		
Result Set 5	Target *hsp70*	8 µM $CdCl_2$	27.87	0.05	1.06
	Reference β-tubulin	8 µM $CdCl_2$	23.46		

Relative quantification of Aspergillus terreus *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 10

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp70* Calibrator	25°C	35.08	0.04	1.00
	Reference β-tubulin Calibrator	25°C	30.48		
Result Set 2	Target *hsp70*	30°C	36.02	0.03	0.69
	Reference β-tubulin	30°C	30.89		
Result Set 3	Target *hsp70*	35°C	37.05	0.01	0.26
	Reference β-tubulin	35°C	30.50		
Result Set 4	Target *hsp70*	40°C	29.67	0.07	1.63
	Reference β-tubulin	40°C	25.77		
Result Set 5	Target *hsp70*	8 µM $CdCl_2$	38.68	7.40E-3	0.18
	Reference β-tubulin	8 µM $CdCl_2$	31.60		

Relative quantification of Cladosporium cladosporioides *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 11

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp70* Calibrator	25°C	23.90	0.25	1.00
	Reference β-tubulin Calibrator	25°C	21.92		
Result Set 2	Target *hsp70*	30°C	27.12	0.17	0.67
	Reference β-tubulin	30°C	24.56		
Result Set 3	Target *hsp70*	35°C	26.78	0.17	0.67
	Reference β-tubulin	35°C	24.24		
Result Set 4	Target *hsp70*	40°C	26.79	0.14	0.56
	Reference β-tubulin	40°C	23.97		
Result Set 5	Target *hsp70*	8 µM $CdCl_2$	26.13	0.23	0.91
	Reference β-tubulin	8 µM $CdCl_2$	24.03		

Relative quantification of Penicillium chrysogenum *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 12

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp70* Calibrator	25°C	38.08	5.22E-6	13.41
	Reference β-tubulin Calibrator	25°C	20.54		
Result Set 2	Target *hsp70*	30°C	46.50	1.53E-8	0.04
	Reference β-tubulin	30°C	20.54		
Result Set 3	Target *hsp70*	35°C	42.86	1.63E-7	0.42
	Reference β-tubulin	35°C	20.31		
Result Set 4	Target *hsp70*	40°C	46.68	1.10E-7	0.28
	Reference β-tubulin	40°C	23.57		
Result Set 5	Target *hsp70*	8 μM $CdCl_2$	41.29	3.89E-7	1.00
	Reference β-tubulin	8 μM $CdCl_2$	19.99		

Relative quantification of Trichophyton mentagrophytes *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets. . Under certain LC-software conditions, T.mentagrophytes

Table 13

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp70* Calibrator	25°C	22.47	5.58	1.00
	Reference β-tubulin Calibrator	25°C	24.95		
Result Set 2	Target *hsp70*	30°C	22.74	2.06	0.37
	Reference β-tubulin	30°C	23.79		
Result Set 3	Target *hsp70*	35°C	23.69	3.31	0.59
	Reference β-tubulin	35°C	25.42		
Result Set 4	Target *hsp70*	40°C	22.92	3.37	0.60
	Reference β-tubulin	40°C	24.68		
Result Set 5	Target *hsp70*	8 µM $CdCl_2$	26.31	0.56	0.10
	Reference β-tubulin	8 µM $CdCl_2$	25.47		

Relative quantification of Scedosporium apiospermum (Pseudallescheria boydii) *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets.

Table 14

	Paired Samples Types	Incubation-Temperature °C	Real-time PCR Crossing points	Real-time PCR Concentration Ratio	Real-time PCR Normalized Ratio
Result Set 1	Target *hsp70* Calibrator	25°C	38.08	5.22E-6	13.41
	Reference β-tubulin Calibrator	25°C	20.54		
Result Set 2	Target *hsp70*	30°C	46.50	1.53E-8	0.04
	Reference β-tubulin	30°C	20.54		
Result Set 3	Target *hsp70*	35°C	42.86	1.63E-7	0.42
	Reference β-tubulin	35°C	20.31		
Result Set 4	Target *hsp70*	40°C	46.68	1.10E-7	0.28
	Reference β-tubulin	40°C	23.57		
Result Set 5	Target *hsp70*	8 μM $CdCl_2$	41.29	3.89E-7	1.00
	Reference β-tubulin	8 μM $CdCl_2$	19.99		

Relative quantification of Trichophyton mentagrophytes *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets. Under certain LC-software conditions, T.mentagrophytes Result set 5 was set to 1.00 for all other Result sets.

	Paired Samples Types	Incubation Temp °C	Aspergillus fumigatus	Aspergillus terreus	Cladosporium cladospor.	Penicillium chrysogenum	Scedosporium apiospermum	Trichophyton mentagrophytes
Result Set 1	Target *hsp70* Calibr. Reference β-tubulin Calibrator	25°C 25°C	1.00	1.00	1.00	1.00	1.00	13.41
Result Set 2	Target *hsp70* Reference β-tubulin	30°C 30°C	1.52	1.30	0.69	0.67	0.37	0.04
Result Set 3	Target *hsp70* Reference β-tubulin	35°C 35°C	2.57	2.22	0.26	0.67	0.59	0.42
Result Set 4	Target *hsp70* Reference β-tubulin	40°C 40°C	2.28	12.60	1.63	0.56	0.60	0.28
Result Set 5	Target *hsp70* Reference β-tubulin	8 µM $CdCl_2$ 8 µM $CdCl_2$	2.15	1.06	0.18	0.91	0.10	1.00

Compendium of the Relative quantification values of A.fumigatus, A.terreus, C.cladosporioides, P.chrysogenum, S.apiospermum and T.mentagrophytes *hsp70* mRNA expression showing normalization ratio, the housekeeping gene β-tubulin serves as reference. Result set 1 was set to 1.0 and served for all other Result Sets. Under certain LC-software conditions, T.mentagrophytes Result set 5 was set to 1.00 for all other Result sets.

6. Discussion

The goal of this study was to obtain new scientific knowledge about the identification and characterisation of the heat shock protein 60 and 70 of common environmental fungi of medical interest.

Recent investigations demonstrated the intense impact of numerous fungi on the human immune system. Allergenic molds, like *Alternaria alternata* and *Cladosporium herbarum* are recognized as common inducers of immunogenic diseases like bronchial asthma or chronic rhinosinusitis (CRS) (Havaux 2005). Mouse models showed that chronic intranasal administration of mould spores lead to allergic lung inflammation, hyper-reactivity and mucosa remodeling (Denis 2007). Men sensitized to different fungi present with a more severe phenotype of epidemic asthma with a significant higher mortality rate to thunderstorm related asthma than non-sensitized individuals (Dales 2003, Pulimood 2007). This reports fit to the findings of Buzina et al. that a few immunogenic molds show a significant higher spore level during late summer and early autumn (Buzina 2003). Fungi are a normal content of human nasal mucus, but in CRS patients clusters of eosinophils and fungal elements are considered to be a marker of the disease (Braun 2003, Lackner 2005). Environmental fungi like *Alternaria* and *Penicillium* are able to induce activation and degranulation of human eosinophils and induce the production of IL-8 (Inoue 2005). Investigations by Shin et al. showed that the stimulation of peripheral blood mononuclear cells (PBMC) with common environmental fungi lead to a release of pro-inflammatory cytokines like IL-5 and IL-13 (Shin 2004). In the collective of 238 CRS patients and 23 healthy controls 88 different fungal genera were found (Braun 2003). The current expertise shows that numerous different fungi play an intense role in human diseases and Hsp60 was isolated from several fungi like *Histoplasma capsulatum* and *Trichophyton mentagrophytes* and successfully used for a protective vaccination in some animal models (Gomez 1995, Raska 2004).

The detection of Hsp60 and Hsp70 in all of our six investigated fungi confirms the general acceptance that heat shock proteins have been found in all organisms

so far (Matthews 1998, Gunther and Walter 1994). Our results showed that both Hsp60 and Hsp70 are found in fungi which showed a temperature related response but they were also been detected in fungi which didn`t react with an up-regulation of the expression of Hsp60 or Hsp70. Hence, Hsp60 and Hsp70 were constitutively expressed in all of our six investigated fungi.

Due to the fact that different heat shock proteins respond to numerous stressors, recent investigations showed that fungal Hsp60 is both, constitutively expressed and as an up-regulated response to thermal stress (Lindquist 1988, Jäättelä 1999, Pfister 2005). That confirms your findings of Hsp60 mRNA in all of our six fungi. Five of the investigated fungi including *A. fumigatus, A. terreus, P. chrysogenum, T. mentagrophytes* and *S. apiospermum* showed a heat inducible response of Hsp60 mRNA, wherein *C. cladosporioides* no significant temperature dependent response was observed. *C. cladosporioides* is a highly immunogenic fungus and due to our findings it has to be considered that the heat inducible Hsp70 mRNA of *C. cladosporioides* may be more likely involved in immunogenic processes than Hsp60 (Pulimood 2007). The immunological analyses and mass spectrometry by Li et al. considered among others a 72 kDa and a 61 kDa protein as one of the allergens from *C. cladosporioides* (Li 2008).

We observed that most of our investigated fungi showed the highest expression levels of Hsp60 and Hsp70 at an incubation temperature of 35°C and 40°C, except the expression of Hsp70 of *T. mentagrophytes* which showed a 13.4 fold up-regulation at an incubation temperature of 25°C. Due to the fact that some *Aspergillus species* have a higher temperature optimum for conidiation the results of A. fumigatus and A. terreus may explain the highest expression levels of Hsp60 and Hsp70 at 35°C and 40°C incubation temperature. In a study by Kumar et al. a recombinant heat shock protein of *A. fumigatus* reacted with immunoglobulin E and immunoglobulin G antibodies in the sera from patients with allergic bronchopulmonary aspergillosis and expressed a 65 kDa protein of *A. fumigatus*. This shows the immunogenic nature and the homology to human heat shock proteins, which may play an important role for this protein in protective immunity and autoimmunity (Kumar 1993).

Higher concentrations of cadmium chloride ($CdCl_2$) inhibited the growth of Aspergillus nidulans (Guelfi 2003). Cytotoxicity studies showed that McCoy cells exposed to a concentration of 1 µM cadmium significantly increased the cell activity and concurred with the production of high levels of Hsp70 (Damelin 2000). In the present study, *A. fumigatus* was the only fungus that showed a significant up-regulation of the Hsp70 mRNA expression when incubated with 8 µM $CdCl_2$ at 25°C in comparison to 25°C without $CdCl_2$. All other fungi developed no significant response to the incubation with 8 µM $CdCl_2$. A study by Georg et al. showed that the exposure to cadmium in the aquatic fungus *Blastocladiella emersonii* causes oxidative stress and apoptosis in this fungus (Georg 2007).

The expression of Hsp70 mRNA was inducible in a temperature dependent fashion in *A. fumigatus, A. terreus, C. cladosporioides* and *T. mentagrophytes,* but *P. chrysogenum* and *S. apiospermum* showed no temperature response. Thus it appears that the class of the 70-kDa HSP family consists of two major members of the family. One is constituted as the stress inducible Hsp70 with a molecular mass of 72 kDa and the other is a constitutively expressed Hsc70 with approximately 73 kDa (Lindquist and Craig 1988). The differences of the expression levels between *A. fumigatus* with the highest induction of a 2.6 fold up-regulation incubated at 35°C and *A. terreus* with the highest enhancement of a 12.6 fold up-regulation incubated at 40°C showed that the characterization for the stress induced response is strongly differing between the specimens. Furthermore, the response between yeasts and dermatophytes differed essentially in our study. Surprisingly, *T. mentagrophytes* exhibited the highest induction of a 13.4 fold up-regulation of the Hsp70 mRNA incubated at 25°C.

The comparison of our sequence data showed a high similarity of the Hsp60 and Hsp70 mRNA with published sequences of other fungal Hsp60 and Hsp70. This underlines the evolutionary highly conserved nature of the heat shock proteins.

The real time RT-PCR was performed on the LightCycler© Instrument (Roche). The different primers and hybridization probes were designed to perform a LightCycler© protocol for Hsp60 and Hsp70 for all fungi with two tailored cycling

conditions, in order to acquire a reduced processing time and a faster turn-around. This means that one cycling process contained at least six fungi to indentify and characterize the specific Hsp60 or Hsp70 and the corresponding housekeeping gene β-tubulin. The facility to accomplish a melting curve analysis after expired PCR discriminates unspecific PCR products like the formation of primer dimers. Hence, the use of hybridization probes instead of a TaqMan probe is expedient (Raggam 2005).

The translation of the nucleotide sequences of our investigated fungi is to be considered as essential and will be done, but weren`t translated at the time of finishing this diploma thesis. Due to the fact that amino acid substitutions are relatively conserved even in the variable C-terminal regions of Hsps (Zang 1996), the recent findings by Buzina et al. (personal communication), revealed that even the alteration of one amino acid can be the cause of an anti-fungal drug resistance.

In conclusion, all of the six investigated fungi expressed both Hsp60 and Hsp70 and showed a temperature dependent induction of at least one of the immunogenic heat shock proteins, whereas *A. fumigatus, A. terreus* and *T. mentagrophytes* actually exhibited an increase of both heat shock proteins. Due to the strong differences in expression levels in comparison to the diverse temperature gradients and the heavy metal exposure of $CdCl_2$ it shows the ubiquitous importance of the heat shock proteins in the field of medicine. This findings support the consequence of further investigations of the allergenic function of different Hsps and the demand of a better understanding of the pathogeneses pathways for inflammatory processes of the respiratory tract in humans and animals.

8. References

1. Ainsworth G.C., Sparrow F.K., Sussman A.S. The fungi, an advanced treatise. Academic Press, New York, 1973.
2. Alexopoulos C.J. Introducción a la micología. Endeba Edit. Universitario, Buenos Aires, 1966.
3. Ashburner, M. Pattern of puffing activity in the salivary gland chromosomes of Drosophila. V. Response to environmental treatment. Chromosoma 1970. **31**:356-376.
4. Aukrust L., Borch S.M., Einarsson R. Mould allergy: spores and mycelium as allergy sources. Allergy 1985; **40**:43- 48.
5. Benagiano M., D`Elios M.M., Amedei A., Azzurri A., Van der Zee R., Ciervo A., Rombola G., Romagnani S., Cassone A., Del Prete G. Human 60-kDa heat shock protein is a target auto-antigen of T-cell derived from atherosclerotic plaques. J Immunol 2005; **174**:6509-6517.
6. Bent J.P., Kuhn F.A. Diagnosis of allergic fungal sinusitis. Otolaryngol Head Neck Surg. 1994; **111**:580- 588.
7. Berendes. The induction of changes in chromosomal activity in different polytene types of cell in Drosophila hydei. Developmental Biology 1965. **11**:371-384.
8. Braun H., Stammberger H., Buzina W., Freudenschuss K., Lackner A., Beham A. Incidence and detection of fungi and eosinophilic granulocytes in chronic rhinosinusitis. Laryngo-Rhino-Otol 2003; **82**:330-340.
9. Bromuro C., La Valle R., Sandini S., Urbani F., Ausiello C.M., Morelli L., Fé D`Ostiani C., Romani L., Cassone A. A 70-kilodalton recombinant heat shock protein of Candida albicans is highly immunogenic and enhances systemic murine candidiasis. Infection and Immunity 1998; **66**:2154-2162.
10. Bukau B., Horwich A.L.: The Hsp70 and Hsp60 chaperone machines. Cell 1998; **92:**351-366.
11. Buzina W., Braun H., Freudenschuss K., Lackner A., Habermann W., Stammberger H. Fungal biodiversity- - as found in nasal mucus. Med Mycol 2003; **41**:149-161.

12. Buzina W., Raggam R.B., Paulitsch A., Heiling B., Marth E. Characterization and temperature-dependent quantification of heat shock protein 60 of the immunogenic fungus Alternaria alternata. Med Mycol 2008; in press.
13. Codina R., Lockey R.F. Possible role of molds as secondary etiologic agents of the asthma epidemics in Barcelona, Spain. J Allergy Clin Immunol 1998; **102**:318- 320.
14. Dales R.E., Cakmark S., Judek S., Dann T., Coates F., Brook J.R. The role of fungal spores in thunderstorm asthma. Chest 2003; **123**:745- 750.
15. Damelin L.H., Vokes S., Whitcutt J.M., Damelin S.B., Alexander S.J. Hormesis: a stress response in cells exposed to low levels of heavy metals. Hum Exp Toxicol 2000; **19**:420-430.
16. Denis O., van den Brŭle S., Heymans J., Havaux X., Rochard C., Huaux F., Huygen K. Chronic intranasal administration of mold spores or extracts to unsensitized mice leads to lung allergic inflammation, hyper-reactivity and remodeling. Immunology 2007.
17. DeShazo R.D., Swain R.E. Diagnostic criteria for allergic fungal sinusitis. J Allergy Clin Immunol. 1995; **96**:24- 35.
18. Fackler, K., Gradingerm, C., Hinterstoisser, B., Messner, K., Schwanninger M. Lignin degradation by white rot fungi on spruce wood shavings during shorttime solid-state fermentations monitored by near infrared spectroscopy. Enzyme Microb. Technol. 2006; **39**:1476-1483.
19. Gehrmann M. Untersuchung zur Modulation von Hitzeschockprotein 70 (Hsp70)-Expression und Identifizierung Hsp70-assoziierter Moleküle auf der Zellmembran. Dissertation 2003; WWW: http://www.bibliothek.uni-regensburg.de/abteil/erwerb/disser/2004/diss.htm
20. Georg R.C., Gomes S.L. Transcriptome analysis in response to heat shock and cadmium in the aquatic fungus Blastocladiella emersonii. Eukaryot Cell 2007; **6**:1053-1062.
21. Gomez F.J., Allendoerfer R., Deepe G.S. Jr. Vaccination with recombinant heat shock protein 60 from Histoplasma capsulatum protects against pulmonary histoplasmosis. Infect Immun 1995; **63**:2587-2596.

22. Gosepath J., Brieger J., Vlachtsis K., Mann W.J. Fungal DANN is present in tissue specimens of patients with chronic rhinosinusitis. Am J Rhinol 2004; **18**:9-13.
23. Graveson S. Fungi as a cause of allergic disease. Allergy 1979; **34**:135-154.
24. Grigoriu D., Delacretaz J., Borelli D. Lehrbuch der medizinischen Mykologie. Verlag Hans Huber Bern 1984. p 24.
25. Guelfi A., Azewedo R.A., Lea P.J., Molina S.M.G. Growth inhibition of the filamentous fungus Aspergillus nidulans by cadmium**:** an antioxidant enzyme approach. J Gen Appl Microbiol 2003; **49**:63-73.
26. Guillemette T., Sellam A., Simoneau P. Analysis of a nonribosomal peptide synthetase gene from Alternaria brassicae and flanking genomic sequences. Curr Genet 2004; **45**:214-224.
27. Guine J., Peláez T., Alcalá L., Bouza E. Outdoor environmental levels of Aspergillus spp. conidia over a wide geographical area. Medical Mycologie. 2006; **44**:349-356.
28. Gunther E. and Walter L. Genetic aspects of the hsp70 multigene family in vertebrates. *Experientia* 1994*;* **50**:987-1001.
29. Guy B. Marks and Robert K. Bush. It`s blowing in the wind: New insights into thunderstorm-related asthma. J Allergy Clin Immunol 2007; **120**:530-532.
30. Habich C., Kempe K., Gomez F.J., Lillicrap M., Gaston H., Van der Zee R., Kolb H., Burkart V. Heat shock protein 60: Identification of specific epitopes for binding to primary macrophages. FEBS Lett. 2006; **580**:115-120.
31. Hartl F.U. Molecular chaperones in cellular protein folding. Nature 1992. **381**:571–579.
32. Hartl F.U. and M. Hayer-Hartl. Molecular chaperones in the cytosol: from nascent chain to folded protein. Science 2002*;* **295**:1852-1858.
33. Havaux X., Zeine A., Dits A., Denis O. A new mouse model of lung allergy induced by the spores of Alternaria alternata and Cladosporium herbarum molds. Clin Exp Immunol 2005; **139**:179-188.
34. Hauet-Broere F., Wieten L., Guichelaar T., Berlo S., van der Zee R., Van Eden W. Heat shock proteins induce T-cell regulation of chronic inflammation. Ann Rheum Dis 2006; **65**:65-68.

35. Hof Herbert, Rüdiger Dörries. Medizinische Mikrobiologie, 3. Auflage, Duale Reihe. 2005. p 458- 496.
36. Hightower L.E., Hendershot L.M. Molecular chaperones and the heat shock response at Cold Spring Harbor. Cell Stress Chaperon. 1997; **2**:1-11.
37. Inoue Y., Matsuwaki Y., Shin S.H., Ponikau J.U., Kita H. Nonpathogenic, environmental fungi induce activation and degranulation of human eosinophils. J Immunol 2005; **175**:5439-5447.
38. Jäättelä M. Heat shock proteins as cellular lifeguards. Ann Med. 1999; **31**:261-271.
39. Jensen R.E., Johnson A.E. Protein translocation is Hsp70 pulling my chain? Curr Biol. 1999; **9**:R779-R782.
40. Kaliner M.A., Osguthorpe J.D., Fireman P., Anon J., Georgitis J., Davis M.L., Naclerio R., Kennedy D. Sinusitis: bench to bedside. Current findings, future directions. Otolaryngol Head Neck Surg 1997; **116**:S1-S2.
41. Katzenstein A.L., Sale S.R., Greenberger S.A. Allergic Aspergillus sinusitis: a newly recognized form of sinusitis. J Allergy Clin Immunol. 1983; **72**:89-93.
42. Kumar A., Reddy L.V., Sochanik A., Kurup V.P. Isolation and characterization of a recombinant heat shock protein of Aspergillus fumigatus. J Allergy Clin immunol 1993; **91**:1024-1030.
43. Lanza D.C., Kennedy D.W. Adult rhinosinusitis defined. Otolaryngol Head Neck Surg 1997; **117**:S1- S7.
44. Lackner A, Stammberger H, Buzina W, Freudenschuss K, Panzitt T, Schosteritsch S., Braun H. Fungi: A normal contant human nasal mucus. Am J Rhinol 2005; **19**:125-129.
45. Li L., Li Q., Liu Z., Wu H. Immunological analysis and mass spectrometry identification of the major allergen from Cladosporium cladosporioides. Wei Sheng Yan Jiu 2008; **37**:50-2.
46. Lindquist S., Craig E.A. The heat shock proteins. Annu Rev Genet. 1988; **22**:631-677.
47. MacIntosh D.L., Brightman H.S., Baker B.J., Myatt A.T., Stewart H.J., McCarthy J.F. Airborne fungal spores in a cross-sectional study of office buildings. Journal of Occupational and Environmental Hygiene.2006; **3**:379-389.

48. Nucci M., Marr K.A. Emerging fungal diseases. Clin Infect Dis 2005; **41**:521-526.
49. Offermanns et al. Molecular Pharmacology. Springer Verlag.
50. O`Hollaren M.T., Yunginger J.W., Offord K.P., Somers M.J., O`Connel E.J., Ballard D.J. Exposure to an aeroallergen as a possible precipitating factor in respiratory arrest in young patients with asthma. N Engl J Med 1991; **324**:359- 363.
51. Pfister G., Stroh C.M., Perschinka H., Kind M., Knoflach M., Hinterdorfer P., Wick G. Detection of HSP60 on the membrane surface of stressed human endothelial cells by atomic force and confocal microscopy. J Cell Sci 2005; **118**:1587-1594.
52. Pleis J.R., Lethbridge-Ceiku M.. Summery health statistics for U.S. adults: National health interview survey. Vital Health Stat. 2005; **10**:1-153.
53. Ponikau J.U., Kern E.B., Homburger H.A., Frigas E., Gaffey T.A., Roberts G.D. The diagnosis and incidence of allergic fungal sinusitis. Mayo Clin Proc 1999; **74**:877- 884.
54. Pulimood T., Corden J., Bryden C., Sharples L., Nasser S. Epidemic asthma and the role of the fungal mold Alternaria alternata. J Allergy Clin Immunol 2007; **120**:610- 617.
55. Raggam R.B., Leitner E., Berg J., Mühlbauer G., Marth E., Kessler H. Single-run, parallel detection of DANN from three pneumonia-producing bacteria by real-time polymerase chain reaction. J Mol Diagn 2005; **7**:133-138.
56. Ranford J.C., Henderson B. Chaperonins in disease: mechanisms, models, and treatments. J Clin Pathol: Mol Pathol 2002; **55**:209-13.
57. Raska M., Zemanova E., Kafkova L., Belakova J., Vudattu N.K., Kopecek P., Weigl E. Isolation and characterization of an immunogenic fragment of heat shock protein 60 of Trichophyton mentagrophytes. Mycoses 2004; **47**:482-490.
58. Ray N.F., Baraniuk J.N., Thamer M., Rinehart C.S., Gergen P.J., Kaliner M., Josephs S., Pung Y.H. Healthcare expenditures for sinusitis in 1996: contributions of asthma, rhinitis, and other airway disorders. J Allergy Clin Immunol. 1996; **103**:408-414.

59. Richardson A., Landry S.J., Georgopoulos C. The ins and outs of a molecular chaperone machine. Trends Biochem 1998; **23**:138-143.
60. Ritossa F. A new puffing pattern induced by heat shock and DNP in *Drosophila*. Experientia 1962; **18**:571–573.
61. Ruhnke M. Epidemiology of Candida albicans infections and role of non-Candida albicans yeasts. Curr Drug Targets 2006; **7**:495–504.
62. Shin S.H., Ponikau J.U., Sherris D.A., Congdon D., Frigas E., Homburger H.A., Swanson M.C., Gleich G.J., Kita H. Chronic rhinosinusitis: An enhanced immune response to ubiquitous airborne fungi. J Allergy Clin Immunol 2004; **114**:1369-1375.
63. Tariq S.M., Matthews S.M., Stevens M., Hakim E.A. Sensitization to Alternaria and Cladosporium by age of 4 years. Clin Exp Allergy 1996; **26**:794-798.
64. Tissieres A., Mitchell H.K., and Tracy U.M. Protein synthesis in salivary glands of *D. melanogaster*: relation to chromosome puffs. J Mol Biol 1974; **84**:389–398.
65. Vailes L., Sridhara S., Cromwell O., Weber B., Breitenbach M., Chapman M. Quantitation of the major fungal allergens Alt a 1 and Asp f 1, in commercial allergenic products. J Allergy Clin Immunol 2001; **107**:641-646.
66. Vanbreuseghem R., De Vroey Ch., Takashio M. Guide pratique de mycologie médicale et vétérinaire. Masson, Paris, New York, Barcelona, Milano, 1978 (2. Auflage).
67. Welch W.J. Mammalian stress response: cell physiology, structure/ function of stress proteins, and implications for medicine and disease. Physiol Rev 1992; **72**:1063–1081.
68. Welch W.J. Heat shock proteins functioning as molecular chaperones: their roles in normal and stressed cells. Philos Trans R Soc Lond B Biol Sci 1993; **339**:327-333.
69. Zang L., Muradia G., De Vouge M.W., Rode H., Vijay H.M. An allergenic polypeptide representing a variable region of hsp70 cloned from a cDNA library of cladosporium herbarum. Clin Exp Allergy 1996; **26**:88-95.

Curriculum vitae

Vor- und Nachname:	Helmut Josef Friedrich Salzer
Geburtsdatum:	12.09.1981
Anschrift:	Mitterleitenweg 14c, 4040 Linz
Staatsbürgerschaft:	Österreich
Telefonnr.:	+43650/ 7828679
Email:	helmut.salzer@gmx.net

Schulausbildung:

10/ 1996 – 06/ 2001	Bundesoberstufenrealgymnasium Linz (BORG)
06/ 2001	Reifeprüfung

Zivildienst:

09/ 2001 – 09/ 2002	Sanitäter und Rettungsfahrer, Arbeiter- Samariter- Bund Linz

---

Hochschulstudium:

10/ 2002 – 09/ 2008	Diplomstudium Humanmedizin in 12 Semester Medizinische Universität Graz Neues Curriculum O202
09/ 2008	Promotion zum Dr. med. univ.

Diplomarbeit:	„Characterization and temperature- dependent quantification of heat shock protein 60 and 70 of

	immunogenic fungi.” Betreuer: Priv. Doz. Mag. Dr. Walter Buzina
Zusatzqualifikationen:	
09/ 2007 – 07/ 2008	Wissenschaftlicher Mitarbeiter am Institut für Hygiene, Mikrobiologie und Umweltmedizin
Vortrag:	
05/ 2008	31. Jahrestagung der Österreichischen Gesellschaft für Hygiene, Mikrobiologie und Präventivmedizin (ÖGHMP), Bad Ischl, Österreich
Kongressteilnahmen:	26th Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), Graz, Austria 7th International Symposium on Molecular Diagnostics, Graz, Austria 31. Jahrestagung der ÖGHMP, Bad Ischl, Österreich
Weiterbildungen:	
10/ 2007 – 02/ 2008	Vertiefte Ausbildung, Innere Medizin
06/ 2006	Epidemiologische, ätiologische und therapeutische Aspekte der infektiösen Hepatitiden
03/ 2006 – 06/ 2006	Vertiefte Ausbildung, Pädiatrische Infektiologie
10/ 2005 – 06/ 2006	Basic Medical Communication I + II (English)
Famulaturen:	
2007/ 2005/ 2004	Allgemeines Krankenhaus Linz (AKH): Neurologie, Intern I, Interne II, Chirurgie II
06/ 2007	Kalra Hospital and SRCNC, New Delhi, INDIEN

08/ 2006	Cho Ray Hospital, Department of Infectious Diseases and Tropical Medicine, Ho- Chi- Minh City, VIETNAM

Sprachkenntnisse:	
Englisch:	Sehr gut (=Schrift und Sprache)
EDV- Kenntnisse:	MS Office

Graz, am 13.06.2008 Unterschrift: