MATERIALS AND METHODS

Collection and Isolation

The Candida albicans isolates were collected from dermatological sections of Chulalongkorn Hospital, The Royal Thai Army Hospital and Siriraj Hospital, from June 1974 to June 1975. After initial clinical examination by a dermatologist, the diagnosed and suspected candidosis specimens were handed over for further laboratory investigation.

The specimens were taken from different infected areas by scraping and swabbing. The material of systemic infection were collected from urine and sputum.

The preliminary investigation was directly examined under the microscope for detecting the presence of the fungus. A small portion of collected sample was taken from stock material and placed on a slide. A few drops of 10% KOH were added and then mixed thoroughly. The cover-slip was placed on the mixture, then gently heat the slide in order to make the material soft and clear. On observation under a microscope the appearance of numerous small oval about 3-10 μ in size of yeast cells with occasionally mycelial elements and budding will proved the positive examination of the yeast infection.

Other portions of collected material of each specimens were simultaneously inoculated on Mycosel agar in duplicated slants and let them being incubated at room temperature for few days until the creamy white colony of Candida species were formed. A typical and dominant yeast colony was then transferred to Sabouraud Agar slant and again incubated at room temperature (30°C) for 3-4 days. After that, all of the supposedly pure culture isolates were kept at low temperature for further investigations.

Identification

The identification of C. albicans was based on three specific characters, chlamydospore production, germ tube formation and sugar fermentation.

The study of chlamydospore formation was used the cut slit method (Kligman, 1950). Two kinds of initiating chlamydospore medium were Corn meal agar (Benham, 1931) and Rice agar (Beneke, 1971). Heavy inoculations of isolates were made lines one of each by the use of a gauge loop vertically cut through the medium in the plates, and all lines were placed on top by sterile cover-slips. The process as such was duplicated in a number of plates which were then incubated at room temperature for 3-7 days. Each plate was examined after 4-7 days of incubation. The thick wall chlamydospores

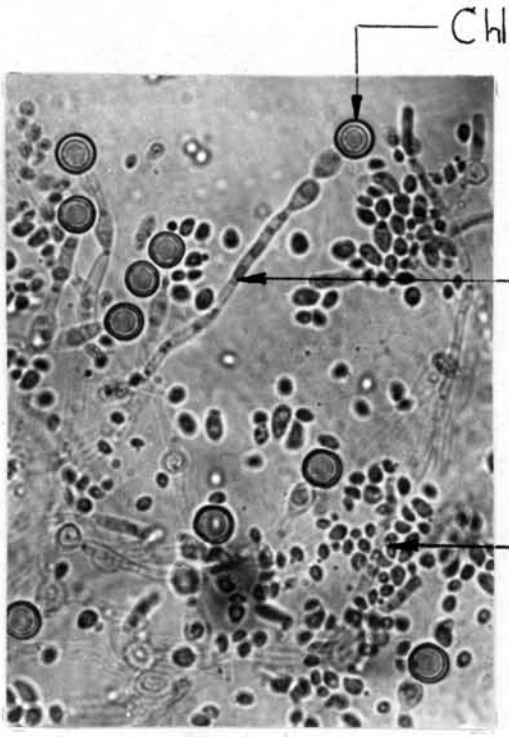

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Figure 2 Chlamydospores formation at terminal of pseudohyphae of C. albicans growing on Corn Meal Agar.

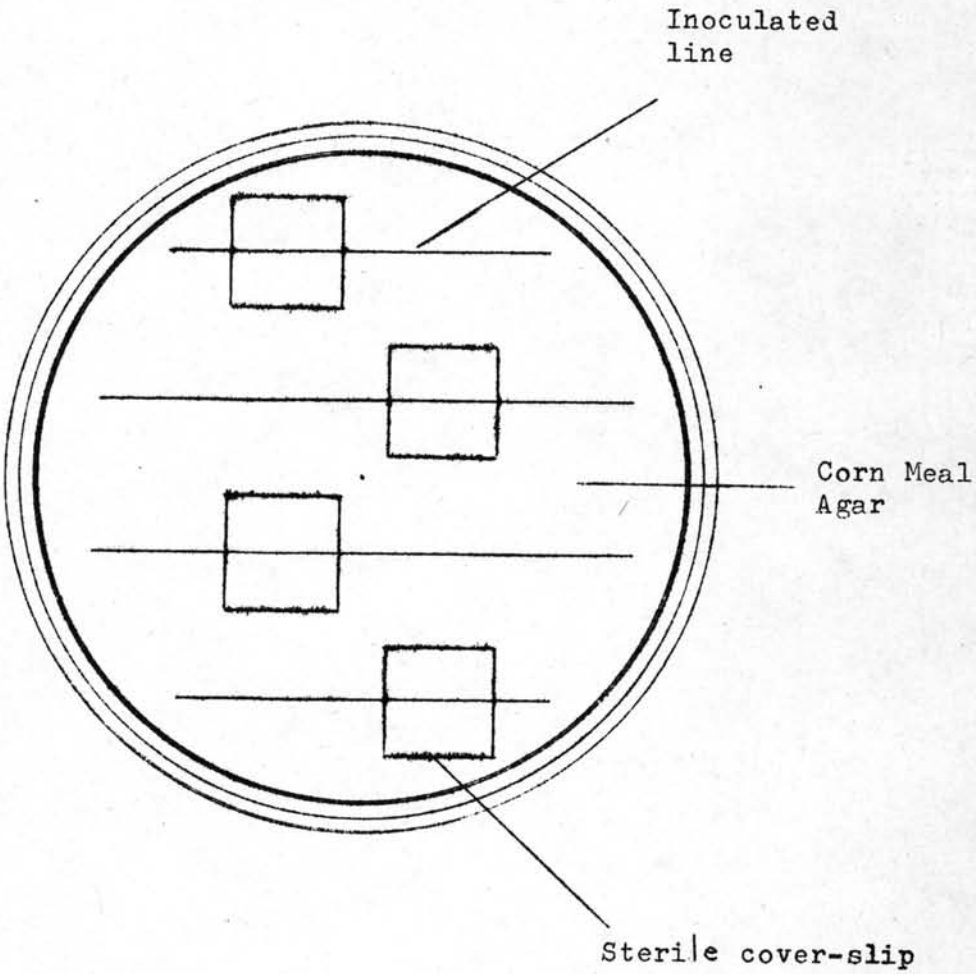

Figure 3 Cut slit method by Kligman 1950.

of C. albicans if present will form at terminal of pseudomycelium on the cover-slip. That marks the positive isolates of chlamydospore formation and could be collected for further testings of germ tube formation and sugar fermentation.

The former was detected by serum technique (Taschdjian, 1960), using one-fold dilution of horse serum with 1 ml. of physiological saline as a medium for inoculation. Each of isolates was done separately. Incubation period was 3-6 hrs., at 37°C then the mixture solution was taken off for examination under the microscope.

Of all two hundred collected isolates which were due to candidosis only forty-two isolates through the observation of chlamydospore production and germ tube formation were identified as C. albicans. In other word 21% was recorded.

In studying the relationship of various localize lesions to the ability of infection twenty-three isolates from different locations (nine strains from vagina, seven strains from sputum, two strains from skin and unknown location and only one strain of each from urine, ear and mouth) were selected and used for testing of sugar fermentation and protein pattern by disc gel electrophoresis.

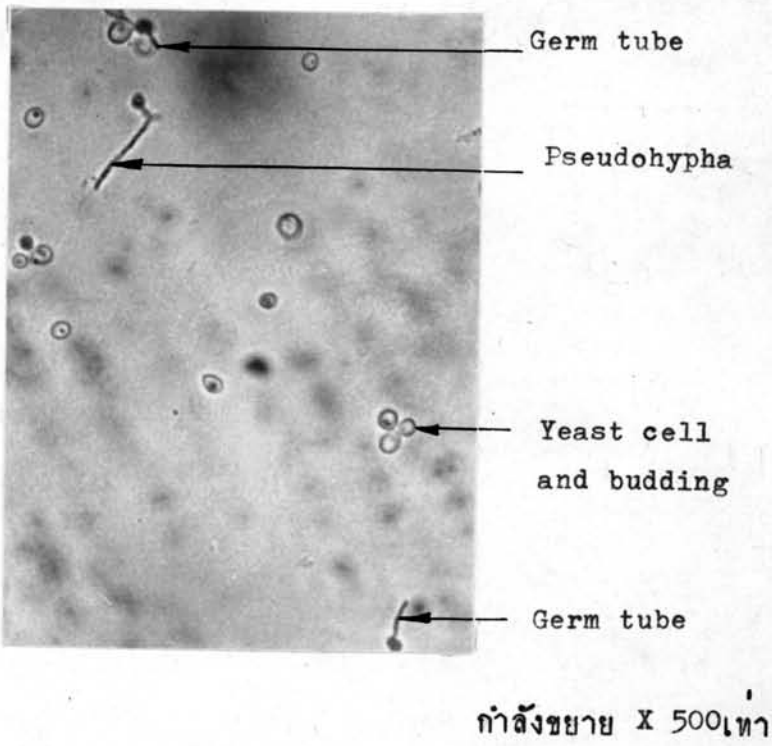

Figure 4 Germ tube of C. albicans when incubated for 3 hrs. in serum at 37°C.

For sugar fermentation different forms of sugar, glucose, maltose, sucrose and lactose were used in experiment by the following procedure.

A set of four test tubes contained 9 ml. of sterile beef extract broth and 1 ml. of 10% of each sugar was added. Durham tube was placed in the medium tube for detecting gas production. Bromcresol purple solution was used as indicator of acid production in the medium. The tube sets were autoclaved at 121°C, 15 lb/inch^2 for 15 minutes.

Each of twenty-three isolates of C. albicans was inoculated into the sugar media, then incubated at 37°C for 24 hrs. . The result of sugar fermentation was recorded after 24 hrs. .

There were also two more strains of Candida species from Northern Regional Research Laboratory (NRRL) C. krusei and C. utilis which were used in the experiment to compare with pathogen. All of stock isolates were maintained on Sabouraud agar slants through the experiment.

Extraction of water soluble proteins

Twenty-three strains of C. albicans and each of C. krusei and C. utilis were grown on Sabouraud agar slants at 37°C for 24 hrs. and then added with 10 ml. of physiological

saline for further preparation of the homogenous suspension of the yeast cells. Such 2 ml. (approximately 3 X 10^8 cells/ml. of yeast determined by optical density at 550 nm.) of the prepared inoculum was again transfered to 500 ml. erlenmyer flask containing 200 ml. of Sabouraud liquid medium (Shechter, 1972). All the flasks were incubated on the rotary shaker at 250 rpm. for 72 hrs. at 30°C before yeast cell harvesting.

Extraction of soluble proteins was followed Gunsalus' method (1955), using acetone powder preparation. The process began with collection of the harvested yeast cells by centrifugation for the period of 10 minutes at 4°C and 2500 rpm. The cells collected from each culture were washed twice with 0.1 M phosphate buffer pH 7.2 at 4°C and resuspended in 10 ml. of cold buffer. The following step was disrupting the cells by rapid freezing and thawing in acetone dried ice for 5 min. so that the final required product would be after filtering through filter paper under vaccuum the protein powder deposited on the filter paper and can be purified by cold acetone at -20°C.

Protein Preparation and Gel Electrophoresis

Protein determination was used Lowry's method (Lowry et. al., 1951) using bovine serum albumin (BSA) for standard curve.

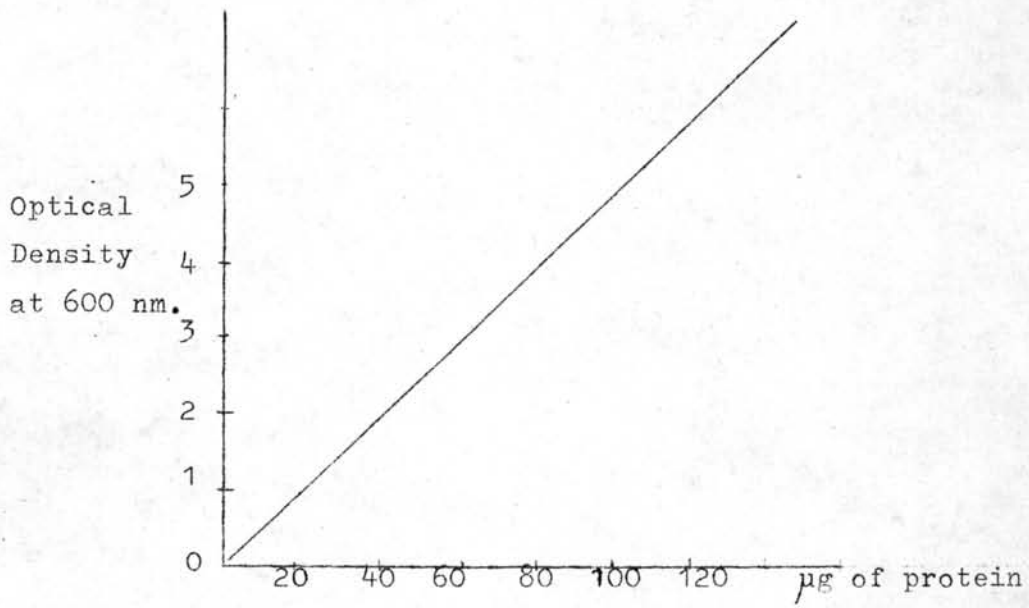

Figure 5 Standard curve of soluble protein with using bovine serum albumin (BSA).

The dried actone powder of each isolates was determined the amount of protein by comparing with standard curve of BSA. That is using 100-200 mg. of dried acetone powder the amount considered enough for running gel electrophoresis. The powder was resuspended in 0.2-0.3 ml. 0.004 M sodium bicarbonate solution and stored overnight at 4°C. The supernative material was collected after centrifuged at 3,000 rpm., 4°C for 15 minutes. For further investigation according to Davis' method (1964) at least 300 µg/0.1 ml. of each protein preparation was used.

The gel column was composed of two parts. The lower part was a small pore gel where the fractionation of proteins took place, the upper part was a large pore gel where the protein became stacked according to their sizes and charges before being separated into the small pore gel.

By this experiment the chemicals reagent in polyacrylamide gel electrophoresis were acrylamide (Merck), N,N'-methylenebisacrylamide (BIS), 2-amino-2-(hydroxymethyl)-1,3 propanediol (TRIS or TRIZMA BASE), N,N,N',N', - tetramethylethylene diamine, riboflavin, hydrochloric acid and ammonium persulfate.

Stock solutions were prepared and stored in brown glass bottle in a refrigerater. The shelf life of these solutions was up to several months.

(A)

1 N HCl	48	ml.
TRIS	36.6	gm.
TEMED	0.23	ml.
water to	100	ml.

(pH 8.9)

(B)

1 N HCl	48	ml.
TRIS	5.98	gm.
TEMED	0.46	ml.
water to	100	ml.

(pH 6.7)

(C)

Acrylamide	28.0	gm.
BIS	0.735	gm.
water to	100.	ml.

(D)

Acrylamide	10.0	gm.
BIS	2.5	gm.
water to	100	ml.

(E)

Riboflavin	4	mg.
water to	100	ml.

(F)

Sucrose	40	gm.
water to	100	ml.

Working Solutions

Small-pore Solution No. 1

1 part A
2 parts C
1 part water

pH 8.8-8.9

Small-pore Solution No.2

Ammonium persulfate 0.14 gm.
water to 100 ml.

Large-pore Solution

1 part B
2 parts D
1 part E
4 parts F

pH 6.6-6.8

Stock-buffer Solution

TRIS 6.0 gm.
Glycine 28.8 gm.
water to 1 liter

pH. 8.3

The details of Davis' method are as follow:

Disc electrophoresis was performed following the method of Davis (1964). Twelve gel tubes 5 X 110 mm. size were cleaned with soft detergent solution and a cotton - tipped applicator stick. The tubes were rinsed and soaked overnight in distilled water. These tubes were drained and allowed to dry before used.

The small pore gel solution composed of 7.5 % acrylamide gel which was defined to separate protein having the molecular weight between 30,000 and 300,000. The preparation of small pore was mixing equal volumn of solutions No. 1 and No. 2. The mixed solution was gently filled into the tube which standed vertically upto the marker at 8.5 cm. by using 10 ml. syringe with long needle. Then thin layer of water was gently added on top of the gel in order to flatten and prevented oxidation of surface of the gel. The rack of gel tubes were placed under the fluorescent lamp in order to polymerize gel. The polymerization of the gel was completed after 30 minutes. Then water was removed from the surface by cotton-tipped applicator stick.

The large pore gel approximately 2.5% polyacrylamide, where the proteins became concentrated was prepared by mixing 1 part of B, 2 parts of D, 1 part of E and 4 parts of F respectively. The solution mixture was gently loaded on top of the

separation gel 1.5 cm. of the height, then few drops of distilled water was gently added on top of gel surface and the water was removed after polymerization.

The polymerized gel tubes were inserted vertically into the grommets of the upper tank of electrophoretic apparatus (Hoefer Scientific Instruments Model DE 102) Figure 6. Then both upper and lower tanks were filled with tris-glycine buffer pH 8.3 which diluted to 1:10 strength with double distilled water. A few drops of 0.03% methylene blue were added into the upper tank for tracking the frontier of protein movement.

Protein sample of C.albicans isolate was added with 0.5 ml. 40% of sucrose for the purpose of increase the density of loading proteins. Soluble protein sample approximately 0.3 ml. of 200-300 ug. was gently loaded just on top of the spacer gel. The power supply (Shandon, Vokam 2541) was connected the cathode to the upper tank and the anode was put down to the lower compartment. The voltage was adjusted 100 volt at the beginning for 15 min., then kept constant at 150 volt through the process. The current was 3 mA. per tube throughout the time of electrophoresis. The negative charge protein molecules would move down along the gel toward the anode. The time required for complete process of each experiment about 3 hours.

Figure 6 Disc-gel electrophoretic apparatus (Hoefer Scientific Instruments Model DE T02).

The gel tubes were removed from the upper tank, when the frontier moved down approximately 8 centimeters. The gel tubes were placed into cold distilled water for few minutes. Then gels were removed from the tubes by injecting cold distilled water in between the gel and the tube. The gel columns were stained overnight in 0.05% coomassie blue (in 25% isopropyl alcohol and 10% acetic acid followed Fairbanks et. al., 1971). After destaining in destained solution which composed of 0.005% coomassie blue solution of 10% isopropyl alcohol and 10% acetic acid for 8 hrs. The gel columns were placed in 10% acetic acid and changed in every 12 hrs. until the sharp bands of soluble proteins were appeared. Staining and destaining had been done at 25°C.

Staining gel columns were stored in 3% of acetic acid test tubes. Gilford 2400 Densitometer was used to scan the protein bands. The chart speed was run 1 inch. per min., and a scanning rate was 2 cm. per min. at 550 nanometer.

The technique of amylase activity detection was followed Beneke et.al. (1975) using 1 % of soluble starch dissolve in ammonium persulfate (Bernfeld, 1951) as a substrate. The soluble proteins was using as the source of intracellular amylase enzyme. After electrophoresis, the gels were removed from the tubes and then incubated in 0.01 M phosphate buffer,

pH 5.8 for 40 min. at room temperature (30°C), quickly immersed in Gram's iodide solution for 1-2 min.. The gels were removed and recorded the Rf value of the clear band, which indicated amylase activity.