ON TWO STRAINS OF YEAST-LIKE ORGANISMS CULTURED FROM DISEASED HUMAN THROATS

BY

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TARTU (DORPAT) 1929

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The object of the present article is to compare the properties, in culture, of two strains of yeast-like organisms isolated from diseased human throats, and also to test their pathogenicity for the common laboratory animals. It is known that yeast-like organisms in conjunction with bacteria are frequently found in the bacteriological examination of the exudates obtained from diseased mucous membranes of the respiratory system. Generally, they are only saprophytes harmless for man and do not cause pathologic changes in test animals. During recent years there have been described various lesions of the throat, bronchi and lungs with numerous yeast-like organisms in the expectoration. The latter were found to be pathogenic for the laboratory animals, and many cases of human diseases came to be looked upon as being caused by these organisms. Other cases, however, were thought to be caused by certain pathogenic bacteria and the yeast-like organisms were regarded as a secondary infection. Bronchomycosis has recently received prominent attention from many observers. Stovall and Greeley[1]) reported 18 cases of pulmonary affections with yeast-like organisms in the expectorations. 12 strains were pathogenic for the laboratory animals. In an examination of the sputa of a number of cases of suspected tuberculosis Marett (Jersey) found that 40 per cent. contained blastomycetes, 40 per cent. blastomycetes and tubercle bacilli, and 20 per cent. tubercle bacilli without blastomycetes. The author points out that chronic blastomycetic bronchial catarrhs are of frequent occurrence and that they are the commonest precursors of pulmonary tuberculosis. A search through the literature of the subject reveals the fact that in similar cases many authors attach no importance to the presence of yeast cells in the sputum, and assume some other cause for the bronchial catarrh.

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1) Stovall, W. D. and Greeley, H. P. J. Amer. Med. Assoc. 1928, 91.

It has long been known that yeast-like organisms are present in the throats of healthy persons, but how far they are responsible for pathological conditions is not yet settled. Our previous bacteriological investigations have shown that in a large proportion of the cases of angina various yeast cells were present. Generally, they were considered as mouth contamination and their pathogenicity for the laboratory animals was not tested. The present two strains of yeast-like organisms were cultured from two patients in whom the clinical features of the throat affection suggested the possibility that the yeasts in association with bacteria might be the factor of the pathologic condition. It seems, therefore, that it might be of interest to study these strains in order to determine their virulence and importance in disease. From a practical point of view it may be mentioned that in cases of blastomycetic affections a special and systematic treatment must be carried out. The successfulness of the treatment, however, is secured by the early recognition of the cause of the pathologic condition.

Strain 1 came from the throat of a 7-year old boy having scarlet fever. At first the child exhibited the clinical features of a mild form of scarlet fever. By the sixth day the malignant pharyngeal symptoms appeared and progressed rapidly. The fauces and tonsils were covered with a thick membranous exudate. Necrosis occurred in the tissues of the throat and the angina was associated with otitis. On bacteriological examination of the throat hemolytic streptococci and numerous yeast-like organisms were found. The Klebs-Loeffler bacillus was not present. The child died within 36 hours with clinical picture of an acute intoxication.

Strain 2 together with the hemolytic streptococcus was isolated from the throat of a 46-year old man. The patient exhibited the clinical picture of an acute and severe pharyngitis. The disease set in with a high fever. The examination of the throat revealed a general congestion of the mucous membrane. The tonsils were covered by a pultaceous exudate. The disease persisted for 5 days and improved after medical treatment.

Cultural characteristics. The strains 1 and 2 of yeast-like organisms grow abundantly on ordinary media; a richer development occurred on sugar media. The beerwort medium was especially favourable. They grow both at room

(20° C) and at incubator (37° C) temperature; 37° C was more favourable for growth than 20° C. No growth occurred in anaerobic cultures.

The growth of the two strains on Sabouraud's acid agar was found to be practically identical. In young cultures the colonies were circular, white, smooth and elevated in the centre, becoming cream-coloured and powdery in old cultures. On glucose agar the cultural characteristics were more varied. Growth was rapid and the cultures covered the entire surface of the slants. The beginning of growth was white, smooth and heaped up. After a few days the white of strain 1 changed to a cream colour, becoming light brown, downy and powdery in old cultures. Filaments were sometimes noted in old cultures radiating out from the edges of the colonies and sheets. On the surface of growth light folds were noted and the underside of the colonies changed to brown.

The growth of strain 2 showed much the same character in young cultures. In old cultures the white slowly changed to a slightly grayish tinge, the powderiness was scantier and no filaments were noted on glucose agar medium. Growth on carrot was abundant, white in young cultures and lightly grayish in old cultures. For the gelatine liquefaction test the organisms were grown in gelatine medium (12%) for thirty days at room temperature (20° C) and in the incubator (37° C). In the gelatine-stick cultures (20° C) growth was very slow. At first, only a thin layer formed on the surface. Later on development occurred along the line of puncture and filaments radiated out from the stab like an inverted pinetree along the line of stab. At the end of the cultivation the set that was grown in the incubator was put into the ice box for three hours. The gelatine was not liquefied.

These yeast-like organisms produced much the same type of growth in all liquid sugar media. At first, growth appeared at the bottom of the tubes. After a few days of growth a flocculent grayish-white deposit was visible at the bottom of the tubes. After a few weeks a flocculent cloudiness become apparent in the liquid medium and a granular deposit along the sides of the tubes. A grayish-white culture-ring developed on the walls of the tubes encircling the surface of the medium. No pellicle developed on the surface of the cultures.

M o r p h o l o g y. These yeast-like organisms stain readily in the young state with ordinary dyes, they are Gram-positive. The shape and size of cells varied markedly even in the same culture. In the young cultures (48 hours at 37° C) oval and slightly elongate budding forms were more often observed. Some sausage-shaped and very long forms were found in young cultures of strain 1. No mycelia were present in the early cultures. In the unstained preparations the yeast cells were strongly refractive (See Figs. 1 and 2).

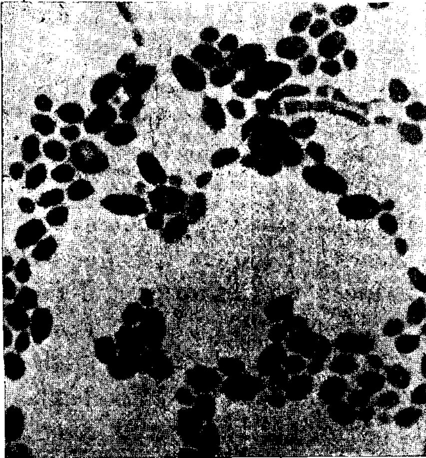

Fig. 1. Strain 1 from dextrose agar, 48 hours at 37° C.

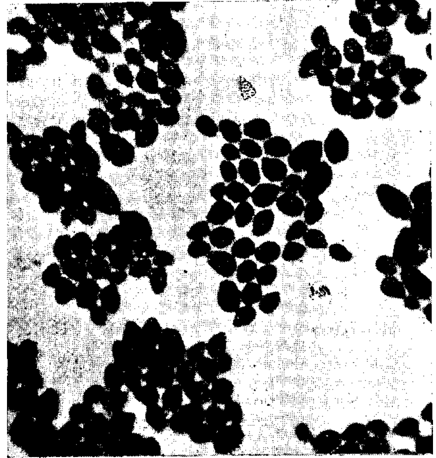

Fig. 2. Strain 2 from dextrose agar, 48 hours at 37° C.

The yeast cells from the old cultures stained very irregularly. The size and shape of the cells varied enormously. Each yeast cell contained a more or less definite nucleus, which was demonstrated by the hæmatoxylin. In the glucose agar cultures incubated for 30 days at 37° C numerous large round, encapsulated cells were present. With basic stains the cytoplasma of these was clear, except for a small bit of rounded or oval material which took the stain. Strain 1 produced numerous elongate yeast cells on this medium as well as some long septate mycelia. Strain 2 formed mycelia reluctantly on glucose agar (see Figs. 3 and 4).

Strains 1 and 2 produced abundant mycelia in the old sugar broth cultures. Carrot peptone broth was found to be a

good medium for the production of mycelium. The mycelia were septate and continued to reproduce by budding only. Short branches were present arising anywhere along the mycelium.

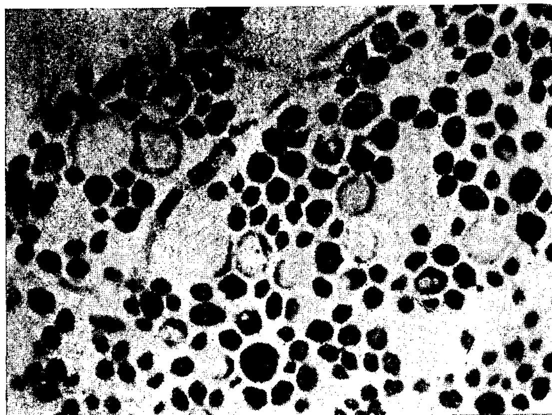

Fig. 3. Strain 1 from dextrose agar, 30 days at 37° C.

Lateral and terminal conidia were present. At the end of many mycelia a rounded large body was visible, with doubly refractive membrane and granular cytoplasm. With basic stain the cyto-

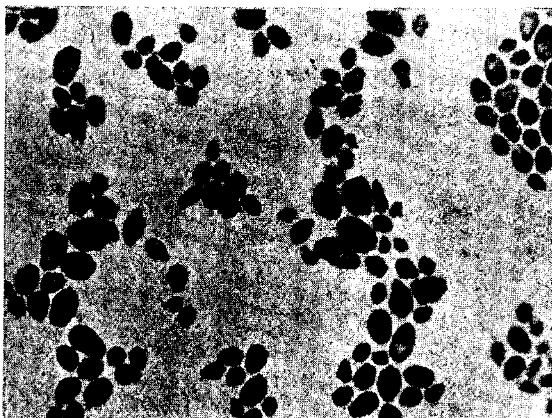

Fig. 4. Strain 2 from dextrose agar, 30 days at 37° C.

plasm was clear, except for small bits of material which took the stain (see Figs. 5 and 6).

For demonstration of the formation of spores Moeller's and Fraenkel's staining methods were used. No spores were

found in cultures on ordinary media. An attempt was then made with various elective media. Young cultures in beerwort media were transplanted to carrot, to Gorodkova's medium and

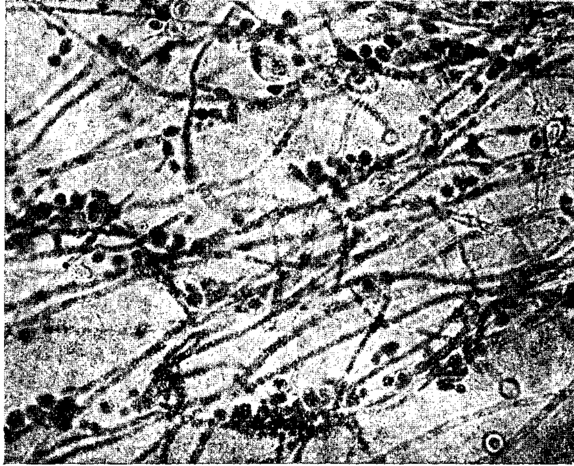

Fig. 5. Strain 1 from 10% sucrose broth, 37 days at 37° C.

to gypsum blocks. The cultures were incubated for 3 months at 37° C. No spores were formed on these elective media.

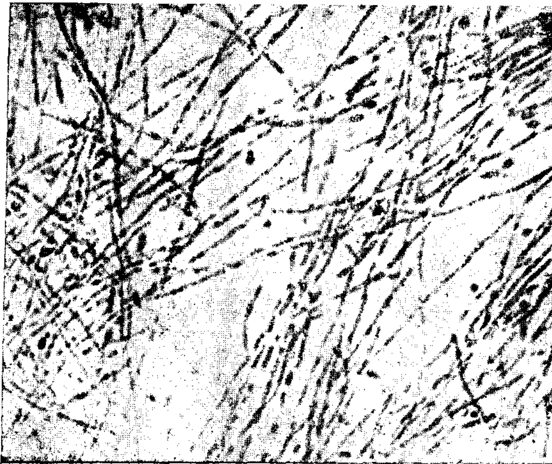

Fig. 6. Strain 2 from 10% sucrose broth. 37 days at 37° C.

Sasakawa [1]), in his extensive work, tested the formation of spores in numerous strains of pathogenic blastomycetes. His experiments have shown that only one species of pathogenic yeasts, Saccharomyces canis Sanfelice, possesses the property of forming spores. Many irregular acid resisting granules and bodies were demonstrated, however, in several strains by Moeller's and Fraenkel's staining methods. We, too, have found similar acid resisting bodies in the cells of strain 1 incubated on carrot for three months.

Fermentation tests. As fermentation tests with carbohydrates, the ordinary macromethods and the micromethod recommended by Guilliermond [2]) were used. Two per cent. of each of the carbohydrates in broth medium was used in this series with litmus as indicator. The cultures were always incubated for ten days before being discarded as negative. Table 1 gives the reactions with the carbohydrates studied. The Hopkins [3]) medium containing lactose and saccharose was tried out for testing the pathogenicity of the fungi in vitro. After growing for three weeks they did not ferment either of these sugars. The results obtained with our strains 1 and 2 are thus in agreement with Hopkins' theory that pathogenic yeasts will not ferment these two sugars. Generally, the present yeast-like organisms possess very weak zymogenic properties. They did not change the litmus milk; no indol or hydrogen sulphide was formed by them.

Table 1. Fermentation tests of the two strains.

Yeast-like Organism	Glucose	Levulose	Maltose	Galactose	Saccharose	Lactose	Mannitol	Dulcitol	Dextrin	Arabinose	Inulin	Litmus milk	Glycerine	Gelatine
Strain 1	AG	0	AG	sA	0	0	0	0	0	0	0	alk	0	0
Strain 2	AG	0	AG	sA	0	0	0	0	0	0	0	alk	0	0

0 = no reaction; A = acid; G = gas; s = slight; alk = alkaline.

1) Sasakawa. Centralbl. f. Bakteriol. etc. Abt. I, Orig. Bd. 88, S. 269.

2) Guilliermond. Clef dichotomique pour la détermination des levures. Paris, 1928.

3) Hopkins, J. G. and Iwamoto, K. Arch. f. Dermat. u. Syphil. 1923, 8.

Thermal resistance. Thermal death point tests were tried out at 48° C and at 56° C. Sterile test tubes each containing 5 cc of sterile physiologic salt solution were heated in the water bath at 48° C and at 56° C. After 10 minutes 1 cc of 24-hour growth suspended in sterile salt solution was added to each tube and the tubes were heated in the water bath. The fragments were cultured on glucose agar after 5-, 10-, 20-, 30-, 45-, 60- and 90-minute exposures. The final results after 72-hours growth at 37° C were recorded. The time in minutes required to kill the organisms is shown in table 2.

Table 2. Time and temperature necessary to kill the yeast-like organisms.

Strain	Temperature	Minutes							
		5	10	15	20	30	45	60	90
1	48° C	+	+	+	+	+	—	—	—
1	56° C	+	+	+	—	—	—	—	—
2	48° C	+	+	+	+	+	—	—	—
2	56° C	+	+	+	+	—	—	—	—

+ indicates growth; — no growth after the exposure.

Ricketts[1]) has reported that the pathogenic yeasts were killed in two to five minutes at 54° to 58° C. Spring[2]) and Mc Kinney[3]) have found that numerous strains of blastomycetes can survive 48° C for twenty minutes. It appears that the pathogenic yeasts thus rank among the more resistant organisms as far as heat is concerned.

Animal tests. As soon as the yeast-like organisms could be grown in pure culture, they were injected into test animals to determine their virulence. Cultures grown on sugar agar for 24 hours at 37° C were suspended in sterile physiologic salt solution, and 0.5 cc to 1 cc of the emulsion of each of the strains were injected subcutaneously, intraperitoneally and intravenously. When abscesses resulted from the injection, they were cultured. The animals that died were necropsied and the heart blood, spleen, kidney, liver and lungs were cultured.

1). Ricketts, J. Med. Research 1901, 6, p. 537.
2) Spring, D. Journ. of Infect. Diseases 1929, 44, p. 169.
3) Mc Kinney, M. Journ. of Infect. Dis. 1929, 44, p. 47.

When the yeast-like organism was recovered from an animal, it was identified by carbohydrates fermentation tests.

The rabbit which received 1 cc of an emulsion from strain 1 into the marginal ear vein died within 48 hours, and the rabbit which received an emulsion from strain 2 died within 3 days. No lesions were to be seen, but the identical organisms were recovered from lungs, spleen, liver and heart blood. It may be noted in passing that the death of the rabbits is due to organ insufficiency rather than to the virulence and multiplication of the organisms. Intraperitoneal injection into rabbits caused no real visceral changes. In rabbits killed 40 days after the injection a few nodules were seen in the tissue at the root of the mesentery. No yeast-like organisms were found. The rabbits which received subcutaneously 1 cc of a suspension from strains 1 and 2 developed a marked infiltration in the subcutaneous tissue. Within 10 to 14 days a fluctuant abscess was formed which subsequently ulcerated and discharged quantities of greenish-yellow pus. Identical yeast-like organisms were recovered from the pus. The ulcers improved spontaneously, and there was no tendency to local extension, much less to metastasis.

The guinea-pigs which received intraperitoneally 0.5 cc of an emulsion remained well. When killed four weeks later, there were usually found adhesions between several loops of bowel. Small caseous nodules were present in the abdominal cavity which had a predilection for the root of the mesentery. Only one of the guinea-pigs, which was inoculated with strain 1, had an abscess in the liver, from which the identical organisms were recovered. All the guinea-pigs that were injected subcutaneously with 0.5 cc of an emulsion developed a marked infiltration and later a local abscess was formed, from which the identical organisms were cultivated. The guinea-pigs improved spontaneously and there was no tendency to metastasis.

The white rats which were inoculated intraperitoneally with strains 1 and 2 remained well. When killed four weeks later and examined at necropsy, the animals exhibited small caseous nodules near the root of the mesentery. There was no evidence of generalized blastomycosis. All the rats that received subcutaneously 0.5 cc of an emulsion developed a local infiltration which exhibited a tendency toward spontaneous healing.

As to the susceptibility of different animal species, it appeared that the white mice were the most susceptible of all the laboratory animals. Several mice which were inoculated intraperitoneally with strains 1 and 2 died within a few days. No lesions were to be seen, but the kidneys, liver, spleen and lungs contained numerous yeast-like organisms. The remainder of the injected mice died within 25—35 days. Multiple miliary blastomycetic abscesses were recovered in most of the organs. The mice which were inoculated subcutaneously died within 20—30 days. Local infiltrations and ulcers were sometimes to be found. When examined at necropsy, there was evidence of generalization in some cases. The examination confirmed that in the mouse the yeast-like organisms had the power of proliferation.

Classification. A review of the preceding data shows that the two strains of the yeast-like organisms are closely related. That they are not of the true yeasts or saccharomycetales is suggested by their failure to produce ascospores under favourable conditions and also by their failure to reproduce sexually. All the pathogenic yeasts are called blastomycetes because of their frequent method of reproduction by means of budding. The position which the blastomycetes occupy in systematic biology has not yet been exactly determined. In recent years they have been grouped with the ascomycetes (Buschke [1]), but this view was contested by Guillermond. In general, the blastomycetes can be regarded as modified yeast forms and because of this they are designated as Fungi imperfecti (Non-saccharomyces of Guillermond, Hyphomyces of other authors). The fungi imperfecti may or may not form a mycelium. Ricketts divided the yeast-like organisms which form mycelia into a group which forms mycelia reluctantly, and another which forms abundant mycelia. The yeast-like organisms which multiply by budding and form mycelia under certain conditions are variously classified as Monilia (Plaut [2]), Persoon), Blastomyces (Gilchrist [3]), Oidia (Ricketts), Oospora (Saccardo)

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1) Buschke. Handb. der pathog. Mikroorg. Bd. V. 1928.

2) Plaut. Handb. d. pathog. Mikroorg. von Kolle u. Wassermann, Bd. 32, 1913.

3) Gilchrist. Bull. John Hopkins Hosp. 1896, I, p. 269.

or Parasaccharomyces (Anderson [1]). Those which do not form mycelia are classified as Torula (Stoddard and Cutler [2]), or Cryptococcus (Castellani and Chalmers [3]). On the morphological basis it seems to be evident that our strains 1 and 2 should be placed with the budding organisms which form mycelia, and they could therefore be called Monilia. Castellani [4]) thinks that since mycelial elements are present, classification among the Monilia is most satisfactory.

Classification of the Monilia is undertaken by means of biochemical characteristics by Castellani and Chalmers, Nye, Zerfas and Cornwell [5]), and similar tables of the sugar fermentations were constructed by them. If the carbohydrates fermentation reactions are chiefly considered, it appears that our two strains 1 and 2 would belong to the same species. Castellani, [6]) however, points out that many Monilia after a few transplantations can alter their fermentation characters. Fermentation tests may, therefore, form a basis for separating genera; they are unreliable for separating species. Many authors (Anderson etc.) have arranged a classification of yeast-like organisms ignoring carbohydrates fermentation tests altogether. The sugar fermentations of our two strains do not exactly agree with those in Castellani's table, and the nature of these pathogenic yeasts does not yet admit of definite classification.

In recent years many authors have suggested that serologic reactions might aid in the identification and classification of yeast-like organisms. Animal immunization with yeasts and immune reactions in those animals have been studied by Hines [7]), Van den Linden [8]), Castellani [9]), McKinney [10]). Generally, it was found that fungi behave like bacteria in pro-

1) Anderson. Journ. of Infect. Dis. 1917, 21, p. 341.

2) Stoddard and Cutler. Monog. 6. Rockefeller Inst. for Med. Res. 1916.

3) Castellani and Chalmers. Manual of Tropical Med. 1920, p. 1082.

4) Castellani. Arch. Derm. u. Syphil. 1926, 14, p. 291.

5) Nye, Zerfas and Cornwell. Am. J. M. Sci. 1928, 175, p. 153.

6) Castellani. Arch. f. Dermat. u. Syphil. 1927, 16.

7) Hines. Journ. Infect. Dis. 1924, 34, p. 529.

8) Van den Linden. Compt. rend. Soc. de biol. 1926, 95, p. 881.

9) Castellani. Brit. Med. Journ. 1923, 2, p. 238 and Arch. f. Derm. u. Syphil. 1927, 16.

10) Farah. Journ. Trop. Med. 1923, 26.

ducing specific agglutinins, but that non-specific agglutinins are also present. It was more difficult to immunize rabbits against the yeast-like organisms than against bacteria. Mc Kinney states that the agglutination and complement fixation reactions indicated a marked serologic difference between the non-pathogenic commercial yeast and the Monilia isolated from human sources. The serologic reactions show only a slight group or species specificity and thus do not classify the yeast-like organisms within the pathogenic group. Specific agglutinins in patients serum during blastomycotic diseases have been found by Widal and Abrami. Farah found positive agglutination and complement fixation reactions with patients serum during bronchomoniliasis. In general, the serologic reactions with patients serum during the diseases produced by the pathogenic yeasts are not yet sufficiently studied and they remain a source of confusion to serologists.

Summarizing discussion. A review of the characters by which the yeast-like organisms are classified shows that the organisms described could be called Monilia. The cultural characteristics, pathogenic and zymogenic properties of these organisms do not yet admit of definite identification of our two strains. They are virulent to some degree for laboratory animals. In general, the virulence for the common laboratory animals was low and the symptoms of disease were featureless, even when injected into the peritoneal cavity. The degree of pathogenicity of our strains 1 and 2 varied from production of inflammation and abscesses to death. As to the susceptibility of different animal species, it appears that rabbits were but slightly susceptible, generalization occurred in no instance. Guinea-pigs were comparatively resistant. White rats were susceptible, although they were not killed by the disease. White mice were very susceptible to these blastomycetic organisms and several mice died within a few days of the inoculation. In the case of the mice, there were but a few instances of generalization. On the whole, it appears that the white mouse is an animal of choice for diagnostic tests of the pathogenic yeasts. Spring points out that the testicle of the rat and mouse is the situation of choice for inoculation. In appraising the pathogenicity in animal tests death should not be expected; it is sufficient to achieve local multiplication of the blastomycetic

organisms and the development of abscesses. Ricketts, Stober[1]), Hektoen, Hyde and Bevan[2]) tested many strains of pathogenic yeasts on various laboratory animals and they were never able to secure consistently positive results. The animal tests were planned by many authors towards the reproduction of the blastomycotic disease as it occurred in man. This way of comparison of the pathogenicity, however, is not of much value, because in man a pathogenic yeast can lose a certain degree of the virulence in a short time. On the other hand, incongruous results might be explained by the degree of tissue resistance. It is usual to find that blastomycetic lesions have not developed uniformly enough in the different animal individuals and in the various anatomical parts of the body.

As to the pathogenicity of our two strains to man, it appears that these yeast-like organisms should be looked upon as being a certain etiologic factor of the pathologic condition in the two patients described. On microscopic examination of the stained smears from the throat, great numbers of yeast-like organisms in conjunction with cocci and bacteria were found. In none of the patients with an acute pharyngitis were such great numbers of yeast-like organisms present. One cannot therefore escape the impression that these blastomycetic organisms were able to increase the severity of the disease. It is well-nigh impossible to admit that they are only harmless saprophytes in the throat. Certainly, in these two cases the mucous membranes of the throat were bearers of the pathogenic yeasts, and the tissue resistance was considerably diminished before the symbiotic infection took place.

Conclusions.

Two strains of yeast-like organisms isolated from diseased human throats were studied. The following are the principal results:

1. Culturally, the budding yeast-like organisms were Monilia, producing mycelium and having no ascospores. Irregular acid resisting granules and bodies were found in the yeast cells incubated on carrot for three months.

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1) Stober. Arch. Int. Med. 1914, 13.

2) Hektoen, Hyde and Bevan. Journ. of Dermatol. 1899, 11.

2. The present yeast-like organisms possess very weak zymogenic properties. No indol or hydrogen sulphide was formed.

3. These yeast-like organisms rank among the more resistant microorganisms as far as heat is concerned.

4. They are virulent to some degree for the common laboratory animals. Generalization of infection occurred only in the mouse, which is the animal of choice for diagnostic tests.

5. It appears probable that the yeast-like organisms isolated were a certain etiologic factor of the pathologic condition in the two patients described.