Chapter 1

INTRODUCTION

Yeasts and yeast like organisms are the more common etiologic agents of the mycotic diseases which are being isolated with greater frequency from patients who have alteration of the host's cellular defense and physiological change. Candida albicans and Cryptococcus neoformans are the well known causative agents. The improvement of various medical methods for prolongation of human life have concomitantly increased human susceptibility to colonization and superinfection with other genera and species of yeasts and yeast-like fungi (1,2,3). In 1978, Ahearn (4) had reviewed and reported that the death of patients due to candidiasis was increasing from 39 to 215 cases and accordirg to the cyptococcosis was increasing from 70 to 131 cases. Because of the steadily increase in the incidence of clinically significant disease caused by opportunistic yeasts (4-12).

Yeasts of medically important are including in 5 genera : Candida, Cryptococcus, Trichosporon, Rhodotorula and Geotrichum and about 20-30 species from about 600 known species in the literature are encountered as the etiologic agents (5). And the number of species implicated in the pathogenesis of compromised host has grown steadily (2). Since many yeast species constitute the normal flora in humans and most of the yeast and yeast like will have the same general appearance in tissue section. So that the rapid and correctly identification of these organisms in laboratory is very important for

further diagnosis and clinical management of patient (6). Specific identification of medically important yeasts is mainly based on morphological characteristics, which are primarily used to demonstrate genera whereas physiological characteristics are used to differentiate the various species.

The order to the identification of medical important yeast isolant (3), the first step of presumptive identification is based on microscopic morphology such as presence of germ tube, presence of chlamydoconidia on special media, presence of hyphae and pseudohyphae. The second step is based on some physiological characteristic such as present of phenol-oxidase enzyme, urease, and potassium nitrate assimilation test. When the former two steps could not specified the isolant, the last step is employed. It is based on assimilation and fermentation of carbohydrates for identification and ccnfirmation of isolants. Since the assimilation of carbohydrate was more rapid and accurate than fermentation of carbodrydrate so that this test was well developed for rapid and correct identification (13). During the last 10 years, many commercial kits on carbohydrate assimilation for rapid identification was available and time requirement was in 24-72 hours but their cost are more expensive than the manual (5,13-20). So we try to develope and modify many useful and rapid test for specification medical important yeasts in 24 hours or less.

The purpose of this study, first is the modification of method for carbohydrate assimilation test which can give rapid, low cost and accurate result. Second is to modify other rapid tests, urease test,

nitrate utilization test, L-DOPA paper strip test, that had been reported and used in differentiate species of medically important yeast. Third is collecting all results that have been studies and proposed the flow chart in differentiation and identification of medical important yeast.

REVIEW OF LITERATURE

Yeast and Yeast-Like as the Normal Flora and Saprophytes

Yeasts and yeast-like microorganisms are world-wide distribution. They are found in various sources such as soil, vegetable, honey, food product,breweries and clinical specimens from human and animal, etc. Some are very useful in food industries such as Saccharomyces cereviceae is used for the leavening of bread, for wine and breweries for the alcohol production (29), But some can cause the spoilage of food products, diseases in human and animals (29,30). For example foods and food products , Torulopsis can spoil sweetened condense milk, fruit-juice concentrate and acid foods; Candida lipolytica can spoil butter and oleomagarine, while C. krusei has been grown with diary starter culture to maintain activity and increase longevity of the lactic acid bacteria; Trichosporon found in breweries and on chilled beef; Rhodotorula may cause discoloration on food, e.g., color spot on meat or pink areas in sauerkraut (29).

Of the nearly 600 species of yeasts in the current literatures, only about 25 are commonly associated with human or have been documented as human pathogenic for man (5). The common yeast encountered as contaminants of bacterial culture or as transient flora of the human are the member of the asporogenous genera Rhodotorula, Candida, Trichosporon, and Cryptococcus. The saprophytic species, including several Rhodotorula and Trichosporon spp. are occasionally isolated from human disease and represented opportunistic pathogen

(1). Negroni et.al (31) isolated Rhodotorula from 2000 human specimens, mostly from skin, nails and mucous membrane, and found that 39 were positive cultures. They also found that Rhodotorula was isolated with dermatophytes and Candida but the pathogenicity of Rhodotorula could not be established in any of the cases.

A statistical study of the ecology of Torulopsis spp.in man has showed that T. glabrata was the most important species. It was often found in the genito-urinary tract and its pathogenic nature seemed certain (32). On the survey of yeast flora of the hard palate, mucosa and pooled saliva of schoolchildren, the predominate isolate was Candida albicans (71%), then followed by Saccharomyces spp.(19.7%) and Candida tropicalis (8.6%), but no isolation of Torulopsis or Rhodotorula were recovered (33). Others found that C. krusei 3-6% and T. grabata 26% were isolated too (3).

In most investigation on the incidence of yeast from normal skin, C. parapsilosis and C. guilliermondii lead the list, C. krusei is also common isolated, C. tropicalis and C. albicans are not regularly found on normal skin but can be recovered from anogenital area, from the anorectal area or feces, 50% of isolates were C. albicans and up to 20% were T. glabrata and C. tropicalis. Geotrichum candidum and Trichosporon cutaneum are also present but in small number. In survey of the normal vagina, between 11 and 30% of healthy women found C. albicans and up to 85% of gynecological patients. The other common isolants in both groups were T. glabrata (9-30%), C. parapsilosis, and C. tropicalis. In an investigation of the external

ears C. robusta, C. rhagii, C. parapsilosis and C. guilliermondii were found in normal ears and also associated with a variety of eczematous condition (2,3).

It was not only found these organism from human but also from bovine, porcine and equine (95% of isolated yeasts) and 4.9% from feline avian species (4.9% of isolated yeasts). The gastrointestinal and reproductive tract were the major sources of yeasts and yeast-like organisms representing 26.2% of the total isolates. This survey revealed that C. albicans, C. krusei , C. tropicalis, C. parapsilosis were the most frequent species recovered from clinical specimens representing 71.4% of total Candida isolates (30).

In the survey of air-borne fungi in Thailand, it was found that yeast is the predominate organism and other evidences are supported this finding too (79). From the preliminary survey of yeast from marine habitats it was reported that C. guilliermondii, Cr. albidus, Cr. laurentii, Rhodotorula glutinis, Rhodotorola rubra, Trichosporon cutaneum, Saccharomyces fermentati, Torulopsis spp. were isolated from marine sediment and sand, conch or spiny lobster and no isolation of C. albicans, Cr. neoformans and Torulopsis glabrata were found (34).

Clinical Significances

Yeast and yeast-like infections are among the most common fungal infection in human and have been recorded for approximately 140 years (4,28). Thrush, mostly in new born and the aged, was the first recognized yeast infection. The disease, common in the 19th and early 20th centuries, was associated with malnutrition. The etiologic agent was Candida albicans. Reports of systemic infection by C. albicans occured infrequently prior to 1940. In the late 19th century, Cryptococcus neoformans was reported to associate with disease. Infections were rare and most often manifested as fetal meningitis (4,29,36)

Since 1950, a steadily increasing number of reports have recountered the growirg incidence of yeast and yeast-like infections. The increasing has been associated with many factors and the factors that predispose yeast and yeast-like fungi to colonize or infect human were the following factors (1,2,3,4,28,39,40,41,42).

1. Extreme youth: During the normal process of establishing a resident flora, the restriction factor for Candida may be absent, and a clinical condition is produced (thrush, diaper rash,etc.).

2. Physiological change: Pregnancy appears to affect the carbohydrate content of the vagina and leads to an increase of the population of normal flora yeast.The administration of steriods to males or females also leads to proliferation of yeast and yeast-like.

The patients who have endocrine dysfunction, particular diabetes, also leads to easy colonization and infection.

3. Prolonged administraticn of antibiotics. Much evidence has accumulated associating clinical disease with the use of antibacterial antibiotics. The most important effect is the elimination and alteration of the bacterial flora that holds the population of yeast flora in check. Evidence also suggests that there is some effect of the antibiotic on the host tissue that predisposed it to invasion by the organism.

4. Immunosuppressive agents, cytotoxins and other drugs abrogate the normal defense and various immure defect patients and the slight avitaminosis were the causes of colonization and infection by yeast and yeast-like.

5. The barrier-break such as a prolong catheter, hyperalimentation, peritoneal dialysis,inadequate catheters care, lorg term intravenous therapy, accidental barrier-break such as trauma, burn, shooting, and cut were allowed yeast and yeast-like proliferation at the site of opening skin.

Candidiasis is a primary or secondary infection involving a member of genus Candida, especially C. albicans which shows greatly varied of clinical manifestations. They range from acute, subacute and chronic to episodic. The pathogen may involve in the mouth, throat, skin, vagina, nail, lung, and gastrointestinal tract, or may become

systemic or fungemia, endocarditis and meningitis. Among the species of Candida, Candida albicans causes most of the clinical forms of candidiasis (3,35).

Although, Candida albicans is the most common etiologic agent, in some of the uncommon clinical condition such as endocarditis, arthritis, peritonitis, other species are more frequently isolated as shown in the table 1. From table 1 the other species represent normal flora of the cutaneous and mucocutaneous areas and are of very limited pathogenicity (3). All species may involved in any form of candidiasis, but some are regularly encountered in one particular type (2,3,4,28,40).

The various clinical forms of candidiasis can also be produced by other yeast and yeast-like organisms. These include Rhodotorula glutinis, R. rubra, Torulopsis candida, Trichosporon beigellii, Tr. capitatum and Geotrichum candidum.

Cryptococcosis is now being recognized more frequently both as a primary and secondary infection in man. The causative agent of disease is Cryptococcus neoformans. The pathogen may involve mostly central nervous system, pulmonary, skin, mucocutaneous membrane, bone and joint and many become dissiminated cryptococcosis (2,3,4,12,35,36).

From histopathology of yeast infection, it is difficult to differentiate the genera or species of etiologic agents, since the

Table 1 : Human yeast pathogens.

species	clinical manifestation
Candida albicans	Most commonly isolated and considered by some to be most pathogenic. Clinical syndromes variable and include cutaneous, CNS,UTI,vaginitis,pulmonary and systemic infections.
Candida tropicalis	Arthritis, bronchopulmonary,meningitis, onychomycosis, peritonitis,UTI,vaginitis, systemic infection.
Candida parapsilosis	Endocarditis,otitis externa, fungemia.
Candida krusei	Rarely endocarditis, fungemia UTI, vaginitis.
Candida pseudotropicalis	UTI, vaginitis
Candida guilliermondii	Dermatologic, endocarditis, onychomycosis,UTI.
Candida lusitaniae	Mucocutaneous, fungemia.
Torulopsis glabrata	Endocarditis, fungemia, UTI, vaginitis.
Trichosporon beigellii	White piedra, fungemia, endocarditis, endophthalmitis.
Trichosporon capitatum	Systemic , pulmonary.
Geotrichum candidum	Rare pulmonary geotrichosis.
Rhodotorula rubra	Fungemia, CNS, nephritis.
Rhodotorula glutinis	Blood.

CNS = central nerveous system

UTI = urinary tract infection

general appearance in tissue section is alike (3). Then the specific diagnosis of the disease depends on isolation and identification of the organism on culture. So the rapid and precisian of identification of the organism is very important in clinical laboratory. For this importance, the availability of modified system, together with commercial system for identification of yeasts were developed and should allow more rapid turnaround time with acceptable limits (2).

Mechanism of Pathogenesis

Yeast infection does not depend only on the predisposing factors but also on the agent itself.

In candidiasis, the most common etiologic agent , Candida albicans could produce endotoxin-like substance (canditoxin) which inducedthe releasing of histamine in-vivo (43). Not only the secreted substance may influence the pathogenesis, the whole cell and the cell walls were also caused pyrogenic to rabbit and lethal for actinomycin-D treated mice (44). In addition, another factors that may play role in colonization of yeast is some certain enzymes; phospholipase A, lysophospholipase, and keratinase. The first two enzymes were isolated frcm the developing buds, the cell membrane, and also the cell walls of the vegetative cells (35). It has been reported that phospholipase A damaged cell membrane, whereas, lysophospholipase protected the yeast from the damaging by phospholipase. It was sugested that these enzymes may assist the yeast invasion in host

tissue by disruption the epithelial membrane. Followed by the hypha penetrated through the host cell. Like phospholipase, keratinase could digest keratin in vitro allowing the development of germ tube into host cell (35).

In cryptococcosis, like candidiasis, there is the endotoxin-like substance isolated from the cell extract, may play role in pathogenesis (45). This substance resembled the cell wall component of gram-positive bacteria, based on the study of chemical composition and its activity in animal. Apart from endotoxin, it has been suggested that the urease induced the release of ammonia which may destroy the complement function hinding the host defense and encourage the fungal growth (46).

One of the well defined virulence factor in cryptococcosis is polysaccharide capsule which may acts as specific and potent inhibitor of phagocytosis. Therefore, the failure of capsule engulfment by the macrophage is explainable however the actual mechanism is not known at the present time (47). The common route of cryptococcal infection is dealt with the respiratory tract. Therefore mechanism of infection may depend on the size of the particle. To enter to the lung, it must be compatible with pulmonary deposit. To be clinically important, an aerosol should be composed of particle less than 10 μm in diameter and the particle less than 5 μm in diameter are more compatible with low respiratory tract deposition (48).

Despite of a vast knowledge of the mechanisms of pathogenesis of cryptococcosis and candidiasis, there is still less clear understanding of the mechanisms in other yeast infections for example: geotrichosis, torulopsosis and rhodotorulopsis, also (3,35).

Classification and Identification of Medically Important Yeasts

Yeasts are a heterogenous group of fungi that on simple appearance seem to be homogenous. During their life cycle, yeasts grow in a conspicuous unicellular form that reproduced by fission, budding, or a combination of both (40). Yeastsare separated into three groups according to the methods of sexual reproduction;

1. Blastomycetes (deuteromycotina or fungi imperfecti) with no known sexual reproduction and illustrated by the majority of species of Candida and Torulopsis.

2. Hemiascomycetes (ascomycetes) which produce ascospore as a result of sexual reproduction and are exemplified by the genera Saccharomyces, Endomycopsis and Pichia.

3. Heterobasidiomycetes with formation of basidiospores as a result of sexual reproduction and illustrated by the genera Filobasidiella, Leucosporidium, and Syringospora (3,49).

In the recent monograph,Kreger (41) and Barnett (60) found nearly 600 species of yeast. And for their differentiation using the

physiological and morphological characteristics, Kreger (41) and Barnett (60) suggested about 60 and 83 tests respectively. Apart from the above characteristic, the molecular study, including the type of coenzyme in the electron transport system and on nucleic acid base composition and reassociation, appear promising in determining relationship of yeast and providing more reliable phenotypic properties for classification. The type of coenzyme Q appears to correlate with genetic grouping of yeasts. And the G + C content is very useful in the species delimilation of the imperfect yeasts, especially new species (41).

The yeasts of medical importance are in the class Blastomycetes, Form-Family Cryptococcaceae, and listed below along with the generally accepted classification and basic differential characteristic (3,29,40,49)

Genus 1 : Candida. Candida is reproductive by budding or blastoconidia. They may form pseudomycelium or true mycelium; urease test is generally negative; capsules are not formed; starch or carotenoid pigments are not produced, They do not assimilate inositol.

Genus 2 : Cryptococcus. This genus is characterised by unicellular budding. Most of species are urease positive yeasts. Cryptococcus produces heteropolysaccharide capsule and starch-like compound; carotenoid pigments are usually lacking. Cryptococcus assimilates inositol but sugar are not fermented.

Genus 3 : Trichosporon. Trichosporon is reproduced by blastoconidia and arthroconidia formation. They form mycelium and pseudomycelium.

Genus 4 : Geotrichum. Reproduction is by arthroconidia only. A true mycelium is formed.

Genus 5 : Rhodotorula. This genus produces unicellular budding and rarely produces pseudohyphae. They do not assimilate inositol or ferment sugars. They can produce carotenoid pigments.

Identification of medically important yeasts is based on both morphological and physiological characteristics. The morphological characteristics may include such structures as capsule, germ tube, chlamydoconidia, arthroconidia, blastoconidia, pseudohyphae, and hyphae. The physiological characteristics, useful for differentiation of various species, may include assimilation and fermentation of carbohydrates, production of urease, assimilation of nitrate, caffeic acid reaction, temperature tolerance, cycloheximide resistance and production of film surface on Sabouraud broth (49,51). Each laboratory must develop its own policy about the need to do a complete identification on each yeast isolate.

On demonstration of capsule for rapid presumptive identification yeast of medical important in the genus Cryptococcus, it was easily detected by using wet mount with diluted india-ink (51). The most rapid identification of Candida albicans is done by observing the

formation of germ tube within three hours at 35-37°C in various media such as peritoneal cavity and subcutaneous tissue of mice, in plasma, egg white, saliva, cerebrospinal fluid and tissue culture medium TCC 199, sheep serum and in trypticase soy broth (40,52,55,56).

In 1931, Benham described the use of corn meal agar for stimulation of chlamydoconidia formation. Since then many modifications have been formulated to increase the efficiency of this process. Addition of tween 80 to rice infusion agar and glutinous rice agar greatly improved chlamydoconidia production (55,56). It was found that partial anaerobism and poorly utilizable carbon sources were among several factors to favor filamentation and chlamydoconidia formation by C. albicans (55,56,58). Cycloheximide sensitivity test had shown that the final concentration of cycloheximide in medium for using in routine isolation of yeast was 0.05%. Barnett et al used 0.1% cycloheximide supplemented medium in the differentiation of some Candida species (60). Campbell et al (51) suggested that production of film surface on Sabouraud broth could use in differentiation of C. tropicalis from C. albicans. They showed that C. tropicalis produced thin film surface and C. krusei produced width film surface but C. albicans, T. glabrata did not produce film surface.

Zimmer, and Roberts (61) had developed rapid urease test for presumptive identification of Cr. neoformans within 15 min. They used concentrated Christensen's urea agar base treated swab and combined with 1% benzalkonium choloride pH 4.86+0.01. They suggested

that pH of benzalkonium chloride was very important.

Robert, et al (62) investigated urease test by using urea broth and urea R broth in multiwell test. All the urease producing yeasts, Cryptococcus and Trichoporon, showed positive outcome in 4 hours by using urea broth. And 60% of these yeasts yielded the positive reaction in 30 minutes by using urea R broth

Hopkins and Land (63) had developed rapid method for determining nitrate utilization by yeasts. In their study, they used concentrate potassium nitrate and phosphate buffer treated swab and could demonstrate nitrate utilization by yeast within 10 min.

Odd, and Abbott, (42) had investigated the growth of Candida on acidic pH medium. They suggested that a positive result in the pH 1.55 tolerance test allowed presumptive identification of a yeast isolate C. albicans. Later, the same authors found that one isolate of C. krusei could grow in this pH, so they proposed the use of pH 1.44 for further simple system in the presumtive dentification of C. albicans (64).

Since Staib (65) reported that Cr. neoformans cultures deveolped a brown color when grewn on media into which an aqueous extract of Guizotia abyssinica had been incorporated. He did not observe coloration in other species of Cryptococcus or in species of the genera Candida, Trichosporon, Rhodotorula. The ability to form dark color was therefore a unique characteristic of Cr. neoformans.

This technique has become a very important method for detection of colonies of Cr. neoformans in mixed culture. It has also proved to be a reliable tool for the rapid identification of Cr. neoformans. So many author had developed this pigment formation on extract of G. abyssinica (65-74). The 3,4-dihydroxycinnamic acid (or caffeic acid, an O-diphenol), when isolated from extract of G. abyssinica seed, was found to produce brown coloration of Cr. neoformans colonies in the presence of iron compound such as ferric citrate within 6 hours when incubated at room temperature (70,72). Paliwal, and Randhawa, (74) developed rapid pigmentation of Cr. neoformans in 30 miniutes by using shaked cultures of Cr. neoformans in a phosphate buffer, L-β- 3,4 dihydroxyphenylalanine (L-DOPA)-ferric citrate medium at 37°C. Later, Kaufman and Merz (73) developed this test by using non-medium based test, the paper strips saturated with a buffered L-DOPA ferric citrate solution. Only Cr. neoformans produced pigment within 60 to 90 miniutes and all other yeasts remained negative.

The ability of a yeast to assimilate (use) carbohydrates is determined by the observation of growth or acid production when only a carbohydrate is provided in a simple yeast nitrogen base. These tests may be done either in a liquid or a solid medium (49-51). The Wickerham's tubes, with each individual carbohydrate incoporated into a basal liquid medium, are the most accurate and easy to read but this method takes too much time (75). Auxanographic plates, with the carbohydrate absorbed into a paper disc placed on a plate of inoculated agar which contains indicator, gives almost as accurate

results as (approximate 90% correlation) the tubes and is easier to prepare (76, 78). Time required for this method is about 24-72 hours. On the further development of this method by Michelsen et al (77). They used heavy inoculum of unstarved yeast, autoclaving yeast nitrogen base and diluted yeast nitrogen base recieved satisfactory result by these modifications (77).

Up to date there are many kits providing by various companies. The BBL Mini-Tek system is a modified auxanographic technique, as is the Corning Uni-Yeast-Tek system (68-70). These two systems consist of a few kinds of carbohydrate and need incubation period for 3-6 days. The API 20 C, Abbott MS-2 and Vitek Yeast Biochemical Card test system which consist of single used plastic strip or card containing 20 lyophilized tested substrate in chambers, are based on primarily on the Wickerham tube system (7,71-74). The time requirement in these system are 24-72 hours. For all systems the inoculum is a suspension of an isolated yeast colony in sterile distilled water or saline or, in the case of the API 20 C system, in a specially prepared medium that must be held at a temperature of 50°C during inoculation. Qadri et al (7), have compared the cost, time requirement and accuracy of the results among Abbott MS-2, API 20 C, Uni Yeast Tek, and conventional dye poured plate method. The identification accuracies with all the commercial systems are in the range of between 92.3% to 97.5%. These tests are often definitive at 24 hours. However, the cost of commercial kits are higher than of the manual.