Identification of *Candida albicans* from Clinical Specimens Obtained from Lebanese Hospitals By Fluorescence Resonance Energy Transfer and Probe Melting Curves.

By

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Abstract

Identification of *Candida Albicans* from Clinical Specimens Obtained from Lebanese Hospitals By Fluorescence Resonance Energy Transfer and Probe Melting Curves.

The fungi are an independent group as plants and animals. Some fungi including the yeast are pathogenic to mammals and particularly humans. The increasing frequency of invasive fungal infections and the high mortality associated with disseminated fungal diseases have underscored the importance of rapid detection of these pathogenic fungi – most common of which are the *Candida Spp.* Thus, prompt detection and accurate speciation may help to improve fungal disease management as a whole and lead to more rational use of anti-fungal. Traditional identification methods are often time-consuming and depend largely on the skill and experience of the technician.

In light of the above, the aim of the experiment was to evaluate a more recent real-time PCR technique that has been developed for the identification of many microbial organisms including the Candida spp and comparing that to results obtained from Lebanese hospitals. This technique relies on Fluorescence Resonance Energy Transfer and Probe Melting Curves. The above was done by using a Lightcycler Candida Kit from Roche Applied Science. The technique was used on 100 samples presumed to be *C.albicans* gathered from the Lebanese hospitals and its results were compared to those given by the hospital and to the results of a germ tube test repeated at LAU.

24 samples out of the 100 appeared negative on the light cycler. This discrepancy was quite significant with a $p < 0.001$. Further more, 6 samples gave false positive results with germ tube test and 17 showed a false negative result. Both, these results are significant with respect to the rate of false positivity and negativity.

The real-time PCR is rapid, simple and specific and offers many advantages in reference to the conventional methods. Many discrepancies occurred in the result between the hospital and the repetition of the germ tube itself. Thus, in light of the results hospitals are advised to reconsider their techniques of identification.

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INTRODUCTION

1.1 Kingdom overview

The fungi constitute an independent group equal in rank to that of plants and animals. They share with animals the ability to export hydrolytic enzymes that break down biopolymers, which can be absorbed for nutrition. Rather than requiring a stomach to accomplish digestion, fungi live in their own food supply and simply grow into new food as the local environment becomes nutrient depleted (Alexopoulos et al., 1996). They include mushrooms, rusts, smuts, puffballs, truffles, morels, molds, and yeasts, as well as many less well-known organisms. About 70,000 species of fungi have been described; however, some estimates of total numbers suggest that 1.5 million species may exist (Hawksworth et al., 1995).

As all eukaryotes fungi contain membrane bounded organelles (such as mitochondria and nuclei). Fungi also have a cell wall composed of chitin instead of cellulose. This cell wall entails the fungi's antigenic determinant. The fungal kingdom is composed of five phyla based on reproduction, type of spores and recently some DNA and rRNA sequencing (Bruns et al., 1993).

These phyla are:

- Chytridiomyces: most primitive fungi mainly include water parasites.
- Zygomycetes : mainly include molds such as the black bread molds.

- Ascomycetes: include the yeasts having both sexual and asexual cycles (most known member is saccharomyces cerevisiae).
- Basidiomycetes: includes mushrooms having only a sexual cycle.
- Deuteromycetes: imperfect fungi, since they have no known sexual cycle (most common member is the yeast *Candida*).

Fungi are vital for their ecosystem functions. In addition, a number of fungi are used in the processing and flavoring of foods (baker's and brewer's yeasts, Penicillia in cheese-making) and in production of antibiotics and organic acids. Other fungi, however, are pathogenic to mammals and particularly humans such as opportunistic yeasts or filamentous fungi. Infection could happen by direct invasion of the host or by producing secondary metabolites such as aflatoxins that may be potent toxins and carcinogens in food of birds, fish, humans, and other mammals (Bruns et al., 1993).

1.2 Fungal infections

1.2.1 Incidence

The incidence of fungal infections has dramatically increased in the last decades. In 1964, there were 2 fungal infections for each 1000 hospital discharges. Data from the National Nosocomial Infection Surveillance showed that nosocomial fungal infection rate - the rate of infection acquired in the hospital - almost doubled from 1980 to 1990, increasing from 2,0 to 3,8 for each 1000 hospital discharges (Beck-Sague and

Jarvis, 1993). Unfortunately, no similar data on Lebanese hospital patients is available.

1.2.2 Reasons of increase

During the past few decades, advances in medical technology, chemotherapeutics, cancer therapy (including radiotherapy), organ transplantation, and the development of new diagnostic and treatment approaches have had a major impact on reducing the morbidity and mortality of life-threatening disease and have increased the life expectancy of critically ill patients. Thus, the increase in the number of fungal infections is clearly related to an increase in the incidence of risk factors for these kinds of infections (Write and Wenzel, 1997).

The risk factors mentioned above include greater use of immunosuppressive drugs (for transplant patients and patients with autoimmune diseases), prolonged use of broad spectrum antibiotics , widespread use of indwelling catheters (particularly in patients of advanced age and in patients with chronic disabling illness), the acquired immunodeficiency syndrome (AIDS), increase number of patients undergoing hemodyalisis and surgeries, and the increased number of patients that undergo severe trauma and extensive burns but are saved and treated. In addition, there are risk factors that have been constant in their incidence of predisposition such as pregnancy (Wenzel, 1995).

1.2.3 Major etiological agent:*Candida species* .

Candida spp. cause the huge majority of fungal infections (around 85% of them) and several multicentre studies showed that it was, nowadays,

one of the most frequent etiologies of nosocomial infections (Beck-Sague and Javris, 1993; Vincent et al., 1995; Pfaller et al., 1998), being responsible for 8-15% of the hospital nosocomial blood stream infections and for about 10% of the Intensive Care Unit (ICU) blood stream infections (Jarvis and Maratone, 1992; Valles et al., 1997). Two very large studies, the EPIC in Europe (Vincent, 1995) and the Pfaller-Wenzel in the USA, showed that *Candida* was the fifth most predominant nosocomial pathogen. *Candida albicans* accounted for 76% of all*Candida species* . infections (Pfaller and Wenzel, 1992).

Not only is *Candida* one of the most frequent causative agents of infections, but it is also becoming one of the most frequent isolated microorganisms: more than 50% of the ventilated patients and about 40% of the patients with a urinary catheter acquire *Candida* in bronchial and urinary specimens, respectively. Oropharyngeal colonization is also found in 30-55% of healthy young adults, and*Candida species* may be detected in 40-65% of normal fecal florae (Hidalgo, 2005).

In addition to the increase in incidence of invasive candidosis , this type of infection have proved to carry the highest mortality of all pathogens and Pittet (1997) showed that*Candida species* . were the only organisms independently influencing the outcome of a population of patients with nosocomial blood stream infections. Invasive candidosis causes a high crude mortality that depends on the population analyzed but is usually between 50 and 60% in critically ill non-neutropenic patients, while related or attributable mortality varies from 21 to 38% (Pittet et al., 1997).

1.3*Candida species*

In sake of its clear predominance *Candida* has become one of the most studied infectious organisms. This is reflected in the number of publications with subject heading as *Candida* and candidiasis or with a specific focus on treatment of *Candida*. During the 5- year interval from 1980 to 1985 there were 3000 publications, but they reached the peak between the years 1995 to 2000, in which the publications mounted up to 6,900 more than double the amount in the first interval (Odds, 1988a).

1.3.1 General characteristic

Candida spp. is yeast-like ubiquitous fungi. They are small (4-6 μm), unicellular, thin-walled, ovoid cells (blastospores) that reproduce by budding with no known sexual cycle. Most members of the genus also produce a filamentous type of growth (pseudohyphae where one cell buds and then elongates retaining a somewhat ellipsoidal form); in addition, *Candida albicans* and *C. dubliensis* also form true hyphae where cells grow by apical expansion. The above two species are also characterized by showing under particular conditions thick walled spherical cells arising as terminal cells on pseudohyphae, these cells are called chlamydospores (figure 1). Thus, almost all species of *Candida* are considered polymorphic (Odds, 1988b).

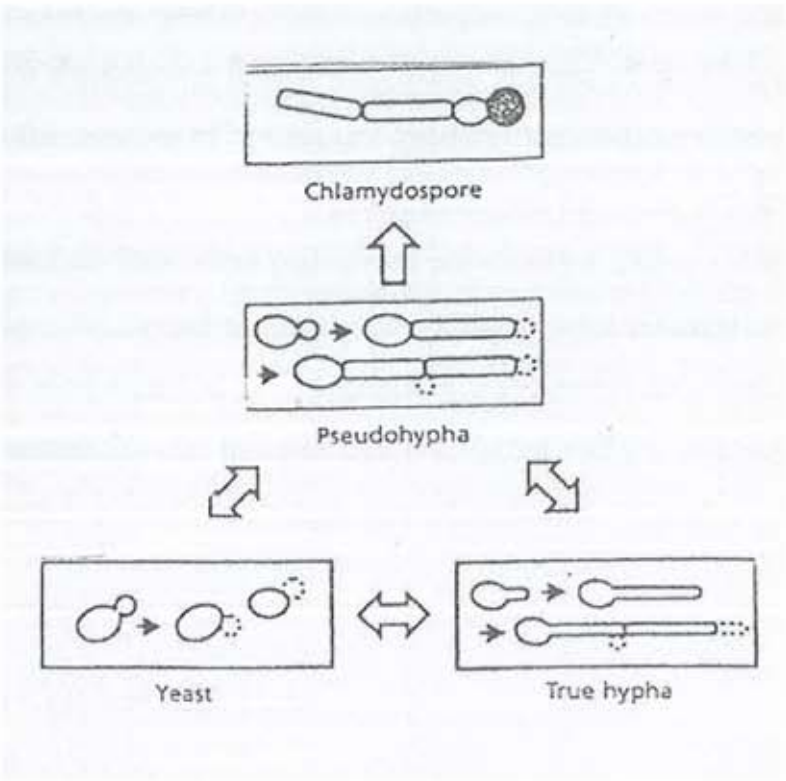

Figure 1: Morphological forms of*Candida species*
Source: Odds, 1988b

1.3.2 Pathogenicity

Candida genus includes more than 150 species and 11 of them have already been isolated in the human and considered to be pathogenic. These include *Candida albicans* (50-60%), *C. guilliermondii* (<5%), *C.krisei* (1-3%, with intrinsinc resistance to ketocanazole), *C.parapsilosis* (10-20%), *C.stellatoidea* (<5%), *C.tropicalis* (6-12%), C.*Pseudotropicalis, C.lusitaniae* (not very common but resistant to amphotericin B), *C.rugosa, C.glabrata*(15-20%), *C.dubliniensis* (recovered from patients who are positive for Human Immunodeficiency Virus (HIV)).*Candida species* . are considered opportunistic infectious agents that is they take advantage of every weakness in the body to attack. Thus, under regular conditions *Candida* is part of the normal flora

in humans. They are commonly found on skin and through out the GI tract. They are also commonly found in expectorated sputum and throughout the female genital tract (Hidalgo, 2005).

Any marked increase in the intestinal population of *Candida*, by ingestion of fungi or by yeast overgrowth in the gastrointestinal tract, contributes to the development of *Candida* infections. Alterations in host defenses (immunocomprimisation) lead to yeast overgrowth which is associated to the altered intestinal barrier function and decreased reticuloendothelial system function characteristics of the critically ill patients, and may result in life-threatening fungal infections. The use of broad-spectrum antibiotics, which eliminates many bacteria of the gastrointestinal tract that confer colonial protection, is also a major risk factor that potentiates colonization. After colonization, there is proliferation, translocation through the mucosa with invasion and colonization at a remote site after a period of unapparent Candidemia and finally apparent Candidemia. Thus, colonization is an independent risk factor and also a prerequisite for *Candida* infection and is a result of its opportunistic character (Pittet et al., 1994).

1.3.3 *Candida* infections

Infections due to*Candida species* can manifest in a wide spectrum of clinical syndromes. The clinical presentation can vary depending on the type of infection and the degree of immunosuppression. Clinical syndromes associated with *Candida* infection are usually subdivided into

four main categories, each having its own sub division (Hidalgo, 2005) as follows:

1-Cutaneous or superficial candidiasis syndromes: there are four common infections.

- Oropharyngeal candidiasis: oral thrush, first to be discovered. Mainly common in older people, neonates, and more recently in HIV infected patients; it is characterized by burning mouth or tongue, dysphagia, whitish, thick patches on the oral mucosa. Physical examination reveals a diffuse erythema and white patches that appear on the surfaces of the buccal mucosa, throat, tongue, and gums (figure 2 and 3).
- Vulvovaginal candidiasis: This is the second most common cause of vaginitis. The patient's history includes vulvar pruritus, vaginal discharge, dysuria, and dyspareunia. Approximately 10% of women experience repeated attacks of VVC without precipitating risk factors. Physical examination findings include a vagina and labia that are usually erythematous, a thick curdlike discharge, and a normal cervix upon speculum examination (figure 4).
- *Candida* balanitis: Patients report itchiness of the penis. Lesions and whitish patches are present. *Candida* balanitis is acquired through sexual intercourse with a partner who has VVC. Physical examination reveals vesicles on the penis that develop later into patches resembling thrush. The rash may spread to the thighs, gluteal folds, buttocks, and scrotum (figure 5).
- Intertrigo: Pruritic red rash occurs in patients with a history of intertrigo affecting any site where the skin surfaces are in close proximity, providing a warm and moist environment (figure 6).

2-Chronic mucocutaneous candidiasis: Chronic mucocutaneous candidiasis describes a group of *Candida* infections of the skin, hair, nails, and mucous membranes that tend to have a protracted and persistent course and are encountered simultaneously in several areas. Most patients survive for prolonged periods and rarely experience disseminated fungal infections. Physical examination findings reveal disfiguring lesions of the face, scalp, hands, and nails. This is occasionally associated with oral thrush and vitiligo.

3- Systemic Candidiasis: Involves the infection by *Candida* of deep tissue and organs, but usually one organ only is involved . It can be divided into subgroups according to the location.

- GI tract candidiasis
- Respiratory tract candidiasis
- Genitourinary tract candidiasis
- Hepatosplenic candidiasis
- *Candida* endophthalmitis:
- Renal candidiasis
- CNS infections due to*Candida species*
- Myocarditis-pericarditis:
- *Candida* splenic abscess and hypersplenism

4- Dissiminated Candidiasis: This is frequently associated with multiple deep organ infections. Unfortunately, of patients with disseminated candidiasis, as many as 40-60% may have blood culture results negative for*Candida species* . The history of a patient with presumptive disseminated candidiasis reveals a fever unresponsive to broad-spectrum antimicrobials and negative results from blood culture. Physical

1.4 Anti - fungal Therapy

In light of the wide spectrum of infection and the diversity of the location and severity deciding when and which type of anti-fungal therapy to start is therefore problematic, especially given the high morbidity and mortality associated with fungal infections.

There are four broad approaches to the use of antimicrobial therapy - prophylaxis, pre-emptive therapy, empirical therapy and definitive therapy - in terms of fungal infection (British Society for Antimicrobial Chemotherapy Working Party, 1994):

1- Prophylaxis: the preventive therapy of a whole population regardless of individual risk factors.

2- Pre-emptive therapy: is the treatment of individual patients thought to be at high risk of developing deep candidosis, identified by clinical or laboratory markers, to prevent the disease.

3- Empirical therapy is the treatment of patients thought to have established deep candidosis without microbiological, histological or serological confirmation. As fungal diagnosis is difficult, empirical is and will continue to be necessary in some occasions. The Working Party proposed the following indications for empirical therapy: (a) clinically unstable or deteriorating premature neonate with skin breaks from which *Candida* has been grown or positive urine microscopy or culture for yeast was found; (b) candiduria in high-risk patient with deteriorating clinical status; (c) patients with prosthetic valve endocarditis likely to be due to *Candida*, even with negative blood cultures.

4- Definitive therapy is the treatment of established deep candidosis, which requires microbiological or histological evidence of

examination reveals fever (may be the only symptom) with an unknown source and sepsis and septic shock.

Source : (Hidalgo, 2005)

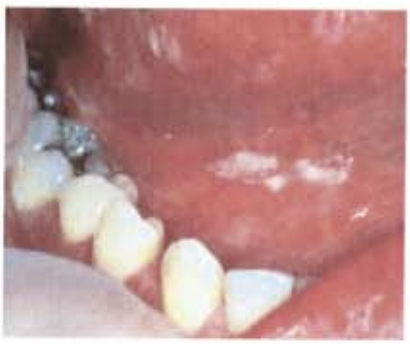

Figure 2: White patches of oral thrush

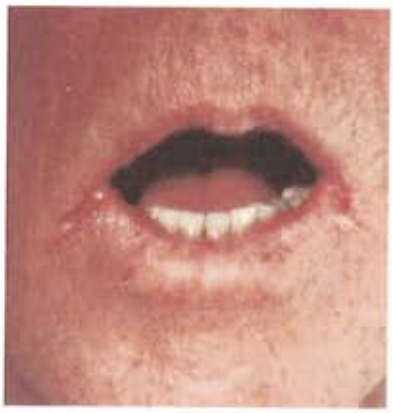

Figure 3: Inflamation of Oral mucosa

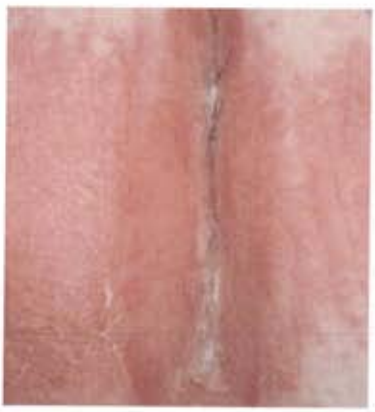

Figure 4: Vaginal discharge of *Candida*l infection

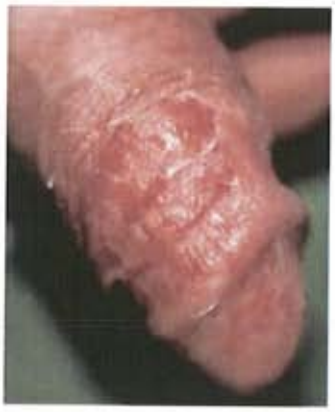

Figure 5: vesicles on the penis

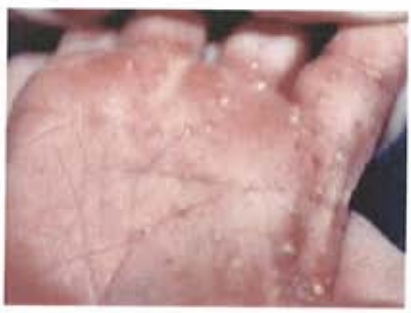

Figure 6: Pruritic rash on the foot

fungal infection.

The currently available systemic antifungal agents useful include Amphotericin B, lipid-based preparations of Amphotericin B, Fluconazole and, although less useful, Itraconazole and Flucytosine. At least four studies showed that in stable non-neutropenic patients, fluconazole may be considered as an alternative to amphotericin B for first-line therapy of Candidemia, unless it is caused by fluconazole-resistant yeasts like *Candida kruzei* and often *Candida glabrata* or in patients that have been previously under fluconazole. Fluconazole is the best agent for urinary tract candidosis, due to its high urinary concentrations and for infections caused by *Candida lusitanae.* Amphotericin B should be preferred in any clinically unstable, deteriorating patient, in patients with high-grade Candidemia or in patients with hematogenous deep-organ infections, like severe endophthalmitis, suppurative phlebitis, endocarditis, pericarditis, osteomyelitis and meningitis (British Society for Antimicrobial Chemotherapy Working Party, 1994).

Apart from pharmacology, adequate therapy includes further strategies. Fair amount of data shows that the removal or change of intravenous catheters is essential and reduces mortality in candidosis.

The first three aspects of treatment are not applicable for several reasons. Each*Candida species* has a different degree of susceptibility to common antifungal agents. For instance, *Candida krusei* is innately resistant and *Candida glabrata, Candida guilliermondi* and *Candida dubliniensis* are less susceptible to fluconazole than other*Candida species* .Emergence of secondary resistance in *Candida lusitaniae* to Amphotericin B has also

been observed and monitored closely. In addition, there is great concern that such practices would result in the emergence of new resistant fungal pathogens. Further more, many drugs have severe side effects, example, the use of conventional amphotericin B is restricted by dose-limiting side effects, including infusion-related symptoms and nephrotoxicity (Hsu et al. et al., 2003).

As more and more alternative antifungal agents with various spectra of activities and increasing specificity to a single species are developed and become available, treatment according to accurate diagnosis has become extremely important. Therefore, rapid and accurate species identification will be more critical for effective disease therapy and control (Hsu et al., 2003).

1.5 Diagnosis

1.5.1 Conventional methods

Below are the conventional diagnostic techniques used in clinical and mycology laboratories world wide despite of the apparent side effects of each. Most of these studies concentrate on identification of *Candida albicans* from other species since it is till now the most common etiological agent of infection.

Serological tests are the primary tests done upon suspicion of *Candida* infection taking into consideration the rapidity of the results. These tests include antibody and antigen detection. Serological tests have certain limitations, e.g. antibody response may be lacking or varied, since the patients most at risk of fungal infections are often immunosuppressed.

As for antigen tests, there are no widely accepted *Candida* antigen tests and most cannot differentiate among*Candida species* (Hsu et al., 2003).

The germ tube test is another commonly employed test in the diagnostic mycology laboratory to identify isolates of *Candida albicans* or *C. dubliensis* rapidly from patients. A germ tube is defined as a filamentous extension from a yeast cell that is about one half the width and three to four times the length of the cell. It is initiated by *Candida albicans* and *C.dubliensis* in germination conditions that include temperature above 35 degrees celcius and a PH of 6.5 to 7 or slightly alkaline (100% fetal bovine serum at 37 degrees celcius is an ideal representative of these conditions) (Odds, 1988b).

Growing yeast on Chrom agar is another way to identify *Candida albicans*; it will show green pigment formation. Chrom agar contains peptone (10.2 g/L), chloramphenicol (0.5 g/L) and chromogenic mix (22.0 g/L), agar (15.0 g/L), pH: 6.1. This chromogenic media allows the presumptive differentiation of yeasts since it contains various substrates for the enzymes of yeast species. It has been demonstrated that ß-N-acetylgalactosaminidase which was produced by *Candida albicans* enables the chromogenic substrates to be incorporated into the medium and the isolates of these species are seen as green colored colonies (Table 1). Growing the specimens on chrom agar and identification via this technique takes at least 24 to 48 hrs (Yücesoy and Marol, 2003).

The disadvantage of the above technique lies in the subjectivity of interpreting or identifying similar colors with different shades. Many

species for example have a pink colored colony but are differentiated by the shade of pink (pink to purple vs. pale pink). Further more, if you consider *Candida albicans* and *C.dubliniensis* they both give green color but with two different shades (figure 7).

Table1: Identification of clinical yeast isolates by growth characteristics on Chrom agar.

Species (no. of strains)	**Colony characteristics on Chrom agar**
Candida albicans (50)	Apple green colonies; consistent distinctive appearance
C. glabrata (116)	Large pale pink to purple glossy colonies
C. tropicalis (87)	Dull blue, sometimes pink, colonies; all developed purple halo of pigment that diffused into surrounding agar; distinctive appearance
C. krusei (6)	Large, flat, spreading, pale pink colonies with matt surfaces; distinctive appearance
C. guilliermondii (5)	Small pink to purple colonies; variable appearance
C. dubliniensis (1)	Dark green colonies
Trichosporon spp. (37)	Tiny, rough, dry-looking, dirty blue-to-grey colonies, distinctive appearance
C. neoformans (2)	Pink colonies, sometimes mucoid
S. cerevisiae (2)	Small purple colonies

Source: Koehler et al., 1999

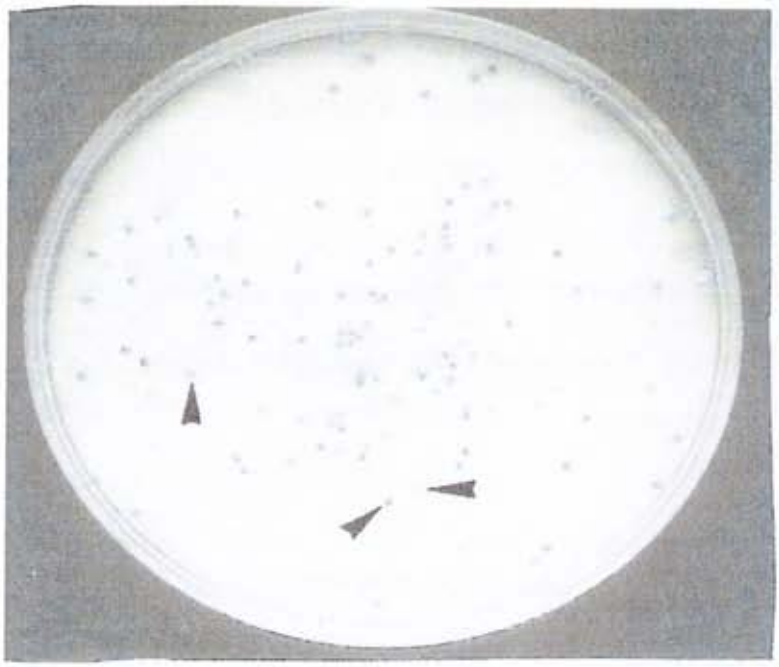

Figure 7: The above picture is a primary culture of an oral rinse on Chrom agar *Candida* after 48 hrs at 37 degrees celcius showing three different shade of green (arrowheads), all finally identified as *C. dubliniensis.*
Source: Tintelnot, 2000

The API 20C yeast identification strip (Analytab Products, Div. ofAyerst Laboratories, Plainview, N.Y.), was one of the first commercial products to be introduced for testing medically important yeasts. It provided media in dehydrated form for testing both carbohydrate fermentation and carbohydrate assimilation. Then a clinical evaluation of the system with an extensive data base consisting of at least 20, and in some cases over 100, isolates of each of 16 yeast taxa is performed. With this system, a binomial profile number can be derived from growth patterns on 19 substrates. This profile number provides a convenient method for comparing unknown yeast isolates with those numerical profiles in the data base in order to derive an identification of the unknown. These properties, when used in combination with morphological characteristics, permitted identification of many yeast species with a respectable level of

reliability (Land et al., 1979). However, this method is also time consuming and cumbersome.

The biolog microstation system is another newly developed yeast identification system. Isolates are put in aqueous suspensions in microplates and then the system uses 94 biochemical tests to distinguish the various yeast TAXA contained with in its Database. However, this method is very unreliable since the system is incapable of excluding isolates not included in its Database, and more importantly the identity of some isolates change substantially depending on the incubation period (McGinnis et al., 1996).

1.5.2 Identification of *Candida albicans* from *C.dubliniensis*

Various reports of yeast species *Candida dubliniensis* indicate a worldwide occurrence of this fungus, which is phylogenetically closely related to *Candida albicans* (figure 8). Evidence for the inducibility of stable fluconazole resistance in vitro in *C. dubliniensis* strains may indicate an emerging pathogen for immunocompromised patients receiving long-term fluconazole prophylaxis. Thus the correct identification of these two species is very important (Tintelont et al., 2000)

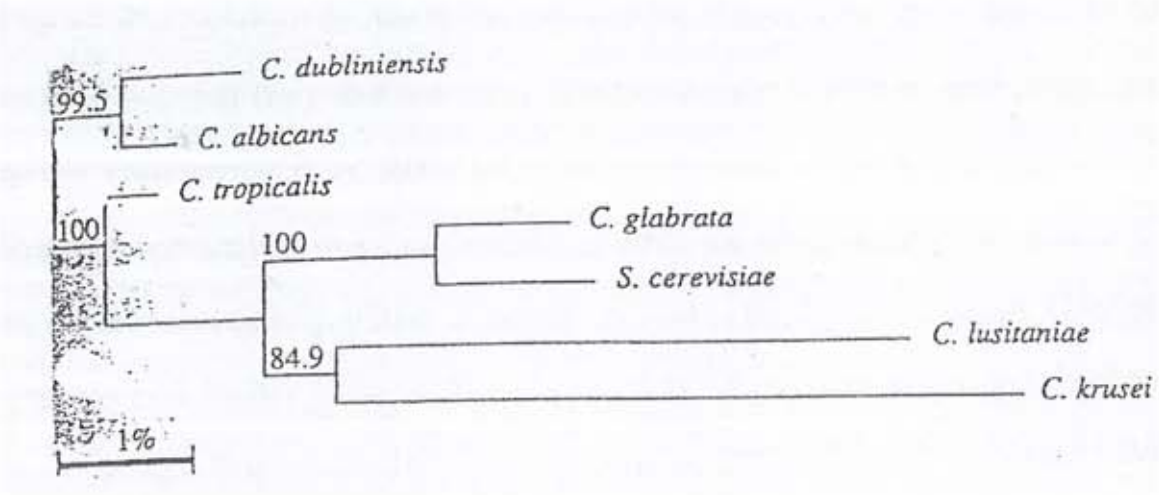

Figure 8: Phylogenetic tree showing the close evolutionary relation between *Candida albicans and C.dubliniensis*
Source: Gilfillan et al., 1998

Since *C. dubliniensis* is normally found in mixed cultures with *Candida albicans*, several methods for the identification of *C. dubliniensis* and discrimination from *Candida albicans* have been reported, (Tintelnot et al., 2000) these include:

i- The formation of dark green colonies on Chrom agar *Candida* mentioned earlier.

ii- No or strictly reduced growth of *C. dubliniensis* at 45 degrees celcius (Pinjon et al., 1998):

The data from the study done on 120 isolates of *Candida dubliniensis* and 98 of *albicans* and the reproducibility of the results indicate that growing isolates at 30 and 45 degrees celcius is a simple reliable method to differentiate these two strains since *C. dubliniensis* cannot grow at 45 degrees celcius.

iii- *C. dubliniensis* often shows characteristic chlamydospore formation (Tintelnot et al., 2000):

Both species *Candida albicans* and *C.dubliniensis* form chlamydospores which are found when cells are subjected to extremely poor media (corn-meal agar) in combination with decrease in oxygen availability, coverslip). The physiological status of chlamydospores is unknown. There are no reports of there germination nor are they found in clinical specimens. They are fragile structures compared to yeast cells and can be easily crushed under the microscope. Therefore, they seem to have no property to suggest that they are robust and adapted for survival and dormancy like those of bacteria (Sullivan et al., 1995).

Chlamydospore formation in *C. dubliniensis* is more abundant than that in *Candida albicans*, with chlamydoconidia often attached in pairs, triplets, or larger clusters to the same suspensor cell rather than singly at the ends of hyphae or pseudohyphae as in *Candida albicans*, although this characteristic may not be present in all strains. Other *Candida species* may also show characteristic morphology on corn meal agar (table 2) which may play a role in their identification (Koehler, 1999).

Table2: Identification of clinical yeast isolates by growth characteristics on Corn-meal agar.

Species (no. of strains)	**Morphologic features on Cornmeal agar**
Candida albicans (50)	Chlamydospores present, although >2 days required for some strains; abundant pseudohyphae, some true hyphae, clusters of blastospores along pseudohyphae; distinctive appearance
C. glabrata (116)	No pseudohyphae or hyphae; yeast cells are ~2.5 × 4 μm; consistent distinctive appearance
C. tropicalis (87)	Abundant pseudohyphae often radiating with clusters of blastoconidia at the center; variable appearance
C. krusei (6)	Extensive branched pseudomycelium with chains of elongate cells giving tree-like appearance; clusters and chains of blastospores along pseudohyphae; consistent distinctive appearance
C. parapsilosis (47)	Branched chains of elongated cells with clusters of blastospores along them; occasional giant cells; variable appearance
C. guilliermondii (5)	Pseudohyphae with clusters of blastospores; variable appearance
C. dubliniensis (1)	Abundant chlamydospores present; abundant pseudohyphae, some true hyphae, clusters of blastospores along pseudohyphae
S. cerevisiae (2)	Oval cells with very short multilateral budding

Source: Koehler, 1999

1.5.3 Molecular techniques

Advances in molecular biology in the last 2 decades have allowed the development of rapid molecular genotyping techniques for clinical and epidemiological analysis. Several molecular typing methods have been developed to differentiate *Candida albicans* strains, including electrophoretic karyotyping and the use of species-specific probes such as Ca3 or 27A in restriction enzyme analysis (Sampio et al., 2003). It is note worthy to state that almost all molecular advances in the techniques developed for *Candida albicans* are derived from studies done on *Saccharomyces cerevisiae.* This is the case because it is a model organism for experimental investigation in molecular biology. It has a short life cycle, is easily amenable to modern biochemical and genetic techniques, and shares a wide array of basic cellular processes with higher eukaryotes, including human. On the other hand *Candida albicans* is a diploid with no known sexual cycle and thus no haploid stage to simplify genetic analysis (Froche et al., 99).

A new important method depends on short tandem repeats (STRs) or microsatellites has assumed increasing importance as molecular markers in fields so diverse as oncogenetics, population genetics, and strain identification and characterization. Microsatellites occur in several thousands of copies dispersed throughout the genome and display high polymorphism, Mendelian codominant inheritance, and PCR typing simplicity. Only a few polymorphic microsatellite loci have been identified so far in the *Candida albicans* genome, most of them located near or inside coding regions and exhibiting a high discriminatory power However, it is known that the degree of polymorphism is much higher in microsatellite loci from noncoding regions, and to date, few studies have

been developed for the analysis of loci from those regions in *Candida albicans* (Sampio et al., 2003).

Although these published PCR methods are quite useful for identification of fungal species, they still require a minimum of several hours for DNA amplification and visualization. More recently, real-time PCR techniques have been developed for the detection of fungal pathogens such as*Candida species* (Hsu et al., 2003). This technology combines rapid thermocycling with glass capillaries with online fluorescence detection of the PCR amplicon. Cycling is achieved by alternating heated air and air of ambient temperature inside the light cycler (Roche Diagnostic, Germany). The detection system is based on fluorescence resonance energy transfer (FRET) with two different specific oligonucleotides. Hybridization probe 1 (FL) is labeled with fluorescein, and hybridization probe 2 (LC) is labeled with the fluorophore Light Cycler Red 640 (Loeffler et al., 2000a). Both probes could hybridize in a head-to-tail arrangement, bringing the two fluorescent dyes into close proximity (figure 9).

During fluorescence energy resonance transfer (FRET), fluorescein is excited by the light source of the LightCycler instrument. The excitation energy is transferred to the acceptor fluorophore, LightCycler-Red 640, and the emitted fluorescence is measured by the photohybrids of the instrument. The fluorescence is monitored while the temperature is slowly increased; the Fluorescence decreased when one of the probes melted off and the two fluorescent dyes were no longer in close proximity. Any thing not matching the sequence of the probe would not attach them and thus no signal is detected or if there is attachment but

not with a perfect match the probe will melt off at a lower temperature than the designated one (Loeflller et al, 2000b).

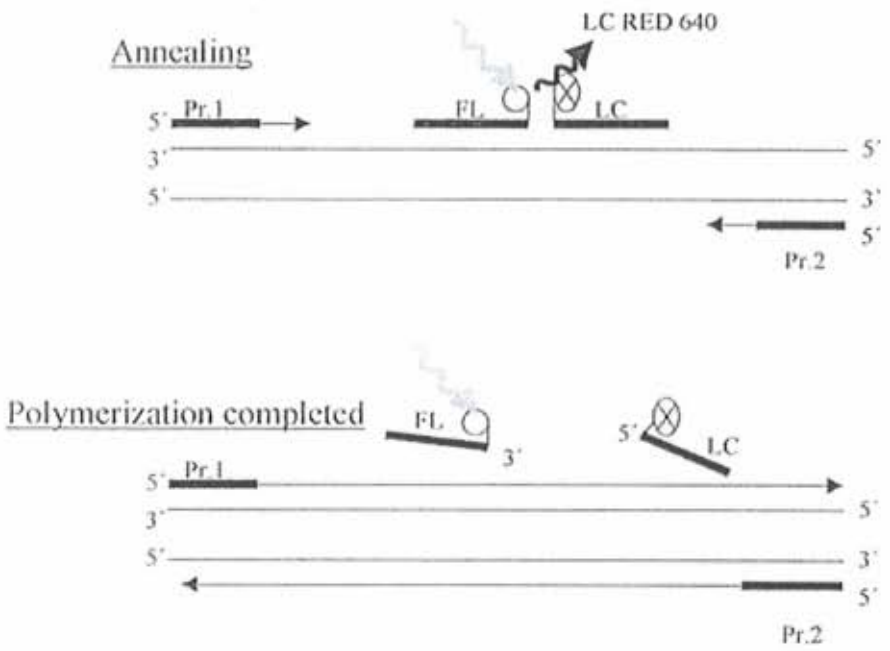

Figure 9: Hybridization probe format overview. Probe FL is labeled with fluorescein, and probe LC is labeled with Light Cycler Red 640 fluorophore. During annealing, the excitation energy is transferred to the acceptor fluorophore, Light Cycler Red 640 fluorophore.

Source: (Loeffler et al., 2000a)

It is note worthy to state that identification of microorganism via the real time PCR has been established for several years. Many infectious agents such as Hepatitis B, *Bordettella pertusis, helicobacter pylori* and many others already have techniques for lightcycler identification that are being thoroughly used (Meuer et al., 2001).

The Advantage of the real time PCR technique and particularly the one used for *Candida* identification lies in the following. The real time PCR

is relatively fast. Sample preparation may take up to 20 minutes (versus few minutes in normal PCR), however, running the samples will not take more than 70 to 90 minutes (versus several hours with the normal PCR).

The specificity of this PCR technique lies also in the primers used. In the particular case of identifying *Candida albicans* via a kit from Roche Diagnostic, Germany, the primers are derived from Internal Transcribed Spacer region (ITS) region of the fungal genome. As seen in figure 10; the Internal Transcribed Spacer region is divided into two parts, the ITS1 lying between the 18s and the 5.8s rDNAs and the ITS 2 lying between the 5.8s rDNA and the 28 s rDNA (Hsu et al., 2003).

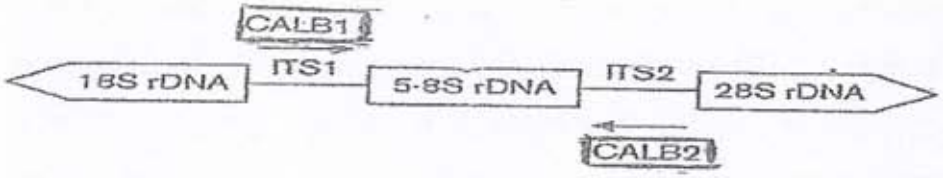

Figure 10: The figure represents the ITS region and the specific primer sites for the real time PCR. CALB1 is the forward primer, CAlB 2 is the revers primer. Thus, totally PCring the 5.8S rDNA.
Source: Hsu et al., 2003

In a study performed by Chen et al. DNA sequence analysis of amplified ITS1 region DNA from 79 isolates revealed species-specific ITS1 alleles for each of the 40 pathogenic species examined. This provided identification of unusual clinical isolates, and 53 diagnostic ITS1 sequences were deposited in GenBank. Phylogenetic analyses based on ITS sequences showed a similar overall topology to 26S rRNA gene-based trees. However, different species with identical 26S sequences

contained distinct ITS alleles that provided species identification with strong statistical support. Together, these data indicate that the analysis of ITS polymorphisms can reliably identify 40 species of clinically significant yeasts including*Candida species* (Chen et al., 2001).

The lightcycler system offers another advantage of analysis of the melting temperature of the amplicon. The melting temperature of the amplicon is dependant on the G+C content, sequence length and compositional variation in the nucleotide bases. Each fungal species has a characteristic Tm, which helps further to confirm its identity.

Further more, real time PCR assays dramatically decrease the risk of false positive results, because the PCR and detection systems are coupled and conducted in a closed system and no laborious post-PCR analyses are required and no cross contamination (Hsu et al., 2003).

1.6 Purpose

The purpose of this experiment is to identify *Candida albicans* from clinical samples based on the real time light cycler PCR using the Lightcycler *Candida* Kit. The results are then compared with those given to us by the Lebanese hospitals and to those of the germ tube test performed at the Lebanese American University. All this is done in aim of assessing the accuracy of *Candida albicans* diagnosis done by the Lebanese hospitals, keeping in mind that accurate diagnosis is essential for healthier and more effective treatment.

Furthermore, the positive samples will be cultured at 30 degrees celcius and 45 degrees celcius and the results will be compared. This will be done to prove that there is no cross reaction in this technique between *C.dubliniensis* and its closely related relative *Candida albicans*.

Chapter 2

MATERIALS AND METHODS

2.1 Sample collection

One hundred samples were collected from four different Lebanese hospitals: American University of Bierut MC, Hotel Dieu de France, St. Georges, and Sahel hospital (the majority of which use the germ tube test, mainly and occasionly the Chrom agar to identify *Candida albicans*). I was granted full authorization by the collectors of the samples Ghida Barada and Raida Basma to use the samples as needed and to publish any data obtained. In addition, I was granted to permission to publish and discuss results of the germ tube test done by them on these samples in sake of comparison. The samples were purified after collection and stored in the deep freeze at -80 degrees celcius.

2.2 DNA extraction

The samples were obtained from the deep freeze and immediately cultured on Potato Dextrose Agar (PDA) at 30 degrees celcius for 48 hrs. The agar was prepared by adding 19.5 g of ready made PDA and 10 g of glucose along with 500 ml of distilled water in a flask. The whole mixture was autoclaved at 121 degrees celcius for 15 minutes. After cooling the flask, the media was poured in sterile dishes and kept to dry overnight.

Then one colony was taken and grown overnight at 30 degrees celcius in 5 ml of Potato Dextrose Broth (PDB) in sterile centrifugable tubes. This was done to obtain a pure culture of the same species with no contamination. The PDB was prepared by adding 20 g PDB with 10 g glucose along with 500 ml distilled water in a flask. The whole mixture was autoclaved at 121 degrees celcius for 15 min and left to cool overnight.

DNA extraction steps were done as follows:

1- Centrifuge the tubes for 5 min at 12000 rpm and decant the supernatant
2- Wash the pellet with 5 ml of a sterile solution of 1M Sorbitol and 0.1M Na2EDTA- PH 7.5
3- Centrifuge the mixture for 5 min at 12000 rpm, then decant supernatant
4- Resuspend the pellet in 0.5 ml of the same solution of step2
5- Transfer the content into a micro centrifuge tube
6- Add 1 µl B- mercaptoethanol to each tube.
7- Add 100 µl lyticase (10 mg/ml)
8- Incubate at 37 degrees celcius for 2 hrs and 30 min
9- Centrifuge the tubes for 1 min at 12000 rpm then carefully remove the supernatant
10- Resuspend the pellet in 0.5 ml of a filtered solution containing: 50mM Tris-HCl (ph: 7.5) , 20mM Na2EDTA , and 1 % SDS
11- Incubate at 60 degrees celcius for 30 min
12- Add 0.2 ml 5 M Pottassium Acetate (KAc)
13- Keep the mixture on ice for 60 min

14- Centrifuge the tubes for 5 min at 12000 rpm then transfer the supernatant into a new tube

15- Add 0.5 ml PCIA (Phenol 25 : chloroform 24 : isoamyl alcohol 1) , vortex and centrifuge for 5 min at 12000 rpm

16- Add 0.5 ml of CIA (chloroform 24: Isoamyl alcohol 1) to the supernatant , vortex , spin for 2 min at 12000 rpm

17- Transfer the supernatant to a new tube and add 1 ml Ethanol (ETOH) (95%) , keep on ice for 10 min

18- Centrifuge for 5 min at 12000 rpm, remove the supernatant, and add 1 ml ETOH (75%)

19- Centrifuge for 2 min at 12000 rpm, dry the pellet, resuspend it in 50 µl double distilled sterile water

20- Add 2 µl Rnase- A (10mg/ml)

21- Incubate for 30 min at 37 degrees celcius

22- Add 250 µl double distilled sterile water

23- Repeat step15 to 19

The tubes were then stored at – 20 degrees celcius until the PCR procedure was initiated

2.3 Lightcycler machine preparation:

The lightcycler instrument (Roche Diagnostic Science, Germany) was programmed before starting the experiment and preparing the working solution as follows:

The protocol for the lightcycler instrument consists of four separate programs summarized in tables 3 to 6 (Roche Diagnostic Sciences, Germany)

Table3: Program 1, Denaturation, set the values as follows:

Cycle Program Data	**Value**
Cycles	1
Analysis mode	None
Temperature target	**Segment 1**
Target temperature	95
Incubation time (min.s)	10:00
Temperature transition rate	20
Secondary target temperature	0
Step size (c)	0.0
Step delay (cycles)	0
Acquisition mode	None

Table 2: Program 2, PCR amplification

Cycle program data	**Value**		
Cycles	45		
Analysis mode	Quntification		
Temperature target	**Segment1**	**Segment2**	**Segment 3**
Target temperature	95	50	72
Incubation time (min.s)	10	15	10
Temperature transition rate	20	20	20
Secondary target temperature	0	0	0
Step size (c)	0.0	0.0	0.0
Step delay (cycles)	0	0	0

Acquisition mode	None	None	None

Table 5: Program 3, Melting curve

Cycle program data	**Value**		
Cycles	1		
Analysis mode	Melting curve		
Temperature target	**Segment1**	**Segment2**	**Segment 3**
Target temperature	95	40	80
Incubation time (min.s)	60	60	0
Temperature transition rate	20	20	0.1
Secondary target temperature	0	0	0
Step size (c)	0.0	0.0	0.0
Step delay (cycles)	0	0	0
Acquisition mode	None	None	None

Table 6: Program4, Cooling

Cycle Program Data	**Value**
Cycles	1
Analysis mode	None
Temperature target	**Segment 1**
Target temperature	40
Incubation time (min.s)	0:30
Temperature transition rate	20
Secondary target temperature	0
Step size (c)	0.0
Step delay (cycles)	0
Acquisition mode	None

The fluorescence channel are then set before or after the run at display mode: f2/ back-f1

2.4 PCR Procedure

Before starting the procedure one should be sure to have:

1. sterile filter eppendorph tips along with their appropriate pipettes
2. a box of sterile lightcycler glass capillaries
3. A pre-cooled centrifuge adapters for the capillaries
4. a bucket of ice
5. the *Candida* lightcycler kit containing the ingredients mentioned in Table 7

After preparing the above, the master mix was prepared by multiplying the amount in the Volume for one PCR column by the number of reactions to be run, plus one to allow for aliquoting discrepancies.

Then the PCR procedure was followed as mentioned in Table 8.

Table 7: Kit ingredients

Vial/cap	**Label**	**Contents/function**
1a red cap	Reaction mix a	32 µl enzyme soulution containing faststart taq polymerase
1b colorless cap	Reaction mix b	2 x 80 µl solution containing faststart taq DNA Polymerase reaction buffer , and the dNTP mix
2 green cap	Detection mix	2 x 80 µl solution of primers and hybridization – probe mixture ,specific for *Candida albicans* and the *Candida* – specific internal control
3 purple cap	Internal control	360 µl stabilized solution of plasmid DNA
4 yellow cap	Positive control	50 µl stabilized solution of plasmid DNA
5 colorless cap	H20 , PCR-grade	1000 µl nuclease-free, water for dilution of reaction mixtures

Table 8: PCR procedure

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>• store the reaction mix a on ice
• thaw the remaining ingredients and the samples , mix gently ,and store on ice</td></tr>
<tr><td>2</td><td>In a 1.5 ml reaction tube on ice , add the following components in the order mentioned bellow
<table>
<tr><th>Componenet</th><th>Volume for one PCR</th></tr>
<tr><td>Vial 5</td><td>11 µl</td></tr>
<tr><td>Vial 2</td><td>2 µl</td></tr>
<tr><td>Vial 3 (1 in 10 dilution)</td><td>1 µl</td></tr>
<tr><td>Vial 1 b</td><td>1.67 µl</td></tr>
<tr><td>Vial 1 a</td><td>0.33 µl</td></tr>
<tr><td>Total volume</td><td>15 µl</td></tr>
</table></td></tr>
<tr><td>3</td><td>• mix gently by pipetting. Do not vortex
• pipette 15 µl master mix into lightcycler capillary
• add 5 µl of the template DNA (sample DNA, Lightcycler Candida positive control, and negative control,(distilled water))</td></tr>
<tr><td>4</td><td>• seal each capillary with a stopper and place the adapters, which contain the capillaries into a standard microcentrifuge
• centrifuge for 5s at 3000 rpm</td></tr>
<tr><td>5</td><td>Transfer the capillaries to the sample carousel of the lightcycler instrument</td></tr>
<tr><td>6</td><td>Cycle the sample as described in the program above</td></tr>
</table>

According to the kit, to specify whether the result is positive or negative two resulting graphs must be analyzed on the lightcycler computer system. The results of the quantification curve and that of the melting curve must both be analyzed on the display mode of channel f2/ back-f1 for the samples and f3/back-f1 for the internal control.

For the quantification curves the results from both display channels f2 and f3 are compared and the results are interpreted as seen in table 9.

Table 9: Interpretation of the results

Candida albicans **(channel f2)**	**Internal control (channel f3)**	**Result interpretation**
+	+	Sample positive
–	+	*Sample negative*
+	–	Sample positive
–	–	*Invalid result*

However, complete analysis is based on the melting curve. Thus, melting curve is necessary for the correct interpretation of results. Once the result is positive then the sample should display a melting curve with a peak Tm at 57.5 + or – 2 degrees in the f2 channel which is the melting temperature for the probes that are specific for *Candida albicans* with a perfect match.

The invalid results mentioned might be considered true negative (not *Candida albicans*) despite the fact that the internal control (IC) is also negative in certain conditions mentioned below.

The IC is a synthetic double stranded DNA molecule with primer binding sites identical to the *Candida albicans* target sequence, which contains a unique Hybridization Probe binding region that differentiates the IC from the target amplicon (figure 11).

The internal control is detected in channel F3, whereas the *Candida albicans* DNA is detected in channel f2. In order to avoid influencing PCR sensitivity the IC is spiked into the sample in a low concentration. In case of a *Candida albicans* – negative sample (samples that contain no DNA), the IC must be positive (which was the case here). In *Candida albicans* positive samples, the internal control is allowed to give negative.

Results maybe viewed were the IC is negative and the sample is negative which can be due to high amounts of DNA from other *Candida* than *Candida albicans* or from other fungal species; including those mentioned in the specificity list. DNA from these species could be amplified by the lightcycler kit but would not be detected by the hybridization probes. In these cases, amplification of the Internal Control might be suppressed, due to its competitive behavior (Roche Applied Science, Lightcycler *Candida* Kit).

Once the positive control run along with the samples was positive and the negative control which contained no DNA (distilled water instead of the sample) was negative and its internal control was detected positive (no inhibition) then one can safely assume that the negative result of the samples are valid.

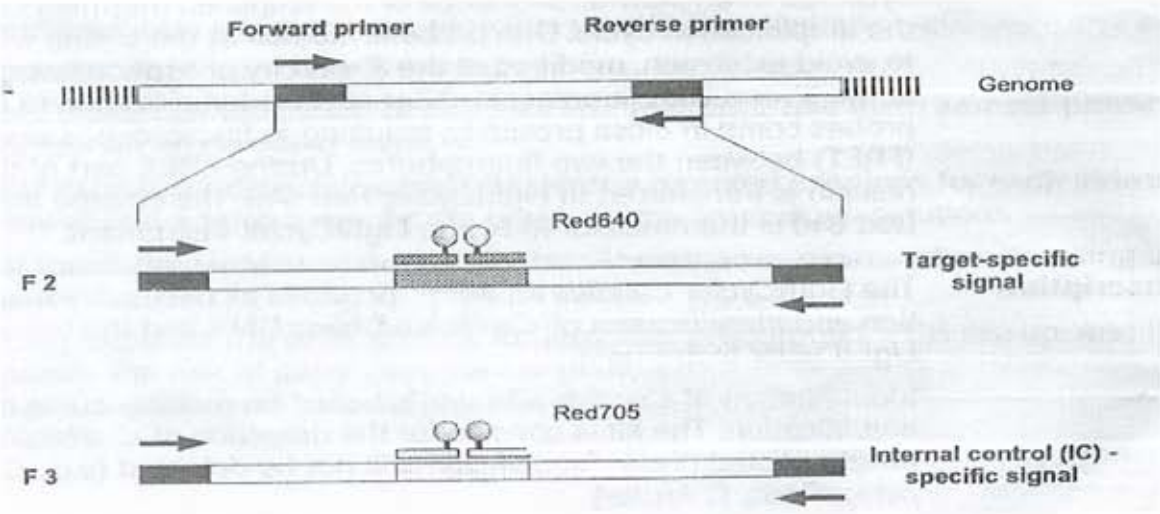

Figure 11: Internal Control Concept

Source: light cycler *Candida* Kit, Roche Applied Science; Germany

2.5 Growth at 45 degrees celcius:

All the samples were initially grown at 30 degrees celcius on PDA for DNA extraction. Once the results were obtained, one colony was taken from the cultures of all positive samples and grown overnight in 5 ml PDB. Then each sample was replated on PDA and put for 48 hrs at 45 degrees celcius. Growth was checked after 48 h.

2.6 Chi- square test:

A Chi- square test was performed on the data collected using Sigmastat computer software to asses the significance of the total discrepancies.

RESULTS

3.1 A sample of resulting curves:

Figures 12, 13 and 14 constitute a complete graph analysis of four samples along with a positive control and a negative control all in one run.

Figure 12 represents 6 melting curves analyzed at channel f2/ back f1 Sample 24 and the negative control are showing no melting curve peak between 55.5 and 59.5 degrees Celsius. However, samples 1, 7, and 15 along with the positive control are showing four separate melting peaks lying between the above mentioned temperatures. On the other hand, figure 13 is the quantification analysis curve in the F2/ backf1 channel. This graph indicates a positive control that is positive along with three other samples 1, 7 and 15; and a negative control that is negative along with sample number 24. Finally, figure 14 represents the quantification analysis graph in the f3/back f1 channel (that of the internal control). This graph shows the internal control of all the samples as positive except that of sample 24.

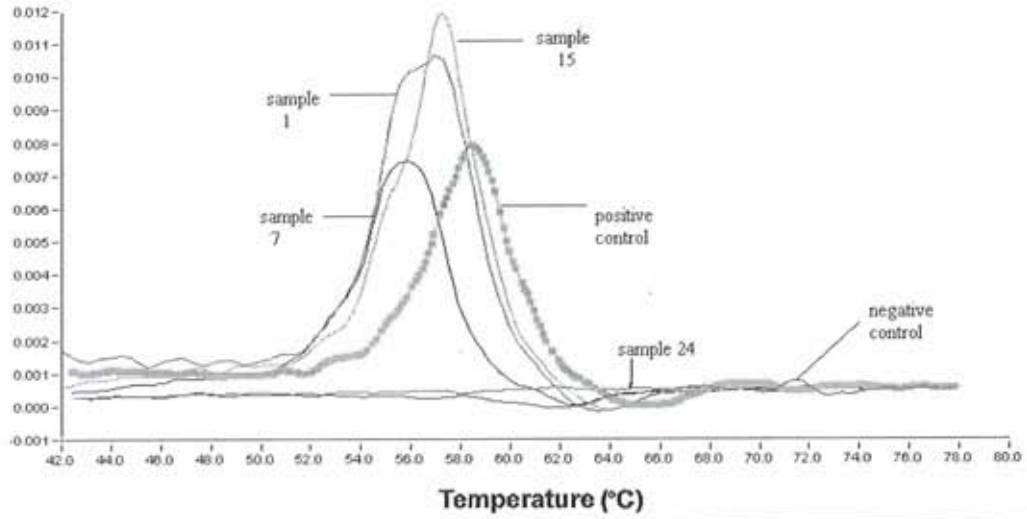

Figure 12: Melting temperature analysis curve
Samples 1, 7, and 15 are showing a positive melting peak. Sample 24 is negative showing no melting peak.

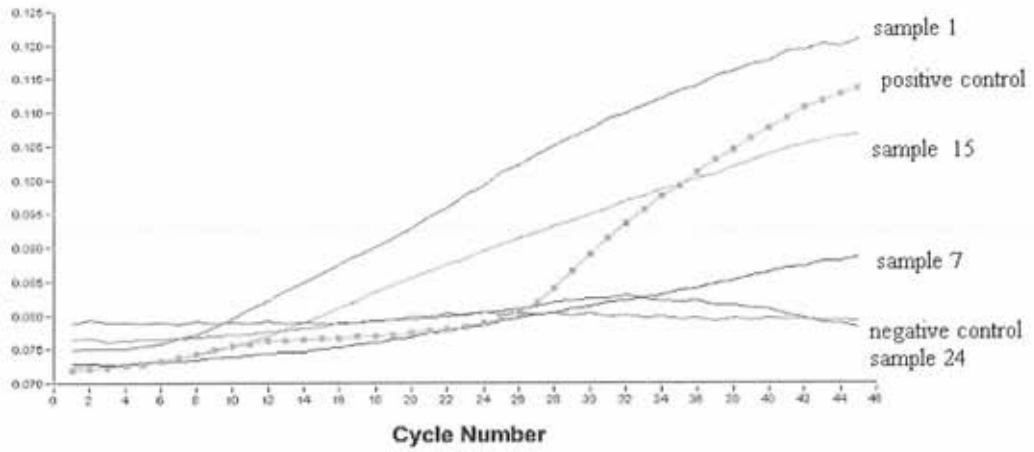

Figure 13: Quantitative analysis Curve at channel f2

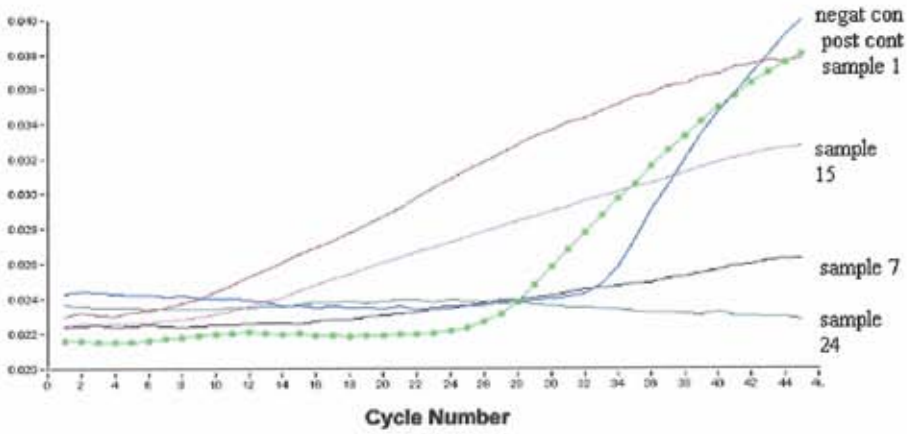

Figure 14: Quantitative analysis curve at channel f3

3.2 Results of growth at 45 degrees celcius

Figure 15 and 16 represent the same sample of *Candida albicans*, the first grown at 30 degrees celcius and the other grown at 45 degrees celcius. Both showed equal growth, indicating that it is not *C. dubliniensis.*

A total of 76 samples that were identified as *Candida albicans* by the light cycler were cultured and showed similar result to this particular sample (permissive to growth at both temperature).

Figure 15: sample 1 at 30 degrees
Showing growth

figure 16: sample1 at 45 degrees
showing the same amount of growth

3.3 Summary table of all the results

After analyzing all melting and quantification curves along with the internal control of each sample the following results were obtained summarized in tables 10, 11 and 12. The tables also include result of a germ tube test (done by the two students who collected the samples).

N.B: All the samples collected were diagnosed as *Candida albicans* by the Lebanese hospitals

Table 10: light cycler results versus the hospital

Sample location	Sample number	LC results	Hospital results
Urine	3	-	+
	16	+	+
Bronchial alveolar lavage	2	_	+
	7	+	+
Abdominal	2	+	+
Sputum	7	_	+
	28	+	+
Deep tracheal asp	1	-	+
	3	+	+
Stool	10	_	+
	9	+	+
Wound	4	+	+
Vaginal	3	+	+
Tooth	1	_	+
Peritoneal fluid	1	_	+
Pus	1	+	+
Blood	1	+	+
Nail	1	+	+
Total	100	24 – 76 +	0 - 100 +

Thus, we totally have 24 samples that show discrepancies between the light cycler and the hospital results. A Chi- Square test was done on the data in the last raw. The data was found to be highly related and significant with $p < 0.001$.

Table 11: comparison of the results of both tests depending on body locations

Sample location (total number)	Number of samples	Light cycler result	Germ tube result	Remarks
Urine (19)	1	+	–	
	3	–	+	3 showed true hyphae
	15	+	+	
Bronchial alveolar lavage (9)	2	–	–	
	7	+	+	
Abdominal swab and fluid (2)	2	+	+	
Sputum (35)	2	+	–	
	6	–	+	
	26	+	+	
	1	–	–	
Deep tracheal aspirate (4)	1	+	–	
	2	+	+	
	1	–	–	
Stool (19)	1	+	–	
	6	–	+	1 showed true hyphae and was considered positive
	8	+	+	
	4	–	–	
Wound (4)	1	+	–	

	3	+	+	
Vaginal (3)	3	+	+	
Tooth (1)	1	_	+	It showed true hyphae but was considered positive
Peritoneal fluid (1)	1	_	+	
Pus (1)	1	+	+	
Blood (1)	1	+	+	
Nail (1)	1	+	+	
Total	100	76 + 24-	87 + 13 -	

Table 12: comparison between the total results of light cycler and those of germ tube

Number of samples	**Light cycler (LC) results**	**Germ tube (GT) results**	**Chi- square test P**	**Remarks**
6	+	_	**= 0.024**	
17	_	+	**< 0.001**	5 showed true hyphae were considered positive
7	_	_		4 showed pseudo hyphae were considered negative
70	+	+		3 showed true hyphae were considered positive

The raw data is found in table 13 in the appendix.

Chapter 4

Discussion

The increasing frequency of invasive fungal infections and the high mortality associated with disseminated fungal diseases have underscored the importance of rapid detection of pathogenic fungi. Prompt detection and accurate speciation may help to improve fungal disease management as a whole and lead to more rational use of anti-fungal drugs. Traditional identification methods based on phenotypic features are often time-consuming and depend largely on the skill and experience of the technician (Hsu et al., 2003).

Therefore, we tried to evaluate the Light cycler *Candida albicans* identification kit which is based on the PCR of the rDNA region followed by melting-curve analysis for identification in comparison with one of the most used method for *Candida albicans* identification in hospitals- the germ tube test.

Lightcycler versus hospital identification:

The one hundred samples used in this study given to us by the Lebanese hospitals were all presumably identified as *Candida albicans* and thus the patients were treated on this basis. The samples as seen in the tables were gathered from different body locations.

However, according to the results obtained from the lightcycler 24% of the samples where not identified as *Candida albicans*. Thus, they gave a negative result by showing no signal emission at the f2 detection channel, indicating no amplification product and thus no annealing of the specific probes in a head to tail manner to emit a signal. Further more, no peak was observed in the melting curve analysis at 57.5 + or – 2 degrees celcius, since the specific probes where unable to anneal head to tail and emit a signal that can be traced as the temperature increases. Although, their internal control was negative that is because all the samples contain a large number of fungal DNA which was identified by the hospital as *Candida albicans.* The internal control was inhibited as explained in the materials and methods (refer to page 36).

Once the Chi- square test was performed comparing the two results, the significance p was <0.001. This indicates that the results are highly related and the discrepancy is very significant.

The largest percentage of sample discrepancy was seen in the stool samples. More than 50% of the samples were misidentified as *Candida albicans*. It is important to note that the stool has the largest number of microorganisms that inhabit the intestine as part of the normal flora *Candida species* are one of the many species that inhabit the intestine thus various types might be present in the stool. These *Candida species* might resemble *Candida albicans* in the various tests that depend on phenotypic characteristic.

Further more, urine and sputum are also other locations were we have a significant amount of misidentification. This is probably due to the same reasons mentioned above.

Out of the 24 samples proven to be negative, 7 were confirmed negative by simply repeating the germ tube test. Another 5 were seen directly at the true hyphae stage and were considered *Candida albicans* as is the case in most hospitals identification. This should not be the case because many species other than *Candida* filament giving filaments that resemble the true hyphae. The isolates were only observed between 2 and 4 hrs post gern tube induction in an effort to mimic hospital identification technique. It is most probable that if the test was repeated carefully (by looking at the microscope every 2 to 3 minutes) these samples would have not passed through the germ tube stage before filamentation. Thus, around 12 samples did not show a definite germ tube and might be considered true negative, thus, they are in support of the light cycler result.

Lightcycler versus germ tube results:

Once the samples from the hospitals were received, the germ tube test was repeated at LAU by two of my colleagues. The microscopic examination occurred at 4 hrs.

17 samples showed a false positive result in the germ tube test. This difference appeared to be significant on the Chi-square test was performed. They showed a p less than 0.001. On the other hand, 6 samples showed a false negative result. The discrepancy in this case was not as significant but it did show minor significance with a p of 0.024.

Although, this test is highly specific and has been relied on for years, it is still highly subjective. The new cellular material that composes a germ tube represents true hyphae that do not show points of constriction. A constricted germ tube represents pseudohyphae formation derived from a budding process of the blastoconidia. Both constricted and nonconstricted "germ tubes" may be seen in the germ tube test for *Candida albicans*. However, if the preparation appears to contain only constricted germ tubes, one should seriously consider the possibility of other *Candida species* like, for example *Candida tropicalis* that forms constricted germ tube exclusively (figure 15). We have 12 samples that presumably showed germ tubes but were later identified as non-*Candida albicans*.

Again the samples recovered from the stool showed the highest rate of false positivity, followed by the urine and sputum. This is also the case because the contamination of the sample by various *Candida species* from the normal flora that might show a constricted germ tube and cause confusion.

One should also be able to view the germ tube at exactly the right time between 2-4 hrs before the stage is missed and the cell has already developed pseudohyphae or true hyphae because almost all non- albicans *Candida* form one form of filamentation (pseudo or true hyphae) without passing through the germ tube state. 5 samples were already in a true hyphae stage when viewed for this test and were considered positive but later proven by the LC to be negative. 4 out 5 of these samples were samples of stool and urine.

Its note worthy to say that *Candida albicans* should not form pseudohyphae at 37 degrees Celsius (the temperature of the GT test). The four samples that were seen at the pseudohyphae stage in the GT test were all proven negative for *Candida albicans* by the light cycler. The former statement is more proof of the accuracy of the LC and proof of misidentification by the hospitals.

Further more, it has been found that more than 5% of *Candida albicans* species that have been identified to be germ tube negative (Crist et al., 1996). Out of the 100 samples, 6 samples (6%) showed no germ tube formation but were proven to be positive for *Candida albicans.* Thus, via the LC we obtained around 5% *Candida albicans* that were germ tube negative which similar to the known statistics regarding this matter. One can also notice that the germ tube negative samples are scattered between all body locations (urine, stool, sputum, deep tracheal aspirate, wounds). Thus, this is logical because this is related to a kind of *Candida* and should not have preferences between body locations.

Moreover, in addition to being subjective this test cannot differentiate between *Candida albicans* and *C. dubliniensis* since they both form germ tube (Tintelnot et al., 2000), thus another explanation for the discrepancy.

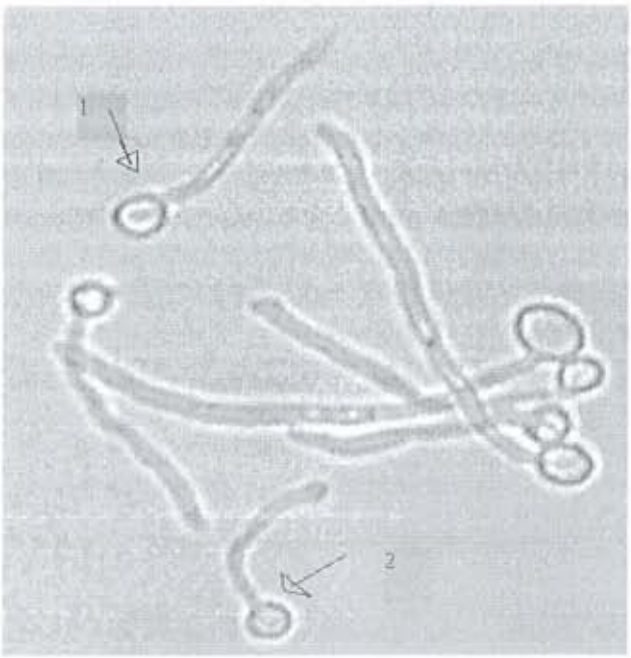

Figure 17: a germ tube test for *Candida albicans*
1 is constricted germ tube (not true)
2 is a nonconstricted true germ tube

Light cycler and *Candida dubliniensis*

Since the Kit does not mention any possible cross reactivity testing with *Candida dubliniensis*, all positive results were cultured at 45 and 30 degrees celcius. All the samples showed growth in both temperature conditions. This is further proof of the specificity of the kit and the technique. These results are in concurrence with the results of the study done by Chen et al that proves the ability of the ITS region polymorphism to differentiate between *Candida albicans* and *C. dubliniensis*

In conclusion, the real time PCR technique used is simple, rapid, specific and sensitive. Considering the above mentioned facts, it is recommended that Lebanese hospitals reconsider their diagnostic techniques. Out of the 100 samples, 24 were proven to be negative (13 of which are also proven to be negative for *Candida albicans* once the germ tube test was repeated).

It may not be practical or feasible for Lebanese hospitals to consider identification via molecular techniques yet. The molecular machinery such as the light cycler may not be available and is still considered expensive especially factoring in the cost of materials and kits. However, in the long run it will become cost-effective, taking into consideration two important facts. First of all, anti-fungal drugs are quite expensive and thus excessive use of these drugs wrongfully, followed by switching the drug until the correct treatment is reached will eventually outweigh the cost of the lightcycler or other molecular identification. Second and more importantly, one must consider the impact of false identification on the public health. Emergence of new drug resistant species of pathogenic fungi due to wrong and prolonged treatment is detrimental to the society especially to immunocompromised individuals. Funding research to find new drugs that target these resistant species is extremely expensive, and thus, would also easily outweigh the cost of molecular identification.

Thus, in the short run Lebanese hospitals should use cheap conventional methods in couples (two techniques in parallel) for better conformation of their results until the more precise molecular techniques become more readily available.

In the end, one could recommend that a wide comparative study could be done that factor in all conventional diagnostic assays along with at least two molecular techniques on samples obtained from the Lebanese hospitals. This set of experiments would give the above study the necessary credibility and would further reinforce the advantages and the accuracy of molecular identification.

In addition, the molecular identification could be further broadened to include identification of *Candida albicans* subspecies found in Lebanese hospitals. This could be done by comparing DNA fingerprint profiles of all isolates. This study could help in the development of targeted drugs that are subspecies specific and hence more efficient.

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Appendix

Summary of all data

Below is a table summarizing all the result of the experiments done on 100 samples gathered from four Lebanese hospitals presumably identified *as Candida albicans*.

Table 13: Raw data

sample location	**sample number**	**lightcycler ID as *Candida albicans***	**internal control**	**type of filamentation**
urine	1	Positive	positive	germ tube
	2	Positive	positive	germ tube
	3	Positive	positive	germ tube
	4	Positive	positive	None
	5	Positive	positive	germ tube
	6	Positive	positive	germ tube
	7	Positive	positive	germ tube
	8	Positive	positive	germ tube
	9	Positive	positive	germ tube
	10	Positive	positive	germ tube
	11	Positive	positive	germ tube
	12	Negative	negative	true hyphae
	13	Negative	negative	true hyphae
	14	Negative	negative	true hyphae
	15	Positive	positive	germ tube
	16	Positive	positive	germ tube
	17	Positive	positive	germ tube
	18	Positive	positive	germ tube
	19	Positive	positive	germ tube
bronchial	20	Positive	positive	germ tube
alveolar lavage	21	Positive	positive	germ tube
	22	Positive	positive	true hyphae

	23	Positive	positive	germ tube
	24	Negative	negative	psuedo hyphae
	25	Negative	negative	psuedo hyphae
	26	Positive	positive	germ tube
	27	Positive	positive	germ tube
	28	Positive	positive	germ tube
abdominal swab	29	Positive	positive	germ tube
abdominal fluid	30	Positive	positive	germ tube
sputum	31	Positive	positive	germ tube
	32	Positive	positive	germ tube
	33	Positive	positive	germ tube
	34	Positive	positive	None
	35	Positive	positive	germ tube
	36	Negative	negative	psuedo hyphae
	37	Positive	positive	germ tube
	38	Positive	positive	germ tube
	39	Positive	positive	germ tube
	40	Positive	positive	germ tube
	41	Positive	positive	germ tube
	42	Positive	positive	germ tube
	43	Positive	positive	germ tube
	44	Positive	positive	germ tube
	45	positive	positive	None
	46	positive	positive	germ tube
	47	positive	positive	germ tube
	48	positive	positive	germ tube
	49	positive	positive	germ tube
	50	positive	positive	germ tube
	51	positive	positive	germ tube
	52	positive	positive	germ tube
	53	positive	positive	germ tube
	54	positive	positive	germ tube
	55	positive	positive	germ tube
	56	positive	positive	germ tube
	57	positive	positive	germ tube
	58	negative	negative	germ tube
	59	negative	negative	germ tube

	60	positive	positive	germ tube
	61	negative	negative	germ tube
	62	positive	positive	germ tube
	63	negative	negative	germ tube
	64	negative	negative	germ tube
	65	negative	negative	germ tube
deep trachial asp	66	positive	positive	germ tube
	67	positive	positive	None
	68	positive	positive	germ tube
	69	negative	negative	None
stool	70	positive	positive	true hyphae
	71	negative	negative	germ tube
	72	positive	positive	germ tube
	73	positive	positive	germ tube
	74	positive	positive	germ tube
	75	positive	positive	germ tube
	76	negative	negative	None
	77	positive	positive	None
	78	negative	negative	psuedo hyphae
	79	positive	positive	germ tube
	80	negative	negative	germ tube
	81	negative	negative	true hyphae
	82	negative	negative	germ tube
	83	negative	negative	None
	84	positive	positive	germ tube
	85	negative	negative	germ tube
	86	positive	positive	germ tube
	87	negative	negative	germ tube
	88	positive	positive	germ tube
neck wound	89	positive	positive	germ tube
wound swab	90	positive	positive	germ tube
wound	91	positive	positive	None
	92	positive	positive	germ tube
vaginal	93	positive	positive	true hyphae
	94	positive	positive	germ tube
	95	positive	positive	germ tube
tooth	96	negative	negative	true hyphae

abscess				
peritonial fluid	97	negative	negative	germ tube
pus	98	positive	positive	germ tube
blood	99	positive	positive	germ tube
nail	100	positive	positive	germ tube
	wild type strain	positive	positive	germ tube