THE ISOLATION AND IDENTIFICATION OF YEAST-LIKE FUNGI IN NON-ACTIVE TUBERCULOSIS PATIENTS IN ROWAN COUNTY, KENTUCKY

by

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TABLE OF CONTENTS

List of Tables

List of Figures

INTRODUCTION

The isolation of yeasts from tuberculoid patients as well as non-tuberculoid individuals is common (5, 14, 17, 20, 24, 39, 42, 47, 48, 52). Yeasts have become common since the introductiom of such treatments as steroids (2, 17, 49) and antimicrobial drugs (2, 3, 19, 22, 23, 25, 29, 33, 34, 36, 42, 43, 45, 49, 51). This study was done in an attempt to identify the yeasts most commonly accompanying tuberculosis or resulting in the lungs of patients undergoing chemotherapy or other treatment after tuberculosis.

The two genera of yeast discovered most commonly in research are the two genera generally tested for in the course of this study, although all yeast isolates were identified.

Since yeasts are common in normal individuals (5, 24, 39, 47, 48, 52), the purposes of this study were:

1) to detect and isolate individual yeast species from sputum of non-active tuberculosis patients

2) to identify to specie, specimens of yeasts which were isolated

3) to determine the frequency of the yeasts in tuberculosis patients

4) to determine the frequency of the yeasts in normal persons not under antibiotic or drug treatment.

REVIEW OF LITERATURE

Several studies conducted over the last three decades have produced evidence that the common usage of antimicrobial or antibacterial drugs and steroids in therapy of various diseases and conditions have caused an increase in the number of yeast-like fungal infections accompanying these diseases (2, 8, 19, 22, 28, 29, 33, 41, 43). In the event of a debilitating disease, such as tuberculosis, these factors seem to multiply the appearance of the yeasts (37).

Seven genera of yeasts are medically important: Cryptococcus, Torulopsis, Pityrosporum, Rhodotorula, Candida, Trichosporon, and Geotrichum. The most common yeast-like fungi to appear in such instances as those mentioned above are the genera Candida and Cryptococcus.

Candidosis is a primary or secondary infection caused by any of the genera Candida. Normally candidosis is primarily a result of Candida albicans as this is the principle pathological agent of this genera, but other members of the

genera are occasionally pathogenic. These, C. stellatoidea, C. tropicalis, C. pseudotropicalis, C. krusei, C. parapsilosis, and C. guilliermondii, are infrequent in clinical conditions.

Candida albicans is a normal saprophytic fungi common to the human body. It has been considered by some (47) to be part of the normal human flora. A wide variety of factors are pre-disposing to infection by Candida albicans which include: diabetes (20, 37), leukemia (20, 31, 37), obesity, malnutrition, pregnancy, dentures, surgery, accidental introduction (37), contraceptives (20, 37), treatment with steroids (2, 7, 37, 43, 49), antibiotic therapy (2, 8, 19, 22, 23, 25, 29, 33, 34, 36, 42, 43, 45, 49, 51), and the infections such as tuberculosis (37).

The body has a natural resistance to infection by Candida albicans. This resistance is lowered by any factor which causes alterations of the metabolism as in hormonal disturbances or a breakdown of normal body defenses as in tuberculosis (37).

Administration of corticosteroids enhance Candida albicans. Steroids interfer with the mechanism that localize infection by

decreasing phagocytic mobilization and intracellular digestion, and at the same time depress the synthesis of gamma globulin (40).

Much research has been done on the effect of antibiotics on the enhancement of yeasts in the body. Broad-spectrum antibiotics refer to drugs such as chloramphenicol and the tetracyclines. These are the drugs which have gained a prominent seat in the use of antibiotics to treat bacterial disorders and disturbances in the last twenty-five years. Chloramphenicol depresses the intestional flora by destroying the sensitive gram positive cocci and gram negative bacilli (6,22). The drug seems to only depress the intestional flora while the patient is on the drug (6). The disturbance of this normal flora may result in a rampant growth of yeasts not only in the intestines, but throughout the body. Terramycin may also act the same way as it destroys intestinal gram positive and negative bacteria (22).

Aureomycin hydrochloride is one antibiotic that has been highly researched for its effects on Candida albicans growth. For this reason, there are contradictory reports concerning the

effects of aureomycin. Aureomycin also depresses intestinal flora, especially coliform growth as long as the patient is on the drug. Aureomycin is said to have a stimulatory effect on Candida albicans (18), while others contend it neither stimulates or inhibits the growth of Candida albicans. Papenfort contends the resultant growth of Candida albicans after aureomycin treatment is due to avitaminosis caused by destruction of the normal bacterial flora (34).

Streptomycin, like other mold-produced antibiotics, depresses the intestional flora (6). Keefer says streptomycin stimulates gram positive cocci (22). Streptomycin has been used to treat tuberculosis since 1948. From initial studies done at that time, it was concluded that streptomycin did not effect the incidence of Candida albicans in the sputum of treated patients (14), but later evidence seems to indicate streptomycin treatments do cause an increase in Candida albicans growth (40).

Much controversy has occured over penicillin's ability to enhance yeast growth. Bierman (6) says penicillin does not affect

the intestional flora while on the other hand, Smith (45) has shown that penicillin inhibits gram negative bacteria and eliminates the normal inhibitors of yeast (primarily the gram positive cocci), and thus accelerates the growth of yeast. Other studies have shown a marked loss of susceptible gram positive cocci from the mouth and nasopharynx (22) and outbreaks of yeast following penicillin therapy (10).

As mentioned earlier, the incidence of Candida albicans in normal individuals is high. Candida albicans is frequently found in sputum samples. Candida albicans invades the bronchial wall under certain conditions, such as ulcerations of the bronchial mucosa which may be resultant of tuberculosis. Candida stellatoidea has been found to represent 2% of all yeasts isolated from normal sputum (13). Candida pseudotropicalis has been cultured from patients suffering from respiratory diseases. Candida krusei has also been isolated from the respiratory tract (13).

The Cryptococcus genera has one primary pathogenic member, Crypotcoccus neoformans. In man, infection with Cryptococcus

neoformans often remain subclinical. Usually the budding yeast attack the meninges of the brain, but infrequently, Cryptococcus neoformans may be encountered in the lungs. Most cases occur in persons with previously good health. However, susceptibility to Cryptococcus neoformans is increased in persons with diseases of the liver, lymph glands, spleen, and bone marrow (1).

Through the year 1970, only fifteen cases of tuberculosis and Cryptococcus neoformans occuring simultaneously have been reported (35). This would seem to indicate that tuberculosis is not predisposing to Cryptococcus neoformans infections.

Torulopsis glabrata, normally a non-pathogenic fungus, is occasionally found in the oral and respiratory tract but rarely is reported to invade human tissue. It has been shown to cause human pulmonary infections. Torulopsis is associated with cancer, diabetes mellitus, treatment with antibiotics, steroids, or chemotherapeutic agents for malignant diseases (32). In one study of yeast isolated from normal persons, Torulopsis glabrata represented 14% (47) which has been blamed on the increase in use

of antimicrobial drugs. In none of the articles researched (7,15,16,26,47,50) was Torulopsis glabrata or any Torulopsis specie associated with tuberculosis.

The drugs commonly used today to treat tuberculosis are isoniazid, rifampin, ethambutol, para-amino-salicylic acid, pyrazinamide, cycloserine, ethionamide, streptomycin, kanamycin, viomycin, and capreomycin. Of these, the most popular are isoniazid, para-amino-salicylic acid, and streptomycin. Any of these drugs could enhance the development of yeasts. Since yeast infections flourish due to bacterial disturbances or annihilation of the bacteria by treatment with antibiotics, the increase growth of yeast could arise with the treatment by any of the bactericidal (isoniazid, rifampin, pyrazinamide, streptomycin, or ethionamide) or bacteriostatic (ethambutol, para-amino-salicylic acid, cycloserine, or capreomycin) chemotherapeutic agents (38).

Several theories have been set forth to explain the enhanced growth of yeasts following antibiotic therapy:

Theory I - The administration of antibiotics upsets the equilibrium in which normal bacterial and mycotic flora exist, and results in the elimination of the susceptable microorganism. Consequently, the number of organisms competing for the food supply is decreased, which permits the resistant specie to grow rampantly (19, 40). The main danger with antibiotics seems to be their ability to inhibit Escherichia coli (33). Prolonged use of any antibiotic can completely suppress both gram positive cocci and gram negative bacilli (45).

Theory II - Normal flora of the intestines supply certain nutrients to the host. A disturbance in the host's nutritional supply lowers resistance to organisms not normally able to invade healthy human tissue. Streptomycin (11, 33) has been shown to lead to secondary vitamin deficiencies which could result in rampant yeast growth.

Theory III - Several authors have demonstrated that some yeast are stimulated by certain antibiotics (8, 30, 34) while others contend this is not possible (33, 51).

Studies conducted to show the frequency of Candida albicans in normal individuals and tuberculosis patients have yielded interesting results. In a study by Baum (2) of thirty patients with tuberculosis, eighteen were positive for Candida species, eight of which were Candida albicans. Of a control group, numbering thirty-four, twelve gave Candida specimens, five of which were Candida albicans. Results showed 55% of those tested has Candida species in their sputum. In the control group, positive cultures were found in 36%. The thirty patients were receiving antituberculosis chemotherapy.

A study conducted in 1927 showed 10% of 1002 non-tuberculin students obtained yeast-like "monilia" cultures without recent history of sore throat or upper respiratory infections. Haler found "monilia" in throat cultures in a group of 20% of a group of normal children. Fisher and Arnold in a 1936 study with seventy-six normal individuals (28 adults, 48 children) showed 75% of the adult population had a fungal infection and 52% of the children. Of those with fungal infections, 43% of those adults showed Candida albicans as did 63% of the

children (13).

In several other studies of patients with tuberculosis or undergoing chemotherapy for treatment of tuberculosis, an increase of occurance of Candida albicans and Candida specie were noted. Weedon in 1937 examined fifty-five sputum samples, 9.9% of which showed presence of Candida albicans (13). Bert and Ketchem in 1941 examined 693 specimens and found 36% contained Candida albicans. Bojalil and Gonzales-Mendoza in 1957 examined 200 specimens and found 20.5% contained Candida albicans (13).

In a study conducted on normal persons, Stenderup (47) in 1962, found 53% of all yeast isolates were Candida albicans, 14% Torulopsis, and 1% Cryptococcus with the remaining 32% being mostly other members of the genera Candida.

A large portion of the control groups or normal patient groups in these studies showed high percentages of Candida albicans as did those tuberculin patients which would appear to indicate Candida albicans occurs in nearly all individuals.

Research into the effect of "new" antituberculin drugs have revealed startling results. Rifampin, along with amphotericin B, has shown a synergistic effect on Candida albicans with a reduction in viable cells of up to 97% (3). Of course, amphotericin B is an antifungal drug with great powers of its own. Thus, the use of amphotericin B along with the new drugs have almost completely halted the occurence of mycosis in tuberculosis patients.

MATERIALS AND METHODS

Isolation and Maintenance of Sputum Samples

All isolates were obtained from routine sputum cultures taken from tuberculosis patients in Rowan County, Kentucky between April and October, 1975. Controls were taken in Rowan and Meade Counties during the same time period. The individuals were of varied ages, sexes, and backgrounds.

Sputum samples were collected in sterile, screw cap jars immediately after the patient had arisen in the morning and completed his oral hygeine procedures. Sputum was a single deep cough specimen. In some tuberculosis patients, samples were collected by the Rowan County Health Department in the above manner except later in the day.

Under sterile conditions, sputum samples were streaked with sterile cotton swabs on Bacto-Sabouraud Dextrose (SAB) agar with and without antibiotics (cyclohexamide and chloramphenicol - Difco) and blood agar (BHI). The above media was prepared from commercial preparations (Difco) and autoclaved for fifteen minutes

at 121°C and 15 pounds per square inch (psi). The media was poured aseptically to a depth of 2 mm in sterile glass petri dishes. Antibiotics were filtered by Millipore filter and added to media asentically as was the blood.

The yeast were isolated and maintained at 37°C.

The yeast isolates were initially identified by characteristic colonial morphology and microscopic appearance in a crystal violet stain. One isolate of each specie from any one patient source was subjected to followup confirmation tests.

The media for pure culture isolation and maintenance of the yeast was Bacto-Sabouraud Dextrose Agar manufactured by Difco. The powdered agar contains neopeptone (10 grams), dextrose (40 grams), and agar (20 grams) and was diluted with one liter of deionized water. The media was autoclaved at 15 psi and 121°C for fifteen minutes. The pH of the media was 5.6. After autoclaving, the agar was placed on a slant board and allowed to harden for twenty-four hours.

The media was inoculated with the yeast isolates by standard aseptic technique. The isolates were allowed to grow at 37°C for seven days. At this time, serum germ tube tests were conducted before biochemical tests were began as a follow-up identification method.

All moldy isolates were discarded.

Identification of Yeasts from Sputum Samples

The following are the procedures used in identification of yeast isolates:

A. Serum germ tube test

B. Sugar assimilation test

C. Sugar fermentation test

D. Urease test

E. Nitrate assimiliation test

F. Chlamydospore production test

A) Serum germ tube test - The serum tube method as described by Mackenzie (27) was employed. A 5 ml test tube of the organism was inoculated with 0.5 ml of blood serum. The test tubes were incubated at 37° C for 2 hours 50 minutes. Slides were prepared

from each inoculum and checked for germ tubes or short filamentous outgrowths from the rounded or oval cells of Candida albicans. The germ tube test is characteristic of this specie.

Fresh or inactivated serum and deep-frozen stored serum are satisfactory as well as rabbit, guinea pig, horse or bovine serum. Serum used in these tests was human serum obtained from Saint Claire Medical Center, Morehead, Kentucky.

The production of germ tubes by a concentration of Candida albicans in serum tubes at 0.5 ml of serum gives a rapid identification in three hours (4). Several authors have also suggested germ tubes can be produced by C. stellatoidea, C. utilis, C. rugosa, and Schizosaccharomyces fragilis (27).

A number of factors may prevent the formation of germ tubes including too high a concentration of the yeast cells, temperatures above 41°C or below 31°C, and heat coagulated serum.

B) Sugar assimiliation test - Two percent water agar (Difco) was made and despensed into test tubes and autoclaved at 15psi

and 121°C for fifteen minutes. After autoclaving, the agar was kept at 53°C in a water bath until its usage.

A 10x solution of yeast nitrogen broth (Difco) was prepared and sterilized via Millipore filter and refrigerated until needed.

An 80% physiological saline solution was prepared, dispensed into test tubes, and autoclaved at 15 psi and 121°C for fifteen minutes. After cooling, the saline solution was inoculated with one loop of the test yeast organism and shaken to dispense the yeast. 5 ml of the saline-yeast solution was placed in a sterile petri dish by pipette. 5 ml of the yeast nitrogen broth was added to the other side of the plate by pipette. The cooled two percent agar solution was poured into the plate and the plate swirled to mix the ingredients. The plates were allowed to harden and cool. Prepared carbohydrate discs (Difco) of maltose, mannose, raffinose, meliobiose, trehalose, sucrose, lactose, glucose, galactose, and inisitol were added with sterilized tweezers, five different discs per petri dish. The plates were inspected in 48 hours for

growth of yeast colonies around the individual discs.

Diagnosis of isolates was confirmed by charts in Beneke's Medical Mycology Manual.

C) Sugar fermentation tests - Phenol red indicator broth (Difco) - 16 mg/1000ml - was dispensed into screw top test tubes containing a Durham tube (fermentation tube) to a depth of 13 ml. After the tubes were autoclaved at 15 psi and 121°C for fifteen minutes, the tubes were cooled to 37°C, and 1.3 ml of a carbohydrate in a 10% solution was added. The carbohydrates used were maltose, sucrose, galactose, raffinose, trehalose, dextrose, and lactose (Difco). Carbohydrates were sterilized by Millipore filter technique and added to the phenol red broth by standard aseptic technique with pipette.

A loop of the test organism was added to a test tube of 80% physiological saline solution. .3 ml of the yeast suspension was added to each carbohydrate tube. The tubes were tightly sealed. The tubes were inspected in ten to fourteen days for production of gas in the fermentation tube. If no bubbles were visible in the

fermentation tube, the tube was slightly tapped with the forefinger and watched for bubbles to rise from the bottom of the tube. If this procedure failed, the screw cap was loosed while listening for the escape of gases (not a reliable technique and seldom employed). A change in color from pink to red indicates acid but is not sufficient to use in identification, therefore the production of gas is essential for identification purposes.

Diagnosis again was aided by Beneke's Medical Mycology Manual.

D) Urease test - This media is used to distinguish Cryptococcus from Candida. Cryptococcus is urease-positive and Candida is urease negative. Christensen's agar (Difco) was prepared and autoclaved at 115°C and 15 psi for fifteen minutes. The agar, having been dispensed into test tubes before sterilization, was allowed to cool and then 15 ml of urea was added per tube. The urea was previously sterilized via Millipore filter. The tubes were then slanted. The hardened media was inoculated with a minute amount of the yeast and incubated at room temperature. The tubes were read in seven days. A deep red color through the media indicated a positive reaction.

E) Nitrate assimilation test - Two percent water agar (Difco) was prepared and dispensed in tubes and autoclaved at 15 psi and 121°C for fifteen minutes. After autoclaving, the agar was kept in a 53°C waterbath until used.

A 10x concentration of yeast carbon broth (Difco) was prepared and sterilized via Millipore filter and refrigerated until used.

An 80% physiological saline solution was also prepared, dispensed into test tubes, and autoclaved at standard temperature and pressure for fifteen minutes. The tubes, after cooling, were inoculated with one loop of yeast organism. 5 ml of the yeast suspension and 5 ml of the yeast carbon base were added aseptically to sterile petri dishes. 20 ml of water agar was added and the plate swirled to disperse the yeast suspension and yeast carbon base. The plates were allowed to cool.

Observation for yeast growth was made in twenty-four hours.

F) Chlamydospore production test - This media stimulates

chlamydospore production in Candida albicans. The agar, commerically prepared by Difco, was mixed and autoclaved at standard temperature and pressure for fifteen minutes. After cooling, the agar was inoculated with the test yeast by barely cutting the surface with the inoculating needle. A glass cover slip, sterile, was then added, and the plate examined in seventy-two hours under the microscope for the production of chlaymdospores.

RESULTS

Following completion of the physical and biochemical tests on the yeast isolates from the sputum samples, the presence of three yeast specie were noted. Although all three are generally saprophytic (9), they are considered pathogenic in these instances as they were found to accompany tuberculosis.. The three yeasts isolated were Candida albicans, Candida stellatoidea, and Cryptococcus neoformans. Candida albicans, where it appeared, was in heavy concentrations, whereas Candida stellatoidea and Cryptococcus neoformans were in light concentrations on initial media cultures of SAB and BHI.

Fifty-two initial sputum samples from tuberculosis patients were cultured. Twenty-one of these or 40% were found to produce yeast-like fungal isolates (Table I). One sample of each yeast colony was then isolated for further identification. Candida albicans was found to exist in 57% of the initial unidentified pure yeast isolates after serum germ tube tests were conducted (Table III). Since the serum germ tube test is not always

conclusive, the remaining 43% plus some suspected Candida albicans were put through biochemical identification tests with the results given in Table IV.

Table I. Frequency of yeast isolates in tuberculin and control individuals.

	Number of Sputum Samples	Number with Yeast Present	Percentage with Yeast Present
Tuberculin individuals	52	21	40%
Control	25	5	20%

Table II. Occurence of yeast in sputum isolates of tuberculosis patients receiving and not receiving chemotherapy.

	Number of Sputum Samples	Number with Yeast	Percentage with Yeast
Receiving chemotherapy	29	11	37%
Not receiving chemotherapy	23	10	43%
Control (non-tuberculin)	25	5	20%

Table III. Frequency of Candida albicans appearance in initial yeast pure culture isolates by serum germ tube procedure.

	Isolates Tested	Number of Candida albicans	Percentage Candida albicans
Tuberculin patients	54	31	57%
Control	25	3	12%

Table IV. Frequency of yeast appearances in sputum isolates of tuberculosis patients identified by biochemical tests.*

Yeast Isolates	Number Found	Number of Sputum Isolates Tested	Percentage in which Yeast were Found
Candida albicans	17	30	56%
Candida stellatoidea	1	30	3%
Cryptococcus neoformans	1	30	3%
Yeast-like bacteria	11	30	36%

* Urease, nitrate assimiliation, sugar assimiliation, and sugar fermentation tests.

Table V. Frequency of yeast appearances in sputum isolates of control patients identified by biochemical tests.*

Yeast Isolates	Number Found	Number of Sputum Isolates Tested	Percentage Which Yeast Were Found
Candida albicans	5	5	100%
Candida stellatoidea	0	0	0
Cryptococcus neoformans	0	0	0
Yeast-like bacteria	0	0	0

* Urease, nitrate assimiliation, sugar assimiliation, and sugar fermentation tests.

Table VI. Diagnostic reaction of yeasts which produce gaseous fermentation of sugars and are associated with the human body.*

	Candida albicans	Candida stellatoidea	Cryptococcus neoformans	Torulopsis glabrata
Dextrose	+	+	-	+
Galactose	VF **	VF	-	-
Lactose	-	-	-	-
Maltose	+	+	-	-
Sucrose	+	-	-	-
Trehalose	VF	VF	-	+
Raffinose	-	-	-	-

* after Beneke (4).
** variable fermentation

Table VII. Diagnostic reaction of yeasts which assimiliate sugars and are associated with the human body.*

	Candida albicans	Candida stellatoidea	Cryptococcus neoformans	Torulopsis glabrata
Maltose	+	+	+	-
Melibiose	-	-	-	-
Raffinose	-	-	V**	-
Galactose	+	+	+	-
Lactose	-	-	-	-
Trehalose	+	V	+	+
Dextrose	+	+	+	+
Sucrose	+	-	+	-
Inósitol	-	-	+	-

*After Beneke (4)
** V=strains vary in assimiliation

DISCUSSION

In the normal person, the trachobronchial tree is considered to be clean due to the ciliary action of the mucosa. Thus it is surprising to find an organism as large as Candida albicans or Cryptococcus neoformans at this site. The appearance of these yeast in the sputum may be due to contamination by the mouth as the sputum is being expectorated. An increase in the number of yeast in the mouth could account for an increase in the amount of yeast in the sputum. The circumstances of excess yeast in the mouth can arise when a person suffers from a chronic debilitating disease, such as tuberculosis, prolonged use of antibiotics or corticosteroids, or nutritional deficiencies (6,11,12,14,18,19 22,25,28,29,30,33,34,36,41,43,44,45,46,49,50,51). Therefore sputum cultures positive for Candida albicans, Cryptococcus neoformans, or any other yeast are poor evidence for the presence of these yeast in the lungs.

The individuals involved in this study who were receiving chemotherapy were taking isoni . id in units of 100 mg daily.

In no instance in my research did I find a case where the use of this chemotherapeutic drug led to an increase in the yeast population of the user. Also, those patients not receiving chemotherapy for their inactive tuberculosis had a higher percentage of yeast present than did those receiving therapy. Data as to the length of time since the chemotherapy was discontinued was not available.

Only one case each of Candida stellatoidea and Cryptococcus neoformans was identified. This may have been due to inadequate or faulty research attempts. This would seem to indicate that, by far, Candida albicans is the prominant yeast to invade the the body under lowered resistance and serves to confirm other writers who say that other yeasts seldom become pathogenic.

Figure 1.

Questionnaire sent to all participants in the study.

MEDICAL HISTORY

Name or No. _____ Date _____

Address _____ Occupation _____

_____ _____

_____

Age _____ Sex _____ Race _____

Present Medication _____ Length of time _____

_____ _____

_____ _____

History of tuberculosis yes ____ no ____

History of mycosis yes ____ no ____

Additional comments _____ _____

_____

_____

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