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STUDIES ON CERTAIN YEASTS ASSOCIATED  
WITH ANIMALS

BY

ABDELHAFEEZ HASSAN NIMIR, B. V. Sc.

Thesis submitted for the degree of Doctor of  
Philosophy in the Faculty of Veterinary Medicine,  
the University of Glasgow.

Department of Veterinary Pathology,  
University of Glasgow.

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## SUMMARY

The objectives of this study were to investigate the significance of yeasts as animal disease agents in the West of Scotland, to study the commensal association of yeasts with animals and, in particular, to assess the possibility of applying serological tests in the diagnosis of feline cryptococcosis.

Examination of pathological specimens from various animal sources revealed that animal diseases attributable to yeasts were not common in this area. Apart from otitis externa in dogs, with which Pityrosporum pachydermatis was associated, other disease conditions were only sporadically encountered. Candida albicans was associated with 2 cases of oral thrush in adult guinea-pigs and with 3 cases of gastrointestinal ailments in puppies. Torulopsis pintolopesii was incriminated in causing a fatal gastrointestinal and systemic infection in a racing pigeon.

No previous systematic studies have been done to investigate the commensal existence of yeasts on and in dogs and cats. In this study, cultural surveys were undertaken to investigate the prevalence of yeasts in those hosts. It was established that yeasts might occur as commensals on normal canine skin and in the nasal cavities of apparently normal dogs and cats.

Besides its association with canine otitis externa, Pit. pachydermatis was found in association with skin infections in dogs.

The association of C. albicans with oral infection in 2 guinea-pigs prompted a cultural study to see if this and other yeasts were prevalent in the oral cavity of healthy guinea-pigs. Candida albicans and

Saccharomyces telluris were found to exist as normal inhabitants in this site.

The value of serological methods in the diagnosis of animal cryptococcosis has not previously been determined. A serological survey was undertaken to test sera from apparently normal cat and dog populations for the presence of antibody to Cryptococcus neoformans. Three tests, namely, counterimmunoelectrophoresis, immunodiffusion and agglutination tests were employed. Counterimmunoelectrophoresis proved more sensitive than the other tests. Using it, precipitating cryptococcal antibody was demonstrated in a proportion of cats and dogs. The antibody response was thought to have resulted from exposure to the yeast in the environment. The use of serology in the diagnosis of feline cryptococcosis was evaluated in experimentally infected cats. The 3 tests were used to examine sera and other body fluids from experimental cats for the presence of antibody and/or antigen. It was established that the presence of serum cryptococcal antigen was proof of active cryptococcosis in the cat. On the other hand, the presence of circulating cryptococcal antibody alone could not be regarded as evidence of such disease. Its presence, however, may suggest early or past infection, or simply exposure to the organism.

The role of black yeasts as disease agents in domestic animals is not known and has not previously been investigated. In this study, 3 black yeast isolates from animal sources were identified and their pathogenicity for mice determined. One isolate, a Cladosporium species, was found to be non-pathogenic. Two isolates of Fonsecaea dermatitidis were highly virulent to mice and had a marked neurotropic affinity.

Although not yet determined, the possibility that this species is pathogenic to domestic animals, could not be dismissed.

The literature on diseases caused by yeasts in animals is reviewed.

INTRODUCTION

Van Leeuwenhoek is credited with the first observations of yeasts microscopically. In 1680, he sent descriptions and drawings of yeast cells to the Royal Society in London, some four years after he had made public his observations on bacteria. It was not until the first half of the 19th century that significant progress was made towards an understanding of the biology of yeasts and through this to an appreciation of their physiology and biochemistry. According to Rose and Harrison (1969), Cagniard-Latour in 1837 demonstrated that beer yeasts were spherical bodies capable of multiplying and that they belonged to the vegetable kingdom.

Pasteur proved that fermentation was due to living cells and pointed out the role of oxygen in alcoholic fermentation (1866, 1876).

After Pasteur, there followed a period of intense activity during which yeast taxonomy and morphology flourished. Jørgensen (1886) wrote on yeast fermentations. In 1912, the first treatise devoted entirely to yeasts was published by Guilliermond. He was the first to include keys for the identification of yeasts. Advances in yeast taxonomy and systematics have, in recent years, been dealt with comprehensively by workers in Delft, Holland (Stelling, 1931; Lodder, 1934; Diddens and Lodder, 1942; Lodder and Kreger-van Rij, 1952; Lodder, 1970). Other valuable texts dealing with the biology and/or taxonomy of yeasts include those by Ingram (1955), Roman (1957), Cook (1958), Reiff, Kautzmann, Llers and Lindemann (1960; 1962), Rose and Harrison (1969; 1970) and Barnett and Pankhurst (1974).

Over the years, more and more yeast genera and species have been described and Lodder (1970) has listed 341 species in 39 genera. Later, Barnett and Pankhurst (1974) added 10 new genera and 93 new species to those already reported, thus giving a total of 434 species in 49 genera that are presently accepted universally.

Yeasts are ubiquitous, occurring from the polar to the tropical regions and are found in terrestrial and aquatic environments (Carmo-Sousa, 1969; Lodder, 1970; Ahearn, 1974). They inhabit the soil, plants, atmosphere, fresh and salt water, insects and vertebrates. They are known to exist as commensals on or in the animal body and have been isolated from domesticated, zoo and wild animals. Isolations have been made from the digestive tract and faeces of cattle (van Uden and Carmo-Sousa, 1957), horse, sheep, goat and swine (van Uden, Carmo-Sousa and Farinha, 1958; van Uden and Carmo-Sousa, 1962), camels (Saez and Rinjard, 1973), from zoo and wild animals (Krabisch and Amtsberg, 1974; Saez, Hugot and Traore, 1974), and in the ears of apparently healthy dogs, cattle, horses and pigs (Gustafson, 1960; Fraser, 1961; Smith, 1968). They have been recovered from the digestive tract and droppings of poultry and other birds (Verona, 1972; Swinne-Desgain, 1975).

Yeasts have long been recognised as causal agents of disease in man and lower animals. According to Wilson and Plunkett (1965), Lagenbeck in 1839 demonstrated a yeast-like fungus in the lesions of human thrush, and his findings were confirmed by Gruby (1842) and the causal organism was named Oidium albicans by Robin (1853). Zopf (1890) named the fungus Monilia albicans and for many years the disease was known as moniliasis. However, after the organism was renamed Candida albicans



by Berkhout in 1923, the disease name candidiasis was adopted. Presently the term candidiasis is the name generally accepted by medical mycologists.

The early data regarding animal candidiasis was, apart from that on poultry, regarded as scanty and unsatisfactory (Ainsworth and Austwick, 1959). Eberth in 1858 first reported candidiasis in chickens and the first major epidemic of this disease was recorded in the United States of America by Gierke (1932) who described 8-20% mortality in young turkeys. In the following year, also in the U.S.A., Jungherr (1933) reported the loss of several thousand chicks in a commercial hatchery. Outbreaks of candidiasis among turkeys and fowls in Australia were recorded by Hart (1947) and in Britain a series of epidemic outbreaks among turkey poults were reported by Blaxland and Fincham (1950). Avian candidiasis has also been reported from Germany (Schlegel, 1912; 1921), Sweden (Plazikowski, 1935), France (Salgues, 1937), New Zealand (Salisbury, 1949), Poland (Borkowska-Trippenbach and Szwejkowski, 1950) and Italy (Tappi, 1952-53).

Reports on infections of farm animals attributed to C. albicans are more recent. Fatal oral candidiasis of suckling pigs was described by Kovalev (1947) and in 1952 Quin recorded a similar incident and attributed it to the effect of antibiotic residues in animal feed. Oral candidiasis in pigs has also been reported by McCrea and Osborne (1957) and Gitter and Austwick (1959) in Britain. Systemic bovine candidiasis was first reported in feed-lot cattle by McCarty in 1956 and in young calves on prolonged antibiotic therapy by Mills and Hirth (1967).

The genus Candida accommodates most of the pathogenic yeast species

and many species of Candida other than C. albicans have been associated with animal disease. Candida tropicalis has been associated with mycotic abortion in cattle and was isolated from bovine fetuses by Austwick and Venn, (1957) and Schulte and Scholz (1962). Catarrhal and purulent endometritis was produced in heifers by intra-uterine and intra-vaginal infection with C. tropicalis, C. krusei and C. parapsilosis by Hajsig, Kopljar and Steficic (1964). Austwick, Pepin, Thompson and Yarrow (1966) isolated C. tropicalis from an aborted foetus and incriminated it as the cause of abortion. These authors infected pregnant cows intravenously with their isolate and produced uterine infection and abortion; C. tropicalis was isolated from the stomach contents of the aborted fetuses. Wohlgemuth and Knudtson (1973) reported this yeast as the cause of abortion in a cow in the 6th month of gestation. The lesions were thickening of the allanto-chorion, placentitis and partial attachment of the caruncles to the cotyledons. Candida tropicalis was recovered from foetal abomasal contents and placenta and Periodic acid-Schiff positive "bodies" were demonstrated in the placental villi and vessels. It has also been associated with bovine mastitis (Austwick et al., 1966; Sebryakov, Velitok, Zavgorodnyi and Tikhonov, 1971; Sebryakov, 1973; Segers and Craenen, 1976).

Sudaric, Agonovic and Hadzimuratovic (1970) isolated C. tropicalis from pulmonary lesions of a pig with pneumonia and demonstrated "Candida blastospores" in sections from the lesions. They believed that C. tropicalis was the cause of pneumonia in this case. Kuttin, Beemer, Bock and Perlstein (1975) attributed an outbreak of respiratory tract infection among 1,000 turkeys, of which 50% died, to C. tropicalis and

the authors suspected that the addition of tetracycline and furazolidone in food and this antibiotic and chloramphenicol in water might have predisposed to infection. This yeast was isolated from the lungs and airsacs of infected birds.

Volintir, Popa, Moga-Minzat, Jivanescu, Garoiu and Onica (1967) reported an epizootic of gastroenteritis in unweaned piglets which evolved parallel to mastitis in the sows. A mortality rate of 80% was recorded among the piglets and C. pseudotropicalis was isolated from gastrointestinal tissues of the piglets and from the sow's udders and was demonstrated invading the damaged intestinal mucosa of piglets histologically. Sidkar, Singh, Banerjee and Sharma (1972) implicated this species as a cause of abortion in mares after isolations from placentas, foetal stomachs, lungs and liver. They also reported that the isolate was fatal to mice. This yeast has also been incriminated as a cause of bovine mastitis (Artecki, Zmarlicki and Gawel, 1970; Trolldenier, Boretins and Schultz, 1970; Danko, 1972).

Candida parapsilosis was isolated by Bisping, Refai and Trautwein (1964) from a case of mycotic abortion in a cow in the 3rd month of pregnancy and by Prasad and Prasad (1966) from a cow with acute mastitis which had developed 10 days after antibiotic treatment for clinical Staphylococcal mastitis. Dale (1972) attributed a case of canine dermatitis, manifested as alopecia and ulceration of the tail, to this yeast and reported successful treatment with topical application of a nystatin-containing cream.

Candida krusei has been associated with abortion in cattle (Hajsig

et al., 1964; Austwick et al., 1966), with mastitis in cows (Sebryakov, 1973; Segers and Craenen, 1976) and with enteritis in calves (Austwick et al., 1966; Hofmann, Kaufer and Weber, 1975).

Although C. guilliermondii is regarded as less pathogenic than certain other Candida species, it has been incriminated by Nicolet, Steck and Gerber (1965) as causing disseminated skin sclerosis in a mare with chronic skin granulomas, abdominal oedema and mastitis. The yeast was isolated from biopsy material from the skin lesions. Austwick et al., (1966) isolated it from aborted bovine fetuses and Silverio and Camargo (1975) isolated it, together with C. albicans, C. stellatoidea and C. tropicalis, from 20 of the 1,113 samples of semen or uterine secretions from English thoroughbred horses with genital diseases. These authors thought that the yeasts which they isolated might have been of significance in the disease conditions.

C. slooffii has been reported solely from disease conditions in pigs. Austwick et al. (1966) found it to be capable of invading the epithelium of the oesophagus and the oesophageal zone of the stomach as evidenced by hyphal penetration. Smith (1967) reported it invading the epithelium in the pars oesophageal area of the stomach of 35 of 53 pigs, stated that the majority of affected pigs appeared clinically normal and no deaths because of candidiasis were encountered.

The yeast Cryptococcus neoformans was recognised as an animal pathogen late in the 19th century. Following his isolation of a yeast from peach juice in 1894, which he named Saccharomyces neoformans, Sanfelice (1895a,b) isolated a similar yeast from the lymph node of an ox in Italy.

He named this latter isolate S. litogenes. Cox and Tolhurst (1946) believed that this isolate was Cr. neoformans. Hence Sanfelice's isolate represented the first record of cryptococcal infection in an animal. In 1901, Vuillemin found cryptococci in a pulmonary lesion in a pig and recognising that the lack of ascospore formation differentiated it from the ascosporogenous yeasts, transferred it to the genus Cryptococcus. Klein (1901) described a pathogenic yeast recovered from cow's milk. Weis (1902) considered this to be identical with strains of Cr. neoformans of human and plant origin.

The first case of equine cryptococcosis was described by Frothingham (1902) in Massachusetts. He isolated Cr. neoformans from a myxoma-like lesion in the lung of a horse which had a persistent nasal discharge of a year's duration. In 1913, Meyer isolated, from a myxoma-like nasal growth of a horse in Pennsylvania, a yeast-like fungus which was designated by him, and later by Harrison (1928), as Torula nasalis. A similar isolation was made by Schellner (1935) in Germany and according to Ainsworth and Austwick (1973) it is probable that all these yeasts, and those obtained by Kikuchi (1923) from a horse in Japan and by Weidman (1935) from the lip of a horse in Philadelphia, were strains of Cr. neoformans.

The main disease caused by Cr. neoformans in animals is bovine mastitis. An impressive outbreak was reported from Maryland by Pouden, Anderson and Jaeger (1952) who described the clinical aspects. In the course of 12 months, 106 of 235 cows of a Holstein-Friesian herd became infected. Innes, Seibold and Arentzen (1952) described the pathology of the condition and Emmons (1952) identified the yeast as

Cr. neoformans. A further outbreak of cryptococcal mastitis in the United States of America in which 50 of 280 cows were affected was reported by Simon, Nichols and Morse (1953). Although these authors did not determine the initial source, they attributed the spread of infection within the herd to the use of an antibiotic diluent which was contaminated with the yeast.

Prior to 1950, cases of cryptococcosis in animals were reported only sporadically. About this time, a mounting interest in animal mycoses developed and case reports appeared with increasing frequency. Holzworth (1952) reported the first case of feline cryptococcosis in the U.S.A. in a mature male cat which, at necropsy, was found to have a generalised infection. In 1953, Holzworth and Coffin reported a second case in a mature female cat. Both animals had histories of chronic respiratory disorders of some duration. Subsequent reports of feline cryptococcosis from the U.S.A. have been recorded by McGrath (1954), Kaplan, Ajello, Bitetto and McDonough (1961), Trautwein and Nielsen (1962), Olander, Reed and Pier (1963), from Canada by Beauregard and Malkin (1970); Brasil (Carvalho, Mello and Ferreira, 1974); Hawaii (Palumbo and Perri, 1975). In Europe, cases have been described from Great Britain by Howell and Allan (1964), from France by Thery (1969) and from Austria by Kohler, Gialamas and Kuttin (1976). In the Southern hemisphere, feline cryptococcosis has been recorded from Japan by Yamamoto, Ishida and Sato (1957), from Australia by Curtis (1957) and by Johnston and Lavers (1963) and from Papua, New Guinea by Humphrey, Fordham and McKerrow (1977). Further details of cases of feline cryptococcosis are given in table 10.

The first case of canine cryptococcosis was reported from the United States of America by Seibold, Roberts and Jordan (1953). Subsequent reports of cases were given by McGrath (1954), Schwabe (1954), Kavut (1958), Rubin and Craig (1965), Price and Powers (1967) and Wagner, Pick and Krigman (1968).

Within the genus Cryptococcus, only Cr. neoformans is considered to be pathogenic to man and animals. Other species are usually regarded as contaminants when isolated from clinical specimens. However, there are a few case records assigning pathogenic significance to at least 3 of these supposedly saprophytic species in man and animals. Castellani (1963) isolated an encapsulated yeast from 2 men with chronic ulcerative balanitis of more than one year's duration which involved the glans penis and the inner surface of the prepuce. The yeast was obtained in pure culture from unopened pustules and he named it Cr. genitalis which Lodder (1974) considered to be a synonym of Cr. albidus var. albidus. Cunha and Lusins (1973) described a case of meningitis in a man which they attributed to Cr. albidus as they repeatedly demonstrated the yeast in spinal fluid and also obtained it in culture. Silva-Hutner (1970) described Cr. laurentii and Cr. luteolus as rare causes of infection of the central nervous system in man. Cryptococcus albidus was reported by Galli (1965) as the cause of an outbreak of mastitis in a dairy herd. The cows had been treated with penicillin during the dry period and 12 of 54 cows showed clinical signs of mastitis 12-13 days post parturition. The main clinical signs were severe swelling and tenderness of the mammary glands and hypertrophy of the supramammary and deep inguinal lymph nodes. The milk from affected cows was viscous,

greyish-white and flaky. The yeast was seen microscopically in milk sediments and isolated in culture, in the absence of other causal agents of mastitis. Galli produced lethal infection in guinea pigs and mice by inoculating them subcutaneously and intracerebrally respectively with a sample of infected milk. A genital infection manifested as nodular lesions on the prepuce of a 5 year old stallion was attributed to Cr. albidus by Codazza, Bertoldini and Sampieri (1973).

Until relatively recently, only a few yeasts were considered as potentially capable of causing diseases in man and animals. However, following increased use of antibiotics, immunosuppressive drugs and other medical and surgical procedures which affect host resistance, and in addition, among farm animals, such factors as malnutrition, malmanagement and poor husbandry, more and more yeast species are being recognised as potential pathogens in man and animals. Today, at least 15 species of yeasts, in 6 genera, namely, Candida, Cryptococcus, Torulopsis, Pityrosporum, Trichosporon and Rhodotorula are designated as medically important (Gentles and La Touche, 1969; Silva-Hutner, 1970; Ainsworth and Austwick, 1973; Ahearn, 1974; Segal and Ajello, 1976; Haley and Callaway, 1978).

In the genus, Pityrosporum, only Pit. pachydermatis is considered to be of veterinary significance; the other species Pit. ovale and Pit. orbiculare are mainly associated with the human skin, both as commensals and as pathogens. Pit. pachydermatis is largely associated with otitis externa of the dog. It has been reported to be present in the diseased ear canal in over 50% of cases of otitis externa in dogs by Baxter and Lawler (1972), Sharma and Rhoades (1975) and by Baxter



(1976). Other investigators also noted a high frequency of the yeast in diseased ears and Gustafson (1955) succeeded in experimentally producing otitis externa by instilling cultures of the yeast into the ear canals of dogs. Some authors, however, are not certain about the significance of Pit. pachydermatis in the aetiology of canine otitis externa because it is frequently isolated from apparently normal ears (Fraser, 1961; Grono and Frost, 1969). This yeast has also been isolated from normal and otitic ears of the cat (Baxter, 1976) and from normal ears of pigs, cattle and horses by Gustafson (1960).

In the genus Trichosporon, the species most frequently associated with animal disease, is Tri. cutaneum. Ainsworth and Austwick (1955 a,b) isolated it from the throat of a dog suffering from fever, coughing and swollen lymph nodes, in the absence of tubercle bacilli. It has also been incriminated as causing keratitis in a dog (Negroni, Quintana and Piccico, 1960). Kaplan (1959) reported it as a cause of white piedra in horses, mainly on the thick hair of the mane and tail; and Bertschinger, Schweizer and Scholer (1964) reported it as a cause of bovine mastitis.

One species of Rhodotorula, Rh. mucilaginosa has occasionally been associated with disease conditions in animals. Galati (1960) attributed chronic catarrhal angiocholitis in pigs to this species and Hajsig and Jakovac (1960) associated it with bovine mastitis. It has also been incriminated as a cause of an epizootic of dermatitis of the feathered parts of chickens in Israel by Beemer, Schneerson-Porat and Kuttin (1970).

Although the pathogenicity of members of the genus Torulopsis which have been isolated from human and animal sources has been much disputed,

the frequency with which some species, namely T. glabrata and T. candida (formerly T. famata) have been isolated from these sources inclines some investigators to attribute to them, at least tentatively, a pathogenic role (Gentles and La Touche, 1969). Austwick et al (1966) isolated T. glabrata from the digestive tract of piglets suffering from tympany. White, Lindsay and Ash (1972) indicated that, by feeding glucose in fat-free milk to lambs and piglets, T. glabrata, normally present in the digestive tract, could proliferate and produce high levels of ethanol in the plasma of lambs through fermentation, causing obvious symptoms of intoxication. T. glabrata has also been associated with bovine mastitis (Scholer, 1963). Knudtson, Ruth, Kirkbride and Tinaut (1976) isolated it from abomasal contents and lungs of an aborted bovine foetus with diffuse pneumonia and demonstrated the cells of the yeast within bronchiolar macrophages.

That T. candida is a commensal in animals has been shown by Batista, Vasconcelos, Fischman and Silva (1961) and Kaben and Preuss (1967). However, this species has been recovered from the mouths of pigs with vitamin A deficiency by Hajsig, Riznar and Marzan (1962) and from the udders of cows with mastitis by Funke (1960).

For a number of reasons, the true incidence of yeast infections in animals is not known. Infections with these organisms are not notifiable, are irregularly reported and, even when reported, the species or even genus of the isolate is frequently not given. This lack of information makes it difficult to connect individual yeast species with disease conditions. Moreover, yeast infections may often be wrongly diagnosed and the cause of the disease incorrectly attributed to other aetiological agents.

MATERIALS AND METHODS

## MEDIA AND REAGENTS

The following media were used in the study and maintenance of yeast species; for isolation from pathological specimens, chloramphenicol was incorporated as an antibacterial agent.

Commercial media were prepared according to the manufacturer's recommendations.

### Actidione (Cycloheximide) Resistance Agar

Bacto-yeast nitrogen base (Difco)	67.0 g.
Glucose	20.0 g.
Actidione (cycloheximide) (BDH)	100.0 mg.
Agar (Oxoid Technical)	15.0 g.
Distilled water	1000 ml

#### Preparation

The ingredients were heated to dissolve, dispensed in 10 ml volumes in universal bottles and autoclaved at 10 lbs/sq. in. (115°C) for 20 minutes. pH 5.5.

### Arbutin Agar

Arbutin (Hydroquinone- $\beta$ -D-glucopyranoside)	5.0 g.
Yeast extract (Oxoid)	5.0 g.
Agar	15.0 g.
Distilled water	1000 ml
1% aq. ferric ammonium citrate solution	

### Preparation (Lodder, 1974)

Arbutin and agar were dissolved in 0.5% oxoid yeast extract by heating, dispensed in 7 ml aliquots in universal bottles and autoclaved at 15 lbs/sq. in. ( $121^{\circ}\text{C}$ ) for 15 minutes. Immediately after sterilization, 3 drops of Seitz-filtered 1% ferric ammonium citrate solution were added aseptically to the liquified agar in each bottle, carefully shaken to mix and slanted.

### Christensen's Urea Agar

Bacto-urea agar base (Difco)	29.0 g.
Agar	15.0 g.
Distilled water	1000 ml

### Preparation

29 g. of Bacto-urea agar base were dissolved in 100 ml distilled water and sterilized by Seitz filtration; 15 g. of agar were dissolved by heating, in 900 ml distilled water, sterilized by autoclaving at 15 lbs/sq. in. ( $121^{\circ}\text{C}$ ) for 15 minutes, cooled to  $55^{\circ}\text{C}$ , the sterile Bacto-urea agar base was added aseptically, the medium mixed and dispensed into sterile universal bottles. pH 6.9.

### Corn Meal Agar

Corn meal extract (Oxoid)	2.0 g.
Agar No. 3 (Oxoid)	15.0 g.
Distilled water	1000 ml

### Preparation

17 g. of the dehydrated medium were suspended in a litre of distilled

water, brought to the boil to dissolve and sterilized by autoclaving at 15 lbs/sq. in. (121<sup>o</sup> C) for 15 minutes. pH 6.0.

Czapek-Dox Agar (Modified) + Tween 80

Czapek-Dox agar (Oxoid)	33.4 g.
Tween 80 (polyoxyethylene sorbitan mono-oleate)	10 ml
Distilled water	1000 ml

Preparation

33.4 g. of the dehydrated medium were dissolved in a litre of distilled water, 10 ml of Tween 80 added, and the medium sterilized by autoclaving at 10 lbs/sq. in. (115<sup>o</sup> C) for 20 minutes. pH 6.8.

50% Glucose Agar

D-glucose monohydrate (BDH)	50.0 g.
Yeast extract (Oxoid)	1.0 g.
Agar	2.0 g.
Distilled water	100 ml

Preparation (Lodder, 1974)

The ingredients were dissolved by heating, distributed in 10 ml volumes in universal bottles and sterilized by autoclaving at 10 lbs/sq. in. (115<sup>o</sup> C) for 15 minutes.

Guizotia abyssinica (Niger Seed) Agar

Guizotia extract	200 ml
D-glucose	10.0 g.

Creatinine (BDH)	0.78 g.
Chloramphenicol	0.05 g.
Agar	20.0 g.
Diphenyl (Biphenyl) (BDH)	0.1 g.
Distilled water	800 ml

Preparation (Rippon, 1974)

70 g. of Guizotia abyssinica seeds ground in a Waring blender were added to 350 ml distilled water and autoclaved at 10 lbs/sq. in. (115°C) for 10 minutes, then filtered through cheesecloth. The medium was prepared by dissolving the ingredients, except diphenyl, by heating, autoclaving at 15 lbs/sq. in. (121°C) for 15 minutes, and allowing to cool to 50°C in a water bath, when 0.1 g. of diphenyl dissolved in 10 ml of absolute alcohol was added aseptically. pH 7.0.

V-8 Juice Agar

V-8 Vegetable Juice	360 ml
Calcium carbonate	8.0 g.
Agar	20.0 g.
Distilled water	640 ml

Preparation

The agar and calcium carbonate were dissolved in distilled water by heating, the V-8 vegetable juice added, the medium dispensed in 10 ml volumes into universal bottles and autoclaved at 10 lbs/sq. in. (115°C) for 20 minutes. pH 6.8.

### Nutrient Gelatin

Lab-Lemco Powder (Oxoid)	3.0 g.
Peptone	5.0 g.
Gelatin	120.0 g.
Distilled water	1000 ml

### Preparation

128 g. of dehydrated medium were dissolved by heating in a litre of distilled water, distributed into universal bottles in 10 ml volumes and sterilized by autoclaving at 15 lbs/sq. in. ( $121^{\circ}\text{C}$ ) for 15 minutes; the medium was allowed to gel in a vertical position and stored at  $4^{\circ}\text{C}$  till used. pH 6.8.

### 3% Peptone Agar

Mycological peptone (Oxoid)	30.0 g.
Agar	20.0 g.
Distilled water	1000 ml

### Preparation

Peptone and agar were dissolved in distilled water by heating, distributed in 10 ml aliquots into universal bottles and sterilized by autoclaving at 15 lbs/sq. in. ( $121^{\circ}\text{C}$ ) for 20 minutes .

### Potato Dextrose Agar

Sliced potatoes	200.0 g.
Dextrose	10.0 g.
Agar	15.0 g.
Distilled water to	1000 ml



### Preparation (Haley and Callaway, 1978)

Small pieces of potato were simmered in distilled water at 60°C for an hour, filtered through crude filter paper, dextrose and agar added to the filtrate and dissolved by heating; volume restored to 1000 ml and the medium autoclaved at 15 lbs/sq. in. (121°C) for 10 minutes. pH 5.6.

### Sabouraud Dextrose Agar (SDA) supplemented with chloramphenicol

Mycological peptone (Oxoid)	10.0 g.
Dextrose (D-glucose)	40.0 g.
Agar No. 1 (Oxoid)	15.0 g.
Chloramphenicol	0.05 g.
Distilled water	1000 ml

### Preparation

65 g. of dehydrated medium were suspended in a litre of distilled water, dissolved by heating, chloramphenicol added, mixed and sterilized by autoclaving at 15 lbs/sq. in. (121°C) for 15 minutes. pH 5.6

### Sabouraud Dextrose Broth supplemented with chloramphenicol

As for Sabouraud dextrose agar supplemented with chloramphenicol, omitting the agar.

### Starch Production Agar

Yeast-nitrogen base (Difco)	10.0 g.
Dextrose (D-glucose)	30.0 g.
Yeast extract (Difco)	3.0 g.
Agar	20.0 g.
Distilled water	1000 ml

Preparation (Widra and Long, 1970)

Ingredients were dissolved by heating and sterilized by autoclaving at 10 lbs/sq. in. (115<sup>o</sup>C) for 20 minutes.

Vitamin-free Medium

Bacto-vitamin-free yeast base (Difco)	16.7 g.
Agar	20.0 g.
Distilled water	1000 ml

Preparation

16.7 g. of the dehydrated medium were suspended in a litre of distilled water, the agar added, dissolved by heating, dispensed in 10 ml volumes in universal bottles and sterilized by autoclaving at 10 lbs/sq. in. (115<sup>o</sup>C) for 20 minutes. pH 4.5.

Yeast Carbon Base

Bacto-yeast carbon base (Difco)	117.0 g.
Agar	15.0 g.
Distilled water	1000 ml

Preparation

117 g. of yeast carbon base powder were suspended in a litre of distilled water, agar added, dissolved by heating, dispensed and sterilized by autoclaving at 10 lbs/sq. in. (115<sup>o</sup>C) for 20 minutes. pH 4.5.

Yeast Nitrogen Base

Bacto-yeast nitrogen base (Difco)	67.0 g.
Agar	15.0 g.
Distilled water	1000 ml

## Preparation

67 g. of yeast nitrogen base powder were suspended in a litre of distilled water, agar added, dissolved by heating, dispensed and sterilized by autoclaving at 10 lbs/sq. in. (115<sup>o</sup> C) for 20 minutes. pH 4.5.

## Carbohydrate and Nitrate Assimilation (Auxanographic) Tests

Saturated aqueous solutions of: (1) glucose, (2) maltose, (3) sucrose, (4) lactose, (5) D-galactose, (6) inositol, (7) trehalose, (8) D-manitol, (9) raffinose, (10) melibiose, (11) cellobiose, (12) D-xylose, (13) citric acid, (14) erythritol, (15) melezitose, (16) inulin, (17) salicin, (18) ribitol, (19) D-ribose, (20) L-sorbose, (21) L-rhamnose, (22) galactitol, (23) lactate, (24) succinate, (25) D-glucitol, (26)  $\alpha$ -methyl-D-glucoside, (27) D-arabinose, (28) L-arabinose, (29) glucono- $\delta$ -lactone, (30) 2-Ketogluconate, (31) 5-Ketogluconate, (32) sorbitol, (33) arbutin, (34) soluble starch, (35) ethanol and (36) glycerol, were prepared and pipetted on 6 x 6 mm filter paper discs in petri dishes, the excess solution removed and the discs dried at 56<sup>o</sup> C. These impregnated discs were used as carbohydrate sources in the assimilation tests.

In the case of glycerol, ethanol and soluble starch, aqueous solutions were used in the tests. A well was cut in the seeded assimilation agar with a sterile cork borer (size 3) and filled with the solution.

Paper discs impregnated with saturated solutions of potassium nitrate ( $KNO_3$ ) and mycological peptone were used as nitrogen sources in the assimilation tests. Peptone was used as a positive growth control for nitrate assimilation.

## Fermentation Medium

### Sugar Medium Broth Base (Oxoid)

Lab-Lemco beef extract	5.0 g.
Peptone (Bacto)	10.0 g.
Sodium chloride	3.0 g.
Di-sodium hydrogen phosphate	2.0 g.
Distilled water	1000 ml

### Indicator

Bromothymol blue (granules)	0.1 g.
0.1 N NaOH	2.5 ml
Distilled water	47.5 ml

### Test Sugars

D-glucose, D-galactose, sucrose, maltose, lactose, raffinose, cellobiose, melibiose and inulin were used in the fermentation tests.

### Preparation (Lodder, 1974)

The fermentation of all sugars used was tested in 2% solutions in 75 x 10 mm cotton-plugged test tubes containing Durham tubes (15 x 6 mm), except in the case of raffinose where a 4% solution was employed. The sugars were dissolved in a 0.5% solution of Oxoid beef extract as the basal broth medium, incorporating bromothymol blue as an indicator.

Ingredients of the broth were dissolved by steaming, pH adjusted to 7.2-7.3, indicator added (12 ml/litre), and the broth sterilized by autoclaving, in 100 ml volumes, at 15 lbs/sq. in. (121°C) for 15 minutes.

After sterilization, the sugar powders were added to the individual bottles containing the basal medium to the final concentration of 2%,

except in the case of raffinose where a final concentration of 4% was used. 1.5 ml aliquots of the sugar solutions were finally dispensed into sterile cotton-plugged test tubes containing inverted Durham tubes and sterilized by tyndalization, by steaming for 20 minutes each day for 3 consecutive days.

Phosphate Buffered Saline (PBS) (pH 7.3)

Sodium chloride	8.0 g.
Potassium chloride	0.2 g.
Disodium hydrogen phosphate	1.15 g.
Potassium dihydrogen phosphate	0.2 g.
Distilled water	1000 ml

Preparation

10 tablets (Oxoid) were dissolved in a litre of distilled water and autoclaved at 10 lbs/sq. in. (115°C) for 10 minutes.

Veronal (Barbitone) Buffer (pH 8.2)

Diethylbarbituric acid (barbitone)	3.32 g.
$(C_2H_5)_2 C.CO.NH.CO.NH.CO$	
Sodium diethyl barbitone (barbitone sodium)	25.52 g.
$(C_2H_5)_2 C.CO.NH.C.(ONa)$	
Distilled water	2000 ml

Preparation

Barbitone was dissolved in 200 ml hot distilled water and barbitone sodium in 1500 ml distilled water; the two solutions were mixed, made

up to 2 litres and 2 g. (0.1%) of sodium azide ( $\text{NaN}_3$ ) added as a preservative.

#### Coca's Saline

Sodium chloride ( $\text{NaCl}$ )	5.0 g.
Sodium bicarbonate ( $\text{Na HCO}_3$ )	2.75 g.
Phenol crystals	4.0 g.
Distilled water	1000 ml

The solution was sterilized by autoclaving at 15 lbs/sq. in. ( $121^\circ\text{C}$ ) for 15 minutes .

#### Formol Saline

4%	100 ml 40% formaldehyde solution
	900 ml 0.85% saline ( $\text{NaCl}$ )
2%	50 ml 40% formaldehyde solution
	950 ml 0.85% saline

#### Gel-washing Fluid

Di-sodium tetraborate (Borax)	4.0 g.
( $\text{Na}_2 \text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ )	
Sodium chloride ( $\text{NaCl}$ )	4.0 g.
Distilled water	1000 ml

#### 0.1% Amido Black Stain for Gel

One g. of amido (naphthalene) black was dissolved in a litre of methanol-glacial acetic acid-water solvent (5:1:4).

### Gel De-staining Fluid

Methanol	500 ml
Glacial acetic acid	100 ml
Distilled water	400 ml

### Mountants and Stains

The following were used for mounting and staining body fluids, smears and histological sections.

Lactophenol cotton blue

20% potassium hydroxide solution

Gomori's Methenamine-silver-nitrate stain (Grocott modification for fungi)

Gram stain

Haematoxylin and Eosin (H & E)

Malachite Green-Safranine stain (Lodder, 1974)

Mayer's mucicarmine

Periodic Acid Schiff (P.A.S.)

out on SDA containing chloramphenicol, then single colonies were selected. These pure cultures were used for the preparation of inocula and identification, which was carried out according to the criteria defined by Lodder (1974) and Barnett and Pankhurst (1974).

### Colony Characteristics

Yeast isolates were grown on SDA plates at 28<sup>o</sup> C for 3-4 days, colonial characteristics were noticed, then the plates were left at room temperature for up to 3 weeks before the final observation. The surface of the growth, margin, appearance, consistency, smell and pigments were all recorded.

### Morphology of vegetable cells

Wet mounts from 3-4 days old pure cultures were examined to determine the size, shape and type of budding of the vegetative cells.

### Mycelium or pseudomycelium production

The ability to form pseudo- or true mycelium was determined by culture on corn meal agar. Plates were inoculated from young yeast cultures by streaking over the agar surface. A sterile cover slip was placed over a portion of the streak. Inoculated plates were incubated at 24<sup>o</sup> C for 7 days, with daily examination for the presence of filamentation.

### Formation of ascospores

A slant of V-8 juice agar and of 3% peptone agar was inoculated with each isolate, incubated at 24<sup>o</sup> C for 3 days before being examined microscopically for the first time. Cultures that did not sporulate were then



maintained at room temperature and examined at weekly intervals for up to 3 weeks. Ascospore formation was verified by staining with aqueous malachite green — safranin stain (Lodder, 1974).

#### Formation of chlamydospores

Streaks from young cultures were made on the surface of Czapek-Dox agar containing Tween 80, and sterile cover slips applied. Plates were incubated at 24°C for 7 days and examined daily for chlamydospore production.

#### Germ tube test

0.5 ml of sterile horse serum was inoculated with a small inoculum from a young culture, incubated at 37°C for 4 hours and examined for germ tube production.

#### Demonstration of capsule

This test was performed by making an aqueous suspension from the yeast culture in a drop of distilled water, a drop of India-ink was added, mixed and the suspension was microscopically examined.

### Auxanographic tests

#### Assimilation of Carbohydrates

The ability of a yeast species to utilize a specific carbohydrate compound as a sole carbon source was tested by the auxanographic method using Difco yeast nitrogen base as the basal medium.

Fourteen carbohydrate compounds (Nos. 1-14) were used in the primary tests for every yeast isolate. An additional 22 carbohydrate compounds (Nos. 15-36) were used in the confirmatory assimilation tests.

Inocula for the assimilation tests were prepared by making aqueous suspensions of the yeasts, from 3-4 days old cultures on SDA slants in universal bottles grown at 28<sup>o</sup> C, by adding 3 ml sterile distilled water and carefully washing the yeast growth from the agar surface. Twelve drops of the yeast suspension were then added to 20 ml of melted yeast nitrogen base agar previously cooled to 45<sup>o</sup> C in a water bath, using a sterile Pasteur pipette. The yeast suspension and agar were well mixed, poured into 2 sterile Petri dishes (10 ml inoculated medium per plate) and allowed to solidify. The carbohydrate-impregnated discs were then placed on the agar surface with sterile forceps, using 7 discs per plate, one central and 6 peripheral (fig. 1), with the sugar numbers marked on the back of the plate. Soluble starch, glycerol and ethanol were pipetted into wells cut in the agar, as was described in the materials and methods.

The inoculated plates were incubated at 28<sup>o</sup> C, with daily examination, for 7 days. A heavy yeast growth around the disc was regarded as positive (fig. 1). In doubtful cases, the test was repeated.

#### Assimilation of nitrate

The ability of a yeast species to utilize nitrate as a sole nitrogen source was tested by the auxanographic method using, as a basal medium, Difco yeast carbon base.

Potassium nitrate (KNO<sub>3</sub>), in the form of impregnated discs was used, with peptone as a positive growth control.

To 10 ml volumes of melted yeast carbon base agar cooled to 45<sup>o</sup> C, 6 drops of the yeast suspension were added using a sterile Pasteur pipette. The yeast suspension and agar were mixed, poured into a sterile

Petri dish and allowed to solidify. The nitrate and peptone-impregnated discs were placed on the inoculated agar surface and the plates were incubated at 28°C, with daily examination, for a week. A heavy growth around the nitrate disc was regarded as positive.

#### Sugar fermentation

Six sugars: glucose, galactose, sucrose, maltose, lactose and raffinose were used in the primary fermentation tests with all isolates, and 3 sugars: cellobiose, melibiose and inulin were employed, when necessary, in the confirmatory tests.

The same yeast inoculum, used for the assimilation tests, was used to inoculate the fermentation tubes. Two drops of the yeast suspension were added to each sugar fermentation tube. The inoculated tubes were gently swirled between the palms to mix, and were incubated at 28°C, with daily shaking and examination, for up to 2 weeks. The reactions were finally recorded as production of gas, production of acid and gas, or no apparent fermentation. A test was regarded as positive when there was acid and gas production. When raffinose was fermented, the volume of gas contained in the Durham tube was estimated, since differential fermentation of raffinose was considered to be of diagnostic value in yeast speciation (Lodder, 1974).

#### Supplementary tests

##### Splitting of arbutin

Arbutin agar, as slants in universal bottles, was inoculated with a loopful of yeast from a 3-4 days old culture and incubated at 28°C for at least a week, with daily examination. A dark brown colour developing in the agar was regarded as an indication of splitting of arbutin (Lodder, 1974).

### Growth in vitamin-free medium

Bacto-vitamin-free yeast agar was used as the test medium, in the form of slants, in universal bottles. Each slant was inoculated from a 3-4 days old culture and incubated at 28<sup>o</sup> C for a week. A heavy growth was regarded as an indication of ability to grow in the absence of an external vitamin source and the test was considered positive.

### Growth on media of high osmotic pressure

The ability to grow on a medium of high osmotic pressure was used as a differential criterion between osmotophilic and non-osmotophilic yeast species. 50% glucose-yeast extract agar, slanted in universal bottles, was streaked with a loopful of yeast from a young culture and incubated at 28<sup>o</sup> C for a week. A good growth was regarded as positive.

### Growth at 37<sup>o</sup> C and elevated temperatures

All yeast isolates were tested for growth at 37<sup>o</sup> C, on SDA plates held for 3-4 days. A visible growth was regarded as positive. In case of weak growth, the test was repeated.

When it was necessary to test for growth at temperatures above 37<sup>o</sup> C, subcultures were made on SDA slants in universal bottles which were incubated in a water bath adjusted to the required temperature. After 3-4 days incubation, the slants were examined for the presence of growth, and doubtful results were repeated.

### Starch production

The ability of a yeast to produce starch or starch-like substances (extracellular amyloids) was tested by using both 3% glucose (Lodder, 1974) and starch production agar (Widra and Long, 1970). The 3% glucose solution was dispensed in 10 ml volumes in universal bottles and the

starch production agar dispensed in 20 ml volumes in Petri dishes.

Both bottles and dishes were inoculated with a loopful of yeast from a 3-4 days old growth and incubated at 28<sup>o</sup> C for 2 weeks, after which time the presence of starch or starch-like substances in the medium was tested, by adding 2-3 drops of a Lugol's iodine solution to the growth in the liquid medium or by flooding part of the solid agar medium with the iodine solution. A blue colour was regarded as positive.

#### Urea hydrolysis

The yeast isolates were inoculated on Christensen's urea agar slants and incubated at 28<sup>o</sup> C, with daily observation for 7 days. The appearance of a deep pink colour was regarded as positive.

#### Actidione (Cycloheximide) resistance

The resistance or sensitivity of a yeast isolate to the antibiotic actidione (cycloheximide) was tested in Bacto-glucose-yeast-nitrogen base (2% glucose) agar containing actidione to the final concentration of 100 mg/1000 ml of medium. Slants, in universal bottles, were inoculated from young yeast cultures and incubated at 28<sup>o</sup> C, with daily examination, for a week. A yeast isolate which grew well was regarded as actidione resistant.

## PREPARATION OF ANTIGENS

### Whole Cell Antigens

Whole cell antigens were prepared according to the methods of Evans (1976).

### Cryptococcus neoformans

Whole cell antigens were prepared from Cr. neoformans serotypes A, B, C and D and strains V359, CXH Symmers, KCH and Huntington.

Yeast cells, cultured on slants of SDA at 28<sup>o</sup> C for 3-4 days, were washed from the agar with 2% formol saline. The cell suspension was left at room temperature for at least 18 hours, centrifuged at 3,000 rpm for 10 minutes, the supernatant discarded, the yeast cells suspended in SNS and heated in a 56<sup>o</sup> C water bath for 30 minutes. Washing by centrifugation was repeated 3 times and a loopful of the suspension was cultured on SDA to check that the cells were non-viable. The cells were counted using an improved Neubauer counting chamber and the concentration adjusted to  $1 \times 10^9$  cells/ml.

### Candida albicans

Whole cell antigen was prepared from C. albicans strain 107. The yeast, grown as for Cr. neoformans, was washed from the agar surface with 4% formol saline. The cell suspension was left at room temperature for a minimum of 4 hours and washed by centrifugation as before, resuspended in a minimal volume of SNS and cultured to check for non-viability. The cell suspension was then estimated using a micro-haematocrit tube (centrifuged at 3,000 rpm for 5 minutes in a Hanksley micro-haematocrit centrifuge) and adjusted to 2% packed cell volume (PCV) using a Hanksley micro-haematocrit reader.

### Culture Filtrate (CF) Antigens

The method of Proctor (1976) was used to prepare CF antigens from the 4 serotypes and 4 strains of Cr. neoformans and one strain of C. albicans mentioned previously.

200 ml of Sabouraud dextrose broth (SDB) in a 300-ml flask were inoculated from a young SDA slope culture of the yeast and cultured at room temperature (20-22°C), with constant stirring, for 3-4 days. Yeast cells were harvested by centrifugation at 3,000 rpm for 15 minutes at 4°C in sterile centrifuge tubes, supernatant decanted and the cell pellet retained.

These cell pellets were used to inoculate a 1-litre conical flask containing 750 ml of sterile SDB. The culture was stirred at room temperature for 2 weeks and centrifuged at 3,000 rpm for 30 minutes at 4°C. The culture filtrate thus obtained was dialysed for 48 hours against running tap water, followed by dialysis against distilled water at 4°C for 48 hours, the distilled water being changed twice a day. The dialysate was then sterilized by Seitz filtration and concentrated x10 in 20M polyethylene glycol (carbowax) at 37°C and filtered through a 0.45  $\mu$  millipore filter.

### Somatic Extract (S) Antigens

Coca's saline was used to extract soluble somatic antigens from the serotypes and strains of Cr. neoformans and C. albicans used previously.

Yeast cells, grown on SDA slants at 28°C for 3-4 days were harvested and washed 3 times in SNS by centrifugation. Thick cell suspensions were made in sterile Coca's saline and held at 4°C, with frequent daily shaking. After 24 hours of storage and thereafter subcultures were

made on SDA to ensure the death of yeast cells, and direct slide mounts and India-ink preparations were made to examine for morphological changes in the yeast cells. Storage for 24 hours in Coca's saline completely killed the cells and induced morphological changes such as cellular disruption, disintegration and capsular fragmentation in the encapsulated strains of Cr. neoformans. Storage was continued for a week until maximal cellular disruption (80-90%) was obtained. The cell debris was removed by centrifugation at 3,000 rpm for 30 minutes at 4<sup>o</sup>C, and the resultant supernatant was dialysed for 24 hours against running tap water and for another 24 hours against 2 changes of distilled water at 4<sup>o</sup>C. The dialysate was frozen at -20<sup>o</sup>C for 24 hours to aggregate any insoluble materials, centrifuged at 20,000 rpm for one hour at 4<sup>o</sup>C, decanted, concentrated x10 by 20Mi polyethylene glycol (carbowax) at 37<sup>o</sup>C and sterilized by Seitz filtration.



Antisera to Cr. neoformans and C. albicans were raised in mature white New Zealand rabbits (2 kg. av. body wt.) after the methods of Proctor (1976) and Murray and Buckley (1966) respectively.

#### Antisera to Cr. neoformans

Attempts were made to raise rabbit antisera against Cr. neoformans serotypes A, B, C and D and strains V359, CXH Symmers, KCH and Huntington.

Antiserum with a reasonably satisfactory titre was obtained only against Str. V359. The procedure described hereafter refers to this strain. A rabbit was inoculated intravenously, into the marginal ear vein, with 1 ml suspension of formalin-killed whole cells ( $2 \times 10^8$  cells) daily for 5 days a week for 2 consecutive weeks, followed by a week's rest. A test bleed showed an agglutination titre of 1:128 and the rabbit was exsanguinated.

Trials, by the same method using other Cr. neoformans strains were unsuccessful, because rabbits died of anaphylactic shock early in the course of the immunization schedule.

A modification in this procedure, using the method recommended by Evans (1976), in which a starting dose of  $25 \times 10^6$  cells/ml was given, with gradual increase to  $25 \times 10^7$  cells/ml by the 10th day, was also of no avail. Animals died before, or just after, the 10th injection.

Another unsuccessful trial was made by injecting rabbits subcutaneously with 2 ml of equal volumes of Freund complete adjuvant and formalin-killed whole yeast cells ( $1 \times 10^9$  cells/ml), from serotypes A, B, C and D, and boosting them intravenously with whole cells 2

weeks later. The rabbit injected with serotype A died soon after the first booster dose; other rabbits, which did not give detectable agglutinins after a week's rest, died after the second booster dose.

Injections with adrenaline prior to boosting injections did not protect the animals against shock. No further attempts to raise antisera against other Cr. neoformans strains were made.

#### Antisera to C. albicans

Antiserum to C. albicans strain 107 was raised by IV injection of a rabbit with formalin-killed yeast cells adjusted to a concentration of 2% PCV. It received 2 injections, 3 days apart, and rested for a week. A test bleeding showed an agglutination titre of 1:512 and the animal was exsanguinated.

### TITRATION OF ANTIGENS

Culture filtrate and somatic antigens from Cr. neoformans serotypes and strains and from C. albicans were titrated against rabbit antisera to determine their workable concentrations. Samples from the bulk antigens were concentrated 5, 10, 25 and 50 times their original volumes in Amicon ultraconcentrator cells B15, at room temperature, and tested by counterimmunoelectrophoresis. The antigen concentration giving the maximum number of precipitation lines against the respective antiserum was taken as the working concentration and the rest of the antigen solution was concentrated accordingly.

## SEROLOGICAL METHODS

Agglutination, immunodiffusion (ID) and counterimmunoelectrophoresis (CIE) were used in this study.

The methods of Evans (1976), Kaufman (1976) and Proctor (1976) were adopted, with modifications when necessary.

### Agglutination test

WHO trays (80 wells) were used. Volumes of 0.4 ml of phosphate buffered saline (PBS) were dispensed into the wells with Finnpiettes and serial two-fold dilutions of sera made in the saline by adding equal volumes of sera, to dilutions of 1:256. One drop ( $\approx 0.02$  ml) of a  $1 \times 10^9$  cells/ml or of a 2% PCV whole cell antigen was added to each well, the tray was gently shaken to mix and incubated at 37°C for 2 hours. A positive control serum (rabbit anti-Cr. neoformans or anti-C. albicans) and negative control saline yeast cell suspension were included in each run. After incubation, the test was examined for agglutination with the aid of oblique light from a bench lamp against a dark background. The serum titre was taken as the highest dilution that showed any degree of agglutination (Kaufman, 1976). After the initial reading, the test trays were held at 4°C overnight and re-examined.

### Immunodiffusion (ID) (Ouchterlony) test

The test was run in 1% agarose gel in Veronal (barbitone) buffer pH 8.2. Two g. of agarose (BDH) were dissolved in 100 ml distilled water by gentle heating and 100 ml of freshly prepared buffer, heated to 56°C in a water bath, were added to the molten agarose.

Ten ml volumes of hot molten agarose in buffer were pipetted into plastic Petri dishes, 90 mm in diameter, on a levelling stand and

allowed to set. After solidification, the agarose plates were held at 4<sup>o</sup>C for 10-15 minutes. A central antigen well, 6 mm in diameter, and 8 peripheral serum wells, 4 mm in diameter, were cut in the agarose using cork borers, with interwell distances of 6 mm (edge to edge). Up to 4 well-patterns were made per plate (fig. 2). The wells were filled with the reagents using Pasteur pipettes and the plates were held at 24<sup>o</sup>C in a humid chamber for 3 days with daily examination for precipitation lines. The gels were then washed in gel-washing fluid to eliminate unreacted sera and non-specific reactions. Plates were stood on edge in the washing fluid in a plastic container and gently stirred with a magnetic stirrer for 24 hours, allowing a minimum of 400 ml of fluid per plate. Gels were finally washed in distilled water for 10 minutes, transferred to 8 x 8 cm glass plates and dried overnight in a 56<sup>o</sup>C incubator. When dry, the gels were stained with 0.1% amido black for 10 minutes, rinsed in methylated spirit and decolorized in 2 changes of gel de-staining fluid, 10 minutes in each. They were then air dried and examined on a Kodak transparency viewer for precipitation arcs and kept as permanent records.

#### Counterimmunoelectrophoresis (CIE) test

1% agarose in Veronal buffer pH 8.2 was prepared as was described for the ID test.

Acid-cleaned glass slides (3.9 x 7.6 cm) were used to support the agarose gels. Full strength Veronal buffer was used as electrolyte in the electrophoresis tanks.

Five slides were fixed to a plastic tray with a small amount of agarose and 40 ml of molten agarose were poured onto the tray, on a levelling

stand and allowed to set. This volume of agarose gave a depth of 1mm on the slides. After setting, the agarose gel was held at 4°C for 10 minutes to pre-cool and firm.

Parallel rows of wells : 3 and 5 mm in diameter, 6 mm edge to edge and 6 mm apart, were cut into the agarose with cork borers (fig. 3). The agarose was removed from the wells with a curved needle and the wells were filled to the brims with the reactants, using Pasteur pipettes: the large wells with test sera and the small ones with antigens.

The tray was placed in the electrophoresis tank containing 500 ml buffer per compartment, with the serum wells to the anode and antigen wells to the cathode. The tray was connected to the electrolyte by means of lint wicks, gently pressed down with the finger tips to ensure a good even contact with the gel. A constant electric current was passed through the gel from a D.C. power unit (Shandon) at the rate of 2mA per cm width, i.e. 39 mA for the 5 slides, for 90 minutes. After the run, the slides were cooled at 4°C for 10 minutes, read for precipitation lines and carefully removed from the tray. To wash out unreacted sera and non-specific reactions, the agarose gels were held on the slides by narrow rubber bands, washed, dried and stained as was described for the ID test. Slides were examined on a Kodak transparency viewer and the number and intensity of precipitation lines recorded.

Fig. 1: Primary carbohydrate assimilation plate (*C. albicans*).

Disks were impregnated with sugars Nos. 1-7: glucose (1), maltose (2), sucrose (3), galactose (5) and trehalose (7) were assimilated; lactose (4) and inositol (6) were not assimilated.

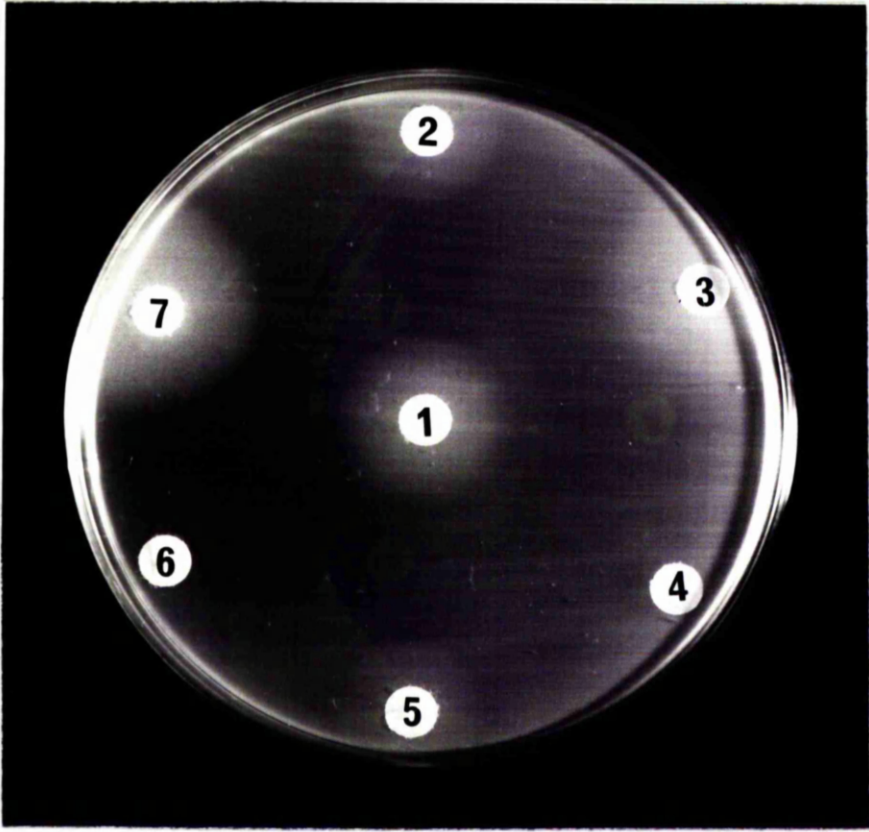


Fig. 2: Immunodiffusion test pattern.

Four central antigen wells (6 mm) and 32 peripheral serum wells (4 mm), with 6 mm interwell distances.



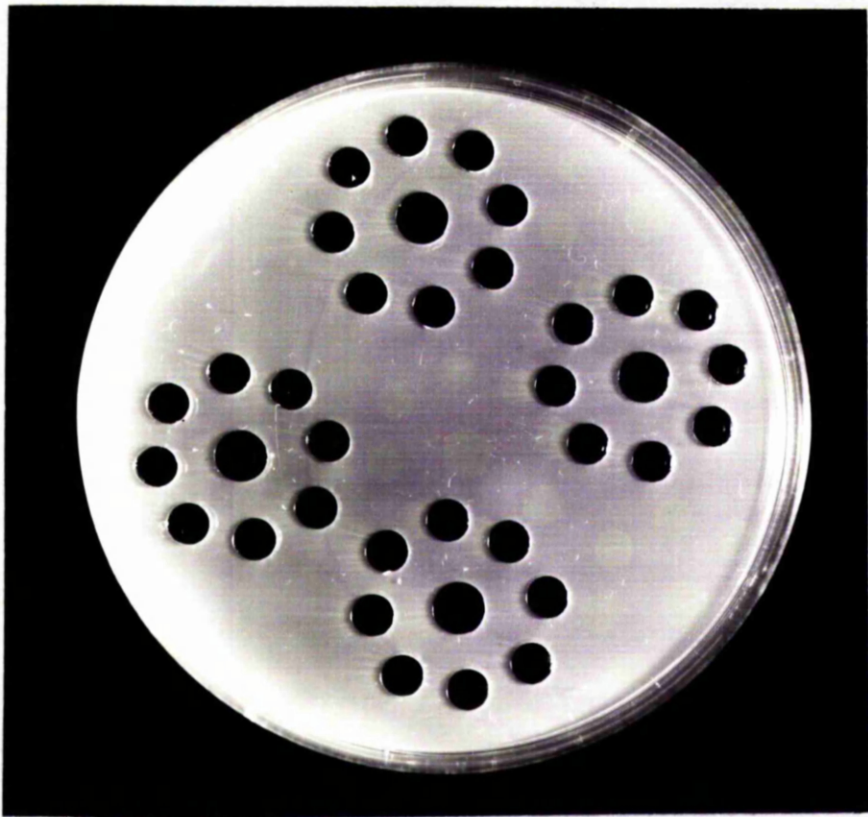
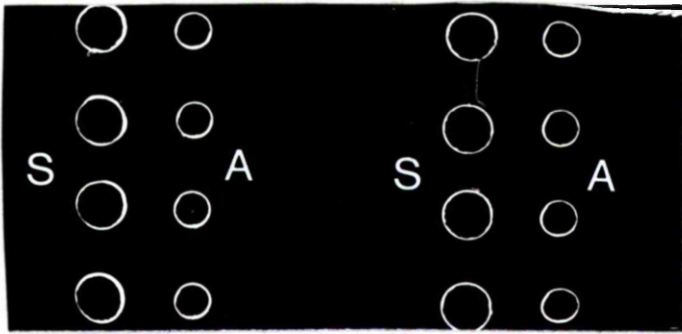


Fig. 3: Counterimmunoelectrophoresis slide.

Two rows of large (5 mm) serum wells (S) and small (3 mm) antigen wells (A), 6 mm edge to edge and 6 mm apart.



ANTIBODY TO CRYPTOCOCCUS NEOFORMANS  
IN SERA FROM APPARENTLY NORMAL CATS  
AND DOGS

## INTRODUCTION

Until recently, serodiagnosis of cryptococcosis was unsatisfactory and patients with this disease were regarded as immunologically inert (Kaufman, 1976). The absence of demonstrable antibodies to Cr. neoformans in serum samples from some proven cases of human cryptococcosis was attributed to poor antigenicity of the fungus (Salvin, 1959), to neutralization of antibodies by an abundance of circulating fungal antigens in the body fluids (Neill, Sugg and McCauley, 1951), or to insensitivity of the available serological tests (Seeliger, 1964).

A number of serological procedures have now been developed. Pollock and Ward (1962) demonstrated anti-cryptococcal antibodies in serum from human patients by the use of an haemagglutination test. Vogel, Seelers and Woodward (1961) successfully used the indirect fluorescent antibody technique. Using the latex-particle agglutination and tube agglutination tests, Gordon and Vedder (1966) detected cryptococcal antigen and anti-cryptococcal antibody in patients with proven cases of cryptococcosis. Kaufman and Blumer (1968) stated that the serological diagnosis of cryptococcosis is best accomplished by the concurrent use of 3 tests, latex particle agglutination to test for antigen and indirect fluorescent antibody and tube agglutination for anti-cryptococcal antibody.

Serological tests are also used to monitor the course of the infection and the effect of treatment. Cryptococcal polysaccharide antigen in a patient's serum or cerebrospinal fluid usually indicates active infection (Goodman, Kaufman and Koenig, 1971). Increasing titres of circulating antigen reflect progressive infection and a poor prognosis, while declining titres indicate good response to chemotherapy and the onset of recovery;

the presence of anti-cryptococcal antibody may suggest early, overt or past infection with Cr. neoformans (Kaufman, 1976).

Despite the heightened awareness of cryptococcosis among farm and pet animals, serology has not yet been applied as a routine in the diagnosis of this disease in such hosts (Ainsworth and Austwick, 1973; Buxton and Fraser, 1977). The only recorded survey of normal animals for the presence of antibody to Cr. neoformans was carried out by Weiland, Böhlm and Sasu (1971 a, b) who examined sera from dogs, horses and town pigeons by an agglutination test.

It is generally accepted that the portal of entry of Cr. neoformans into the body is via the respiratory tract. Evidence that cats have contracted cryptococcosis as a result of inhalation of the yeast from pigeon droppings in their environment has been presented by Yamamoto, Ishida and Sato (1957), Olander, Reed and Pier (1963), and Lau, Reinke and Brown (1971).

As Walter and Atchison (1966) found for pigeon fanciers, it is reasonable to expect that animals may, in some cases, inhale the organism from their environment and mount an immunological reaction without developing clinical infection. Evidence supporting this assumption was obtained from my study on experimental infection of cats in which animals were challenged intranasally with viable and with killed cells of Cr. neoformans. It was found that the yeast could remain viable in the nasal cavity of cats for up to 120 days without causing overt infection and that an immunological response was stimulated in these animals. Killed cells also induced the production of antibody.

Evans (1977) isolated Cr. neoformans from pigeon lofts and roosting

sites in Glasgow and Dumbartonshire. Despite the proven presence of the yeast in this district, over the past 12 years no case of cryptococcosis has been found in the cat, and only one isolation of Cr. neoformans was obtained from the turbinates of a dog with severe nasal aspergillosis. That this dog had concurrent cryptococcosis was, however, not established (Dawson, personal communication).

It was decided that sera from apparently normal cats and dogs in the Glasgow area should be investigated for antibody to Cr. neoformans using antigens representing each of the 4 serotypes to ensure, as recommended by Proctor (1976), that the antigenic spectrum of the yeast was covered.

## MATERIALS AND METHODS

### Sera

One serum sample was taken from each cat. These were collected during a survey on leukaemia virus infection in Glasgow and Dumbartonshire. Of the 275 serum samples, 160 were from females and 115 from males; 227 were from urban cats and 48 from rural cats. Four sera were from cats with leukaemia and the remainder were from non-leukaemic cats. The ages of the cats ranged from 5 months to 12 years.

One serum sample was taken from each dog. The 328 canine serum samples investigated had been sent to the Department of Bacteriology at Glasgow for screening for bacterial antibodies or were from "holding" dogs. Case histories were available for only 171 dogs. The ages of these dogs ranged from 5 months to over 12 years; 87 were males and 84 females and 100 were from urban and 71 from rural environments.

### Antigens and serological tests

All sera were investigated by counterimmunoelectrophoresis (CIE) and by immunodiffusion (ID). Each serum was tested against 8 antigens, a culture filtrate antigen (CF) and a somatic antigen (S) prepared from each of the 4 serotypes of Cr. neoformans. CIE slides and ID plates were read unstained and the readings confirmed after washing and staining with 0.1% amido black.

Agglutination tests were performed on all feline serum samples and on 100 canine sera, including 45 sera shown to have precipitating antibody to Cr. neoformans by CIE. Formalin-killed whole yeast cells prepared from each serotype were used as antigens. The tests were read after 2 hours' incubation at 37° C and re-read after being held at 4° C overnight.

All feline and canine sera positive in CIE tests were titrated against the 8 antigens by CIE, by placing doubling dilutions of the sera in the wells.

### Supplementary tests

The 12 feline and 45 canine sera which were positive for antibody to Cr. neoformans were tested by CIE against CF and S antigens of C. albicans and 100 canine sera, including the 45 positive sera, were also tested by ID against the same antigens.

The 45 positive canine sera and 155 other canine sera were tested by ID against 2 Aspergillus fumigatus antigens.

Sera from 9 dogs with proven nasal aspergillosis, known to be positive for precipitating antibody to A. fumigatus, were tested by CIE against the serotype antigens of Cr. neoformans used in the survey.



## RESULTS

Details of the feline and canine sera investigated and the numbers positive for antibody to Cr. neoformans in the 3 tests are given in table 1.

### Feline sera

Of the 275 feline sera examined, 12 (4.4%) were positive for precipitating antibody to Cr. neoformans by CIE. No evidence of antibody was obtained using ID and agglutination tests (table 1).

In table 2, the results are presented in relation to the environment and to the state of the cats with regard to leukaemia. The sera from the 4 cats in which leukaemia was diagnosed were negative for antibody to Cr. neoformans. Of the 224 sera from non-leukaemic cats from an urban environment, 8 (3.6%) were positive in contrast to 4 (8.5%) sera from 47 non-leukaemic cats from a rural environment.

The age range of the 12 positive cats was from 1 to 8 years (table 3). No cat under one year was positive, 8 of 107 (7.5%) between one and 3 years had antibody, as had 4 (6.9%) of 58 older cats.

Eleven (6.9%) of the 160 female cats were positive for antibody; 8 were from cats from urban and 3 from cats from rural environments. Only one (0.9%) of the 115 male cats was positive. This cat came from a rural environment.

The results of CIE tests on feline sera are presented in table 4: In this test, not more than one line of precipitation was obtained with any serum, and no serum gave a positive result at a 1:2 dilution. The 12 positive sera were all positive with at least one CF antigen, but only 4 (33%) of these sera were positive with S antigen or antigens. No serum was positive only with S antigen.

From table 4, it can be seen that positive reactions to Serotype A antigens were found in 4 (33%) of the 12 positive cats, to B in 5 (42%), to C in 6 (50%) and to D in 7 (58%). Seven cats reacted with only one serotype antigen, in each case that prepared from the culture filtrate; 2 with B, 3 with C and 2 with D. No feline serum was positive with Serotype A only. These "single serotype" sera gave negative results with S antigens.

Sera from 5 cats reacted with antigens from more than one serotype; 2 with 2 serotypes (A & D, C & D), one with 3 serotypes (A, B & D) and 2 with 4 serotypes (A, B, C & D). Of these sera, only one, that reacting with serotypes C & D was not positive with at least one somatic antigen.

Of the 4 positive sera from rural cats, 2 reacted with serotype antigens A, B, C and D, one with A, B and D and one with serotype C only (table 4). In contrast, only 2 sera from urban cats were positive with 2 serotypes, A & D and C & D respectively and 6 were positive with a single serotype. Positive results with S antigen(s) were noted in 3 sera from rural cats, but in only one serum from an urban cat.

None of the 12 feline sera which were positive for antibody to Cr. neoformans by CIE reacted against either of the 2 types of C. albicans antigens by the same test.

#### Canine sera

Of 328 canine sera examined, 45 (13.7%) were positive for precipitating antibody to Cr. neoformans by CIE and 8 (2.4%) were also positive by ID. No evidence of antibody was obtained using the agglutination test (table 1).

In table 5, the results of CIE tests are presented in relation to the environment of the 171 dogs for which this information was available. Of 100 sera from urban dogs, 14 (14%) were positive in contrast to 11 (15.5%) of 71 sera from rural dogs. In ID tests, 4 sera from urban and 2 sera from rural dogs were positive.

The ages of the dogs ranged from 5 months to 17 years and the age range of positive dogs was from 1 to 12 years, with the highest percentage of positive animals in the 10 to 12 year group (table 6).

Antibody to Cr. neoformans was demonstrated in 14 (16.1%) of the 87 sera from male dogs; 10 of these dogs were from urban districts and 4 from rural areas. Of the 84 bitches, 11 (13.1%) were positive; 4 were from urban and 7 from rural environments.

In CIE tests, the number of precipitation lines ranged from 1 to 3 (figs. 4, 5, 6); 32 sera gave one line, 11 sera 2 lines and 2 sera 3 lines. In this test, no serum gave a positive result at a 1:2 dilution. The results of positive tests on canine sera are given in table 7. Forty four of the canine sera were positive with at least one CF antigen; 34 (75.6%) were positive only with CF antigens, 10 (22.2%) with CF and S antigens and one (2.2%) with S antigen only.

Positive reactions to serotype A antigens were found in 33 (73.3%) of sera, to B in 35 (77.8%), to C in 29 (64.4%) and to D in 11 (24.4%). Of the 45 positive sera, 14 (31.1%) reacted with one serotype antigen only; 7 with B, 4 with A and 3 with C. No canine serum was positive with serotype D antigens only. Of these 14 "single serotype" reactions 11 were with CF antigens only, 2 with both CF and S antigens and one with S antigen only. Sera from 31 dogs reacted with antigens from more

than one serotype; 8 reacted with 2 serotypes; (4 with A & B, 2 with B & C and 2 with A & C), one with A, B & D, one with A, C & D, 12 with A, B & C and 9 with A, B, C & D.

The results of CIE tests on sera from the 25 positive dogs with known case histories are presented in table 8. Of 11 positive sera from rural dogs, 3 reacted with all serotype antigens, 2 with A, B & C, 1 with A & B, 2 with B only and 3 with C only. Of the 14 sera from urban dogs, 4 were positive with all serotype antigens, 5 with A, B & C, one with A, C & D, one with A & B, one with A & C and 2 with B & C. Four sera from urban and 3 sera from rural dogs reacted with at least one somatic antigen.

In ID tests 8 (2.4%) of the canine sera were positive, all reacting only against CF antigen. Although 6 of these 8 sera had given more than one line of precipitation in the CIE test, not more than one line was obtained in the ID test (table 9). Furthermore, as can be seen from this table, the spectrum of reaction to serotype antigens was less widespread in the ID test. However, one serum gave a positive result with CF antigen B in ID but was negative with this antigen in CIE. From table 9 it will be seen that in the ID test, antibody to A antigens were found in 5 (62.5%) of sera, to C in 3 (37.5%), to B in one (12.5%) and to D in one (12.5%). No serum reacted with serotype D only. One serum reacted with serotype antigens A and D and one with A and C.

Of the 45 canine sera positive by CIE tests, 3 (6.7%) gave one faint precipitation line when crossed against C. albicans CF and S antigens: by ID, none of the 100 canine sera, including the 45 sera positive for cryptococcal antibody reacted with these antigens.

None of the 45 positive canine sera and 155 negative sera, tested by ID against 2 antigens prepared from A. fumigatus, were positive. Also, the 9 canine sera known to be positive for antibody to A. fumigatus were negative when tested by CIE against all Cr. neoformans CF and S serotype antigens.

## DISCUSSION

The presence of precipitating antibody to Cr. neoformans has, for the first time, been demonstrated in sera from cats and dogs in which there was no evidence of active cryptococcosis. Although none of the animals was available for mycological investigation, Dr. Rogerson, who collected the feline serum samples, stated that there was no sign of cryptococcosis in the cats examined post-mortem and that the others which were not killed were clinically normal at the time they were bled. The canine sera had been sent to the Department of Bacteriology for investigation for antibody to bacteria causing renal disease or were taken from dogs which were to be used as experimental animals or blood donors.

There is other circumstantial evidence suggesting that the animals, from which the serum samples were taken, were unlikely to have cryptococcosis. Although, each year, over 150 cats and 700 dogs are autopsied and, when relevant, microbiological investigations are carried out, no case of cryptococcosis has been diagnosed in Glasgow over the past 12 years. Only one isolation of the yeast, which was of doubtful significance, has been made from a dog (Dawson, 1979; personal communication.)

The agglutination test has been shown to be a reliable method of detecting antibody in human patients with proven cryptococcal infection (Gordon and Vedder, 1966; Kaufman and Blumer, 1968; Kaufman, 1976; Proctor, 1976). It, therefore, seemed likely that this would also be the case in animals. That no serum in the survey was positive by this test also suggests that the animals were free from overt cryptococcal infection.

Exposure to the organism in habitats where it is known to prevail has resulted in the stimulation of demonstrable antibody in man by Walter and Atchison (1966), who found that 22% of 134 sera from pigeon fanciers were positive for antibody to Cr. neoformans compared to 3% of a control group of 36 persons. The authors concluded that the pigeon fanciers had presumably been infected from the environment and that sub-clinical cryptococcosis, as evidenced by the presence of circulating antibody without manifestation of overt clinical infection, was more prevalent than was realised.

That active infection is not essential to the production of antibody to this yeast was shown in the course of my experimental work. Cats inoculated intranasally with killed cells of Cr. neoformans developed antibody just as well as those challenged with viable cells.

The saprophytic existence of Cr. neoformans in the West of Scotland, where the present serological survey was undertaken, has been proved by Evans (1977) who recovered it from the dropping of domesticated and feral pigeons. In Britain, the other records of the isolation of this species from nature are from pigeon habitats (Partridge and Winner, 1965, 1966; Randhawa, Clayton and Riddell, 1965; Randhawa, Staib and Blisse, 1973) and from droppings from habitats used by both pigeons and starlings (Symmers, 1967).

Elsewhere, Cr. neoformans has been isolated from soil (Hasenclever and Emmons, 1963; Rogers and Beneke, 1964; McDonough, Lewis and Penn, 1966), from debris in deserted houses, from rabbit pens (Denton and Di Salvo, 1968) and from excreta of birds such as chickens (Silva, 1960; McDonough et al., 1961; Denton and Di Salvo, 1968), pheasants

(Herceg, Milakovic-Novak and Hajsig, 1973), sparrows (Leone, 1969) and cuckoo (Taylor and Duangmani, 1968). It has also been found to be associated with bat guano (Ajello, Hosty and Palmer, 1967).

The proportion of positive sera varied with the animal species. Antibody to Cr. neoformans was present in 4.4% of the feline sera and in 13.7% of the canine sera in the survey. The response of canine and feline sera to the antigens also differed. In CIE tests all feline sera examined in the survey produced only one line of precipitation, as did all sera from cats challenged experimentally with viable and with killed cells of Cr. neoformans. In contrast, about one third of the canine sera gave more than one line in the CIE test. A difference between the results from dogs and cats was also apparent when the sera were tested by the less sensitive ID method. Using this, no feline serum gave a positive result but 8 (2.4%) of the 45 canine sera, which were positive by CIE, were also positive in the ID test.

There were differences in the numbers of sera positive for cryptococcal antibody among cats and dogs from rural and urban environments: 8.5% of the sera from rural cats were positive as opposed to 3.6% of the sera from urban cats. In dogs the figures are 16.9% and 13% respectively. It seems likely that the higher numbers of positive reactions among rural animals may be due to greater exposure to the yeast in the environment. Town pets, particularly dogs, probably spend the main part of their time indoors, and, when out, are usually accompanied by their owners. They are therefore less likely to gain access to habitats where the fungus may occur. As Cr. neoformans has been shown to be associated with the droppings of chickens, pheasants,



sparrows and other wild birds, it seems likely that dogs and cats living in rural areas may be exposed to the yeast rather more than are urban animals.

In dogs, there was little difference in the number of positives between males (16.1%) and females (13.1%). In cats, the difference was much more definite. Although approximately equal numbers of sera were examined for each sex, sera from 11 females were positive in contrast to one serum sample from a male. One possible explanation for this discrepancy is that the difference might be hormonal. If so, it might be expected that the proportion of positive samples would be different in entire and neutered cats. However, the proportion of sera with antibody was the same (8%) in entire and neutered female cats. The only positive male cat had not been neutered. That more female cats had antibody might also have been due to their being allowed out more frequently than male cats. However, when cat owners were asked if male cats tended to be confined while females were allowed to roam, they stated that this was not so.

Differences in the response of feline and canine sera to antigens prepared from the 4 serotypes were apparent. Antibody to serotype D predominated in cats and to serotype B in dogs.

Sera from 7 cats and 14 dogs had antibody to one serotype only. This suggests that these animals had been exposed to that particular serotype and it may, therefore, be assumed that, as antibody to serotypes A, B, C or D were found singly in some animals, these serotypes are present in Britain.

As far as I am aware, no-one has serotyped the isolates of Cr. neoformans in Britain. Strains from nature, in U.S.A., were serotyped by Walter and Atchison (1966) who found A to be the main serotype from pigeon habitats. Strains isolated from a variety of habitats were studied by Denton and Di Salvo (1968) who found that 91 of 95 strains were serotype A and one, from the floor of a deserted house, was serotype C. Bennett, Kwón—Chung and Howard (1977) typed strains from environmental sources and stated that serotype A predominated in U.S.A. and D in Europe. They failed to find serotypes B or C from the environment and stated that the source of these serotypes was not known.

In view of these results, it is interesting that antibody to serotype B was found in sera from 77.8% of dogs and in 7 of the 14 dogs which reacted to a single serotype only. This serotype was also present in 42% of feline sera and in 2 of the 7 cats reacting only with a single serotype. Antibody to serotype C, the other serotype for which an environmental source is not known, was found in 64.4% of dogs and 50% of cats and 3 dogs and 3 cats reacted only with this serotype.

It is surprising that serotype A, the one that was most frequently recorded from pigeon habitats (Walter and Atchison, 1966) and from avian and rabbit habitats and barns (Denton and Di Salvo, 1968) should be the rarest in feline sera but second in frequency in canine sera. Conversely, serotype D, that stated by Bennett et al., (1977) to be the commonest from environmental sources in Europe, was the rarest in canine sera and no dog had antibody only to this serotype.

This variation in the results between canine and feline sera suggests that there may be a bias towards the development of antibody to different

serotypes in dogs and cats. This might be due to selective exposure to serotypes from the environment or to a host-serotype affinity. The results of my experimental work, in which cats were challenged with the 4 serotypes and were shown to be capable of producing precipitating antibody to each serotype, would suggest that the latter explanation is unlikely.

In the precipitation tests, CF antigens were much more efficient than S antigens. All sera positive in ID tests reacted only with CF antigens and in CIE no feline and only one canine serum failed to react with CF antigen but did so with S antigen. To be certain, therefore, that positive sera are not missed, it is probably advisable that both types of antigen should be used.

Although the efficiency and specificity of CF antigens of Cr. neoformans were confirmed by Murphy, Gregory and Larsh (1973) and Murphy and Pahlavan (1979), the possibility that the antigens used in my serological survey might have produced cross reactions is worth considering. In this country, the likelihood of cross reactions with Blastomyces dermatitidis and Histoplasma capsulatum can be discounted as these fungi have not been shown to exist in Great Britain.

The possibility of cross reaction with Aspergillus fumigatus seemed unlikely in cats as aspergillosis is apparently rare in this species and only 2 cases have been reported (Sautter, Steele and Henry, 1955; Pakes, New and Benbrook, 1967). In dogs, however, cases of nasal aspergillosis do occur. These have been reviewed by Lane and Warnock (1977) who stated that ID was a sensitive and specific test for detection of antibody to A. fumigatus. However, as examination of 200 sera

from dogs in my survey, tested by ID, failed to reveal antibodies to A. fumigatus and as 9 sera from cases of nasal aspergillosis, known to be positive for antibody to A. fumigatus, failed in CIE to produce any lines of precipitation with the Cr. neoformans antigens used in the survey, the possibility that any of the results were due to cross reactions with this fungus seems unlikely.

Candida albicans is a common commensal in animals and, as it does in man (Winner and Hurley, 1966), is likely to produce a low grade immunological response in the host. When the 45 canine sera, positive for cryptococcal antibody, were tested against C. albicans antigens by CIE, a faint broad precipitation line which, according to Evans (1976) may represent the presence of antibody to Candida cell wall mannan was seen in 3 sera. Such sera, with low amounts of antibody to C. albicans would not, in my opinion, react with the test Cr. neoformans antigens for 2 reasons. Strong antiserum to C. albicans, raised in rabbits, failed to react with any of the antigens of Cr. neoformans used in the survey. Furthermore, rabbit antiserum to Cr. neoformans did not react with C. albicans CF or S antigens by the same test.

Maccani (1977) found that none of the 3 types of C. albicans antigen (saline suspension, purified polysaccharide, and allergenic extract) or antigens from other Candida species reacted by CIE with rabbit anti Cr. neoformans antiserum. Walter and Atchison (1966) reported that rabbit anti-Candida antiserum did not react with any antigen prepared from Cr. neoformans serotypes A, B and C when tested by the combined complement fixation-fluorescent antibody test.

As species of saprophytic cryptococci were recovered from the nasal cavities of 7 dogs and one cat in the course of this study, the possibility of cross-reactions with saprophytic cryptococci was considered. Unfortunately, the amounts of the serum samples remaining after the other serological tests had been carried out were not sufficient to enable absorption studies to be undertaken.

Most of the feline and canine sera in the survey reacted with CF antigens. These antigens were shown to be specific for Cr. neoformans by Murphy and Pahlavan (1979). These authors were testing the specificity of their fractionation-purified antigen by skin testing guinea-pigs. It elicited positive skin tests in animals sensitised with Cr. neoformans serotypes A, B and C, but not in those sensitised with Cr. albidus.

Maccani (1977) crossed saline suspension antigens from Cr. laurentii and Cr. diffluens, species recovered from the nasal turbinates (table 26), against rabbit anti-Cr. neoformans antiserum by CIE but obtained only weak reactions unlikely to be confused with the strong reactions obtained when Cr. neoformans antigen was used.

The tests used in the survey were chosen for the following reasons. The agglutination test is reliable in detecting antibody to Cr. neoformans in sera from human cases of cryptococcosis (Gordon and Vedder, 1966; Kaufman and Blumer, 1968; Kaufman, 1976; Proctor, 1976). Although ID is not regarded as sensitive in the diagnosis of human cryptococcosis, it was felt that this might not necessarily be the case in animals. Also, this test is simple and suitable for use by veterinary practitioners. The CIE test is recognised as a sensitive, rapid and reliable test, but, as yet, it has not been evaluated as to its diagnostic use in animal cryptococcosis.

The agglutination test failed to demonstrate cryptococcal agglutinins in any of the 275 feline and 100 canine sera tested; contrary to the findings of Weiland et al., (1971a) who claimed to have demonstrated antibody to Cr. neoformans by tube agglutination tests in sera from 68 of 103 dogs. The highest titre they obtained was 1:16 but 31 sera had titres of 1:2 or 1:4. Such titres are generally regarded as of doubtful significance. In the present investigation, reactions at 1:2 dilutions were considered as negative. The failure of the agglutination test to detect antibodies in the feline and canine sera is unlikely to be due to insensitivity of the test, because a titre of 1:128 was obtained when the antigens used in the survey were tested against antiserum to Cr. neoformans raised in a rabbit.

A few investigators have tried ID to demonstrate antibody in human patients, with rather disappointing results. Kaufman and Blumer (1968) employed it in addition to indirect FA, tube agglutination and complement fixation, to detect antibody in humans with proven cryptococcosis. All sera positive by the other tests were negative by ID. Widra, McMillan and Rhodes (1968) used micro-immunodiffusion to detect cryptococcal antibody in human sera and found it inconclusive. Some sera reacted positively but on re-testing, they were either negative or gave non-specific reactions. They attributed this discrepancy to the instability of their polysaccharide antigen. The ID test did not prove to be sensitive in the present study. None of the feline sera was positive and only 2.4% of the canine sera, each giving only one line of precipitation, were positive by it.

The counterimmunoelectrophoresis test proved sensitive in detecting

antibody to Cr. neoformans in feline and canine sera. Antibody to Cr. neoformans was demonstrated in 4.4% of the feline sera and 13.7% of the canine sera, some of which gave 2-3 lines of precipitation.

This is the first record of the use of CIE in detecting circulating antibody to Cr. neoformans in animals. It has been proved useful in the diagnosis of several fungal diseases in man as for example, of candidiasis (Gordon, Almy, Greene and Fenton, 1971; Remington, Gaines and Gilmer, 1972; Dee and Rytel, 1975, 1977); histoplasmosis (Gordon et al., 1971; Kleger and Kaufman, 1973); blastomycosis, coccidioidomycosis and aspergillosis (Gordon et al., 1971; Galussio, Fridman and Negrone, 1973; Papagianis and Shifrine, 1973; Ward and Kohler, 1973).

The diagnostic value of the CIE test in comparison with the ID test, has been reported by other investigators for the diagnosis and serological follow-up of mycotic and other microbial diseases. Gumaa and Mahgoub (1975) found CIE to be superior to ID for diagnosis and follow-up of mycetoma in man, as did MacKenzie and Philpot (1975) for aspergillosis, candidiasis, histoplasmosis and allestheriasis. Odds, Evans and Holland (1975) found CIE to be more sensitive than ID in the detection of Candida precipitins and recommended it for routine testing for such antibodies. Similar findings were also reported by Marier and Andriole (1978).

In this survey, CIE proved more sensitive than ID for detecting precipitating antibody to Cr. neoformans, in both feline and canine sera. Not only were more sera positive by CIE, but more lines of precipitation were obtained by it in the canine sera, and in no instance was a serum

positive by ID and negative by CIE. It was also noticeable that the ID test was usually positive with sera which gave more than one precipitation line by CIE, i.e. in sera with higher amounts of antibody.

Oversensitivity may sometimes be a disadvantage. A highly sensitive test such as CIE may give false positive results due to cross reactions; or as was thought to be the case in the sera from cats and dogs investigated here, it may detect low levels of antibody developed as a result of exposure to the organism in the absence of clinical illness. It may be that the less sensitive ID test will prove of more use in diagnosis but assessment must await the availability of sera from actual disease cases.

The presence of cryptococcal antibody in sera from dogs and cats examined in this survey could have developed as a result of exposure to the yeast in their environment, or, on the other hand, be a reflection of subclinical, past or overt infection. As no evidence of active or past infection with cryptococcosis was found in these animals, it was assumed that the antibody had developed as a result of exposure to the yeast. This fact should be kept in mind when interpreting serological tests as a means of diagnosing cryptococcosis in animals.

The importance of employing CF and S antigens from the 4 serotypes of Cr. neoformans in serological tests has been established. By so doing, there is a better chance of detecting antibody in a greater proportion of the test sera. Also, information about the presence of serotypes in the environment can be obtained.

This survey has given information useful as a background for future studies. It has shown that a significant number of cats and dogs have



been exposed to Cr. neoformans, but as only 3 cases of cryptococcosis in cats have been recorded in Britain, it would appear that the yeast is of low virulence in these animals.

Table 1. Details of feline and canine sera examined and the numbers positive for antibody to Cryptococcus neoformans in CIE, ID and agglutination tests.

Animals	No. Examined	TESTS USED						TOTAL POSITIVE	
		CIE		ID		Agg.		No.	%
		No.+ve	%	No.+ve	%	No.+ve	%		
CATS	275	12	4.4	0	0	0	0	12	4.4
DOGS	328	45	13.7	8	2.4	0	0	45	13.7

Table 2. Precipitating antibody to Cr. neoformans in non-leukaemic and leukaemic cats from urban and rural environments.

Environment	Sera	Non-leukaemic cats			Leukaemic cats		
		No. of sera tested	No.+ve	%	No. of sera tested	No.+ve	%
Urban	227	224	8	3.6	3	0	0
Rural	48	47	4	8.5	1	0	0
TOTAL	275	271	12	4.4	4	0	0

Table 3. Age groups of cats examined in the serological investigation and of those positive for antibody to Cr. neoformans

Age Group	No. of cats Examined	No.+ve for antibody	%
5-11 m.	110	0	(0)
1-3 yrs.	107	8	(7.5)
4-12 yrs.	58	4	(6.9)
TOTAL	275	12	(4.4)

Table 4. Details of sera, from urban and rural cats, positive for antibody to one or more serotypes of Cr. neoformans in CIE tests

Number of sera	Environment	antibody to serotype antigens:							
		A		B		C		D	
		CF	S	CF	S	CF	S	CF	S
2	U	-	-	+	-	-	-	-	-
2	U	-	-	-	-	+	-	-	-
1	R	-	-	-	-	+	-	-	-
2	U	-	-	-	-	-	-	+	-
1	U	-	+	-	-	-	-	+	-
1	U	-	-	-	-	+	-	+	-
1	R	+	-	+	+	-	-	+	+
1	R	-	+	-	+	+	+	+	-
1	R	+	-	-	+	+	+	+	+
12		2	2	3	3	6	2	7	2
TOTAL	12	4 (33%)		5 (42%)		6 (50%)		7 (58%)	

U = Urban  
R = Rural  
CF = Culture filtrate antigen  
S = Somatic antigen

Table 5. Results of examination of sera from urban and rural dogs for antibody to Cr. neoformans by CIE.

Environment	No. examined	No.+ve for antibody	%	No.-ve	%
Urban	100	14	(14)	86	(86)
Rural	71	11	(15.5)	60	(84.5)
TOTAL	171	25	(14.6)	146	(85.4)

Table 6. Age groups of dogs examined in the serological investigation  
and of those positive for antibody to Cr. neoformans.

No. of dogs examined	Age group	No. +ve for antibody	%
19	5-11 mth.	0	(0)
45	1-3 yr.	5	(11.1)
42	4-6 yr.	8	(19)
32	7-9 yr.	3	(9.4)
27	10-12 yr.	9	(33.3)
6	13-17 yr.	0	(0)
171		25	

Table 7. Results of examination of canine sera positive by CIE for precipitating antibody against culture filtrate and somatic antigens from Cr. neoformans serotypes.

Number of sera	Antibody to serotype antigens:								
	A		B		C		D		
	CF	S	CF	S	CF	S	CF	S	
3	+	-	-	-	-	-	-	-	-
1	-	+	-	-	-	-	-	-	-
7	-	-	+	-	-	-	-	-	-
1	-	-	-	-	+	-	-	-	-
2	-	-	-	-	+	+	-	-	-
4	+	-	+	-	-	-	-	-	-
2	-	-	+	-	+	-	-	-	-
2	+	-	-	-	+	-	-	-	-
1	+	-	+	-	-	-	+	-	-
11	+	-	+	-	+	-	-	-	-
1	+	-	+	+	+	-	-	-	-
1	-	+	-	-	+	+	+	+	+
3	+	-	+	-	+	-	+	-	-
1	+	+	+	+	-	+	-	+	+
1	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	-	+	+	+
1	+	-	+	+	+	-	+	-	-
2	+	-	+	+	+	+	+	+	+
45	31	5	35	7	28	7	10	6	
Dogs 45	33(73.3%)		35(77.8%)		29(64.4%)		11(24.4%)		



Table 8. Details of sera, from 25 urban and rural dogs, positive for antibody to one or more serotypes of Cr. neoformans in CIE tests.

Number of sera	Environment	Antibody to serotype antigens:							
		A		B		C		D	
		CF	S	CF	S	CF	S	CF	S
2	R	-	-	+	-	-	-	-	-
3	R	-	-	-	-	+	-	-	-
1	R	+	-	+	-	-	-	-	-
1	U	+	-	+	-	-	-	-	-
1	U	+	-	-	-	+	-	-	-
2	U	-	-	+	-	+	-	-	-
1	R	+	-	+	+	+	-	-	-
1	R	+	-	+	-	+	-	-	-
5	U	+	-	+	-	+	-	-	-
1	U	-	+	-	-	+	+	+	+
1	R	+	-	+	-	+	-	+	-
1	U	+	-	+	-	+	-	+	-
1	U	+	-	+	+	+	-	+	-
1	R	+	+	+	+	-	+	-	+
2	U	+	-	+	+	+	+	+	+
1	R	+	+	+	+	+	-	+	+
25									

R = rural; U = urban

Table 9. Comparison of results of examination of canine sera positive by ID and CIE for antibody to serotypes of Cr. neoformans.

Antibody to serotype antigens:															
Immunodiffusion						Counterimmunoelectrophoresis									
A		B		C		D		A		B		C		D	
CF	S	CF	S	CF	S	CF	S	CF	S	CF	S	CF	S	CF	S
+(1)	-	-	-	-	-	-	-	+(1)	-	+(1)	-	+(1)	-	-	-
+(1)	-	-	-	-	-	+(1)	-	+(2)	+(1)	+(2)	+(1)	+(2)	+(1)	+(2)	+(1)
+(1)	-	-	-	-	-	-	-	+(2)	-	+(2)	-	+(2)	-	-	-
+(1)	-	-	-	-	-	-	-	+(3)	-	+(1)	-	+(2)	-	+(1)	-
-	-	+(1)	-	-	-	-	-	-	-	-	-	+(1)	+(1)	-	-
-	-	-	-	+(1)	-	-	-	+(1)	-	+(1)	+(1)	+(2)	-	-	-
+(1)	-	-	-	+(1)	-	-	-	+(2)	-	+(1)	-	+(2)	-	-	-
-	-	-	-	+(1)	-	-	-	+(1)	-	+(1)	-	+(3)	-	-	-

Figures in parenthesis correspond to the numbers of precipitation lines

Figs. 4-6: Stained slides showing results of CIE tests on 3 canine sera.

The large wells contained the test sera. The small wells contained the antigens: on the left in descending order, A,S; A,CF; B,S; B,CF; on the right C,S; C,CF; D,S; D,CF.

(Amido Black)

Fig. 4:

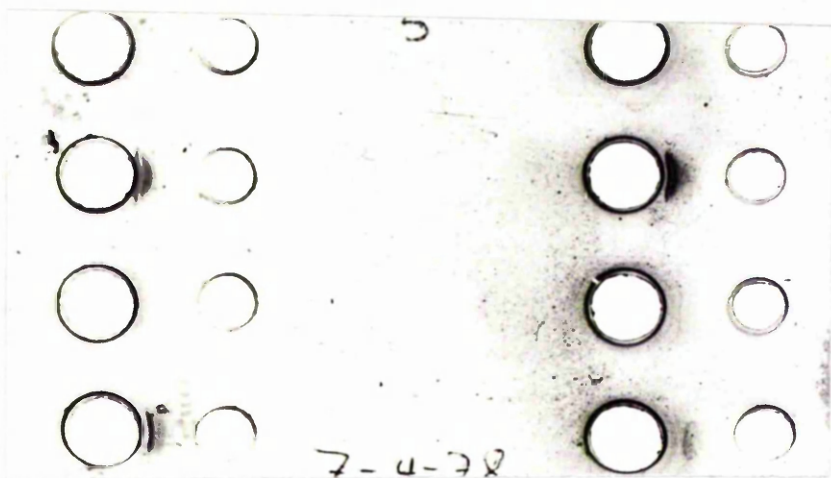
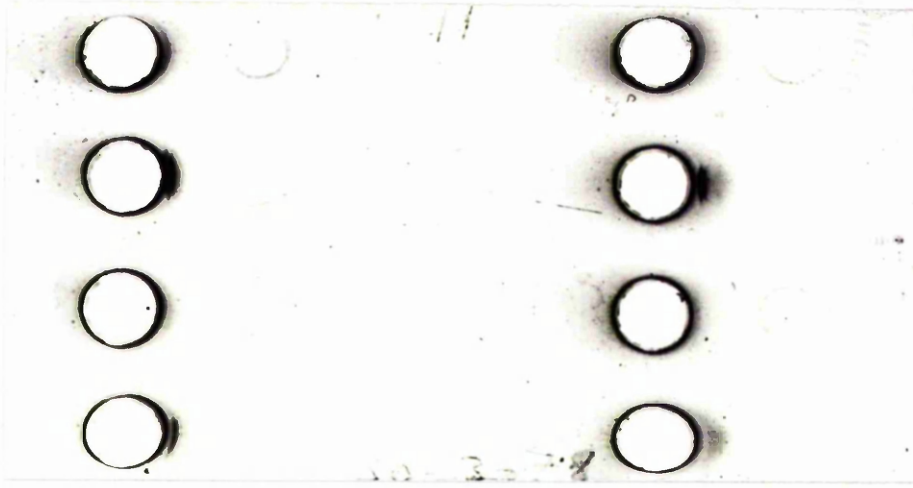
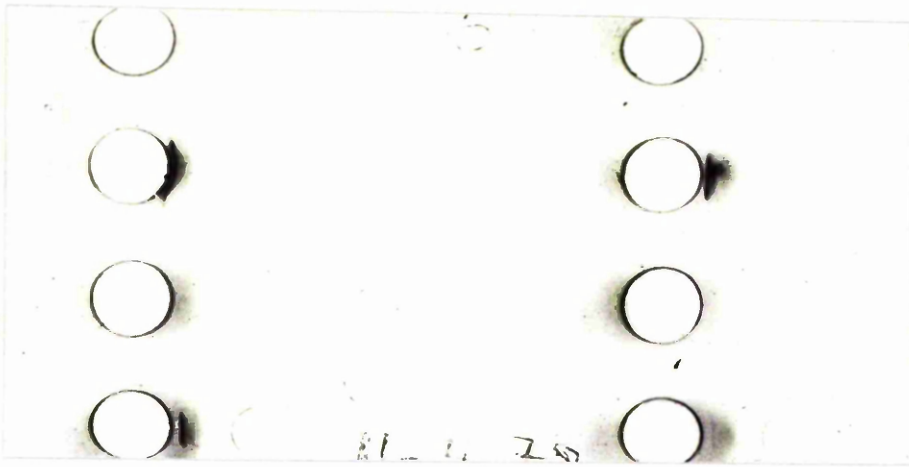
Serum giving one precipitation line with each of CF antigens A, B and C.

Fig. 5:

Serum giving 2 precipitation lines with each of CF antigens A, B and C.

Fig. 6:

Serum giving 3 precipitation lines with CF antigen A, one line with CF B and 2 lines with CF C.



EXPERIMENTAL INFECTION OF CATS  
WITH CRYPTOCOCCUS NEOFORMANS

## INTRODUCTION

Cryptococcosis is considered to be the most common systemic mycosis in the cat (Horne, 1964; Catcott, 1975). The first case of the disease in the cat was reported by Holzworth in 1952 in the United States of America. The subject was a mature male cat with a history of chronic respiratory disorder and, at necropsy, it was found to have a generalized infection. Holzworth and Coffin (1953) reported a second case, also with chronic respiratory involvement. Since then, cases have been reported with increasing frequency from many parts of the world. At least 45 cases have been recorded between 1952 and 1979: from USA (22), Australia (5), Japan (4), Britain (3), New Zealand (3), Brazil (2), Hawaii (2), Austria (1), Canada (1), France (1) and Papua New Guinea (1). These cases, with the relevant references are summarised in table 10.

Cryptococcus neoformans infection in the cat may involve one or more body systems and the clinical signs are determined by the organs affected. The most common sites of involvement are the nasal cavity and lungs. Infections of the upper respiratory tract have been reported by Holzworth (1952); Holzworth and Coffin (1953); Trautwein and Nielsen (1962); Roberts (1964); Fowler et al. (1965); Barrett and Scott (1975); Duckworth et al. (1975); Palumbo and Perri (1975); Rutman et al. (1975); Humphrey et al. (1977) and Weir et al. (1979). Cases of lung involvement have been recorded by Holzworth (1952), Trautwein and Nielsen

(1962), Olander et al., (1963), Cordes and Royal (1967), Ivoghli et al., (1974) and Rutman et al., (1975).

The yeast has been reported infecting the central nervous system (CNS), (Holzworth, 1952; Holzworth and Coffin, 1953; Olander et al., 1963; Johnston and Lucas, 1965; Ivoghli et al., 1974; Rutman et al., 1975); the oral cavity with labial, lingual and palatine lesions (Holzworth, 1952; Holzworth and Coffin, 1953; and Humphrey et al., 1977); the orbit and ocular system (Holzworth and Coffin, 1953; Fischer, 1971; Ivoghli et al., 1974; Barrett and Scott, 1975; Rutman et al., 1975; Gwin et al., 1977); and the skin and subcutaneous tissue (Trautwein and Nielsen, 1962; Olander et al., 1963; Roberts, 1964; Fowler et al., 1965; They, 1969; Rutman et al., 1975; Humphrey et al., 1977; Brown et al., 1978). Invasion of the lymph nodes, with subsequent lymphadenopathy has also been reported (Howell and Allan, 1964; Cordes and Royal, 1967; They, 1969; Ivoghli et al., 1974; Humphrey et al., 1977). It has also invaded the kidney (Cordes and Royal, 1967), spleen (Ivoghli, Anderson and Leipold, 1974), cardiac and skeletal muscles (Cordes and Royal, 1967), and bones (Holzworth and Coffin, 1953; Rutman, Richards and Chandler, 1975).

The primary portal of entry of the organism is considered to be the respiratory tract, by inhalation of dust-borne yeast cells from the environment. Hence, primary pulmonary infection with subsequent haematogenous dissemination is the general rule (Kaplan, 1973; Barrett and Scott, 1975).

There is no evidence of transmission of cryptococcosis from one host to another, but since Barrett and Scott (1975) stated that experi-

mental infection can be induced in the cat by direct inoculation, the possibility of contagion within and between species exists.

The incidence of feline cryptococcosis is unknown, but from the increasing number of cases reported recently, it appears that the disease is more prevalent than is generally believed. The difficulty in assessing the incidence of the disease arises from such factors as lack of awareness of the disease among veterinary practitioners who do not consider it in their differential diagnosis; misdiagnosis as chronic respiratory and other diseases (Cattcott, 1975) and the fact that the disease is not notifiable, so that cases are not always reported (Kaplan, 1973). Although, as in man, the disease is world-wide in distribution, the majority of feline cases have been reported from the USA where veterinarians and diagnosticians are aware of its existence.

Of the 45 recorded cases, 28 have been reported post mortem. This may in part be due to the highly fatal nature of the disease or due to incidental post mortem findings. The remaining 17 cases which were diagnosed ante mortem by microscopy, culture and/or histopathology, mainly originated in locations where the personnel concerned were aware of the disease and were acquainted with its aetiological agent.

There is a marked lack of consistency in the clinical signs exhibited by an infected cat. However, the disease is characterised most frequently by nasal granulomatous lesions, a chronic nasal discharge and focal pulmonary lesions with cough and dyspnoea (Cattcott, 1975). Some animals develop signs referable to CNS involvement, particularly in disseminated infections. These may be somewhat vague, such as listlessness, pupillary dilation and mild ataxia, or be obvious, such as



incoordination, circling and partial or complete blindness, associated with locomotor disturbances (Jungerman and Schwartzman, 1972).

The prognosis of cryptococcal infection in the cat is grave unless the infection is limited to a localised swelling which can be surgically removed but generalised forms of the disease are invariably fatal (Cattcott, 1975). Reports of therapy with amphotericin B, the drug of choice in treatment of human cryptococcosis, present conflicting results. Campbell et al., (1970) reported unsuccessful treatment of a cat with a subcutaneous temporal cryptococcal granuloma, by injection of amphotericin B into the lesion. It has been stated that the drug is too nephrotoxic for use in the cat (Lau, Reinke and Brown, 1971) and that its use in cats is a synonym for euthanasia (Jungerman and Schwartzman, 1972). However, others have recently claimed success with it. Barrett and Scott (1975) reported cure of a cryptococcal nasal granuloma in a cat by intravenous administration of the drug, but not with 5-Fluorocytosine (5-FC) given per os. Palumbo and Perri (1975) treated two cats with naso-granulomatous cryptococcosis by intravenous administration of amphotericin B and reported complete cure in one but failure in the other. Thrall, Rich and Freemyer (1976) claimed to have obtained complete cure with intravenous amphotericin B therapy in a cat with foot ulcerations due to Cr. neoformans. However, because of the high nephrotoxicity, particularly in the cat, all authors emphasised the importance of regularly monitoring the blood urea nitrogen and kidney function during treatment. Recently, Weir, Schwartz and Buergelt (1979) adopted a synergistic regimen with intravenous amphotericin B and oral 5-FC in a cat with nasal cryptococcal granulomas. They reported successful treatment in a 4 week period,

stating that by combining the two drugs, they minimised the inherent risk of nephrotoxicosis resulting from prolonged use of amphotericin B and simultaneously reduced the chances of development of 5-FC-resistant mutants, often encountered in human patients treated with 5-FC alone.

Until recently, data on the serology of cryptococcal infection in the cat and other animal species was lacking (Ainsworth and Austwick, 1973). However, during the progress of the present study, Weir et al., (1979), using the latex-particle agglutination test, demonstrated serum cryptococcal antigen in a cat with nasal cryptococcal granulomas. Their objective was to monitor the progress of the cat and its response to a combination of amphotericin B and 5-FC chemotherapy, by periodically determining the titres of cryptococcal antigen. They reported that an initial titre of 1:16 prior to treatment fell to 1:1 by six months after the start of treatment, indicating successful therapy.

Serology has been established as a rapid and reliable means in the diagnosis and prognosis of human cryptococcosis. Early diagnosis of the disease has often enabled the initiation of specific therapy and successful treatment in human patients that might have otherwise ultimately died of progressive infection. Recent reports on successful treatment of cases of cryptococcosis in cats have stimulated hopes that cats with this disease can be cured. However, before this is possible, it is essential that the disease be diagnosed while in the early stages of infection.

The aim of this experimental study was to infect cats with Cr. neoformans and follow the course of the disease serologically to determine whether serological tests would be useful as a diagnostic tool in the early diagnosis of feline cryptococcosis.

Table 10. Cases of feline cryptococcosis recorded between 1952-1979

<u>Country</u>	<u>Authors</u>	<u>Year</u>	<u>Number of cases</u>
USA	Holzworth	1952	1
	Holzworth and Coffin	1953	1
	McGrath	1954	3
	Kaplan, Ajello, Bitetto and McDonough	1961	1
	Trautwein and Nielsen	1962	1
	Olander, Reed and Pier	1963	2 *
	Roberts, Duncan, Pearson, Hogle and Ramsey	1963	1
	Roberts	1964	1 *
	Chute, Davis and Payne	1965	1
	Fischer	1971	2
	Lau, Reinke and Brown	1971	1
	Ivoghli, Anderson and Leipold	1974	1
	Barrett and Scott	1975	1 *
	Rutman, Richards and Chandler	1975	1 *
	Thrall, Rich and Freemyer	1976	1 *
	Gwin, Gelatt, Hardy, Peiffer and Williams	1977	1 *
	Brown, Nowlin, Taylor and O'Neill	1978	1 *
Weir, Schwartz and Buergelt	1979	1 *	
Brazil	Carvalho, Mello and Ferreira	1974	2
Hawaii	Palumbo and Perri	1975	2 *
Canada	Beauregard and Malkin	1970	1

Table 10 (continued).

<u>Country</u>	<u>Authors</u>	<u>Year</u>	<u>Number of cases</u>
Australia	Curtis	1957	1
	Johnston and Lavers	1963	1
	Johnston and Lucas	1965	1 *
	Clark and Roubin	1970	1
	Howlett, Duff and Allan	1973	1
New Zealand	Cordes and Royal	1967	1
	Wanner and Baird	1974	1 *
	Duckworth, Taylor and Julian	1975	1 *
Papua New Guinea	Humphrey, Fordham and McKerrow	1977	1 *
Great Britain	Howell and Allan	1964	1 *
	Fowler, Forbes and Murray	1965	1
	Campbell, Naylor, Kelly and Esplen	1970	1 *
Austria	Kohler, Gialamas and Kuttin	1976	1
France	Thery	1969	1
Japan	Yamamoto, Ishida and Sato	1957	1
	Saeki, Tokutake and Sato	1964	1
	Chiba, Kato and Nonogaki	1967	1
	Okoshi and Hasegawa	1968	1

\* Diagnosed ante mortem

Intranasal Infection of Cats with *Cr. neoformans* serotypes and one untyped strain

MATERIALS AND METHODS

Four serotypes and one strain of *Cr. neoformans* were used. Cultures of serotypes A, B, C and D were donated by Dr. E. G. Evans of the Department of Dermatology and Microbiology, Leeds General Infirmary, England. He obtained them from the Mycology Reference Laboratory, London, where they had been deposited in 1970 by Dr. M. Gordon of the New York State Department of Health. Since then, they have been maintained in a freeze dried state as numbers 3168, 3169, 3170 and 3171 respectively. Strain V359 was isolated in Glasgow by Dr. C. O. Dawson in 1976 from a nasal swab from a dog and had been maintained in culture.

Inocula were prepared from cultures grown on slants of SDA containing chloramphenicol at 28<sup>o</sup>C for 4 days. Cells were harvested, washed three times in SNS, separated by centrifugation at 3,000 rpm for 5 minutes and finally adjusted to concentrations of  $1 \times 10^8$  cells/ml by haemocytometer counts.

Five male and 5 female, clinically healthy, adult cats were infected, using two cats per serotype/strain of the yeast (table 11). Prior to infection, nasal swabs from all cats were examined by culture for the presence of *Cr. neoformans* and sera were tested for the presence of agglutinating and precipitating antibodies to *Cr. neoformans*.

Prior to inoculation by intranasal instillation (IN) of yeast cell suspensions, the cats were anaesthetized with Vetalar (Ketamine

hydrochloride, Parke-Davis), at the rate of 15 mg/kg body weight, given intramuscularly into the thigh muscle. Inoculation was done by drop by drop instillation from a 1 ml syringe without a needle. With the syringe held in an upright position pointing downward, and the animal facing upward, 1 ml of inoculum ( $1 \times 10^8$  cells) was equally divided between the two halves of the nasal cavity and the animal held in the inoculation position for a few minutes until the inoculum was inhaled.

Animals were examined daily for signs of infection and were bled, under general anaesthesia, at 4 week intervals. At each bleeding, 5-10 ml of blood was withdrawn from the jugular vein of each cat, in a 10 ml sterile syringe which was immediately evacuated into a sterile universal bottle.

On death or euthanasia, the following body fluids, organs and specimens were taken for culture, microscopy, serology and when indicated, histopathology: blood, CSF, urine, nasal turbinates, trachea, lungs, heart, liver, spleen, kidneys, mediastinal lymph nodes, brain, spinal cord and faeces.

At euthanasia, cats were bled out by cardiac puncture. Immediately after withdrawal, 2 ml of blood were evacuated into a sterile vial containing EDTA as an anti-coagulant, for culture, and the rest of the blood was kept for serology.

Cerebrospinal fluid was obtained shortly after death or immediately after euthanasia by cisternal puncture, using the technique of Kay, Israel and Prata (1974). It was then cultured, examined by direct microscopy in India-ink mounts and tested for the presence of cryptococcal antigen and/or antibody.

Urine was collected soon after necropsy, in 20 ml sterile syringes,

by puncturing the wall of the urinary bladder with a 21G1½ in. needle. It was tested as for CSF.

The nasal turbinates, trachea, lungs, heart, liver, spleen, kidneys, mediastinal lymph nodes, brain and spinal cord were aseptically removed shortly after death or euthanasia. All the specimens were cultured and, when indicated, portions of some organs were fixed in 10% neutral buffered formalin for histological examination.

The nasal turbinates were cultivated by cutting them into small pieces with sterile scissors and implanting the pieces into the agar with sterile forceps. The trachea was cut open and the inner surface swabbed with a sterile, plain, cotton swab, which was then streaked on culture medium.

Portions of lungs, heart, liver, spleen, kidneys, lymph nodes, brain and spinal cord were homogenized in 3 ml SNS in tissue homogenizers and cultured by flooding the surface of the agar plate with the fine tissue homogenate. The plate was left for a few minutes and excess fluid was removed with a sterile Pasteur pipette.

Blood samples were treated as follows: half of the sample was cultured directly by flooding the surface of the agar plate as for the tissue homogenates, and the other half was enriched in 10 ml sterile Sabouraud dextrose broth containing chloramphenicol in a universal bottle, incubated at 28°C for 48 hours and then subcultured as for the other half.

Urine was cultured by flooding the surface of the agar plate, and removing excess fluid with a sterile Pasteur pipette after a few minutes standing on the bench.

Faecal samples and CSF were cultured by streaking a loopful on to the surface of the isolation medium.

Cerebrospinal fluid and urine, collected at autopsy, were examined for cryptococcal antibodies by ID and CIE against homologous CF and S antigens, and for cryptococcal antigens by crossing them against rabbit anti-Cryptococcus neoformans anti-serum by CIE.

Sera were tested against homologous antigens for the presence of cryptococcal antibodies by the agglutination, ID and CIE tests; and against rabbit anti-Cryptococcus neoformans anti-serum for cryptococcal antigens by the latter test. In addition, sera from cats infected with serotypes A, B, C and D were tested, by CIE, against heterologous CF and S antigens from each of the other 3 serotypes; and those from cats infected with Str. V359 were tested against CF and S antigens from each of the 4 serotypes.

## RESULTS

Preinfection nasal swabs from all cats were negative for Cr. neoformans, and preinfection serum samples were negative for antibody to Cr. neoformans.

Three cats died early in the course of the experiment. Cat 7 died 3 days postinfection of acute calicivirus infection, and no serum was available for serological examination. Profuse growth of Cr. neoformans was recovered from its nasal turbinates and trachea and a few colonies were obtained from the lungs. Other organs and body fluids did not yield the organism, as may be seen from table 12.

Cat 6 died 33 days post infection, of irreducible intussusception of the ileum. Cryptococcus neoformans was re-isolated in heavy growth from its nasal turbinates and a few colonies were obtained from the lungs



but not from other specimens or organs (table 12). Serum samples collected 28 days post challenge and at autopsy were positive for cryptococcal antibody by CIE, but not by ID or agglutination tests (table 13). However, CSF and urine collected at autopsy were negative for precipitating cryptococcal antibody and antibody and/or antigen by ID and CIE tests respectively. Histological examination of sections from lungs and brain stained with H & E, P.A.S., Mayer's mucicarmine and modified Gomori-Grocott did not reveal the presence of Cr. neoformans in these organs.

Cat 8 became seriously ill with acute streptococcal pneumonia and was killed 48 days post infection. A few colonies of Cr. neoformans were recovered from the nasal turbinates, but no growth was obtained from other specimens (table 12). Cryptococcal antibody was demonstrated in serum samples collected 28 days post challenge and at autopsy, by CIE but not by ID or agglutination tests (table 13). Urine and CSF collected at autopsy were negative for antibody and antigen.

The remaining 7 cats were clinically observed till the end of the experiment, 122-128 days post inoculation. None developed clinical symptoms suggestive of cryptococcal infection and at necropsy none showed any gross abnormality.

Serological results from surviving cats which were bled at 28 day intervals, and then at autopsy are presented in table 13. As may be seen from this table, precipitating cryptococcal antibody was demonstrable by CIE in all 7 cats, as early as 28 days post challenge and persisted until death 122-128 days after inoculation. By ID, antibody was demonstrated in cats 1, 2 and 5, 112 days post challenge, but not at

the time of autopsy 10-13 days later. Each positive serum gave a single precipitation line in both tests. Agglutinating antibodies were not detected in any of the cats during the observation period. Urine and CSF, taken at autopsy from the 7 cats, were negative for antibody and antigen.

The results of examination of sera against heterologous serotype antigens are presented in table 14. Cross reactions with heterologous serotype antigens were obtained in sera from cats infected with the serotypes. With the exception of those from cats 6 and 8 which were positive with both homologous antigens and at least one heterologous antigen at 28 days post inoculation, sera from other cats (1, 2, 3, 4 and 5) remained positive only with the homologous antigens up to 84 days post inoculation when cross reactions with heterologous antigens were noticed. Such cross reactions were obtained with one or more heterologous antigen. None of the sera examined, from cats 9 and 10, infected with Str. V359, reacted with any of the serotype antigens.

The results of cultural examination of body fluids, specimens and organs from these 7 cats, taken at necropsy, are summarised in table 12. Cryptococcus neoformans was re-isolated from the nasal turbinates of 3 cats, (5, 9 and 10). It was recovered in profuse growth from cat 5, 125 days post infection, and a few and numerous colonies were obtained from cats 9 and 10 respectively, 128 days post infection. Other specimens and organs from these 3 cats and those from the other 4 cats, (1, 2, 3 and 4) did not yield the fungus.

Table 11. Details of cats inoculated intranasally with  $1 \times 10^8$  cells of strain V359 and each of the 4 serotypes of Cr. neoformans

Cat No.	Sex	Infecting serotype/strain
1	F	Serotype A
2	F	
3	F	Serotype B
4	M	
5	F	Serotype C
6	M	
7	F	Serotype D
8	M	
9	M	Str. V359
10	M	

Table 12. Isolation of Cryptococcus neoformans at autopsy from organs and specimens from cats challenged intranasally with serotypes A, B, C and D and Str. V359

Cat	Serotype Strain	Time of autopsy (days)	Blood	CSF	Brain	Spinal Cord	Nasal Turbinates	Trachea	Lung	Mediastinal L. Nodes	Kidney	Urine	Liver	Spleen	Heart	Faeces	
1	A	122	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2		123	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3	B	124	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
4		124	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5	C	125	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-
6		33	-	-	-	-	++	-	+	-	-	-	-	-	-	-	-
7	D	3	-	-	-	-	+++	+++	+	-	-	-	-	-	-	-	-
8		48	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
9	V359	128	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
10		128	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-

- no growth  
+ 1-20 colonies  
++ 21-50 colonies  
+++ over 50 colonies

Table 13. Serological follow-up by ID and CIE tests of cats infected with Cr. neoformans Str. V359 and Serotypes A, B, C and D

Cat	Homologous Antigen	Post infection bleedings (days):												Time of Autopsy (days)
		28		56		84		112		At autopsy				
		ID	CIE	ID	CIE	ID	CIE	ID	CIE	ID	CIE			
9	Str. V359	-	+	-	+	-	+	-	+	-	+	-	+	128
10		-	+	-	+	-	+	-	+	-	+	-	+	128
1	Serotype A	-	+	-	+	-	+	+	+	-	+	-	+	122
2		-	+	-	+	-	+	+	+	-	+	-	+	123
3	Serotype B	-	+	-	+	-	+	-	+	-	+	-	+	124
4		-	+	-	+	-	+	-	+	-	+	-	+	124
5	Serotype C	-	+	-	+	-	+	+	+	-	+	-	+	125
6		-	+									-	+	33
8	Serotype D	-	+									-	+	48

\* All sera were negative by the agglutination test

Table 14. Results of CIE tests, using antigens from each serotype, on sera from experimental cats infected with serotypes and with str. V359.

Cat	Infecting serotype/strain	Days post infection when bled	Serotype antigens:							
			A		B		C		D	
			CF	S	CF	S	CF	S	CF	S
1	A	28	+	+	-	-	-	-	-	-
		56	+	-	-	-	-	-	-	-
		84	+	+	+	-	-	-	-	-
		112	+	-	+	-	-	-	-	-
		122	+	-	+	-	-	-	-	-
2	A	28	+	+	-	-	-	-	-	-
		56	+	-	-	-	-	-	-	-
		84	+	+	+	-	-	-	-	-
		112	+	-	+	-	+	-	-	-
		123	+	-	+	-	-	-	-	-
3	B	28	-	-	+	+	-	-	-	-
		56	-	-	+	+	-	-	-	-
		84	+	-	-	+	+	-	-	-
		112	+	-	+	-	-	-	-	-
		124	+	-	+	+	-	-	-	-
4	B	28	-	-	+	+	-	-	-	-
		56	-	-	+	-	-	-	-	-
		84	+	-	-	+	-	-	-	-
		112	+	-	+	+	-	-	-	-
		124	+	-	+	+	-	-	-	-

Table 14 (continued)

Cat	Infecting serotype/strain	Days post infection when bled	Serotype antigens:							
			A		B		C		D	
			CF	S	CF	S	CF	S	CF	S
5	C	28	ND		ND		+	+	ND	
		56	-	-	-	-	+	+	-	-
		84	+	-	+	-	+	+	-	-
		112	+	-	+	-	+	+	-	-
		125	+	+	+	-	+	+	-	-
6	C	28	+	-	ND		+	-	ND	
		33	-	-	-	-	+	-	-	-
8	D	28	+	-	+	-	-	-	+	-
		48	-	-	-	-	-	-	+	-
9	V359	28	-	-	-	-	-	-	-	-
		56	-	-	-	-	-	-	-	-
28		-	-	-	-	-	-	-	-	
10		56	-	-	-	-	-	-	-	-

MATERIALS AND METHODS

Three strains of Cr. neoformans were used in this experiment. They were donated by Dr. B. Partridge of Charing Cross Hospital, London. Strain CXH Symmers had been isolated from a human patient; str. KCH was isolated from a patient with meningeal infection at King's College Hospital in 1959 and str. Huntington had come from Huntington Research Centre in 1973. Pathogenicity tests showed the 3 strains to be pathogenic for mice, with strain differences. Strain KCH was the most virulent.

Inocula were prepared as in the previous experiment and adjusted to a final concentration of  $1 \times 10^7$  cells/ml by haemocytometer counts.

Seven female and 5 male, clinically healthy adult cats were infected, using 4 animals per strain of Cr. neoformans. Later in the course of the experiment, 2 animals (17, 18) were killed and were replaced by 2 others. (table 15). Three adult cats and 3 kittens, were used as exposure controls by letting them mix with inoculated cats in the same room.

Two cats in each group were infected intranasally and 2 intravenously, each receiving  $1 \times 10^7$  yeast cells. Intranasal inoculation was carried out as described previously and intravenous inoculation was made via the jugular vein. Infection in all cats was undertaken under general anaesthesia with Vetalar.

Animals were examined daily for the development of any signs of clinical infection.

Pre-infection serum samples were collected from all infected and



control cats and screened for antibody to Cr. neoformans. Pre-infection nasal swabs from all animals were cultured for Cr. neoformans and so were post infection nasal swabs taken 2, 7, 14 and 21 days after infection.

Animals were bled at 2 week intervals for serological examination and sera tested against homologous and heterologous formalin-killed whole yeast cells and CF and S antigens by agglutination, ID and CIE tests. Sera that were positive for cryptococcal antibody were also tested, by CIE, against the 8 serotype antigens used in the serological survey.

Rectal swabs from infected cats were taken for culture each time animals were bled. A bronchial washing was collected for culture and microscopy for Cr. neoformans, 8-9 weeks post infection from each of the 6 cats which were infected intranasally.

Chest x-ray was performed on all inoculated cats, (excluding cats 17 and 18 which had previously been killed) after 3 weeks post infection in cats 23 and 24, and 8-9 weeks in cats 11-16 and 19-22.

Body fluids, tissues and organs from all infected and exposed cats were collected at autopsy, processed and examined as was described in the previous experiment.

## RESULTS

Pre-infection sera from all infected and exposed cats were negative for cryptococcal antibody by agglutination, ID and CIE tests, when tested against antigens from Cr. neoformans serotypes A, B, C and D. Pre-infection nasal swabs taken 4 days before infection and streaked on Niger Seed agar were all negative for Cr. neoformans.

The results of culture of post infection nasal swabs from intranasally infected cats are presented in table 16. Swabs from intravenously infected cats were all negative for Cr. neoformans 2 to 21 days post infection. All 6 intranasally infected cats yielded Cr. neoformans 2 days after inoculation, 4 were still positive 7 days after infection and one cat was positive 14 days post infection. All were negative 21 days post infection.

All rectal swabs were negative for Cr. neoformans throughout the course of the experiment.

Sediments from bronchial washings from cats 11, 12, 15, 16, 19 and 20 were all microscopically negative for Cr. neoformans when examined in India ink mounts and in P.A.S.-stained smears. The washing from one cat (19), taken 8 weeks after infection, yielded a few colonies of Cr. neoformans but the washings from the other 5 cats were negative.

Chest x-ray examination did not reveal abnormality in any of the cats examined.

### Cat 18

This animal became ill, 10 days post infection, with depression, anorexia, elevated temperature ( $103.5^{\circ}\text{F}$ ) and emesis. It lost weight rapidly and developed conjunctivitis, with a discharge from both eyes,

and it became semi-blind. A blood sample, on the 10th day following infection, was positive for feline leukaemia virus by virus isolation test, an eye swab and a urine sample taken on the same day yielded Cr. neoformans in culture. The animal was killed on the 17th day after infection. Autopsy revealed an emaciated animal with necrotic hepatic foci (fig. 7), fleshy pale spleen (fig. 8), yellowish grey, tender-to-touch kidneys (fig. 9), overinflated lungs (fig. 10) and haemorrhagic peritonitis involving mainly the mesenteric lymph nodes.

#### Cat 17

This cat was clinically normal at the time cat 18 became ill, but was killed as a non-leukaemic control for cat 18, because they were infected with the same strain via the same route and for the same period of time. A blood sample and smear taken 2 weeks post infection were negative for feline leukaemia virus in this cat. Post mortem examination of this cat showed it to be in good general health and no gross abnormality was seen (figs. 7-10).

The results of cultural examination of organs and specimens from both cats are compared in table 17. As may be seen from this table, Cr. neoformans was re-isolated in heavy to profuse growth from all the specimens from cat 18, except the mediastinal and mesenteric lymph nodes, heart muscle and faeces. Escherichia coli was recovered from the mesenteric lymph nodes and in addition to Cr. neoformans, it was also isolated from the necrotic foci in the liver. On the other hand, only nasal turbinates, lung, kidney, urine and spleen from cat 17 yielded Cr. neoformans in amounts of growth ranging from scanty to heavy.

Sections from the spleen, liver, kidney, lung and brain from both cats, stained with H & E, P.A.S., Mayer's mucicarmine and Gomori-Grocott were examined. The presence of focal necrotising hepatitis was confirmed in cat 18; large areas of necrosis associated with colonies of bacteria were surrounded by polymorphonuclear leucocytes and lymphoid cells with macrophages at the periphery of each lesion. There were fatty changes in the tubules of the renal cortex and mild focal interstitial nephritis with glomerular sclerosis and some calcified debris in the medullary tubules. The presence of Cr. neoformans in the kidney was revealed by P.A.S., Mayer's mucicarmine and Gomori-Grocott stains. It was present in the glomerular capillaries (figs. 11, 12), Bowman's space (fig. 13) and renal tubules (figs. 14, 15). In most cases, there was distension of the capillary or tubule, but no cellular reaction, and the yeast cells were occasionally associated with the lesions of nephritis. Microscopy of the lung revealed the presence of moderate oedema with scattered alveolar macrophages. Occasional yeast cells were demonstrated in the cerebrum, but apart from mild cystic lysis, they were not associated with any obvious inflammatory changes (fig.16).

Apart from mild thickening of the alveolar walls, there was no microscopic abnormality in organs from cat 17 and Cr. neoformans was not seen in any of the sections.

Neat and concentrated sera (x2) taken from cats 17 and 18, at autopsy, were positive by CIE for circulating cryptococcal antigen when crossed against rabbit anti-Cryptococcus neoformans antiserum. In addition, the serum sample from cat 17 was also positive for cryptococcal antibody by CIE, but the serum from cat 18 was negative for antibody

(table 18). Cerebrospinal fluids and unconcentrated and concentrated (x2) urine samples, taken at autopsy, were negative for cryptococcal antibody and/or antigen by CIE and ID in both cats (figs. 17, 18).

#### Cat 24

This cat was killed 34 days post infection because of calicivirus infection. Post mortem examination revealed it to be emaciated, with the nasal cavities occluded with thick, purulent material and there was purulent discharge from the eyes. The lungs were slightly emphysematous. Cryptococcus neoformans was recovered in profuse growth from the brain and kidney homogenates and in heavy and slight growth from spleen and liver homogenates respectively, but not from other organs and specimens (table 17).

Sections of brain, kidney, liver and spleen revealed the presence of occasional P.A.S.-positive yeast-like cells in the interstitial tissue of the renal cortex, but the organism was not seen in other organs.

Serum collected at autopsy was positive for cryptococcal antigen when tested neat and concentrated x.2 by CIE, but was negative for antibody (table 18). Cerebrospinal fluid and unconcentrated and concentrated urine were negative for cryptococcal antibody and antigen (fig. 19).

#### Cat 23

This cat showed nervous signs 7 weeks post infection. It was circling, with the head tilted and showed inco-ordinated locomotion. It then became hyperaesthetic and blind. Both pupils were dilated, with hypopyon (fig. 20) and retinal detachment in both eyes. A blood sample taken on the day the animal first showed clinical signs was negative by culture for Cr. neoformans, and by virus isolation test for feline

leukaemia virus.

The animal was killed 56 days post infection. Post mortem examination showed it to be in good general condition, apart from the eye lesions, and no gross abnormality was discernible in other organs. The eyes were enucleated and fixed for histopathology, other specimens and organs were taken for culture and/or histopathology.

On macroscopic examination, both eyes were essentially similar: there was a purulent mass in the anterior chamber (fig. 21) and the retina was detached and funnel-shaped (figs. 22, 23). In the left and right eyes there were small nodules of pigment and in the right eye a haemorrhagic exudate was visible on the inner surface of Bruch's membrane (fig. 24). In the left eye, the subretinal space contained watery fluid and the anterior chamber, a solid gelatinous exudate (fig. 25).

Microscopic examination of the right eye showed the cornea with peripheral neovascularisation, intracellular epithelial oedema and endothelial vacuolisation. These changes suggested a pre-existing glaucoma, which resulted from inflammatory cell infiltration of the outflow system, which was packed with lymphocytes and plasma cells. The anterior chamber contained a fibrinopurulent exudate (fig. 26). The iris and ciliary body were infiltrated by chronic inflammatory cells, but the reaction was not as intense as that present in the choroid in which there was extensive tissue necrosis (fig. 27). The lens was artefactually fragmented but not involved by the inflammatory or glaucomatous process. The ciliary processes showed cystic degeneration in the epithelium. The vitreous body contained scattered macrophages and lymphocytes. The retina was detached and disorganized and the tissues were extensively

invaded by lymphocytes and macrophages (figs. 28, 29). The subretinal space contained blood and had similar inflammatory cells in addition to iron- and melanin-laden macrophages. The optic nerve showed extensive axonal loss, with beading of the residual axons. This was not paralleled by a corresponding loss of axons in the retina, which suggested an additional toxic optic neuropathy. There was no evidence of an inflammatory reaction in the optic nerve.

The microscopic changes in the left eye were essentially similar to those in the right one, except that there was direct involvement of the retro-ocular blood vessels and the optic nerve, which showed extensive demyelination and axonal loss. Cryptococcal endophthamitis in this cat was confirmed by demonstrating Cr. neoformans in the retina and subretinal inflammatory exudate in both eyes (figs. 30-33).

Sections of kidney, lungs, brain, liver and spleen did not reveal the organism, even with the special stains.

Cryptococcus neoformans was isolated in heavy growth from kidney homogenate and urine, but not from any of the other specimens and organs examined (table 17).

Serum samples collected at autopsy were positive by CIE for circulating cryptococcal antigen when tested neat and concentrated x2, but negative for antibody (table 18). Cerebrospinal fluid and urine collected at autopsy were negative for cryptococcal antibody and antigen by CIE and ID.

#### Cats 12, 16 and 21

These cats were killed 65, 65 and 66 days post inoculation respectively. No ante mortem or post mortem abnormality was seen in any of them.

All were in good bodily condition.

The results of cultural examination of specimens and organs from these cats are presented in table 19. Cryptococcus neoformans was recovered only from the nasal turbinates of cats 12 and 16, in profuse and light growth respectively, but not from other specimens from either cat or any of those from cat 21. Cerebrospinal fluids and urine samples taken at autopsy were all negative for cryptococcal antibody and antigen.

Cats 11, 13, 14, 15, 19, 20 and 22

Those seven cats were observed till the end of the experiment. None of them showed signs of clinical infection during 4 months of observation, and they were killed 117-142 days post infection. All were clinically healthy on ante mortem examination and no gross abnormality was noticed in any of them at post mortem examination. The results of cultural examination of specimens and organs from these cats are summarised in table 19.

Cryptococcus neoformans was re-isolated in heavy growth, only from the nasal cavity of cat 11, 140 days after intranasal inoculation. It was not recovered from other specimens from this cat, nor was it recovered from any of the specimens and organs from the other 6 cats. No cryptococcal antibody or antigen was demonstrated in CSF or urine samples from any of these 7 cats.

Exposure Control Cats: 25, 26, 27, 28, 29 and 30

Four of these cats had to be killed during the course of the experiment due to calicivirus infection. Cats 26 and 27 were killed 34 and 36 days respectively after exposure, and cats 28 and 29 were killed 70 days post exposure. The other 2 cats (25 and 30) were observed till the end of



the experiment and killed at 143 days.

At autopsy, all cats were culturally negative for Cr. neoformans.

Cerebrospinal fluids and urine samples collected at autopsy and tested against CF and S antigens from each of the 3 infecting Cr. neoformans strains, were all negative for antibody and antigen by CIE and ID tests.

#### Results of the Serological Examination

The results of the serological follow-up of infected cats, by agglutination, ID and CIE tests, are presented in table 20. Serum cryptococcal antibody was detectable 2 weeks post infection in all cats, except cat 18. Precipitating antibody in positive sera was detected by CIE, each showing only one line of precipitation. Immunodiffusion tests failed to demonstrate precipitating antibody in any of the cats at any stage after infection. Agglutinating antibody was detected in 3 cats; 2-4 weeks post infection in 1 cat, 4-6 weeks in a second and 4-12 weeks in a third cat, at a titre of 1:2 in each case.

The results of serological examination by CIE are summarised in table 21. Cat 18 which became leukaemic after infection was negative for serum antibody 2 weeks post infection and at autopsy, but was positive for serum antigen at autopsy. Cat 17 was positive for serum antibody 2 weeks post infection and was positive for both serum antibody and antigen at autopsy. Cats 23 and 24 which became clinically ill, were positive for antibody 2 and 4 weeks post infection and became negative till they were sacrificed 34 and 56 days post infection respectively. Sera from both cats collected prior to autopsy were positive

for serum antigen but negative for antibody. Cats 12, 16 and 21 remained positive for antibody, till autopsy 65, 65 and 66 days after infection respectively. Cats 13 and 14 remained positive for antibody up to 84 days after infection, when they became negative. Cats 15, 20 and 22 remained positive up to 98 days post infection before becoming negative. Cat 19 was positive for up to 112 days post infection and became negative thereafter. Cat 11 remained positive for antibody till autopsied 140 days post infection.

Four of the 7 cats, killed later than 10 weeks post infection, were infected intranasally and the remaining 3 were infected intravenously. Two of the 4 intranasally infected cats (15 and 20) remained positive for precipitating antibody for up to 14 weeks post infection, cat 19 was positive for up to 16 weeks and cat 11 remained positive for up to 20 weeks post infection. Two of the 3 intravenously infected cats (13 and 14) were positive for up to 12 weeks and the 3rd cat (22) was positive for up to 14 weeks after infection (table 22).

Sera positive for precipitating antibody were all negative when tested by CIE against heterologous CF and S antigens from the other infecting strains of Cr. neoformans. They were also negative when titrated against homologous and heterologous antigens at 1:2 dilutions. These sera were also negative by CIE against CF and S antigens from serotypes A, B, C and D.

Cerebrospinal fluids and urine samples from all inoculated cats, including the ones that showed clinical signs of infection, were negative for both cryptococcal antibody and antigen by ID and CIE.

Serum cryptococcal antigens were detected in sera collected at

autopsy from the 3 cats with clinical infection (18, 23 and 24) and cat 17, killed at 17 days after infection, by CIE. In cat 17, cryptococcal antibody was also present in the same serum sample (table 18).

Precipitating antibody was demonstrated in 2 of the 6 exposed cats, by CIE, each giving one precipitation arc. Cat 25 reacted against CF antigen from Str. Huntington 4 weeks after exposure and remained positive against the same antigen for another 2 weeks, then became negative. Cat 30 reacted against CF antigen from Str. KCH 4 weeks post exposure and remained reactive against it for up to 8 weeks before becoming negative. There was no evidence of precipitating antibody in sera from the other 4 cats at any stage of the exposure period. Cerebrospinal fluids and urine samples from all these cats were negative for antibody and antigen.

## Intranasal challenge of cats with killed cells of *Cryptococcus neoformans*

Following the findings in the previous two experiments that the presence of viable *Cr. neoformans* cells in the nasal cavities of cats was accompanied by a demonstrable antibody response, it was decided to determine whether the presence of killed yeast cells would provide an antigenic stimulus in the cat.

### MATERIALS AND METHODS

Formalin-killed whole cell suspensions were prepared from *Cr. neoformans* strains KCH and Huntington and adjusted to final concentrations of  $1 \times 10^7$  cells/ml by haemocytometer counts.

Four adult female cats were used; 2 cats per strain of the yeast.

Cats were challenged by the intranasal route as previously, each receiving  $1 \times 10^7$  cells. They were bled 2, 3 and 4 weeks post challenge and sera were tested for the presence of antibody to *Cr. neoformans* by CIE test, using CF and S antigens from the challenging strains.

### RESULTS

Precipitating antibody to *Cr. neoformans* was detected in all cats 2, 3 and 4 weeks after the intranasal inoculation with non-viable yeast cells. One precipitation line was obtained in each case.

## DISCUSSION

The intravenous route of infection was found to be more effective than the intranasal route, for inducing clinical cryptococcal infection in the cat. While none of the intranasally infected cats showed symptoms of infection, although viable yeast cells remained in the nasal cavities of some for a long period after IN challenge, 3 of the intravenously infected animals (18, 23, 24) became clinically ill and were found to be invaded by the yeast at necropsy. In addition, serum cryptococcal antigen was present in all 3 cats, a further evidence of infection. The 4th cat (17) which was killed as a non-leukaemic control for cat 18 was also found to be invaded by the organism at autopsy, although it was not showing signs of illness when killed. However, the presence of both serum cryptococcal antigen and antibody in this animal at necropsy, was regarded as evidence of seroconversion towards the appearance of antigen only and disappearance of antibody. That is to say, this cat might have developed clinical infection had it been allowed to live longer.

Cryptococcal endophthalmitis developed in cat 23 in this study and the main symptoms were bilateral hypopyon, pupillary dilation, retinal detachment, and nervous signs manifested by circling, inco-ordination, hyperaesthesia and blindness. The main loci of infection in both eyes were involving the choroid and retina, with the organism abundantly present in the sub-retinal space. The extensive inflammatory cell infiltration and tissue necrosis observed in both eyes, particularly in the choroid and sub-retinal space, is contrary to the general concept that cryptococcal infection is rarely, if ever, accompanied by an inflammatory response.

From this case, and those described by Fischer (1971), Ivoghli et al., (1974) and Gwin et al., (1977), it appears that cryptococcal endophthalmitis in the cat is often accompanied by a considerable inflammatory response. The infection in the above mentioned cat developed as a primary condition in the absence of predisposing factors.

Cryptococcal endophthalmitis had been reported in the cat (Holzworth, 1952; Holzworth and Coffin, 1953); dog (Rubin and Craig, 1965; Kurtz and Finco, 1970; Gelatt et al., 1973; Lipton, 1973; Carlton et al., 1976); horse (Scott, Duncan and McCormack, 1974) and the mink (Trautwein and Nielsen, 1962). Cryptococcal infection involving the eyes of the cat may occur as a localized condition (Fischer, 1971; Gwin et al., 1977) or following disseminated infection (Holzworth, 1952; Holzworth and Coffin, 1953, Ivoghli et al., 1974). The general symptoms include epiphora, pupillary dilation, corneal opacity, retinal detachment and blindness. Histological findings are usually associated with the choroid and retina, with subsequent choroiditis and retinal disorganisation.

In this study, it was established that feline cryptococcosis might occur concurrently with other diseases in the cat, namely feline leukaemia and calicivirus infection. Cats 18 and 24 subsequently became infected with feline leukaemia virus and calicivirus, respectively, after intravenous inoculation with Cr. neoformans str. KCH. Evidence of invasion by Cr. neoformans was found in both cats at autopsy.

The effect of concurrent feline leukaemia virus infection on the degree of invasion by Cr. neoformans in cat 18 became apparent when the tissue population of the organism in this cat was compared with that in cat 17,

which was infected in the same way as cat 18 and for the same period of time, but was negative for feline leukaemia virus infection. Cat 18 was found to be fungaemic and heavily invaded by the fungus, whereas cat 17 was not fungaemic and the organism was recovered from fewer organs and in smaller populations in contrast to cat 18 (table 17). Furthermore, the fungus was demonstrated microscopically in the kidneys and brain of cat 18, but in none of the organs from cat 17.

Considering the fact that the organism was re-isolated from the lungs, kidneys, spleen and nasal turbinates of cat 17 (table 17), in the absence of clinical illness, and that cats 18 and 24 became acutely ill in a relatively short time, with higher fungal tissue populations, it may be said that the concurrent presence of feline leukaemia and calicivirus infections in these two cats may have activated the fungus in them by lowering their resistance.

Cryptococcosis is often encountered as a secondary complication to conditions other than viral infections, particularly in circumstances when the physiological status or immunological defences of the host are stressed or rendered ineffective. The predisposing factors to cryptococcosis in man are many and variable. The most commonly recognised of these factors include Hodgkin's disease, sarcoidosis, leukaemia, aplastic anaemia, diabetes mellitus, tuberculosis and other debilitating conditions, immunodeficiency, immunosuppressive therapy, prolonged treatment with broad-spectrum antibiotics, organ transplant and open-heart surgery (Zimmerman, 1955; Utz, 1962; Gruhn and Sanson, 1963; Gentles and La Touche, 1969; Emmons et al., 1970; Conant et al., 1971; Lewis and Rabinovich, 1972; Ahearn, 1974; Rippon, 1974;

Gordon, 1975). Whether these conditions are primarily responsible for lowered resistance in the host or whether this results from the effects of certain drugs in use for their treatment, is equivocal. However, the consensus is that treatment for many of these conditions involves corticosteroids and it is well established that these compounds significantly decrease resistance to infections by other fungi (Gentles and La Touche, 1969). Long-term antibiotic treatment may suppress the competitive endogenous bacterial population of the host, thus paving the way for adventitious pathogens such as Cr. neoformans to proliferate and when circumstances allow, overcome the host defences and cause overt infection (Ahearn, 1974; Gordon, 1975). Gentles and La Touche (1969) said of the opportunistic nature of cryptococcosis: "the diagnosis of pulmonary cryptococcal infection in persons without symptoms of pulmonary disease suggests that primary pulmonary infections are not uncommon, and that it is the disseminated form for which a lowering of resistance in the host is perhaps a prerequisite".

It appears that at least some of the predisposing factors in man also predispose to feline cryptococcosis. In cats, such conditions include lymphosarcoma and feline leukaemia virus infection (Barrett and Scott, 1975), immunodeficiency (Gwin et al., 1977), immunosuppressive therapy (Horne, 1964; Palumbo and Perri, 1975), prolonged antibiotic treatment (Horne, 1964), hypervitaminosis A (Clark and Roubin, 1970) and debility and corticosteroid therapy (Barrett and Scott, 1975).

It has been reported that infection with feline leukaemia virus may cause immunosuppression in the cat (Perryman, Hoover and Yohn, 1972; Essex, Cotter and Carpenter, 1973; Jarrett, 1979), and that hypervitaminosis A may delay the activation of the reticulo-endothelial cells



(Clark and Roubin, 1970). That this might be significant was shown by Karauoi, Hall and Larsh (1977) and Graybill and Taylor (1978), who suggested that the cell-mediated immune response was of importance in protection against murine cryptococcosis.

The intranasal route was chosen to simulate the natural route of infection as the respiratory tract is regarded as the main portal of entry of Cr. neoformans into the body, through inhalation from the environment. It has for long been suggested that the majority of cases of cryptococcosis started as pulmonary lesions, with subsequent haematogenous or lymphatic dissemination, particularly to the central nervous system (Freeman, 1931; Cox and Tolhurst, 1946; Walter, 1957; Ajello, 1967, 1970; Kaplan, 1973; Gordon, 1975 and Kelley and Mosier, 1977).

Although no clinical cryptococcosis developed in cats in the present study following inoculation via the IN route, another researcher has reported success with it. Turnquest (1968) infected cats orally, subcutaneously and intraperitoneally without producing clinical infection. However, some of his animals developed the disease after intranasal and intratracheal inoculation, thus providing evidence that the respiratory tract is probably the main route of infection by the yeast in cats.

Cryptococcus neoformans was recovered from the post-infection nasal swabs from all of the 6 intranasally infected cats 48 hours post inoculation, and from one cat 14 days post infection. In contrast, none of the intravenously infected cats yielded the fungus at any of these sampling times.

An interesting finding was that Cr. neoformans was recovered from the nasal turbinates of cats 17 and 18 only 3 days after the last nasal swabbing, which did not yield the organism (tables 17, 19). This observation may suggest that the fungus might have been present in the nasal cavities of at least some of the intravenously infected cats, but could not be obtained by the nasal swabbing technique. However, this should not preclude its use to collect material from a grossly visible and discharging nasal growth. Cr. neoformans was reported to have been isolated from the cat, from nasal swabs taken from such lesions (Barrett and Scott, 1975; Weir et al., 1979).

At necropsy, 3 of the intranasally challenged cats were found to harbour the fungus in their nasal cavities, a long time after they were found negative by the nasal swabbing technique. Cat 11 did not yield the organism in the nasal swab beyond 48 hours post infection. However, when autopsied, 140 days after inoculation, the fungus was re-isolated in heavy growth from its nasal turbinates. Cat 12, which was negative by nasal swab sampling 48 hours following infection, yielded the organism in profuse growth from its nasal turbinates when necropsied 65 days post inoculation. Cat 16, that was negative after the 14th day of infection from the nasal swab, was positive for Cr. neoformans from the nasal turbinates at autopsy 65 days after infection, (tables 16, 19).

There were differences among the cats in the cultural isolations of the fungus at autopsy. While some cats seemed to have eliminated it, others

harboured it for considerably long periods. As may be seen from table 12, the fungus was not recovered from the nasal cavities of cats 1, 2, 3 and 4, 122-124 days post challenge, whereas it was re-isolated from cats 5, 8, 9 and 10 between 48 and 128 days after inoculation. It is evident that the fungus did not only remain viable in the nasal turbinates of the positive cats, but was probably proliferating, at least in cats 5 and 10 from which it was recovered in profuse and heavy growth, respectively, 125-128 days after inoculation.

The recovery of the yeast from the trachea and lungs of cat 7 and lungs of cat 6, 3 and 33 days post infection respectively, indicates that it can reach the lower respiratory tract and lungs at least 3 days after inhalation and remain viable in the lungs for at least a month. Whether the same situation applies following natural exposure of the cat to the fungus in the environment, and inhalation of dry yeast cells, remains to be seen. However, it is reasonable to assume it does. Unfortunately, for safety reasons, it was not possible to substantiate this hypothesis experimentally by challenging cats with viable dry yeast cells.

Only cat 19 yielded Cr. neoformans from the bronchial washing 8 weeks after intranasal inoculation. None of the other 5 cats that were sampled by this method was positive for the fungus, including cats 11, 12 and 16 that yielded it from their nasal turbinates at autopsy.

These findings in effect indicate the inferiority of the ante mortem sampling techniques, viz. nasal swabbing and collection of bronchial washing, to the post mortem method, i.e. cultivation of the nasal turbinates. This is unfortunate in that it would have been beneficial to employ the former two methods to collect specimens for culture in cases

of suspected respiratory infection by Cr. neoformans in the cat.

Another disadvantage of collecting the bronchial washing is that it is laborious, necessitates anaesthetization to restrain the animal and there is the risk of precipitating pneumonia.

The failure to recover the organism from the rectal swabs from any of the cats at any stage of infection, including those cats that were found to be fungaemic at autopsy, perhaps minimises the usefulness of this technique for isolating Cr. neoformans from a diagnostic point of view.

It appears that Cr. neoformans is cleared by cats more rapidly following infection via the IV rather than IN route. As may be seen from table 19, none of the 4 cats, intravenously challenged that did not develop infection, was found to harbour the organism in any tissue at autopsy 66-141 days post infection. In contrast, 3 of the 6 cats infected intranasally were harbouring the organism in the nasal cavity for up to 65-140 days post infection. This finding, that some cats can eliminate the fungus after IN challenge while others harbour it in their nasal cavities for more than 4 months without showing clinical infection, is of epidemiological significance.

There are no authenticated cases of man to man, animal to man or animal to animal transmission of cryptococcosis and the disease is generally considered to be non-contagious. My experimental work with cats suggests that this is incorrect. Viable Cr. neoformans was demonstrated in ocular discharges and in urine from infected cats and as antibody developed in control cats housed with infected cats, the yeast must have entered their bodies. Infected animals may therefore constitute a public health hazard, especially to their owners, and the need

for very careful handling of infected animals, as suggested by Barrett and Scott (1975) and Kelley and Mosier (1977) is emphasised.

The serological study of the cats in this experiment revealed that they could mount a detectable antibody response at most 2 weeks following inoculation with viable or killed cells of Cr. neoformans. It was also interesting that the route of inoculation does affect the degree of antibody response exhibited by the cat against introduction of Cr. neoformans. As is seen from table 22, the maximal period of positivity for cryptococcal antibody was 14 weeks after IV challenge, whereas antibody was detectable in the intranasally challenged cats for up to 14-20 weeks post challenge, even in the absence of the organism in these cats at autopsy. These variations in duration of response may be attributed to differences in the rate of clearance of the organism following infection by either route. Cat 11, perhaps provides additional evidence that this might have been the case. This cat remained persistently positive for cryptococcal antibody throughout the observation period, and was found to harbour the fungus in its nasal cavity at autopsy 140 days after intranasal inoculation.

The persistence of precipitating antibody in the absence of the fungus at autopsy, from cats 1, 2, 3 and 4 confirms that circulating antibody remains detectable in cats for up to 124 days following a previous stimulus.

The presence of circulating antibody to Cr. neoformans for a long time following stimulation has been observed in humans (Kimball et al., 1967; Kaufman and Blumer, 1968; Walter and Jones, 1968).

It was surprising that sera from cats infected with unserotyped strains of Cr. neoformans and known to be positive for cryptococcal antibody against homologous antigens, were all negative for antibody when tested against antigens from serotypes A, B, C and D. Various relevant factors were considered to explain this discrepancy. That it might have been due to the CIE test was disproved by crossing the serotype antigens against rabbit anti-Cr. neoformans antiserum and the test was proved to be working properly. The possibility that antibody in the cat sera might have deteriorated was also considered but re-examination of the sera against homologous antigens disproved this possibility. Positive reactions were again obtained against the homologous antigens.

It was thought that the amounts of the antibody in these non-reacting sera might have been insufficient to react with heterologous antigens. Theoretically the infecting strains should have belonged to one or more of the known serotypes of Cr. neoformans. If this was the case, it was reasonable to expect that at least some of these sera at some stage after infection contained antibodies with titres high enough to react within the currently known antigenic spectrum of the yeast.

Whether the antigenic spectrum of Cr. neoformans is not yet fully elucidated and hence the possibility of there being another serotype or whether some kind of antigenic changes take place in the known serotypes after entry into the cat, remain to be seen. This latter speculation seems unlikely as, in the survey, positive reactions to the recognised serotypes were found, and cats inoculated experimentally with serotypes produced antibody to them.

It is generally believed that cryptococcal antigen appears when circulating antibody is neutralized as a result of proliferation of the fungus in the host's tissues. The presence of antigen in serum or CSF is regarded as indicative of active cryptococcal infection in man (Gordon and Vedder, 1966; Kaufman and Blumer, 1968; Goodman, Kaufman and Koenig, 1971; Kaufman, 1976).

Antigen was present in the sera from 4 cats, in 3 of which (18, 23, 24) cryptococcosis was confirmed. The 4th cat (17), which was killed as a control, had, at the time of autopsy 17 days after inoculation, both antigen and antibody. Three days previously it had been positive for antibody only which suggests that active infection was developing. Conversion from antibody to antigen was observed in cats 23 and 24. Both were positive for antibody up to 28 days after infection. Six days later cat 24, and 28 days later cat 23, had developed antigen.

From a diagnostic point of view, the serological results from these cats are of importance. The presence of serum cryptococcal antigen indicated active cryptococcosis and, for cats 18, 23 and 24 this was confirmed post mortem. This emphasises the importance of testing for antigen as well as antibody in the serodiagnosis of cryptococcosis in animals. Weir et al., (1979) detected circulating cryptococcal antigen by the latex-particle agglutination test, in a cat with nasal cryptococcal granuloma, and reported that by assaying the serum antigen titres, they were able to monitor the cat's response to amphotericin B therapy and progress after therapy. They stated that the cat had a titre of 1:16 prior to treatment and that it decreased to 1:1 by 6 months after the start of therapy.

Precipitating antibody to Cr. neoformans was detected by CIE in all the experimental cats, except that which had leukaemia and was probably immunosuppressed. When the results of serological and cultural examinations of the experimental cats are compared, it is apparent that the presence of antibody does not necessarily mean that the cat has active cryptococcosis or even that the yeast is present within the body. The presence of cryptococcal antibody is, however, definite proof that the animal has been exposed to the yeast and could mean that cryptococcosis was developing. In the absence of clinical signs of infection, one cannot, therefore, diagnose cryptococcosis solely by the demonstration of precipitating antibody.

A high degree of innate resistance to cryptococcal infection is recognised in man (Gordon, 1975) and probably also in animals (Longbottom and Pepys, 1975). Factors regarded as important in influencing the development of infective disease are variation in virulence of the infecting strain, the amount and route of infective material entering the body and differences in the resistance of individuals to the infecting organism. A number of facts suggest that cats are resistant to infection by Cr. neoformans.

The number of cases of clinical feline cryptococcosis is low, although the yeast has been isolated from a variety of natural habitats in many countries. The results of the serological survey showed that cats in this area had been exposed to Cr. neoformans and had not developed the disease and, experimentally, overt disease was induced in cats only by IV inoculation.



A possible theory to explain, at least in part, this resistance to infection was suggested by the results of my studies. The normal route of infection is considered to be by inhalation. Cats infected intranasally developed antibody within 14 days. If this antibody was produced before cells of the yeast had become invasive, would it be protective? If so, the failure to induce infection, especially in those cats which harboured viable yeast cells, from strains known to be pathogenic, within the nasal cavity for long periods, might be explained.

Table 15. Details of cats inoculated intranasally or intravenously with  $1 \times 10^7$  cells of 3 strains of Cr. neoformans

Cat No.	Sex	Wt. (Kgs.) at start of experiment	Route of Infection	Infecting Strain	
11	F	2.7	Intranasal	CXH	
12	M	3.3			
13	M	4.9			
14	F	3.7	Intravenous		
15	F	2.5			
16	F	1.9	Intranasal		
17	M	2.8			
18	M	3.9	Intravenous	KCH	
23	F	3.2			
24	M	3.2			
19	F	2.8			
20	M	3.6	Intranasal		Hunt.
21	F	3.8			
22	F	3.5	Intravenous		

Table 16. Results of culture of Cr. neoformans from nasal swabs from cats infected intranasally.

Cat	Strains	Days after infection		
		2	7	14
11	CXH	+	-	-
12		++	-	-
15	KCH	+	+	-
16		+	+	+
19	Hunt.	++	+	-
20		+	+	-

- no growth  
 + 1-20 colonies  
 ++ 21-50 colonies

Table 17. Sites from which Cr. neoformans strain KCH was cultured at autopsy from cats infected intravenously

Cat	Concurrent Disease	Time of autopsy (days)	Blood	CSF	Brain	Spinal Cord	Nasal Turbinates	Trachea	Bronchi	Lung	Media-stinal L. Nodes	Kidney	Urine	Liver	Spleen	Mesen. L. Nodes
18	leukaemia	17	++	+	++	++	++	+	+	+++	-	+++	+++	+++	+++	-
17*	none	17	-	-	-	-	+	-	-	++	-	++	+	-	++	-
24	Calicivirus	34	-	-	+++	-	-	-	-	-	-	+++	-	+	++	ND
23	none	56	-	-	-	-	-	-	-	-	-	++	++	-	-	ND

\* clinically normal; killed as control for Cat 18

- no growth  
 + 1-20 colonies  
 ++ 21-50 colonies  
 +++ over 50 colonies  
 ND Not Done

Table 18 . Results of investigation by CIE for cryptococcal antigen and/or antibody in sera from cats with clinical infection and one killed as a control (17).

Cat	Time of Autopsy (days)	After challenge - days	
		Serum	
		Antibody	Antigen
17	17	17	17
18	17	-	17
23	56	28	56
24	34	28	34

Table 19. Isolation at autopsy of *Cr. neoformans* from intravenously or intranasally infected cats

Strain	Route of infection	Cat	Time of autopsy	Blood	CSF	Brain	Spinal Cord	Nasal Turbs.	Trachea	Lung	Mediastinal L. nodes	Kidney	Urine	Liver	Spleen	Heart	Faeces	
KCH	IV	17	17	-	-	-	-	+	-	++	-	++	+	-	++	-	-	
		18	17	++	+	++	-	++	+	+++	-	+++	+++	+++	-	-	-	
		24	34	-	-	+++	-	-	-	-	-	-	+++	-	+	++	-	-
		23	56	-	-	-	-	-	-	-	-	-	++	++	-	-	-	-
CXH	IV	15	117	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		16	65	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-
		13	120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hunt.	IN	14	141	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		11	140	-	-	-	-	++	-	-	-	-	-	-	-	-	-	-
		12	65	-	-	-	-	+++	-	-	-	-	-	-	-	-	-	-
		21	66	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hunt.	IN	22	120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		19	142	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		20	142	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

- no growth  
 + 1-20 colonies  
 ++ 21-50 colonies  
 +++ over 50 colonies

Table 20. Results of serological follow-up by agglutination and CIE tests of cats infected intranasally or intravenously with Cr. neoformans strains\*

Cat	Route of infection	Antigen	Post infection bleedings (days)												At autopsy	Time of Autopsy (days)		
			14	28	42	56	70	84	98	112	Agg. CIE	Agg. CIE	Agg. CIE	Agg. CIE				
11	IN	CXH	-	+	-	+	-	+	-	+	-	+	-	+	-	+	140	
12			-	+	-	+	-								-	+	65	
13	IV		-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	120
14			-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	141
19	IN	Hunt.	-	+	-	+	-	+	-	+	-	+	-	+	-	+	142	
20			-	+	-	+	-	+	-	+	-	+	-	+	-	-	142	
21	IV		-	+	-	+	-	+	-	+	-	+	-	+	-	+	66	
22			-	+	-	+	-	+	-	+	-	+	-	+	-	-	120	
15	IN	KCH	-	+	-	+	-	+	-	+	-	+	-	+	-	-	117	
16			-	+	-	+	-	+	-	+	-	+	-	+	-	+	65	
17	IV		-	+	-	+	-	+	-	+	-	+	-	+	-	+	17	
18			-	+	-	+	-	+	-	+	-	+	-	+	-	-	17	
23			+	+	-	-											56	
24			-	+	-	+											34	

\* No serum was positive by ID

\*\* All agglutination tests were positive at 1:2 dilutions.

Table 21. Results of examination by CIE for antibody and antigen of sera from cats infected intranasally or intravenously.

Infection		CAT	Days after challenge		Killed at: (days)
Strain	Route		Serum antibody	Serum antigen	
CXH	IN	11	14-140		140
		12	14-65		65
	IV	13	14-84		120
		14	14-84		141
KCH	IN	15	14-98		117
		16	14-65		65
	IV	17	14-17	17	17
		18	- -	17	17
		23	14-28	56	56
		24	14-28	34	34
Hunt.	IN	19	14-112		142
		20	14-98		142
	IV	21	14-66		66
		22	14-98		120



Table 22. Comparison of the persistence of antibody response in surviving, intranasally and intravenously infected cats, by counterimmunoelectrophoresis

Cats	Route of Infection	Last +ve Antibody Response (days)	Mean time (days)	Time of Autopsy (days)
15	Intranasal (I.N.)	98	112	117
20		98		142
19		112		142
11		140		140
13	Intravenous (I.V.)	84	88	120
14		84		141
22		98		120

Figs. 7-10: Comparison of macroscopic appearance of organs from a cat with cryptococcosis (18) and those from a clinically normal cat (17).

Fig. 7:

Liver, cat 18, showing necrotic foci.

Fig. 8:

Fleshy pale spleen of cat 18.

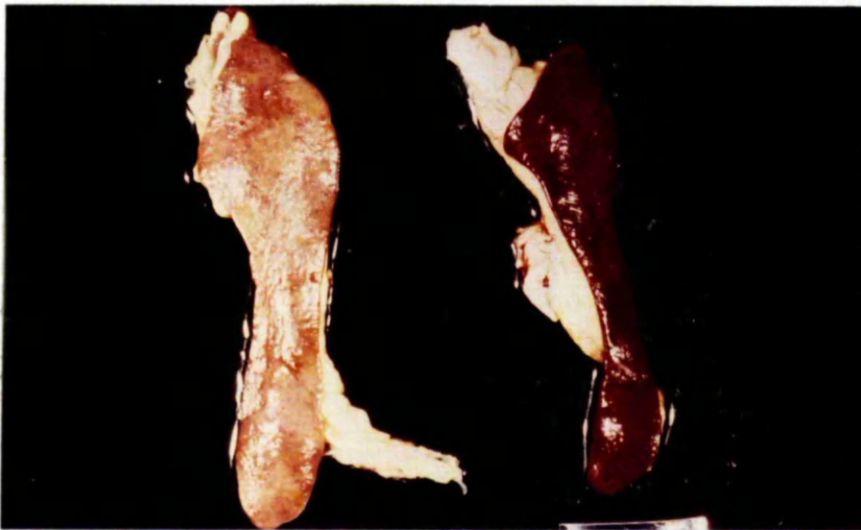
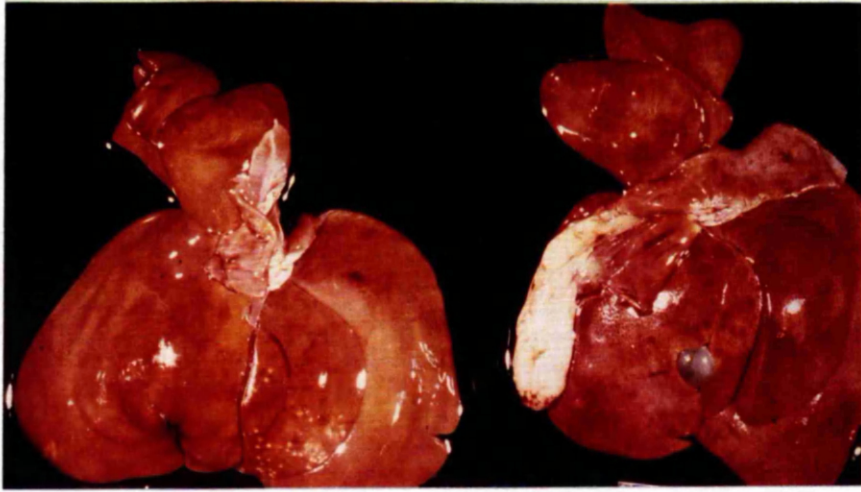
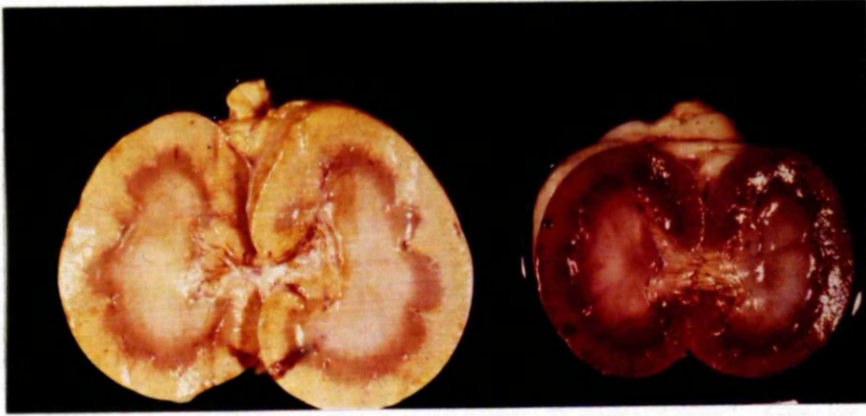


Fig. 9:

Yellowish-grey uraemic kidney of cat 18.

Fig. 10:

Overinflated lungs of cat 18.



Figs. 11-15: Sections of kidney infected with cryptococcosis (cat 18)

Fig. 11:

Encapsulated cells of Cr. neoformans in the glomeruli.

(Mayer's Mucicarmine, x 60)

Fig. 12:

Magnification of Fig. 11, showing infected glomeruli.

(x 240).

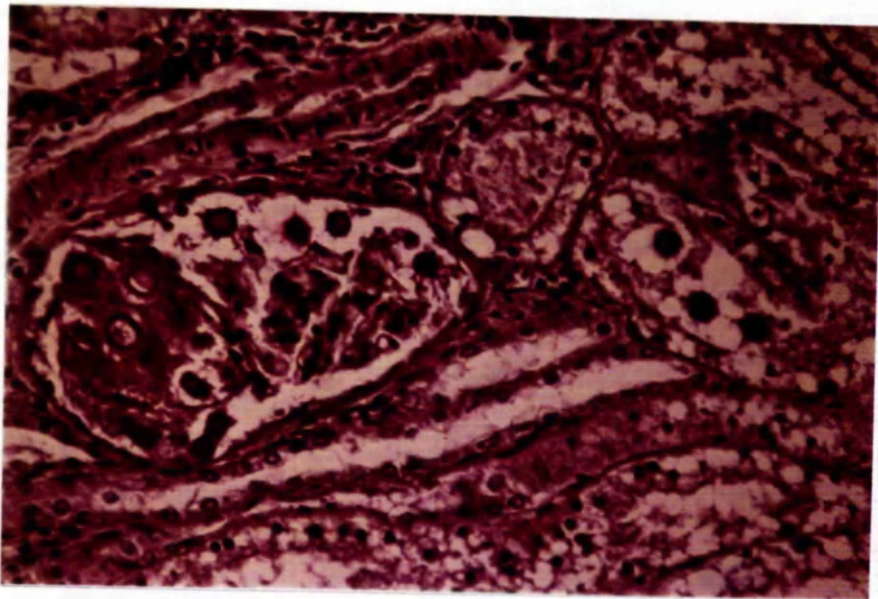
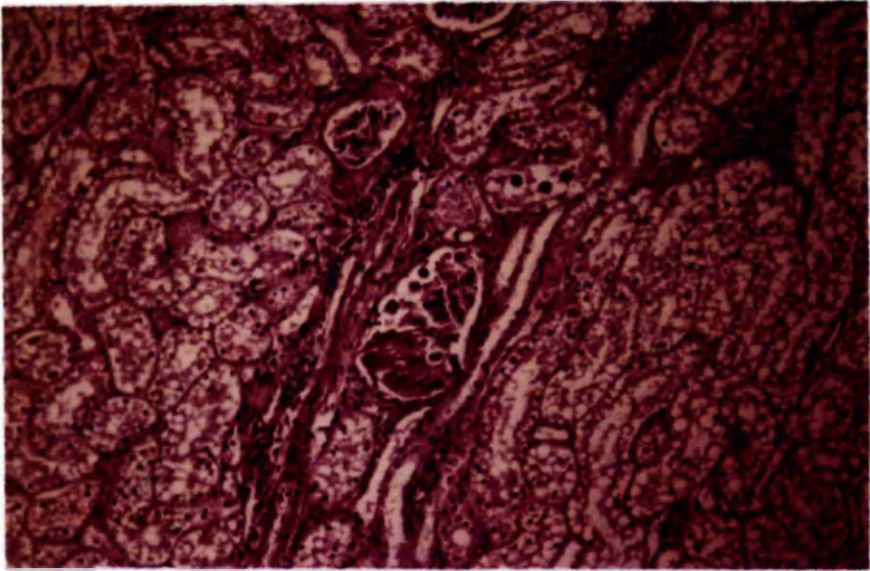


Fig. 13:

Encapsulated and budding cells of Cr. neoformans in Bowman's space.

(P.A.S., x 240)



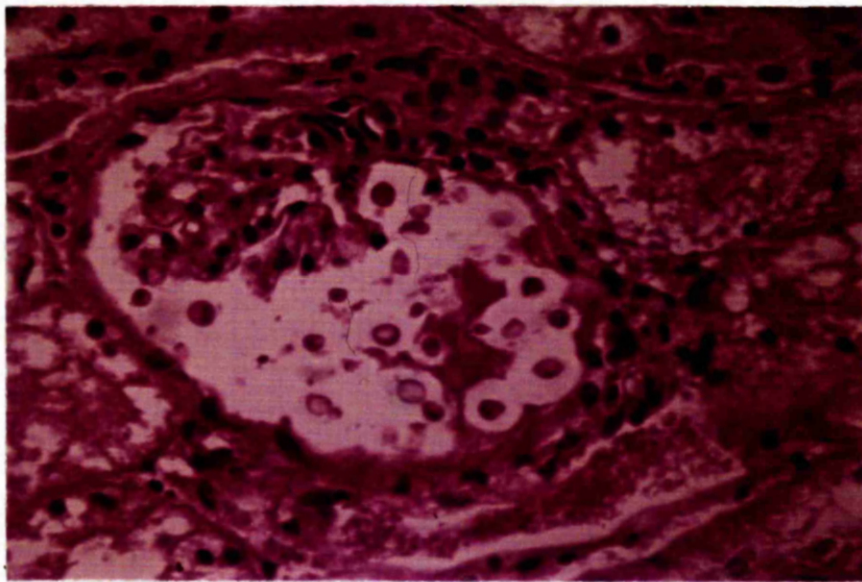


Fig. 14:

Numerous cells of Cr. neoformans in and around the renal tubules.

(Gomori-Grocott, x 50)

Fig. 15:

High power view of Fig. 14.

(x 150)

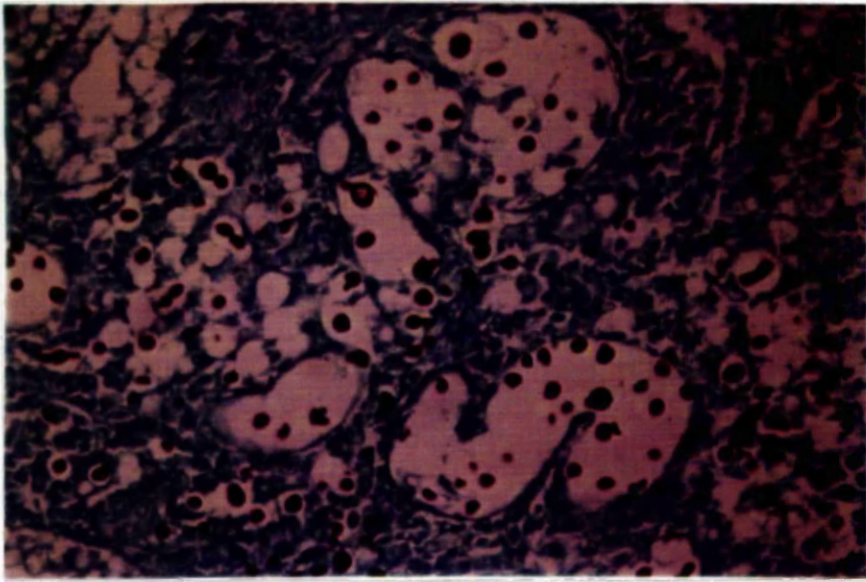
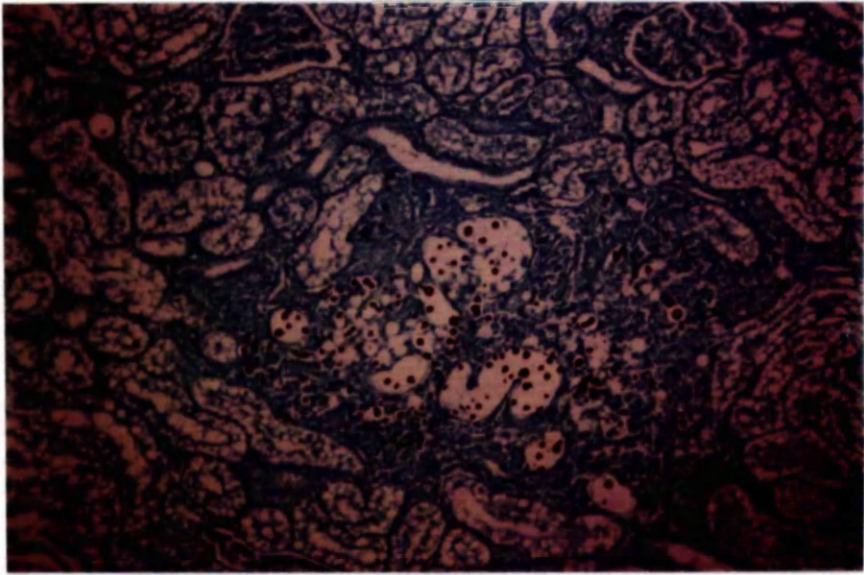
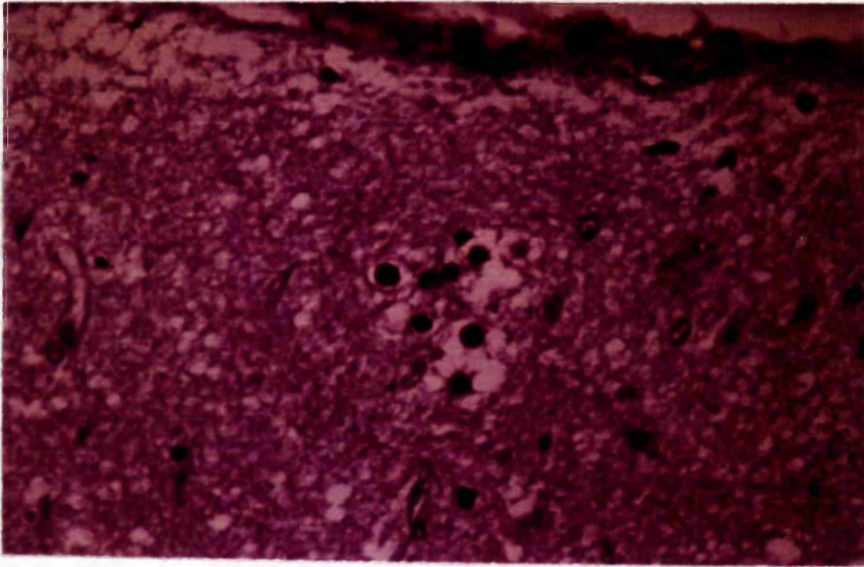


Fig. 16:

Encapsulated cells of Cr. neoformans in the cerebrum of cat 18.

(Mayer's mucicarmine, x 150)



Figs. 17-19: Unstained slides showing results of CIE tests for Cr. neoformans antigen in serum and CSF from 3 cats.

Wells on the left contained rabbit antisera to Cr. neoformans and those on the right, in descending order, serum, serum x 2 and CSF.

(x 4)

Fig. 17: Cat 18.

Antigen present in serum but not in CSF. Note that the lines are stronger than in Fig. 18.

Fig. 18: Cat 17:

Antigen present in serum but not in CSF.

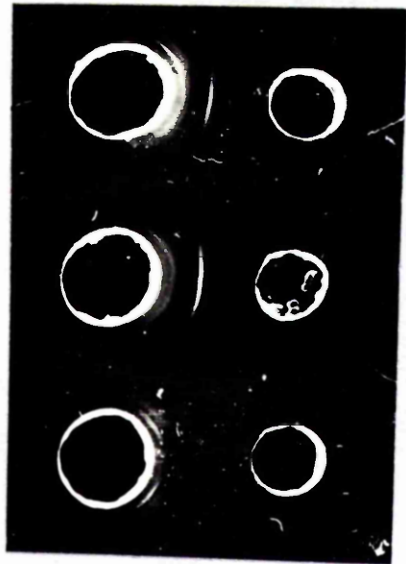
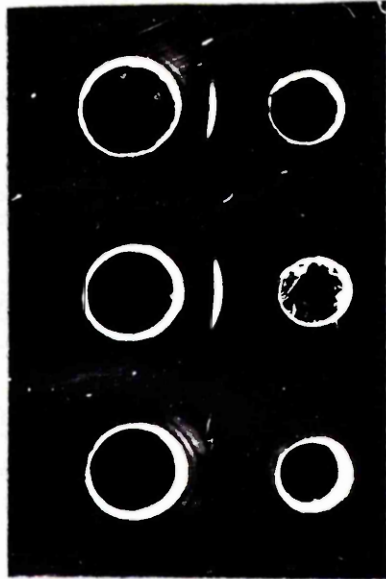
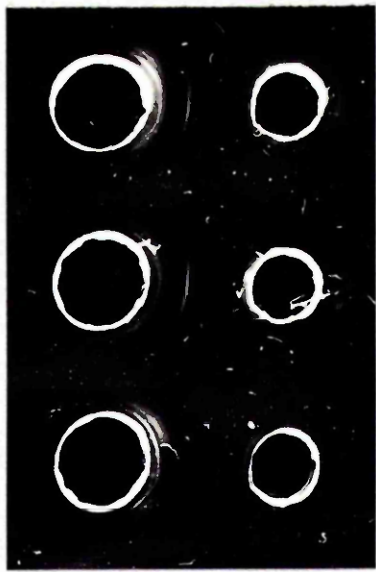


Fig. 19: Cat 24.

Antigen present in serum but not in CSF.





Figs. 20-25: Clinical signs of ocular cryptococcosis in cat 23.

Fig. 20:

Pupillary dilatation and hypopyon in both eyes, 7 weeks post infection.

Fig. 21:

Enucleated eyes showing purulent masses in the anterior chambers, 8 weeks post infection.



Fig. 22:

Sagittal section: right eye with detached, funnel-shaped retina.

Fig. 23:

Sagittal section: left eye showing detached funnel-shaped retina.

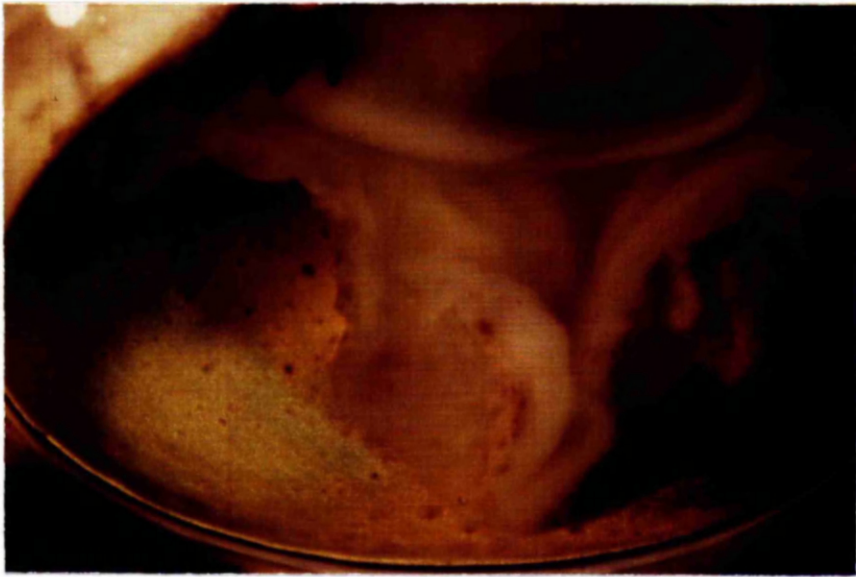
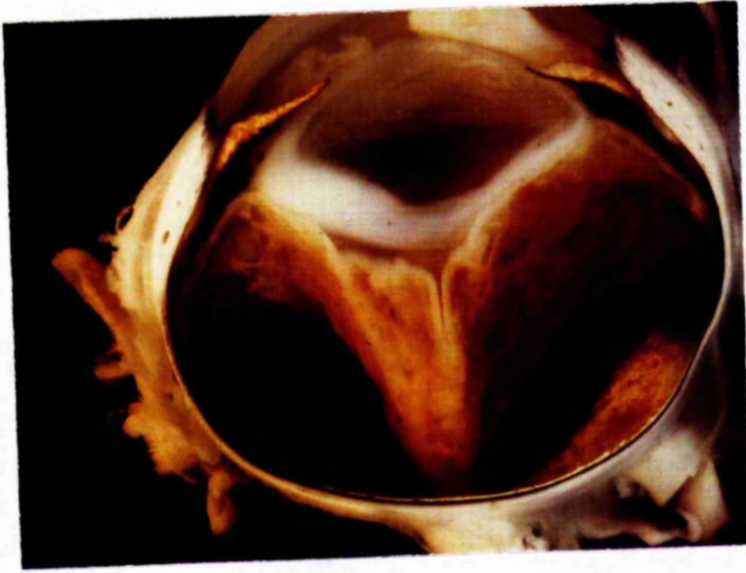
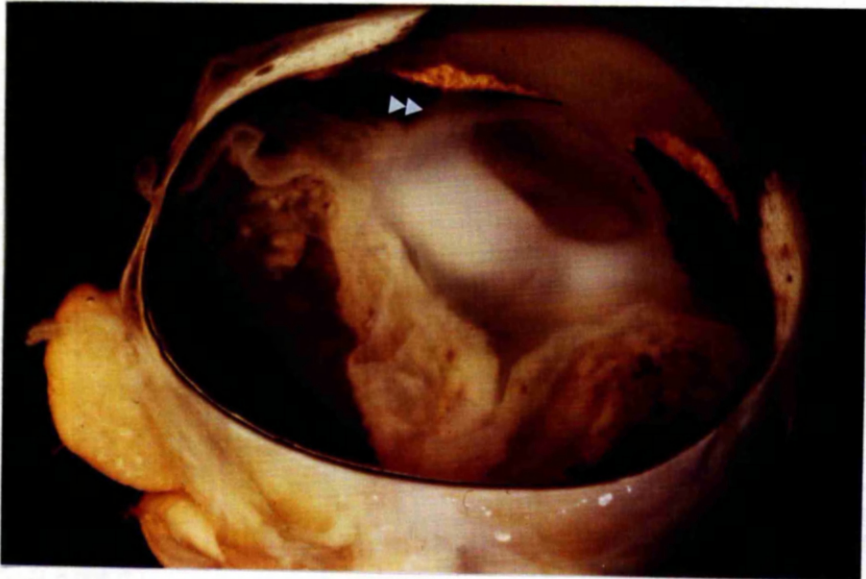


Fig. 24:

Sagittal section: right eye showing small pigmented nodules on the inner surface of Bruch's membrane.

Fig. 25:

Sagittal section: left eye showing a solid gelatinous exudate in the anterior chamber (arrow).



Figs. 26-33: Histopathology of eye lesions in cat 23:

Fig. 26:

Section through the right eye, showing fibrino-purulent exudate  
(star) in the anterior chamber.

(H & E, x 12)



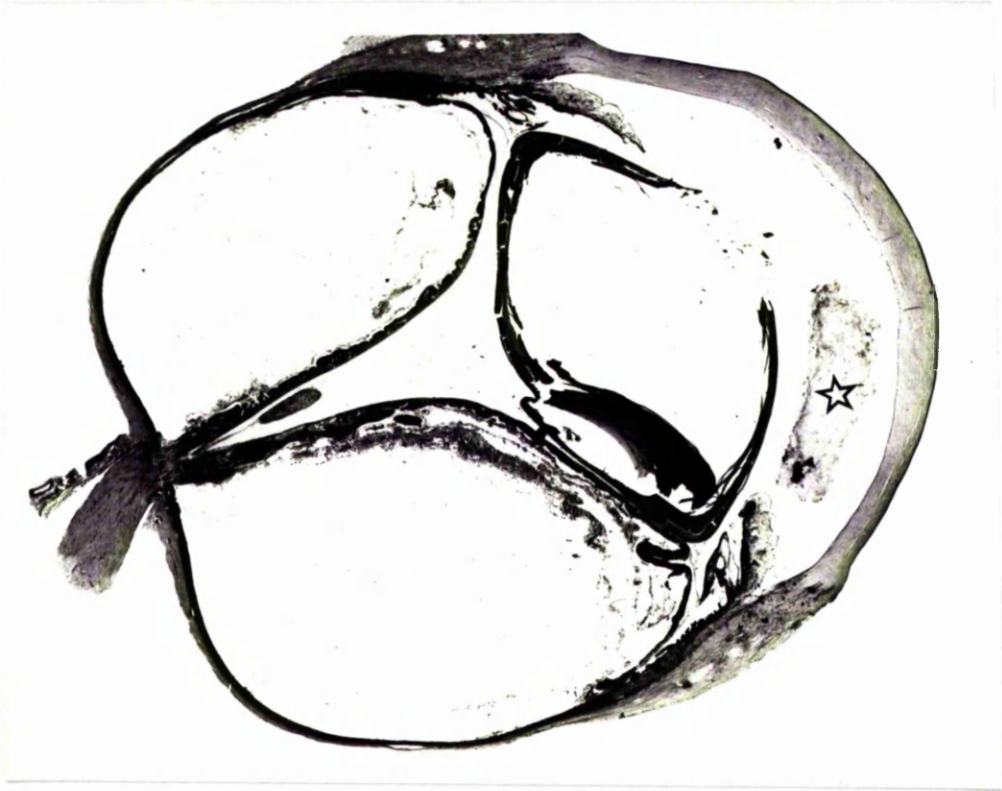


Fig. 27:

Extensive tissue necrosis in the choroid of the right eye.

(H & E, x30)

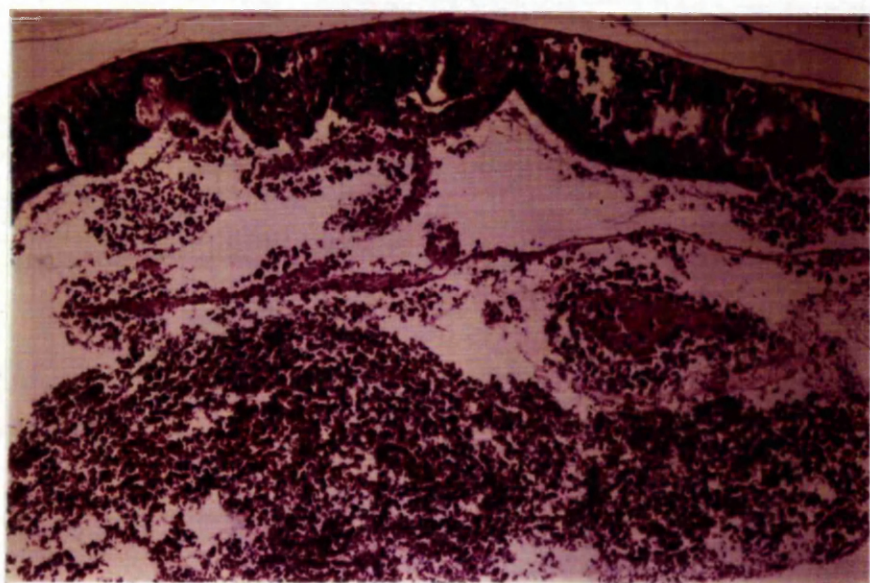


Fig. 28:

Extensive inflammatory reaction in the detached and dis-organized retina of the right eye.

(H & E, x100)

Fig. 29:

Enlargement of Fig. 28, showing infiltration of the retinal tissue by lymphocytes and macrophages.

(H & E, x 1000)

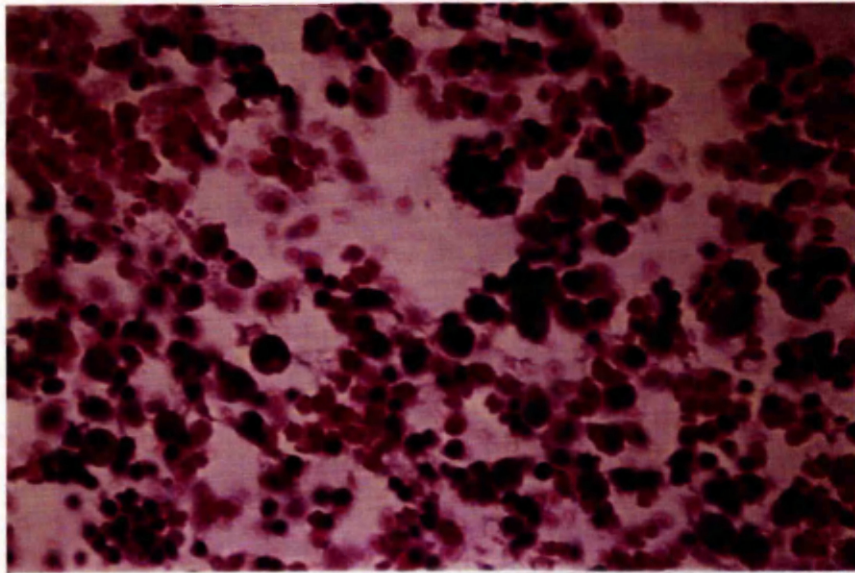
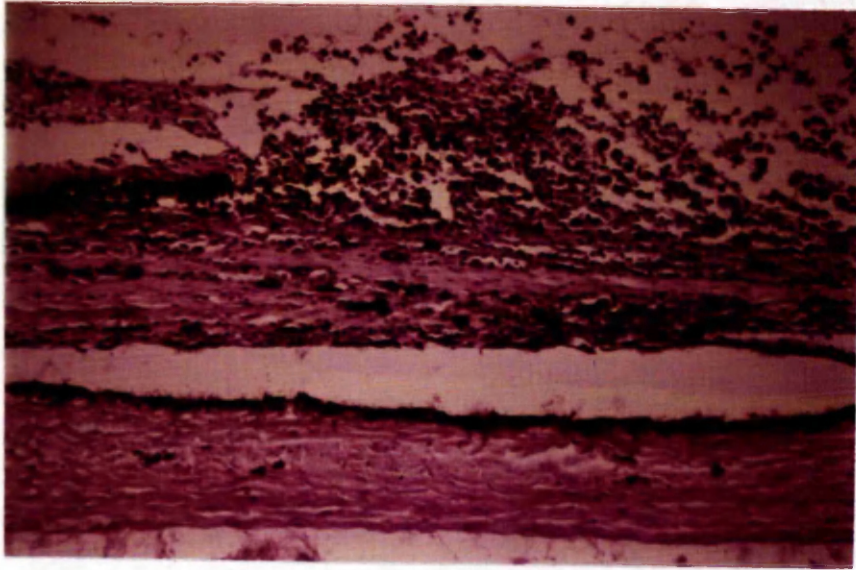


Fig. 30:

Encapsulated and budding cells of Cr. neoformans in the sub-retinal space of the right eye.

(Mayer's mucicarmine, x 250)

Fig. 31:

High power view of Fig. 30.

(x 400)



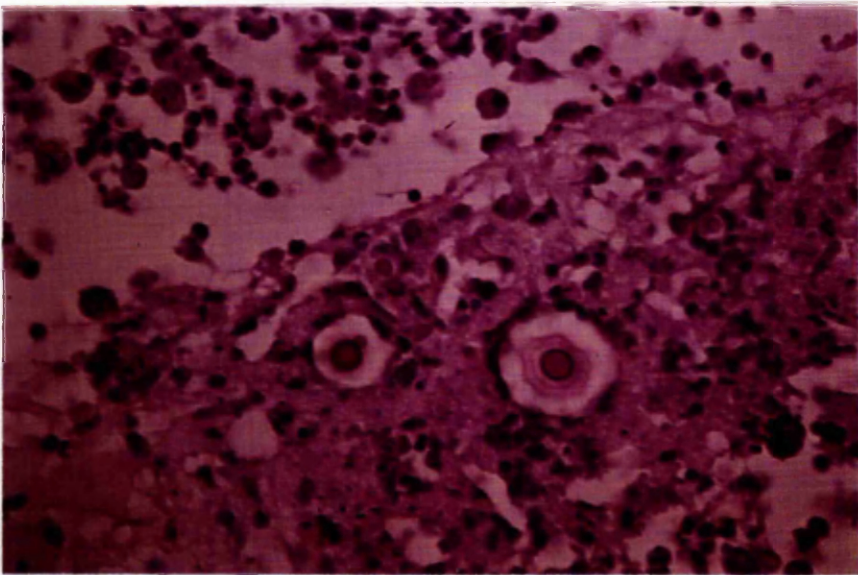
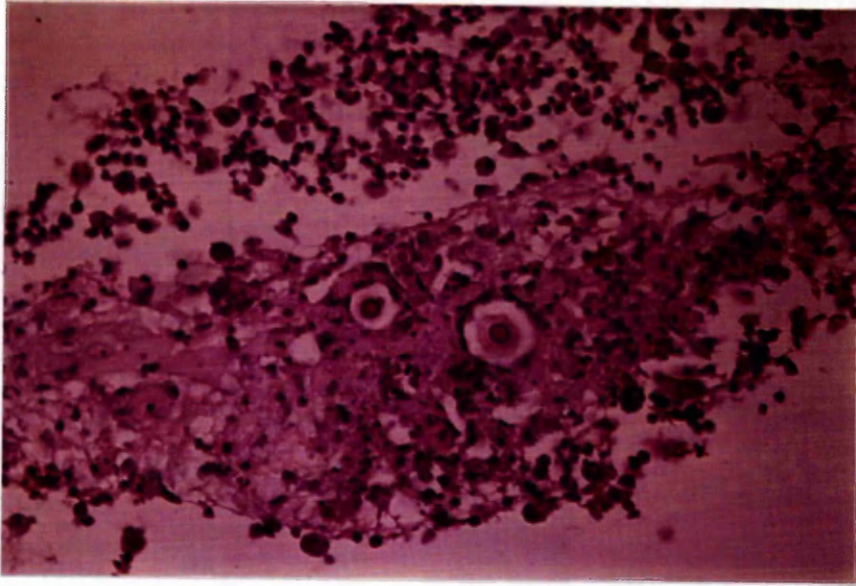


Fig. 32:

Yeast cells in the subretinal space of the right eye.

(Gomori-Grocott, x 100)

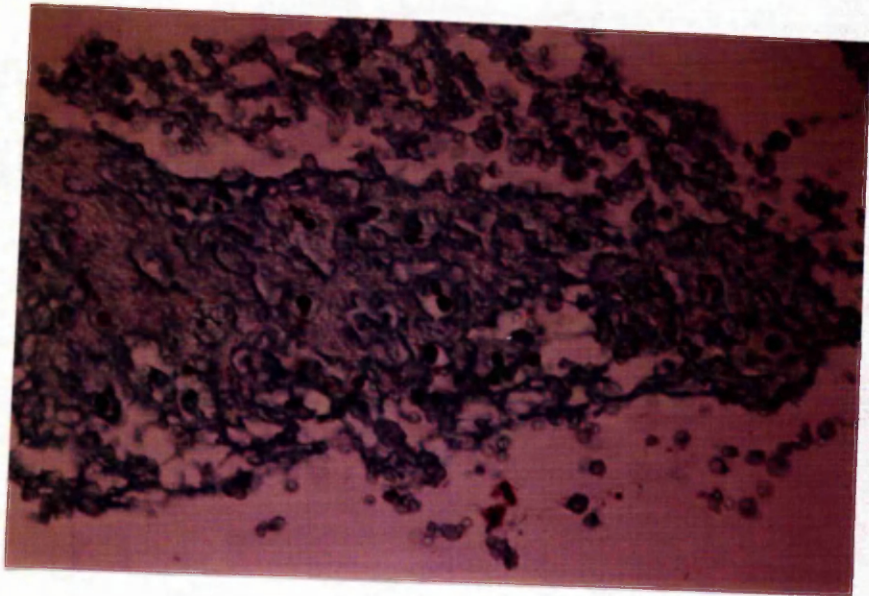
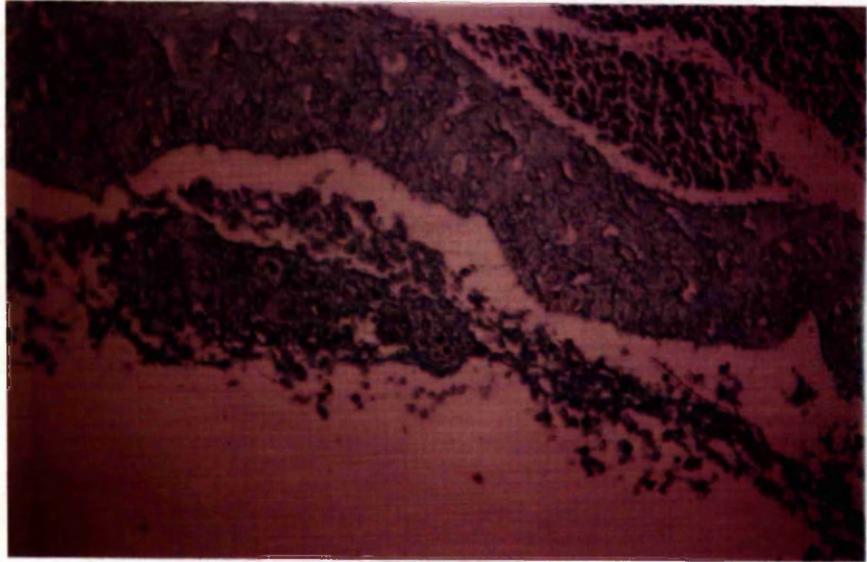
Fig. 33:

Higher magnification of Fig. 32, showing budding cells of

Cr. neoformans.

(x 250)





SURVEY ON YEASTS FROM ANIMAL SOURCES

The available records on isolations of yeasts from domestic animals, sporadic as they are, have been mainly case reports of these organisms as disease agents in animal hosts. A few early investigations had been undertaken on the presence of yeasts as normal inhabitants in the digestive tracts of some animal species. However, there is a lack of systematic studies dealing with the prevalence of yeasts on and in domestic animals, and the nature of such associations.

An investigation was undertaken to study the presence and nature of association of yeasts, on and in animals.

Sabouraud dextrose agar (SDA) supplemented with chloramphenicol was used as the primary and maintenance medium in this study.

The following materials from animal sources have comprised the sources for the investigation:

- (a) Normal and abnormal canine skins
- (b) Nasal turbinates of dogs and cats
- (c) Oral swabs from healthy guinea-pigs

## Investigation of yeasts on normal and abnormal canine skins

As far as is known, there has been no systematic investigation into the presence of yeasts on the canine skin. The published records on the isolation of yeasts from this source originated either from an investigation of skin for the presence of fungi other than yeasts, or merely as case reports from disease conditions. Gentles, Dawson and Connole (1965), during an investigation of dogs and cats for the presence of keratinophilic fungi on their coats, recovered C. albicans from apparently normal dog skins. Only 2 other reports are available, which were from cases of spontaneous canine cutaneous candidiasis, attributed to C. albicans. Kral and Uscavage (1960) reported the isolation of this yeast from a case of generalized skin infection in a dog, following prolonged antibiotic treatment. Kral and Schawrtzman (1964) reported 2 other similar cases, one of which developed after long-term antibiotic therapy.

It was thought worth investigating normal and diseased dog skins for the presence of yeasts on them.

### MATERIALS AND METHODS

The specimens investigated were obtained from dogs submitted for treatment to the Veterinary Hospital and the Cargill Dog and Cat Home Clinic, Glasgow. Those examined for this survey included cases of suspected ringworm, eczema, demodectic mange and alopecia. Dogs with apparently normal skins were also sampled. The animals sampled were of mixed sexes and ages.

Using the brush technique described by Mackenzie (1963), dogs with apparently normal skins were brushed on the head, neck, back, sides,

legs, and abdominal regions, using one brush per animal. Brushings were taken from the lesions of dogs with skin diseases.

On return to the laboratory, each brush was used to inoculate a plate of SDA supplemented with chloramphenicol. The bristles were pressed several times into the agar to give a multiple inoculation of the whole surface. Inoculated plates were then incubated at 28°C, with daily examination, for 7 days.

## RESULTS

A total of 102 dogs was examined, 77 with clinically normal skins and 25 with skin abnormalities. The latter group of animals consisted of 15 dogs with suspected ringworm infection, 8 with eczema, 1 with demodectic mange and 1 with endocrine alopecia.

The results are summarised in table 23. Sixty two (60.8%) of the 102 skin samples were positive for yeast growth and 40 (39.2%) were negative. Forty seven (61%) of the 77 dogs with normal skins yielded yeast growth and 30 (39%) were negative for such growth. Of the 25 dogs with skin conditions, yeasts were isolated from 15 (60%) whereas 10 (40%) were negative for yeasts.

Sixteen yeast species were isolated from healthy skins and 2 from diseased ones. The number of yeast isolates ranged from 1 to 3 species per specimen from normal skins, in contrast to abnormal skins where only one yeast species was recovered per specimen. Of the 47 normal dog skins that were positive for yeasts, 32 (68.1%) yielded 1 yeast species each, 14 (29.7%) yielded 2 yeast species, and one skin (2.2%) yielded 3 yeast species each. In contrast, all the 15 abnormal skins yielded only one yeast species each.

The quantity of yeast growth from skin brushings varied from a few colonies to profuse growth, with the majority of the samples yielding moderate growth. There was no particular pattern of association between any of the yeast species isolated from dog skins in the study.

Yeast isolations are listed in table 24. In all, 78 isolates were recovered from normal and abnormal skins. They belonged to 16 species in 8 genera (Candida, Cryptococcus, Lodderomyces, Rhodotorula, Pichia, Pityrosporum, Torulopsis and Trichosporon).

The species encountered in brushings from normal dog skins were, in descending order of frequency: Torulopsis candida (16; 25.4%), Rhodotorula rubra (9; 14.3%), Trichosporon cutaneum (8; 12.7%), Candida melibiosica (6; 9.5%), C. guilliermondii (5; 7.9%), Rhodotorula glutinis var. glutinis (4; 6.3%), Pichia membranaefaciens (4; 6.3%), Pityrosporum pachydermatis (3; 4.8%), and one (1.6%) isolate each of C. parapsilosis, C. zeylanoides, Cryptococcus uniguttulatus, Lodderomyces elongisporus, Pichia vini var. vini, T. holmii, T. magnoliae and T. vanderwaltii.

Pityrosporum pachydermatis, the main isolate from diseased skins, was recovered 14 (93.3%) times from the 15 positive samples. Candida zeylanoides, isolated once (6.7%) was the other yeast species encountered.

## DISCUSSION

Yeasts are ubiquitous in natural habitats and they also exist as commensals in the digestive tract of animals. Thus, it is perhaps not surprising to encounter them on the animal skin. In this investigation, many yeast species that are known to exist as natural inhabitants as well as animal commensals were isolated.

The following species, recovered from normal canine skin, are known to exist as saprophytes in nature (Lodder, 1974): Torulopsis candida, Lod. elongisporus, Rh. rubra, Rh. glutinis var. glutinis, P. membranaefaciens, P. vini var. vini, T. magnoliae, Tri. cutaneum, C. guilliermondii and Cr. uniguttulatus. On the other hand, some of the yeasts isolated here, have been associated with human or animal sources. Candida melibiosica was isolated from human sputum by Buckley and van Uden (1968). Candida guilliermondii and C. parapsilosis were recovered by van Uden et al., (1958) from the digestive tracts of horses and cattle, horses, sheep and pigs, respectively.

The only record of T. vanderwaltii was its isolation from wine-making equipment in South Africa (Vidal-Leiria, 1966). Its recovery from normal canine skin here probably indicates its possible saprophytic existence in the animal's environment.

Trichosporon cutaneum, although considered to be mainly a soil inhabitant (Ajello, 1962), is also considered to be closely associated with keratinous debris of the skin, since it is isolated mainly from such sources as sludge from drains of swimming pools or communal bathing places and skin scrapings from human feet (Gentles and La Touche, 1969). It was isolated on 8 occasions (12.7% of the total isolates) from normal dog skin. Perhaps this incidence is high enough to consider it as part of the normal flora of the canine skin.

Candida zeylanoides was recovered once from a normal dog and once from a dog with eczema. It was reported to have been isolated from a skin infection in a dog in Austria (Lodder, 1974). However, it was not thought to be responsible for the skin condition here, because only a few

colonies were obtained from the specimen and the frequency of isolation was similar in both normal and diseased skins.

Pityrosporum pachydermatis is generally considered to be associated with the skin and ears of dogs and other animals, including cattle, horse and pig, as a normal commensal (Gustafson, 1960, Carter, 1975).

However, the original isolation of this yeast was made from the inflamed skin of an Indian rhinoceros by Weidman (1925) and Kominami and Soneda (1954) reported another isolation from the skin of an Indian elephant.

In the present survey, Pit. pachydermatis has been recovered from both normal and abnormal canine skin. However, the increased incidence of isolation from dogs with skin conditions as compared to apparently healthy dogs, is probably significant. It was obtained from 3 of 77 normal skins, but from 14 of 25 diseased ones, in each case being the sole isolate.

It was recovered from 10 dogs with suspected ringworm, all of which were found to be negative for dermatophytes; from 2 dogs with eczema, from one with demodectic mange and one with endocrine alopecia. The quantity of growth in all cases ranged from heavy to profuse. In 3 cases of suspected ringworm infection, the yeast was recovered in profuse pure growth and in one of these cases many Pityrosporum-like cells were seen in a Gram-stained smear from skin scales. This latter case was a chronic, crusty skin infection of a year's duration on the bridge of the nose of a dog.

Judging by the frequency of isolation, it may be suggested that the normal canine skin harbours yeasts as part of its normal flora. At least T. candida, Rh. rubra, Tri. cutaneum, C. melibiosica, C. guilliermondii, Rh. glutinis var. glutinis, P. membranaefaciens and



Pit. pachydermatis may be designated as commensals of the normal dog skin. Other yeast species may have been transients due to contamination from the environment.

On the other hand, the increase in incidence of isolation of Pit. pachydermatis from diseased dog skins at nearly 5 times its incidence of isolation from normal skins, probably indicates an abnormal proliferation of this yeast on diseased skins. It may be that pathological changes in diseased skin provide conditions favouring selective multiplication of this yeast. Under these conditions, large numbers of yeasts may irritate the superficial skin layers, thereby aggravating an already existing skin condition. It may also be that Pit. pachydermatis itself was the casual agent in some of these skin conditions. The potential pathogenicity of this yeast has been noticed by other investigators. It has been incriminated as an aetiological agent in otitis externa in the dog (Gustafson, 1955; Manktelow, 1960; Baxter and Lawler, 1972; Sharma and Rhoades, 1975; Gedek et al., 1979).

Table 23. Investigation into the presence of yeasts on normal and abnormal (diseased) canine skins

Skin condition	No. examined	No. +ve	No. of yeast species present:		
			1	2	3
Normal	77	47 (61%)	32 (68.1%)	14 (29.7%)	1 (2.2%)
Abnormal (diseased)	25	15 (60%)	15 (100%)	-	-
TOTALS	102	62 (60.8%)	47 (75.8%)	14 (22.5%)	1 (1.7%)

Table 24. Yeast species isolated from normal and abnormal canine skins.

Species	No. of isolations from:	
	Normal skins (%)	abnormal skins (%)
<u>Candida guilliermondii</u>	5 (7.9)	-
<u>C. melibiosica</u>	6 (9.5)	-
<u>C. parapsilosis</u>	1 (1.6)	-
<u>C. zeylanoides</u>	1 (1.6)	1 (6.7)
<u>Cryptococcus uniguttulatus</u>	1 (1.6)	-
<u>Lodderomyces elongisporus</u>	1 (1.6)	-
<u>Rhodotorula glutinis</u> var. <u>glutinis</u>	4 (6.3)	-
<u>Rh. rubra</u>	9 (14.3)	-
<u>Pichia membranaefaciens</u>	4 (6.3)	-
<u>P. vini</u> var. <u>vini</u>	1 (1.6)	-
<u>Pityrosporum pachydermatis</u>	3 (4.8)	14 (93.3)
<u>Torulopsis candida</u>	16 (25.4)	-
<u>T. holmii</u>	1 (1.6)	-
<u>T. magnoliae</u>	1 (1.6)	-
<u>T. vanderwaltii</u>	1 (1.6)	-
<u>Trichosporon cutaneum</u>	8 (12.7)	-
TOTALS	63	15

## INTRODUCTION

The respiratory tract is generally accepted as the main portal of entry into the body of the organisms causing the major systemic mycoses, including cryptococcosis. Ajello (1967), Kaplan (1973), Rippon (1974), Duperval, Hermans, Brewer and Roberts (1977) and Kelley and Mosier (1977) all believe that, in most instances, human and animal infections with the casual agents of the deep mycoses are acquired as a result of inhalation of fungal spores or other infective elements from the environment. Outbreaks of pneumonitis following heavy exposure to dust containing Cr. neoformans have been reported by Emmons (1960). They occurred in workmen who were cleaning out or demolishing old buildings in which pigeons had lived and pigeon manure had accumulated to a sizeable depth. Cryptococcus neoformans is most frequently associated with man and animals as a pathogen. However, there are reports suggesting a possible commensal existence in man. Howard (1973) isolated the yeast from 3 of 561 sputum samples and suggested that it might be a commensal of the human nasopharynx. Randhawa and Paliwai (1977) isolated it from oropharyngeal washings of a healthy person; and Duperval et. al., (1977) isolated it from the respiratory tract of patients with diseases other than cryptococcosis. These researchers indicated that Cr. neoformans might occur as a transient inhabitant of the upper respiratory tract of healthy persons and those without clinical cryptococcosis.

It is not known whether clinically normal animals, like man, may asymptotically harbour the fungus in their upper respiratory tract,

because no investigation has, so far, been made in this respect.

In the course of this work, circulating antibody to Cr. neoformans was demonstrated in apparently healthy cats and dogs and moreover it was experimentally established that Cr. neoformans could remain viable and probably multiply in the nasal turbinates of cats, following intranasal inoculation, without causing clinical infection in these animals.

Dogs and cats, among other animals, are susceptible to infection with Cr. neoformans. The respiratory tract is the system most frequently involved in these animals. Inhalation of the yeast from their surroundings has often been regarded as the source of infection. However, since no previous investigation has been done on the presence of this and other yeasts in the respiratory tract of these hosts, it was decided to undertake such a survey.

#### MATERIALS AND METHODS

Nasal turbinates were collected from 100 dogs and 75 cats undergoing routine post mortem examination at the Department of Veterinary Pathology, Glasgow. With the exception of one dog with a nasal tumour, no other animal showed any gross abnormality of the upper respiratory tract at the time of the post mortem collection of the specimens.

With the animal lying in the dorsal position, the nasal skin was removed and the nasal bones, together with the dorsal portions of the maxillary bones were removed, using bone forceps, to expose the turbinates. The alar fold of the maxilloturbinate was then grasped with forceps, pulled medially and the lateral attachments of the maxilloturbinate to the maxillary bone cut through. In this way, the intact maxilloturbinate

could be removed from one side of the nasal cavity. The process was repeated to obtain turbinate from the other side.

The turbinates were cut into small pieces with sterile scissors and at least 30 fragments were inoculated on SDA supplemented with chloramphenicol. Plates were incubated at 28° C for 7 days.

## RESULTS

Details of the numbers of animals examined and the numbers from which yeasts were isolated are given in table 25. Of the 100 turbinate samples from dogs, 38 (38%) yielded yeasts. The amount of yeast growth ranged from moderate (1-20 colonies) to profuse (over 50 colonies); with 25 of the specimens giving moderate growth, 7 giving heavy growth (21-50 colonies) and 6 yielding profuse growth.

Fifty yeast isolates were recovered from the 38 positive dogs. Twenty eight (73.7%) of these specimens yielded one yeast species each, 9 (23.7%) yielded 2 species and one (2.6%) specimen yielded 4 species each (table 25). The 50 canine isolates belonged to 24 yeast species in 9 genera, namely: Candida, Cryptococcus, Hansenula, Rhodotorula, Torulopsis, Pichia, Pityrosporum, Saccharomyces and Trichosporon.

Fifteen (20%) of the 75 cats yielded yeast growth. The amount of growth from the positive cats was predominantly moderate (12 specimens), with 3 specimens yielding heavy growth.

Nineteen yeast species were isolated: eleven (73.3%) of the positive specimens yielded one yeast species each, and 4 (26.7%) specimens gave 2 yeast species each. No more than 2 yeast species were obtained from any of the positive feline turbinates. The 19 feline isolates belonged to 12 speci

in the genera: Candida, Cryptococcus, Hansenula, Rhodotorula and Torulopsis.

The species encountered most frequently in the nasal cavities of dogs and cats, although in varying frequencies, were: Torulopsis candida, Candida albicans, Rhodotorula minuta var. minuta and Rh. rubra. Torulopsis candida was recovered 13 times, C. albicans 8 times, Rh. minuta var. minuta 7 times and Rh. rubra 6 times, from both animal species. Table 26, which summarizes the details, shows a generally higher incidence of isolation of these species from the canine nasal cavity. Other yeast species isolated were recovered less frequently. All the species that were obtained from the feline nasal cavity were also recovered from dogs, with the exception of C. zeylanoides, H. californica, Rh. glutinis var. dairenensis and Rh. glutinis var. glutinis, which were isolated only from cats. On the other hand, many of the canine isolates were not recovered from the feline turbinates.

There was no particular pattern of association among the yeast species isolated from either dogs or cats.

#### DISCUSSION

The nasal turbinates were chosen for investigation because it had been established that culturing the nasal turbinates was much more successful than was nasal swabbing, in retrieving Cr. neoformans from the nasal cavity of cats following intranasal instillation.

The difference in frequency of isolation among yeast species and quantity of growth obtained from the canine and feline nasal turbinates may be due to anatomical differences between the 2 animal species. In dogs, the nostrils are wider, the nasal cavity is longer and the

turbinates are more vascular. Hence, yeasts may enter more easily and find a better environment for proliferation there.

Although Cr. neoformans was not recovered from the nasal cavity of either species, saprophytic cryptococci have been isolated. Cr. albidus var. albidus, Cr. laurentii var. laurentii, Cr. luteolus and Cr. uniguttulatus were recovered from the nasal turbinates of dogs and Cr. albidus var. diffluens from the nasal turbinates of both dogs and cats.

The isolation of saprophytic cryptococci and other yeast species from the canine and feline nasal cavities was not unexpected, since most, if not all, of the yeast species isolated here, are known to exist saprophytically in the animal environment. It is, thus, reasonable to expect their presence in the upper respiratory tract of animals as a result of inhalation of dust or atmosphere. Cryptococcus albidus has been isolated from air by Saito (1922) and from soil by Sneller and Swatek (1974). Phaff and Fell (1974) reported that all 17 strains of this species received by them from investigators in various parts of the world had originated from terrestrial sources.

Cryptococcus laurentii was isolated from soil by Phaff and Spencer (Lodder, 1974) and by Sneller and Swatek (1974), and Cr. luteolus was isolated from air by Saito (1922).

Cryptococcus neoformans is a saprophyte that proliferates particularly in avian habitats which provide selective growth requirements, viz. creatinine in bird faeces. One may speculate as to the reason for its absence in the nasal cavities of dogs and cats investigated here. Of the 1751 soil samples collected by Emmons (1954), 1127 collected by Ajello (1958), and 842 collected by McDonough, Ajello, Ausherman,



Balows, McClellan and Brinkman (1961), the number of recoveries of

Cr. neoformans from non-pigeon related soils was less than 1%.

Sneller and Swatek (1974) studying the distribution of the genus Crypto-  
coccus in Southern California soil, isolated Cr. albidus and Cr. laurentii  
from 18 of the 226 natural soil samples, but they did not recover Cr.  
neoformans. These findings indicate that the recovery of Cr. neoformans  
from natural soils is not common, and this may partially explain the  
absence of this species from the nasal cavities of dogs and cats in this  
survey.

However, it may be that the number of animals examined is not big  
enough to draw a statistically significant conclusion, and this should not  
preclude the likelihood of the presence of this yeast in the upper respiratory  
tract of these animals. The majority of animals examined were collected  
from local veterinary surgeons without case histories as to their environ-  
ments. Furthermore, precipitating antibody to Cr. neoformans has  
been demonstrated in apparently normal dogs and cats in this study and  
was thought to have resulted from exposure to the yeast in their environ-  
ment.

Candida albicans was isolated on 8 occasions, as the sole isolate from  
all specimens that yielded it. It was recovered 7 times from dogs, 3  
times in profuse growth, and once from a cat. The presence of this  
yeast in great numbers in the nasal cavities of dogs and cats may indicate  
that it was originally present in quantity. There is the possibility,  
however, that it had proliferated in this site after death.

Torulopsis candida is ubiquitous in nature. It has been isolated from  
air by Saito (1922) and from a wide variety of terrestrial habitats

Lodder (1974). Its predominance among the yeast isolates from both dogs and cats probably is an indication of its saprophytic versatility. Although it was isolated 13 times from 9 dogs and from 4 cats, it was recovered in moderate growth on 10 occasions and in heavy growth on 3 occasions. As was stated in the case of C. albicans, one must take into account the likely proliferation of the yeast after death.

Rhodotorula minuta var. minuta and Rh. rubra, which were isolated from dogs and cats in approximately similar frequencies, are also natural inhabitants in the animal environment. Both have been isolated from soil and atmosphere (Anderson, 1918; Saito, 1922; Lodder, 1974). Rhodotorula sp. are frequently encountered in our laboratory from nasal washings from dogs with nasal aspergillosis. This is perhaps an indication of their prevalence in the environment.

The remaining yeast species were isolated only occasionally, and might have been transient contaminants. In the case of Pit. pachydermatis, there is no record of its isolation from nature, and it has been associated mainly with the skin of dogs, particularly in the ear canal of healthy as well as dogs with otitis externa. Gustafson (1955), Manktelow (1960), Fraser (1961; 1965), Baxter and Lawler (1972) and Sharma and Rhoades (1975) have all reported the isolation of Pit. pachydermatis from healthy and otitic canine ears. It has been isolated from skins of healthy and diseased dogs in the present study. So, the isolates obtained from 2 dogs here may have been contaminants from the skins of the investigated animals.

Judging from the frequency of isolation, and in some cases from the

quantity of growth yielded, it may be said that at least T. candida,  
C. albicans, Rh. minuta and Rh. rubra, among others, do exist and  
may multiply in the upper respiratory tracts of dogs and cats.

Table 25. Investigation into the presence of yeasts in the nasal turbinates of dogs and cats.

Animals	No. Examined	No. +ve	No. of yeast species present:			
			1	2	3	4
Dogs	100	38 (38%)	28 (73.7%)	9 (23.7%)	-	1 (2.6%)
Cats	75	15 (20%)	11 (73.3%)	4 (26.7%)	-	-
TOTALS	175	53	39	13	-	1

Table 26. Yeasts isolated from the nasal turbinates of dogs and cats.

Yeast species	No. of isolations:		Total No. of isolations
	Dogs	Cats	
<u>Candida albicans</u>	7	1	8
<u>C. curiosa</u>	2	1	3
<u>C. parapsilosis</u>	1	1	2
<u>C. pseudotropicalis</u>	1	-	1
<u>C. zeylanoides</u>	-	1	1
<u>Cryptococcus albidus</u> var. <u>albidus</u>	1	-	1
<u>Cr. albidus</u> var. <u>diffluens</u>	2	1	3
<u>Cr. laurentii</u> var. <u>laurentii</u>	2	-	2
<u>Cr. luteolus</u>	1	-	1
<u>Cr. uniguttulatus</u>	1	-	1
<u>Hansenula californica</u>	-	2	2
<u>H. holstii</u>	1	-	1
<u>Pichia membranaefaciens</u>	1	-	1
<u>Pityrosporum pachydermatis</u>	2	-	2
<u>Rhodotorula glutinis</u> var. <u>dairenensis</u>	-	1	1
<u>Rh. glutinis</u> var. <u>glutinis</u>	-	1	1
<u>Rh. minuta</u> var. <u>minuta</u>	4	3	7
<u>Rh. pilimanae</u>	1	-	1
<u>Rh. rubra</u>	4	2	6
<u>Saccharomyces cerevisiae</u>	1	-	1
<u>Sacch. telluris</u>	1	-	1
<u>Torulopsis candida</u>	9	4	13

Table 26 (continued)

Yeast species	No. of isolations:		Total No. of Isolations
	Dogs	Cats	
<u>T. celliculosa</u>	1	-	1
<u>T. ernobii</u>	1	-	1
<u>T. glabrata</u>	1	-	1
<u>T. gropengiesseri</u>	2	-	2
<u>T. magnoliae</u>	2	1	3
<u>Trichosporon cutaneum</u>	1	-	1
TOTALS	50	19	69

## Oral yeast flora of normal guinea-pigs

The finding of 2 cases of spontaneous oral candidiasis in guinea-pigs and the lack of knowledge about the yeast flora of the oral cavity of these animals, stimulated this study. The intention was to investigate the prevalence of C. albicans and other yeasts in the oral cavity of normal guinea-pigs.

### MATERIALS AND METHODS

One hundred guinea-pigs were examined, from a breeding stock kept in the animal house unit of the Department of Veterinary Pathology, Glasgow. All animals were clinically healthy at the time of sampling. They were fed dry food pellets (Peter Pan Specialized Food; manufactured by Hamlyn Angus Milling, Perth) ad lib. and allowed free access to drinking water. In addition, they were given cabbage twice a week and hay ad lib.

One mouth swab was taken from each animal, using sterile plain cotton swabs. The palate, inner cheeks and tongue were all scrubbed with the swab, and the animal allowed to chew the swab until it was wet. Immediately after collection, each swab was streaked onto the surface of SDA supplemented with chloramphenicol. Plates were incubated at 28<sup>o</sup>C, with daily examination, for a week. Food pellets from the stock used for feeding these animals were similarly cultured for yeasts.

### RESULTS

Twenty yeast isolates were recovered from the oral cavities of 19 (19%) of the 100 guinea-pigs investigated (table 27). They belonged to 4 species in 3 genera, which are, in order of frequency of isolation,

Saccharomyces telluris (11; 55%), C. albicans (6; 30%), Trichosporon cutaneum (2; 10%) and C. ciferrii (1; 5%). Single isolates were obtained from 18 mouth swabs, and two isolates from one swab. Primary isolation growth ranged from moderate (1-20 colonies) to profuse (over 50 colonies). Twelve (60%) swabs yielded moderate yeast growth, 5 (25%) yielded heavy growth (21-50 colonies) and 3 (15%) yielded profuse pure growth (table 28). Candida albicans was the only isolate from the 6 swabs that yielded it: on one occasion in moderate growth, in heavy growth on 4 occasions and in profuse pure growth from one swab.

Saccharomyces telluris was the sole isolate from 10 swabs, and with C. ciferrii from one swab. It was obtained in moderate growth on 8 occasions, in heavy growth once and in profuse pure growth twice.

Trichosporon cutaneum was obtained as a single isolate, in moderate growth, from 2 swabs, and C. ciferrii was obtained once, in moderate growth, together with Sacch. telluris.

No yeast isolation was obtained from the stock food pellets.

## DISCUSSION

It is apparent that yeasts constitute part of the normal microbial flora in the oral cavity of guinea-pigs. Nineteen percent of the 100 clinically healthy guinea-pigs investigated here yielded yeast growth. The absence of yeasts in the stock pellets used for feeding the animals eliminates the possibility of their being an external source of the yeast species obtained from their oral cavities.

Candida albicans is recognised as being part of the normal flora of the oral cavity of apparently normal humans (Winner and Hurley, 1966; Winner, 1969); and is generally included with the typical intestinal flora



of warm-blooded animals. Van Uden, Carmo Sousa and Farinha (1958) isolated it from the intestinal contents of apparently healthy horses, sheep, goats and pigs; Kawakita and van Uden (1965) isolated it from the digestive tract of seagulls; and Clarke (1960) recovered it from the bovine rumen. From its isolation in quantity, from 6 of the 19 positive animals in this study, the yeast appears to be a normal resident in the guinea-pig oral cavity. However, it also appears that for some reasons, not clearly understood, this yeast may convert from a normal commensal to a pathogen causing overt clinical infection in guinea-pigs. The finding of clinical oral thrush in 2 guinea-pigs, described elsewhere, during the course of this study, illustrates just that. The fact that the normally commensal C. albicans may, under certain circumstances, become an invasive human pathogen is well realised in the case of human hosts (Winner, 1969; Ahearn, 1974).

Saccharomyces telluris, which was found to constitute 55% of the yeast isolates from the oral cavity of guinea-pigs, is a species that is adapted primarily to a saprophytic existence in man and warm-blooded animals (Lodder, 1974). With the exception of the type strain which was recovered by him in 1956, van der Walt (1974) stated that all known strains of this species have so far been derived either from human or from animal sources. Van Uden et al., (1958) isolated it from the caeca of cattle, horses and pigs and designated it as "an obligate saprophyte that has its natural habitat in the digestive tract of warm-blooded animals".

Trichosporon cutaneum, although considered to be mainly a soil inhabitant, (Ajello, 1962) and closely associated with the keratinous debris of the skin (Gentles and La Touche, 1969), has also been isolated

from faeces and gastrointestinal tract contents of human and warm-blooded animals. Batista and Silveira (1960) isolated it from human faeces; and van Uden et al., (1957, 1958) recovered it from the caeca of cattle, horses and pigs and designated it as "a facultative saprophyte that grows naturally both inside and outside the digestive tract of warm-blooded animals". In the present survey, Tri. cutaneum represented 10% of the total yeast isolates from the oral cavity of healthy guinea-pigs.

Candida ciferrii, recovered once from the mouth of a guinea-pig in this survey, is a relatively recently discovered species. It was first isolated from a wound on the neck of a cow, and was described by Kreger-van Rij in 1965. Other isolations of this yeast have been made from wood in a cow shed and from the throat of a pig (van Uden and Buckley, 1974).

The four yeast species isolated from guinea-pigs in this investigation were able to grow at 37°C. This may partly explain the isolation of at least C. albicans and Sacch. telluris in profuse growth from some animals (table 28), an indication that they could grow and multiply in the oral cavity of guinea-pigs. It may be suggested that at least C. albicans and Sacch. telluris are normal inhabitants of the guinea-pig oral cavity, judging by their frequency of isolation. One can only speculate whether Tri. cutaneum and C. ciferrii are normal inhabitants in the mouths of these animals or not. The numbers of isolation of these latter species were too low to regard them as a normal part of the microbial flora of the oral cavity of healthy guinea-pigs, and it is perhaps reasonable to regard them as transient inhabitants.

Table 27. Yeast species isolated from the oral cavity of 100 normal guinea-pigs.

Species	Isolations	
	No.	%
<u>Candida albicans</u>	6	30
<u>C. ciferrii</u>	1	5
<u>Saccharomyces telluris</u>	11	55
<u>Trichosporon cutaneum</u>	2	10
TOTAL	20	

Table 28. Degree of yeast growth from guinea-pigs oral swabs.

Yeast species	Quantity of Growth:			Total No. of isolations
	Moderate	Heavy	Profuse	
<u>Sacch. telluris</u>	8	1	2	11
<u>C. albicans</u>	1	4	1	6
<u>C. ciferrii</u>	1	-	-	1
<u>Tri. cutaneum</u>	2	-	-	2
TOTALS	12 (60%)	5 (25%)	3 (15%)	20

Moderate : 1-20 colonies

Heavy : 21-50 colonies

Profuse : over 50 colonies

YEASTS IN ASSOCIATION WITH DISEASE

IN ANIMALS

Pathological specimens from animal sources were examined for the presence of yeasts. These included 75 ear swabs from cases of canine otitis; 36 vaginal swabs from bitches with infertility problems; 9 faecal samples from dogs with gastroenteritis; 15 samples of mastitic bovine milk; oral swabs from 2 cases of thrush in guinea-pigs and organs and gut contents from a racing pigeon with haemorrhagic enteritis.

Pityrosporum pachydermatis was recovered, in profuse growth, from 24 (32%) of the 75 otitic dog ears, being the sole yeast isolate in these specimens. The 36 vaginal swabs from bitches were negative for yeast growth. Six of the 9 faecal samples from dogs, 3 of which were further studied, yielded C. albicans. No yeast isolation was obtained from the 15 mastitic bovine milk samples examined.

Six clinical cases were studied because the yeasts from them were either primarily involved in the causation or secondarily contributed to the condition of the diseased animal. They included a case of haemorrhagic gastroenteritis in a racing pigeon, 3 cases of gastrointestinal ailments in puppies and 2 cases of oral thrush in guinea-pigs.

#### Case 1

A young racing pigeon, purchased from the South of Wales and sent by rail to Scotland, became acutely ill and died 24 hours after arrival. No treatment was given. Post mortem examination revealed acute haemorrhagic enteritis involving the whole length of the intestine. The intestinal mucosa was highly inflamed and the contents were blood-tinged. The liver was slightly hyperaemic.

## Laboratory Investigation

The intestinal contents were subjected to bacteriological examination which showed the presence of numerous yeast-like cells microscopically, and the whole bird was referred for mycological investigation, after culturing for bacteria.

Cultures were set up, from an oral swab, crop, gizzard and intestinal contents, liver and spleen, on SDA with chloramphenicol. Crop and gizzard contents were suspended in sterile distilled water, shaken, allowed to settle, and 0.5 ml volumes of the supernatants were streaked onto the agar surface. A loopful of the intestinal contents was streaked directly onto the agar. Liver and spleen were seared with a hot spatula, incised open with a sterile scalpel, and material from the cut surfaces streaked onto the agar surface. Plates were incubated at 37°C. A smear was prepared from the intestinal contents and stained with Gram stain.

Ten wild, apparently healthy, wood pigeons were examined for the presence of yeasts in the digestive tract, liver and spleen.

## RESULTS

No significant bacteria were isolated from the intestinal contents of the racing pigeon. Numerous ovoid yeast cells, budding on narrow bases, were seen in the Gram-stained smear from the intestinal contents. Cultures from all specimens, except the oral swab, yielded brownish, discrete yeast-like colonies, after 48 hours of incubation. Profuse growth was obtained from the intestinal contents and liver, and a heavy growth from the spleen, crop and gizzard contents.

Gram-stained smears from cultures from all organs confirmed the presence of small ovoid yeast cells, budding multilaterally on narrow bases.

The yeast was identified as Torulopsis pintolopesii according to Lodder (1974).

This yeast was not isolated from any of the 10 wood pigeons examined. Torulopsis candida was recovered from the intestinal contents of one bird, but others were negative for yeasts.

#### DISCUSSION

Torulopsis pintolopesii is a thermophilic and acidophilic species that requires a temperature of 37-42°C and a low pH for growth (van Uden and Vidal-Leiria, 1974). It is known to be well adapted to the acid environment of the stomach of mice, rats and other small rodents (Mackinnon, 1959; van Uden, 1963). It has also been isolated from birds and other animal species. Kawakita and van Uden (1965) recovered it from the intestinal tracts of 4 species of gull: lesser black-headed gull (Larus fuscus), slender-billed gull (L. genei), herring gull (L. argentatus) and black-headed gull (L. ridibundus). Among 14 strains of this species studied by van Uden and Vidal-Leiria (1974), 4 strains originated from the intestinal contents of pigeons. Van Uden, Carmo Sousa and Farinha (1958) isolated this yeast from the caecal contents of a horse.

It is reasonable perhaps, to regard the racing pigeon, among other bird species, with a high body temperature and an acidic environment of the gastrointestinal tract, to be an ideal host for the existence and growth of T. pintolopesii. Although the racing and wood pigeons are



not the same species, it is probably not unreasonable to assume that their body temperatures and gut environment may be more or less similar. Hence, it was assumed that the wood pigeons might carry this yeast species in their gastrointestinal tracts. However, the number of birds available for examination, although all negative for T. pintolopesii, was not big enough to be conclusive.

It was not surprising to find T. pintolopesii in the stomach and intestines of a racing pigeon, but the isolation in profuse and heavy growth from the liver and spleen was certainly abnormal. The isolations from those 2 organs and its presence in quantity in the intestinal contents, in the absence of significant bacteria, suggested that it might have caused the death of this bird.

The sequence of events might have been that the bird was stressed by travelling and the yeast, already present in its gastrointestinal tract, proliferated rapidly, with harmful effects on the intestinal mucosa, precipitating acute enteritis. By persorption through the damaged mucosa, it might then have got access to the bloodstream, reaching liver and spleen, with a fatal outcome.

As yet there is no published data on the pathogenicity of T. pintolopesii. However, according to Lodder (1974), van Uden in 1952, isolated this yeast from livers and spleens of 3 mice in Portugal, but it was not stated if those animals were diseased. Van Uden's publication was not available for consultation, but one can reasonably assume that the presence of this species in livers and spleens of those mice might have been abnormal.

## Case 2

An 11-month-old male Alsatian was admitted to the Veterinary Hospital with a history of vomiting and diarrhoea for 3 days, 3 weeks previously. The vomiting stopped soon after admission, but the diarrhoea continued. Treatment prior to admission included Streptoquaine, Oxytetracycline, Thiazole and Enterosorb. On the admission day, the body temperature was 101<sup>o</sup> F, abdominal palpation revealed tender intestines and acute abdominal pain and faeces were soft. A tentative diagnosis of "idiopathic diarrhoea" was made and the animal was hospitalized.

During confinement, the abdominal pain gradually subsided, the diarrhoea stopped and the animal was discharged after 11 days. No record of specific treatment was available.

### Laboratory Findings

A faecal swab taken 3 days before admission by the Veterinary Surgeon was bacteriologically negative, but yielded a profuse pure yeast growth.

A second faecal swab taken 8 days later was negative for Salmonellae, but yielded a growth of non-haemolytic coliforms. A third swab taken 24 hours later gave results similar to the second one. The 2nd and 3rd faecal swabs were not available for mycological examination because they were used to inoculate a tetrathionate broth for Salmonellae.

The yeast isolated from the first swab was identified as C. albicans.

## Case 3

A 3-month-old female Boxer was presented to the Hospital with diarrhoea since it was obtained 2 weeks previously from a Dog and Cat Home. Various treatments (not specified) were given by the Veterinary

Surgeon, without success.

Clinical examination revealed a body temperature of 101.2<sup>o</sup>F and faeces were blood-tinged and mucoid in consistency. A tentative diagnosis of "idiopathic diarrhoea" was made.

For a week in confinement at the Hospital, the animal passed mainly bloody and dark mucoid faeces, with gradual improvement in general condition. It was given Lomotil tablets for 2 days and was discharged after showing clinical improvement.

#### Laboratory Findings

A faecal swab submitted for bacteriological examination on the day of admission was negative for Salmonellae; but yielded non-haemolytic coliforms, together with a heavy yeast growth. Another faecal swab was parasitologically negative.

The yeast isolate was identified as C. albicans.

#### Case 4

The subject in this case was a 5-month-old puppy, with a presumptive diagnosis of diarrhoea. No other clinical details were given by the Veterinary Surgeon who referred a faecal swab for bacteriological examination.

A profuse pure yeast growth was obtained from the faecal swab, and the yeast isolate was identified as C. albicans.

#### DISCUSSION

Candida albicans is a normal inhabitant of the alimentary tract of many animal species where it lives as a budding yeast on the mucosal surface and in the mucosal secretions. (Parle, 1957; van Uden, 1960; Marples, 1966). Under conditions where changes occur in the host

tissues, or in the endogenous competitive microbial flora of the gastrointestinal tract, the fungus may change from a harmless commensal to an invasive pathogen causing overt clinical disease (van Uden et al., 1957; Smith, 1967; Winner, 1969; Ahearn, 1974). Hence candidiasis is generally regarded as an opportunistic infection of endogenous origin, although the role of C. albicans as a primary pathogen in a wide variety of host species, causing various disease symptoms, is well recognised (Ainsworth and Austwick, 1973; Carter, 1975).

Factors predisposing to Candida infection in animals include long-term oral treatment with broad-spectrum antibiotics, systemic treatment with corticosteroids and immunosuppressive drugs (Wilson and Plunkett, 1965); antibiotic residues in animal feed and other dietary factors including feed high in glucose and maltose (McCarty, 1956; Mills and Hirth, 1967; Jungerman and Schwartzman, 1972); and underlying chronic and debilitating diseases, malnutrition and poor husbandry (Jeoffery and Kenzy, 1960; Baker and Cadman, 1963; Wagstaff et al., 1968).

The predisposition to C. albicans infection in animals as a result of prolonged antibiotic treatment is well documented. Mills and Hirth (1967) have reported systemic candidiasis in calves on prolonged antibiotic therapy for diarrhoea; and Kral and Uscavage (1960) stated that "increased incidence of candidiasis attributable to excessive antibiotic administration has been reported in various animal species, manifested as stomatitis and enteritis in cattle or as stomatitis in dogs". Among the theories supporting antibiotic enhancement of candidal growth are: direct stimulation of the fungus, removal of bacterial flora competing

for nutrients or removal of organisms secreting antifungal substances (Jungerman and Schwartzman, 1972). Mills and Hirth (1967) stated that "tissue damage by antibiotics may facilitate local invasion by Candida spp. and destruction of the alimentary flora may cause a vitamin deficiency, thereby lowering resistance of tissues to invasion by C. albicans." They were also of the opinion that the fungus flourishes when the antibiotic is tetracycline, since C. albicans utilises this drug as a source of nitrogen.

In dogs, C. albicans has been associated with disease conditions of the gastrointestinal tract. Austwick, Pepin, Thompson and Yarrow (1966) isolated it in quantity from a group of puppies with diarrhoea; and Carter (1975) stated it as a cause of mycotic stomatitis in dogs.

Common factors in the cases described here are, that the animals were young (3-11 months), with diarrhoeic manifestations of some duration, and at least 2 of them had had some form of treatment. In the first case, the animal had oxytetracycline, with other drugs, for the diarrhoea, and in the second, the puppy had received variable unspecified treatment, probably including antibiotics.

In the first case, C. albicans was recovered from the faecal swab in profuse growth in the absence of any bacteria; in the second case, the yeast was isolated in heavy growth and in the third case in profuse pure growth. It is worth mentioning that all primary isolations were made on bacteriological media (blood agar and Macconkey agar) and under conditions favourable for bacterial growth. The non-haemolytic coliforms isolated in the first case from the faecal swabs that were not examined mycologically and from the second case, together with the yeast growth,

might not have been of aetiological significance. Coliforms are normal commensals in the dog's intestines and the numbers of colonies obtained from the swabs were not suggestive of abnormal multiplication by these organisms.

Unfortunately, it was not possible to examine the form in which the yeast was present (yeast or mycelial form) in the faeces. The swabs were used to enrich a primary isolation tetrathionate broth for Salmonellae.

Although C. albicans was not claimed to primarily have caused the diarrhoea in these puppies, the circumstances were suggestive of abnormal proliferation by the yeast, and it probably played a secondary role in the conditions. Antibiotic treatment, at least in one case, might have caused flourishing of the yeast, thus aggravating the disease condition. It is perhaps worth mentioning that C. albicans was isolated from 3 of the other 6 faecal swabs from diarrhoeic dogs, but not in quantity to suggest more than an ordinary occurrence.

It appears that yeast infections are not usually considered by clinicians in the differential diagnosis of diarrhoea. The three cases of stubborn diarrhoea in puppies studied here, were all diagnosed as "idiopathic diarrhoea", a general term used for disease conditions of unknown aetiology. In all cases, faecal samples were submitted for bacteriological and parasitological, but not mycological examination, and in none of the cases was a significant bacterial or parasitic aetiology discernible.

Clinical oral candidiasis in guinea-pigs - cases 5 and 6

Oral thrush, caused by infection of the mucous membranes by C. albicans, is well known in man, occurring particularly in young children and debilitated adults. In the veterinary field, oral thrush is most frequently encountered in birds affected with avian candidiasis. The condition has been reported in turkeys (Kuprowski, 1960; Tripathy, Kenzy and Mathey, 1967; Saez, 1969, 1974; Bickford, Gallina, Winterfield and Bolte, 1971; Enchev, Aleksandrov, Tsonev, Shishkov, Vranska, Kyurtov, Kolev and Kr"stev, 1975; Tabuchi, Sato, Nakazawa, Muto and Ishizaka, 1976); chickens, (Sansone De Real, 1954; Rozanska and Samorek-Dziekanoska, 1971; Picone and Pena, 1973;) partridges, (Keymer and Austwick, 1961) and peacocks, (Saez, 1974).

Few cases of oral thrush have been reported in mammals. Smith (1967) described erosions in the mouth of a young calf which also had extensive epithelial lesions along the oesophagus, abomasum, rumen and reticulum. Preparations from the lesions demonstrated the presence of abundant pseudohyphae and C. albicans was obtained in culture. Gitter and Austwick (1959), reported an outbreak of candidiasis in piglets in which typical oral and oesophageal thrush lesions were manifested and C. albicans was isolated. Osborne, McCrea and Manners (1960) described the disease in artificially reared piglets. The lesions were of the classical thrush type, involving the tongue, hard palate, oesophagus and the entire length of the alimentary tract. Candida albicans was recovered from the lesions. Similar conditions involving the oral and oesophageal mucosa in piglets due to this yeast have been

described by Austwick, Pepin, Thompson and Yarrow (1966), Coman (1968) and Adamesteanu, Adamesteanu and Carabasiu (1974).

Spontaneous oral thrush due to C. albicans has also been described in monkeys. Kauffman and Quist (1969) described a case of oral candidiasis in a male rhesus monkey (Macaca mulatta), with inanition, anorexia and stubborn diarrhoea over a 2 months period. At necropsy, yellow adherent pseudomembrane lined the entire oesophagus. Candida albicans was demonstrated microscopically and isolated in culture. McCullough, Moore and Kuntz (1977) diagnosed candidiasis in a female monkey which had been experimentally infected with Schistosoma. The lesions involved the nasal, pharyngeal and intestinal mucosal surfaces and a pharyngeal lymph node. The monkey has also been reported to be susceptible to experimental oral candidiasis. Budtz-Jørgensen (1975) induced C. albicans infection of the oral mucosa in adult monkeys by inoculating the yeast under acrylic plates covering the palatal mucosa. Similar infection was produced in monkeys by Olsen and Haanaes (1977) with a similar method to that of Budtz-Jørgensen. In both cases, oral thrush, comparable to denture stomatitis in man, was manifested.

In the guinea-pigs, C. albicans infections have so far only been induced experimentally and have resulted in oral, cutaneous, genital and generalised lesions. Drouhet (1965) induced buccal and vaginal candidiasis in guinea-pigs and reported an effective action of pimaricin in resolving these conditions. But he also, in 1969, stated that experimental thrush in the guinea-pig appeared only after a preparatory antibiotic treatment prior to inoculation.

Cutaneous candidiasis has been experimentally produced in guinea-



pigs following application of C. albicans to scarified and non-scarified skin of the back (van Cutsem and Thienpont, 1971) and under occlusive dressings (Sohnle, Frank and Kirkpatrick, 1976). Both infection methods resulted in cutaneous lesions, characterised by inflammatory cellular infiltration, with crusting and scaling of the keratinized skin layers. These cutaneous lesions were described by the former authors as being comparable to experimental cutaneous candidiasis in man.

The yeast has also been reported to have caused abortion in pregnant guinea-pigs. Lysenko and Miloradovich (1966) induced abortion in 11 of 18 pregnant guinea-pigs, 24 hours to 6 days after intraperitoneal, subcutaneous and rectal infection with C. albicans. Generalised C. albicans infection in guinea-pigs, affecting mainly the kidney and heart, was observed by Hurley and Fauci (1975), following intraperitoneal and intravenous inoculation. However, the guinea-pig has been reported to be refractory to infection with the yeast via the respiratory tract. Voisin, Aerts, Wattel, Tonnel and Petitprez (1971) inoculated C. albicans blastospores intratracheally into a group of guinea-pigs and found that the yeast was phagocytosed and rapidly eliminated from their lungs.

There is no record of natural cases of candidiasis in guinea-pigs. Two cases of spontaneous natural oral thrush in guinea-pigs have been diagnosed and are described here.

#### Cases 5 and 6

Two male, 16-month-old, pet guinea-pigs were presented at the Veterinary Hospital. The animals looked to be in good health, but their upper incisors were continually breaking off and they had difficulty in eating. Both showed crusted lesions on the anterior region of the upper

lips and degeneration of the upper incisors which were discoloured with black debris firmly attached to their inner surface and penetrating the periodontal space (figs. 34, 35). On removal of the debris, a raw, non-bleeding surface was exposed on the gum.

The lesions had persisted for more than 4 months, during which time the animals had been treated topically twice per day with Nystan (Squibb) and Fucidine H (Leo Laboratories) ointments which had been prescribed by the local Veterinary Surgeon.

The owner, who had had the animals since they were very young, could not remember any injury or disease condition which might have predisposed to the infection and, as far as she was aware, there had been no prior treatment with antibiotics. The animals were kept on a varied diet of fruits, vegetables, potatoes and bread. Drinking water was not usually given.

When the condition had been diagnosed as oral candidiasis, Nystatin ointment was prescribed for topical application twice daily. The owner was also advised to clean the affected teeth regularly. After 3 months of treatment, the owner reported that the lip lesions had disappeared and there was a marked improvement in the mouth lesions. At her last report, 7 months after the treatment was begun, the teeth had not broken.

#### Microbiological investigation

On the first visit to the Hospital, a swab was taken from the lip lesion of one of the animals and submitted for bacteriological examination. Culture gave a mixture of bacteria, the predominant bacterial species being Staphylococcus epidermidis and numerous colonies of a yeast which

was identified as C. albicans.

On the second visit, a week later, material from the lip lesions, debris from inside or around the affected incisors and faecal samples were collected from each animal. On microscopic examination of the samples from the teeth, numerous budding yeast cells and pseudohyphae were observed in KOH preparations and in smears stained by Gram (fig. 36). A similar result was obtained in mounts from the lip lesion of one animal; the other was not examined by microscopy due to shortage of material. Microscopic examination of the faeces did not reveal the presence of hyphae or pseudohyphae, but yeast-like cells were seen.

Cultures from all specimens from lip lesions, tooth debris and faeces yielded profuse growth of a yeast which was identified as C. albicans.

#### DISCUSSION

Candida albicans may be present as a commensal on the mucous membranes and in the gastrointestinal tract of man and animals, and, therefore, the isolation of this yeast from clinical specimens may not always be indicative of infection. Definite diagnosis of candidiasis requires the demonstration in tissues and specimens of both blastospores and pseudohyphae, the presence of which is regarded as evidence of transformation from the harmless commensal yeast form to the invasive mycelial form (Soltys, 1963; Mackenzie, 1966; Merchant and Packer, 1967; Gentles and La Touche, 1969; Jungerman and Schwartzman, 1972).

Although C. albicans is capable of causing primary infections, candidiasis is most commonly encountered as a superinfection in compromised human hosts. Predisposing factors to Candida infection are many and

varied, including long-term antibiotic treatment, immunosuppressive therapy, diabetes mellitus, and debilitating conditions, etc. (Winner and Hurley, 1966; Winner, 1969; Ahearn, 1974). It is also recognised as a complication of open-heart surgery and organ transplant (McConnell and Roberts, 1967; Conway, Kothari, Lockey and Yacoub, 1968; Murray, Buckley and Turner, 1969; Evans and Forster, 1976); and as a complication of burns (MacMillan, Law and Holder, 1972; Holder, Kozin and Law, 1977).

In animals, Candida infection has been associated with prolonged antibiotic treatment (Mills and Hirth, 1967), antibiotic residues in animal feed (Quin, 1952), poor husbandry (Baker and Cadman, 1963) and as a superinfection to other diseases, such as gastric mucormycosis in pigs (Gitter and Austwick, 1959).

Drouhet (1969) studied the in vivo effect of prolonged administration of antibacterial antibiotics in relation to experimental oral and systemic candidiasis in guinea-pigs, rabbits and dogs. He concluded that antibacterial antibiotics enhance the development of C. albicans in the mucous membranes, and are responsible for increased incidence and severity of candidiasis. He stated that experimental thrush in the guinea-pig and rabbit and Candida endocarditis in the dog, appear only after a preparatory antibiotic treatment prior to inoculation. His hypothesis was that the suppression of the competitive alimentary bacterial flora upsets the bacteria-yeast equilibrium in favour of the latter, leading to opportunistic pathological complications; and that the antibacterial antibiotics inhibit antibody synthesis and phagocytic activity, thus reducing the host resistance to C. albicans infection.

In the 2 cases of oral thrush in the guinea-pig studied here, it appears that C. albicans was the cause of the conditions, and no evidence of pre-disposition could be discerned. The yeast was demonstrated microscopically in the lesions in both the yeast and mycelial forms and was isolated in profuse growth from all specimens from both guinea-pigs.

Staphylococcus epidermidis was the main bacterial isolate from the lip lesion of one of the animals. However, it is unlikely that it was causing the infection; it is regarded as a non-pathogen commonly occurring as a commensal on the skin of man and animals (Buxton and Fraser, 1977). It might have been a contaminant from the animal skin.

The mouth lesions in these 2 cases are remarkably different from the usual moist, creamy, curd-like patches on inflamed mucous membranes, characteristic of oral thrush in man and animals. The main symptoms were breaking off of the incisor teeth, with black debris sticking to their surfaces and dry, crusted lesions on the lips. A slightly hyperaemic area was present on the gum, on removal of the tooth debris.

Candida albicans has been found, in the present study, to be part of the normal oral yeast flora of healthy guinea-pigs. It may be suggested that the infection in the 2 guinea-pigs might have been of endogenous origin. But what caused essentially similar lesions in both animals at the same time for the same duration is not clear. A similar case of primary Candida infection in a guinea-pig was diagnosed by Lauder and Dawson (1979; personal communication), who observed thrush-like lesions in a young guinea-pig due to C. albicans, with laboratory findings similar to those described in these cases. No underlying abnormality was detected then.

According to the owner of the 2 guinea-pigs, topical treatment was effective; the scabs on the lips disappeared and the teeth had not broken for more than 7 months after starting the treatment. Because of the well-documented specificity of Nystatin as an anti-Candida albicans antibiotic (Kuttin, Beemer and Meroz, 1976; Beveridge, Fairburn, Finn, Scott, Stewart and Summerly, 1977), this cure is yet a further evidence that C. albicans was the cause of primary infection in these 2 cases.

Figs. 34-36: Oral candidiasis in guinea-pigs.

Fig. 34: Tooth lesions

Black debris on the inside surface of the upper incisors and in the periodontal space (left); raw, non-bleeding surface on the gum on removal of debris (right).





Fig. 35: Lip lesion

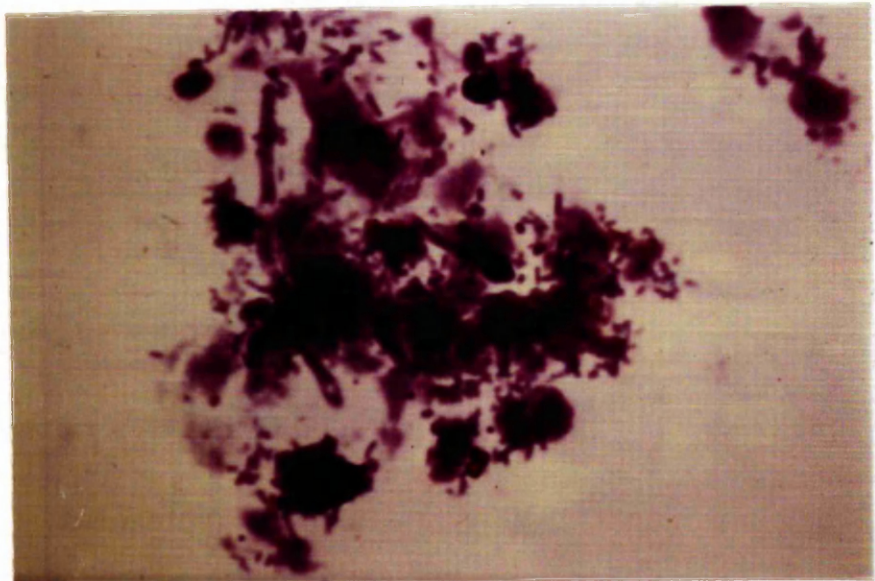
Crusty and scaly lesion on the upper lip (arrow)

Fig. 36:

Gram-stained smear from the tooth lesion in Fig. 34.

Hyphal fragments and blastospores were present amid the debris; Gram-negative bacilli were also visible.

(x 1000)



STUDIES ON SOME BLACK YEASTS

## INTRODUCTION

Black yeasts are a group of olivaceous to black pigmented fungi that belong to the Family Dematiaceae. The term black yeast is derived from the fact that some of them exhibit initially a yeast-like growth which eventually becomes filamentous.

Ajello (1977) has described black yeasts as "dematiaceous, filamentous fungi which, in certain stages of their development and under certain environmental conditions, have a unicellular phase during which multiplication is by a budding process. The colonies at this stage are pasty with some shades of black". Although all black yeasts are by definition dematiaceous fungi, not all such fungi are black yeasts; because not all of them exhibit an initial yeast-like growth. Black yeasts are of interest to medical mycologists because some are potentially pathogenic to humans, and can cause diseases that range in severity from the mild and superficial to those which involve vital organs and hence jeopardise the life of their hosts (Ajello, 1977).

This group of fungi mainly causes subcutaneous and localised diseases such as chromoblastomycosis, phaeoϕyphomycosis, mycetoma and tinea nigra. Infection in these diseases usually results from contamination of traumatised skin from soil, wood and decaying vegetation where these organisms abound. At least one black yeast, Fonsecaea dermatitidis is known to be neurotropic, causing cerebral abscesses. In some systemic infections, particularly those involving the brain, it has been suggested that the primary site of infection might be pulmonary (Rippon, 1974).

Although black yeasts are well recognised as disease agents in man,

little is known about their spontaneous infectivity to domestic animals. Two cases of chromomycosis were reported in domestic animals, one in a dog and the other in a horse. Hoskins, Lacroix and Mayer (1959) described a case in a five year old male dog, in which the first visible sign was a black pigmented nodule between the toes of the right forefoot. The lesion persisted and after two years the prescapular lymph node became enlarged and was found, on incision, to be firm and the cut surface contained numerous brown and black foci. Simpson (1966) reported a case involving the skin of the ventral neck region and the ventral thoracic area of a twelve year old Palomino gelding. The lesions consisted of multiple nodules which periodically broke open and drained. Brown septate hyphae and budding bodies were histologically demonstrated in the granulomatous lesions. However, the dematiaceous fungi incriminated in both cases were identified only as Hormodendrum. It is difficult, therefore, to speculate whether black yeasts were associated with those cases or not.

There are a few reports incriminating black yeasts as disease agents in frogs. Frank and Roester (1970) isolated F. dermatitidis from a frog with cutaneous lesions, and stated that amphibia were carriers of Hormodendrum (Hormiscium) dermatitidis. Elkan and Philpot (1973) isolated a Phialophora sp., "probably P. gougerotii" from frogs suffering from skin conditions, but they were not sure if it was the pathogen. The paucity of case reports of black yeast infections in animals may be due to lack of recognition rather than the absence of disease conditions caused by these fungi.

There is a great deal of controversy regarding the taxonomy of

dematiaceous fungi and much confusing nomenclature. It is not uncommon to find that a dematiaceous fungus is given different names by the same author. This reflects the problems encountered in studying and interpreting the microscopic morphology of these organisms. Rippon (1974) stated that "the taxonomy of the agents of chromomycosis (dematiaceous fungi) has been one of the most confusing in the field of medical mycology".

Currently, three genera are recognised in medical mycology, namely, Phialophora, Cladosporium and Fonsecaea (Rippon, 1974; Haley and Callaway, 1978). In vivo, these fungi are indistinguishable from each other. All occur as brown pigmented branching septate hyphae and/or plantate-dividing, rounded bodies (sclerotic bodies) in tissues, skin scrapings and exudates (Rippon, 1974; Haley and Callaway, 1978). Hence, their specific identification depends on the study of their cultural characteristics, particularly their types of sporulation, percentage of spore types present and details of spore production. At present, three general types of sporulation are recognised in medical mycology (Rippon, 1974; Haley and Callaway, 1978).

In the Phialophora type of sporulation, there is a distinct conidiophore called a phialide, which occurs terminally or along the hypha, (fig. 37). The phialide is generally flask-shaped, with a rounded, oval, or elongate base, a constricted neck and an opening that may have a flaring collarette and lip. The conidia are formed at the end of the flask (semiendogenous sporulation) and are extruded through the neck. They may accumulate around the neck area, resembling "flowers in a vase". The conidia are oval, smooth-walled, hyaline and have no

attachment scars.

In the Rhinocladiella (Acrotheca) type, the conidiophores are simple and sometimes not differentiated from the vegetative hyphae. Oval conidia are produced irregularly on the tip and along the sides of the conidiophore (acropleurogenous sporulation) (fig. 38). They are usually single and do not bud. However, occasionally chains formed by budding and transition to the Cladosporium type sporulation are seen. When the conidia are detached, small bud scars can be found on the conidiophore, and there is a single scar (spicula) on the spore at the attachment site.

The Cladosporium (Hormodendrum) type of sporulation is characterised by a simple stalk that serves as a conidiophore (fig. 39). It is usually slightly enlarged at the distal end. Two or more spores are formed at the tip. These in turn bud and form secondary spores at their distal poles (acropetalous sporulation). Sporulation continues with the formation of long chains. The youngest spore is the one most distal to the conidiophore. Detached spores show a thickening or scar, called a disjuncter, where they were connected to the other spores. All of the spores in the chain will have two or three disjuncter scars, except the terminal spore which will have one. A spore having three disjuncters is described as "shield-shaped".

While members of the genera Phialophora and Cladosporium sporulate almost exclusively by the phialophora and cladosporium types respectively, members of the genus Fonsecaea may produce more than one type of sporulation, the proportions varying with the strain and media used for growth.

A study was undertaken on some black yeasts, isolated from animal

sources, to determine their cultural characteristics, identification and pathogenesis in experimental animals. The identification procedures adopted were those of Rippon (1974) and Haley and Callaway (1978).

## MATERIALS AND METHODS

### Organisms

Three isolates were studied: V413 was isolated from a tongue ulcer and oedematous tissue of a dog; V436 from a necrotic granulation tissue adhering to the pleura in a dog with pleurisy and V497 from a subcutaneous sublingual ulcer in a pony. Although the isolates were recovered in quantity in culture, none of them could be established as the cause of the disease condition in question, since they were not microscopically demonstrable in the lesions.

Isolates were provisionally identified and sent to the Commonwealth Mycological Institute (CMI), Surrey, England, for confirmation. The Herbarium numbers are IMI 235793 (V413), IMI 235794 (V497) and IMI 235795 (V436).

## CULTURAL CHARACTERISTICS

### Macroscopic morphology

Colonial appearances and rates of growth were studied on Sabouraud dextrose agar (SDA) and potato dextrose agar (PDA). Plates were point-inoculated from young (3-4 days old) yeast-phase cultures and incubated at 28°C with frequent examination for 4 weeks. At this time, colonial characteristics were described, rates of growth determined and plates were left on the bench for an extra 4 weeks before final microscopic examination. Streak cultures were also made on



SDA and PDA plates and incubated at 28<sup>o</sup> C for 2 weeks to study the pigmentation and consistency of the yeast-phases. Colour matchings and colonial morphology were described according to Rayner (1970) and Otčenášek and Dvořák (1973), respectively.

#### Microscopic morphology

Types of sporulation and spore sizes were studied on slide-cultures. Blocks of SDA and PDA 2 x 2cm. were inoculated from young cultures, coverslips applied, and incubation carried out at 28<sup>o</sup> C in petri dishes containing moist filter papers, with weekly examination, for 6 weeks. The preparations were then mounted in lactophenol for examination. Teased preparations from 8 week old pin-point cultures were also examined for types of sporulation, in lactophenol-cotton-blue mounts.

#### Growth at 37<sup>o</sup> C and 43<sup>o</sup> C

Streak cultures were set up on SDA plates, from young cultures of the 3 isolates and incubated at 37<sup>o</sup> C for 1-3 weeks.

V413 and V497 were cultured on SDA slants in universal bottles and incubated in a water bath adjusted to a constant temperature of 43<sup>o</sup> C for 4 weeks.

#### Thermotolerance

Yeasts were grown in glucose-peptone broth at 28<sup>o</sup> C for 4 days, then after thorough mixing, 4 ml were dispensed into sterile bijou bottles, warmed at 37<sup>o</sup> C for 10 minutes, and then held at 56<sup>o</sup> C, 60<sup>o</sup> C and 70<sup>o</sup> C in a water bath. Samples were taken for 2 hours at half hour intervals, when 0.5 ml from each culture at each temperature were streaked on SDA plates. Viability was tested for by incubation at 28<sup>o</sup> C for 1 week. The remainder of the broth was re-incubated at 28<sup>o</sup> C for 4 days and then

tested for viability.

#### Gelatin liquefaction

Gelatin gels in universals were inoculated from young cultures, using a straight wire, and incubated at 24<sup>o</sup> C for 2 weeks, at which time the bottles were placed at 4<sup>o</sup> C for 15-30 minutes and examined for liquefaction.

#### Urea hydrolysis

Slants of Christensen's urea agar were inoculated, incubated at 28<sup>o</sup> C and examined daily for urease production.

### MOUSE PATHOGENICITY

#### Inocula

Isolates were grown on SDA slants at 28<sup>o</sup> C for 4 days, harvested and washed 3 times in sterile normal saline (SNS) by centrifugation at 3,000 rpm for 5 minutes. Wet mounts were examined to ensure that all organisms were in the yeast phase. Cell suspensions were then made in SNS and adjusted to a final concentration of  $1 \times 10^6$  cells/ml, by haemocytometer counts.

#### Animals

Three-month-old BLAB C white mice were used for the pathogenicity study.

Nine mice were used per isolate: 3 were infected intraperitoneally with  $2 \times 10^5$  yeast cells in 0.2 ml SNS, 3 subcutaneously in the abdominal region with  $1 \times 10^5$  cells in 0.1 ml SNS and 3 intravenously into the tail vein with  $1 \times 10^5$  cells in 0.1 ml SNS.

Animals were observed for symptoms of illness and mortality. After

death, heart blood, lungs, liver, spleen, kidneys, brain, spinal cord and gross lesions from other sites were cultured on plates of SDA containing chloramphenicol and incubated at 28<sup>o</sup> C for 7 days. KOH preparations and Gram-stained smears were prepared. Portions from organs and tissues of some mice were fixed in 10% neutral buffered formalin, sections cut to 4 m $\mu$  thickness and stained with H & E and P.A.S.

## RESULTS

### Colonial morphology of isolates V413 and V497

These isolates initially exhibited yeast-like growth and remained in that phase for 10-14 days, before the appearance of the filamentous phase. In streak cultures on SDA, both isolates grew as smooth, moist, olive green to black colonies (figs. 40, 42), producing a diffuse olivaceous pigment, with a green-olivaceous reverse. On PDA, both yielded a tenacious mucoid growth, so slimy that it was difficult to hold on the inoculation loop; and India-ink mounts from both isolates revealed the yeast cells to be encapsulated.

In point inoculation cultures, after 4 weeks incubation at 28<sup>o</sup> C on SDA, V413 grew as a circular, flat, fringed colony with a glabrous centre and submerged peripheral mycelium. The centre was black and the periphery greyish-olivaceous; a greyish-velvety sector developed on the periphery and it attained a diameter of 3 cm. On PDA, it developed a circular, slightly raised, radially furrowed colony, with greyish hairy growth at the centre and concentric zoning towards the periphery, intermingled with glistening olive-green, glabrous, yeast-

like growth (fig. 41). The colony was 4.3 cm in diameter. On both media, this isolate produced an olivaceous pigment that diffused into the medium and an olive-green reverse.

Isolate V497 exhibited cultural characteristics similar to those of V413. After 4 weeks on SDA at 28<sup>o</sup> C, the colony was 4 cm in diameter, circular, flat, fringed with peripheral radiation and had a wrinkled, greyish-olive centre surrounded by a glabrous, flat, black zone, and a powdery, radially radiated olivaceous zone peripherally, with greyish-powdery edges. On PDA, it gave rise to a circular, greyish-olive powdery growth, that was slightly raised, with concentric zonation (fig. 43), and a diameter of 4.5 cm was attained after 4 weeks at 28<sup>o</sup> C. It had an olive-green reverse on both SDA and PDA.

#### Microscopic morphology

In wet mounts from young yeast-like cultures, only single, ovoid and occasionally spheroid, hyaline, thin-walled yeast cells were seen.

Mature cells had 1 or 2 bud scars (fig. 44).

In slide culture and teased preparations, both isolates sporulated equally well on SDA and PDA. They produced predominantly Cladosporium-type sporulation with long chains of spores (figs. 45, 47), and occasional Rhinocladiella-type with elliptical spores directly developing on the conidiophores (figs. 46, 48). There were visible apiculae on free conidia. The conidia were thick walled, ovoid to spherical, varying in size from 5.2 m $\mu$  (2.9 - 8.6) x 2.8 m $\mu$  (1.4 - 4.3) in the case of V413 to 6.0 m $\mu$  (3.6 - 8.6) x 3.5 m $\mu$  (2.2 - 4.3) in the case of V497.

#### Growth at 37<sup>o</sup> C and 43<sup>o</sup> C

Both isolates grew well at 37<sup>o</sup> C yielding visible yeast-like growth

after 48-72 hours incubation, but neither of them grew at 43<sup>o</sup> C (table 29).

#### Thermotolerance

Both isolates survived for up to 1½ hours at 56<sup>o</sup> C and 60<sup>o</sup> C, but died after less than ½ hour at 70<sup>o</sup> C and after 2 hours at 56<sup>o</sup> C and 60<sup>o</sup> C.

#### Gelatin liquefaction

None of the two isolates liquefied gelatin after 2 weeks incubation (table 29).

#### Urea hydrolysis

Both isolates hydrolysed urea after 3-5 days (table 29).

#### Mouse pathogenicity

Both isolates were pathogenic to mice and both were neurotropic (table 29).

#### Colonial morphology of isolate V436

This isolate gave an initial yeast-like growth that remained in the yeast phase for 2 weeks when aerial mycelia appeared. Growth was similar on both SDA and PDA. It was tar black, glistening and soft (fig. 49), with a black, diffusing pigment and a black reverse.

This isolate was different from V413 and V497. After 4 weeks of growth on SDA at 28<sup>o</sup> C, the colony was lobulate, with two types of sector: an olive-grey membranous wrinkled sector and a smooth, flat, black one (fig. 50), with a diameter of 2.75 cm. On PDA, it exhibited an essentially similar growth to that on SDA. The colony was lobulate, sectored: a verrucose velvety olive-grey sector and an olive-green wrinkled sector and had a diameter of 2.75 cm after 4 weeks at 28<sup>o</sup> C. It had an olivaceous reverse on both media.

### Microscopic morphology

In wet mounts from young yeast phase cultures, this isolate occurred as elongate, spindle-shaped, hyaline, thin-walled cells, with well-developed transverse septa in mature cells (fig. 51).

It sporulated well on both SDA and PDA, entirely by the Cladosporium-type, with long chains of large, thick-walled, rounded conidia (chlamydospores) that measured  $6.0 \text{ m}\mu$  (4.3 - 8.6) x  $5.6 \text{ m}\mu$  (4.3 - 7.2) (fig. 52).

### Growth at 37°C and thermotolerance

It did not grow at 37°C (table 29), and died after less than  $\frac{1}{2}$  hour at 56°C, 60°C and 70°C.

### Gelatin liquefaction and urea hydrolysis

This isolate liquefied gelatin within 2 weeks and hydrolysed urea after 3 days (table 29).

### Mouse pathogenicity

It was not pathogenic to mice (table 29).

### Identification

Isolates V413 and V497, which exhibited essentially similar in vitro and in vivo characteristics, were considered to be one species. Both were identified as Fonsecaea dermatitidis, by virtue of: growth at 37°C, absence of proteolytic activity, presence of more than one type of sporulation, predominant Cladosporium-type and occasional Rhinocladiella-type; and neurotropic affinity.

Isolate V436 was identified as a Cladosporium sp. by virtue of: inability to grow at 37°C, presence of proteolytic activity, sporulation by the Cladosporium-type alone, and absence of pathogenicity to mice.

### Mouse Pathogenicity of the 3 isolates

Isolate V436 was non-pathogenic. Isolates V413 and V497 were pathogenic by the intravenous (IV), intraperitoneal (IP) and subcutaneous (SC) routes of inoculation.

The results of infectivity study and cultural isolations of organisms from organs and tissues of infected mice are summarised in tables 30, 31. As may be seen from table 30, all the mice infected with isolate V497 died within 7 days after infection; 6 mice died 1-3 days and 3 mice died 5-7 days post infection. Seven of the 9 mice infected with isolate V413 died within 14 days of infection, 3 animals died 1-2 days, 3 died 5-7 days and one animal died 14 days post infection. Two of the 3 mice infected intravenously with isolate V436 died 24 hours after inoculation, but the remaining 7 mice survived till the end of the experiment when they were killed 21-24 days post infection.

As may be seen from table 31, isolates V413 and V497 caused generalized infection in mice, following inoculation via all 3 routes. The fungi were re-isolated from all organs and tissues of mice after death and were microscopically demonstrable in the majority of organs examined. Gross lesions varied from localised subcutaneous granulomata at the injection sites (fig. 53) to acute peritonitis, pneumonia and cerebral haemorrhages. Focal lesions with greenish discoloration were present in the lungs, livers and spleens of most of the infected mice. Brains from some mice were greyish brown.

Both isolates exhibited similar microscopic appearances in organs of infected mice. In KOH squash preparations from the liver, spleen, lungs and kidneys, they were present in the form of short chains of

rounded, swollen cells (moniliform bodies) (fig. 54), in addition to single and budding yeast-like cells; whereas in the brain and spinal cord they were abundantly present in the form of long, branching, slender hyphae with occasional blastospores (fig. 55).

Histopathological examination of brain sections from the mice infected with isolates V413 and V497 revealed the presence of many foci of acute encephalitis. These were composed of accumulations of inflammatory cells, which were mainly neutrophils and there was neural degeneration and gliosis in the surrounding nervous tissue. There was also occasional degeneration and necrosis of cells in the inflammatory foci and generalized perivascular cuffing by mononuclear cells and neutrophils. The organisms were present in the form of long, branching hyphae invading the brain parenchyma (figs. 56-58).

The peritoneal lesions developing at the injection sites, consisted of coalescing areas of necrosis in the fatty peritoneal tissues. The necrotic areas were composed of amorphous debris with pyknotic nuclear fragments. In general, the areas of necrosis were surrounded by a zone of inflammatory cells which were mainly neutrophils with, more peripherally, some large foamy macrophages and a few lymphocytes, producing a granulomatous appearance. Masses of yeast cells and hyphae were present in the necrotic debris. In addition, moniliform-like hyphae were also occasionally seen (figs. 59, 60).

The subcutaneous granulomata were characterised by extensive granulomatous reactions around the organisms, which were present exclusively in the yeast form (figs. 61, 62). The inflammatory process consisted of central areas of necrosis surrounded by a zone of inflam-



matory cells, which were predominantly neutrophils, but more peripherally there were large epitheloid macrophages and lymphocytes. Around and walling off the granulomatous lesions there were zones of fibrosis in which a mixed cellular infiltrate of neutrophils, lymphocytes and macrophages was present.

The 2 surviving mice, that were subcutaneously infected with isolate V413, were killed 16 and 23 days post infection. Both had subcutaneous lesions from which the fungus was recovered. In addition, it was also recovered from the spleen of the mouse killed 16 days after infection. The organ was pale and enlarged.

Isolate V436 was re-isolated from both mice that died 24 hours after IV inoculation, but from none of the organs of any of the surviving mice that were killed 21-24 days post inoculation. No lesions developed at the IP or SC injection sites in any of the mice infected by these routes nor were there pathological changes in the intravenously infected mice.

## DISCUSSION

The slight differences between isolates V413 and V497, regarding colonial morphology, conidial size and virulence to mice, are understandable. These dematiaceous fungi are known to be pleomorphic (Nielsen, 1970; Rippon, 1974), and variation in virulence among strains of the same species occurs (Jotisankasa, Nielsen and Conant, 1970).

The two isolates V413 and V497, differed from Phialophora (Wangiella) dermatitidis in that the latter sporulates exclusively by the Phialophora type, producing phialides (Jotisankasa et al., 1970; Ajello, 1977).

They also differed from P. gougerotii (Exophiala jeanselmei) and P. spinifera (E. spinifera) which sporulate by the Phialophora type producing phialides, exhibit variable growth at 37 °C and are not neurotropic (Rippon, 1974). My isolates were also distinct from Cladosporium werneckii which does not grow at 37 °C and sporulates exclusively by the Cladosporium type (Nielsen, 1970); whereas in isolates V413 and V497, the Rhinocladiella type was occasionally seen, in addition to the Cladosporium type. The olive-green yeast-like growth on SDA distinguishes the two isolates from Aureobasidium pullulans, the young colonies of which are creamy on SDA (Ajello, 1977).

The neurotropic affinity exhibited by isolates V413 and V497, in mice, clearly distinguishes them from other black yeasts and relates them to F. dermatitidis which, so far, is the only black yeast known to be neurotropic (Jotisankasa et al., 1970; Rippon, 1974). Their inability to grow at 43 °C differentiated them from the thermophilic dematiaceous filamentous fungus Cladosporium bantianum, which is also neurotropic in experimental animals (Rippon, 1974).

After provisional identification the 3 isolates were sent to the CMI for confirmation. Dr. B. L. Brady thought V436 was not a Cladosporium sp., but could not place it with certainty elsewhere. She provisionally placed it in the genus Sarcinomyces, as S. crustaceus Lindner, but stressed that it was distinct from this species. De Hoog and Hermanides (1977) who described S. crustaceus, did point out some similarities between this species and the Cladosporium sp, C. werneckii. Such features included chlamydosporulation, production of short protuberances resembling annellated butts and production of conidia of the same size

and shape. The term Cladosporium sp. is generally used in the field of medical mycology to accommodate saprophytic, non-pathogenic dematiaceous fungi that do not grow at 37<sup>o</sup> C, are proteolytic and sporulate only by the Cladosporium type of sporulation (Rippon, 1974; Haley and Callaway, 1978); criteria which isolate V436 fulfills, as may be seen from table 29. Hence, it was thought justifiable placing this isolate as a Cladosporium sp.

The death in 2 mice shortly after IV inoculation with isolate V436 might have been due to mechanical blockage of blood circulation in their vital organs. The yeast cells of this isolate are larger than those of the other isolates. The isolation of the fungus from the organs of those 2 mice 24 hours post inoculation was not unexpected. Since none of the 7 surviving mice became ill following IV, IP or SC inoculation with this isolate, and none of them showed gross lesions at post mortem examination or was culturally positive for the fungus, this isolate was regarded as non-pathogenic to mice.

Dr. Brady placed V413 and V497 in Exophiala pisciphilus, McGinnis and Ajello (1974), but indicated that her identifications were tentative since her findings did not quite conform with descriptions of that fungus. There are obvious differences between my 2 isolates and E. pisciphilus. According to McGinnis and Ajello (1974), E. pisciphilus does not grow at 37<sup>o</sup> C, sporulates poorly on SDA and produces phialides: V413 and V497 grew well at 37<sup>o</sup> C, sporulated well on SDA, and none of them produced visible phialides under any conditions of growth.

My provisional identification for isolates V413 and V497 was that both were Fonsecaea dermatitidis, on the grounds that they grew well

at 37°C, were non-proteolytic, sporulated by more than one type of sporulation and were pathogenic to mice, with neurotropic affinity.

The taxonomy of F. dermatitidis has been debated since it was originally reviewed and described by Carrión (1950), who studied the type culture, isolated and named Hormiscium dermatitidis by Kano in 1937. It was recovered from a chronic lesion on a woman's face. Carrión (1950) was of the opinion that Kano was incorrect in calling his isolate a Hormiscium sp, and concluded that it should be placed in the genus Fonsecaea as F. dermatitidis, because it sporulated by the Hormodendrum (Cladosporium) type, the Phialophora type and occasionally by the Acrotheca (Rhinocladiella) type.

Conant, Smith, Baker, Callaway and Martin (1954) opposed Carrión and placed the fungus in the genus Hormodendrum as H. dermatitidis, and Emmons, Binford and Utz (1963) transferred it to the genus Phialophora as P. dermatitidis. Schol-Schwarz (1968) suggested that Kano's isolate be transferred to the genus Rhinocladiella, along with other human pathogenic species in the genera Fonsecaea and Phialophora. Her reason for adopting this genus was based on the apparent similarities between these organisms and R. atrovirens Nannfeldt, 1934, in which 4 different methods of sporulation (denticulation, annellation, semi-endogenous development and acropetal budding with chain formation) were observed. There are objections to this latter amendment, because, as it is now defined, the genus Rhinocladiella could not accommodate those species in the genus Fonsecaea, again because of the variety of spores produced (Jotisankasa et al., 1970; Rippon, 1974; Silva-Hutner and Carrión, 1975).

Lately, Butterfield and Jong (1976) studied the effect of carbon sources on conidiogenesis in the type culture of Hormiscium dermatitidis (ATCC28869) and indicated that the fungus was polymorphic, producing a Phialophora state in media containing glucose and maltose and a Cladosporium state in media containing galactose and melibiose. They concluded that it was morphologically and developmentally closely related to Fonsecaea pedrosoi and advocated that it should be classified as F. dermatitidis.

McGinnis (1977) studied the type culture of Kano's isolate and found that the conidiophores produced by this fungus were phialides without collarettes. He, on the grounds that members of the genus Phialophora sporulate from phialides with a constricted neck and a terminal collarette, proposed that Kano's isolate could not be classified in this genus. Accordingly, he created the genus Wangiella to accommodate this black yeast, which he called W. dermatitidis.

Rippon (1974) recognises F. dermatitidis (Kano 1937) Carrión 1950 as the species and H. dermatitidis Kano 1937, H. dermatitidis Conant 1954, and P. dermatitidis Emmons 1963 as its synonyms. During the present study, the name F. dermatitidis Rippon 1974 was adopted for isolates V413 and V497. The presence of predominant Cladosporium type sporulation with occasional Rhinocladiella type, in the 2 isolates, is in agreement with the original description of Carrión (1950), although no Phialophora type was observed. Rippon (1974) states that F. dermatitidis produces inconspicuous phialides, that it may sporulate pleurogenously (Rhinocladiella type) and that sporulation of all types by this species is usually sparse.

The pathogenicity of F. dermatitidis to mice has been demonstrated by Jotisankasa, Nielsen and Conant (1970). They infected normal and cortisone-treated mice with 8 human isolates and concluded that all isolates were neurotropic, but that there were differences in virulence. In the present study, isolates V413 and V497 (F. dermatitidis) exhibited unequivocal affinity for the CNS invading brain and spinal cord, with fatal outcome. Invasion of the CNS by both isolates followed IV, IP and SC inoculation, as evidenced by the results of histopathological findings and cultural isolations. It is worth mentioning that Jotisankasa et al., (1970) were unable to induce lethal infections in normal mice by IP inoculation with heavy doses, but death was very common in animals which had been pretreated with cortisone and which received  $25 \times 10^6$  yeast cells intraperitoneally. Isolates V413 and V497 killed normal mice at much lower doses of  $2 \times 10^5$  yeast cells intraperitoneally and  $1 \times 10^5$  cells subcutaneously. These discrepancies may be due to strain differences within F. dermatitidis as was suggested by Jotisankasa et al., (1970) or may be because some of their isolates were so old that they might have become less virulent.

Both isolates V413 and V497 proliferate freely in the brain and spinal cord as long branching hyphae but in other organs such as the liver, lung, spleen and kidney they occur as short chains of swollen cells (moniliform hyphae). This latter form might have developed into the classical tissue form of sclerotic bodies noticeable in spontaneous infections with dematiaceous fungi, had the animals been able to survive for any length of time. In the SC granulomata, both isolates occur in the yeast form only, whereas in the peritoneal connective tissue, they occur in both the yeast and hyphal forms, with occasional moniliform hyphae.

Table 29 . In vivo and in vitro characteristics of the black yeast isolates V413, V497 and V436

Isolates	Growth at 37°C	Growth at 43°C	Gelatin liquefaction	Urea hydrolysis	Mouse pathogenicity	Neuro-tropism	Types of sporulation
V413	+	-	-	+	+	+	Predominantly <u>Cladosporium</u> -type with occasional <u>Rhinocladiella</u> -type
V497	+	-	-	+	+	+	Predominantly <u>Cladosporium</u> -type with occasional <u>Rhinocladiella</u> -type
V436	-	ND	+	+	-	-	Entirely <u>Cladosporium</u> -type

ND = Not done

Table 30 . Comparative virulence of isolates V413 and V497 in mice

Isolates	Route of infection	No. of mice infected	No. of deaths	Post infection time of death (days)						
				1	2	3	5	7	14	
V413	IV	3	3	1	1	-	-	-	-	1
	IP	3	3	-	1	-	-	2	-	-
	SC	3	1	-	-	-	1	-	-	-
V497	IV	3	3	2	-	1	-	-	-	-
	IP	3	3	-	1	2	-	-	-	-
	SC	3	3	-	-	-	2	1	-	-

IV = intravenous  
 IP = intraperitoneal  
 SC = subcutaneous



Table 31 . Pathogenicity to mice of isolates V413, V497 and V436.

Isolate		V413			V497			V436		
Route of infection		IV	IP	SC	IV	IP	SC	IV	IP	SC
No. of mice challenged		3	3	3	3	3	3	3	3	3
No. of mice which died		3	3	1	3	3	3	2	0	0
No. of mice with positive culture from:	Heart blood	2	1	0	3	3	2	2	0	0
	Lungs	3	3	1	3	3	3	2	0	0
	Liver	3	3	1	3	3	3	2	0	0
	Spleen	3	3	2	3	3	3	2	0	0
	Kidneys	3	3	1	3	3	3	2	0	0
	Brain	3	3	1	3	3	3	2	0	0
	Spinal cord	3	3	1	3	3	3	2	0	0
	Peritoneum	ND	3	ND	ND	3	ND	ND	ND	ND
SC lesions	ND	ND	3	ND	ND	3	ND	ND	ND	

IV = intravenous  
 IP = intraperitoneal  
 SC = subcutaneous  
 ND = not done

Fig. 37:

Phialophora type of sporulation.

(After Rippon, 1974)

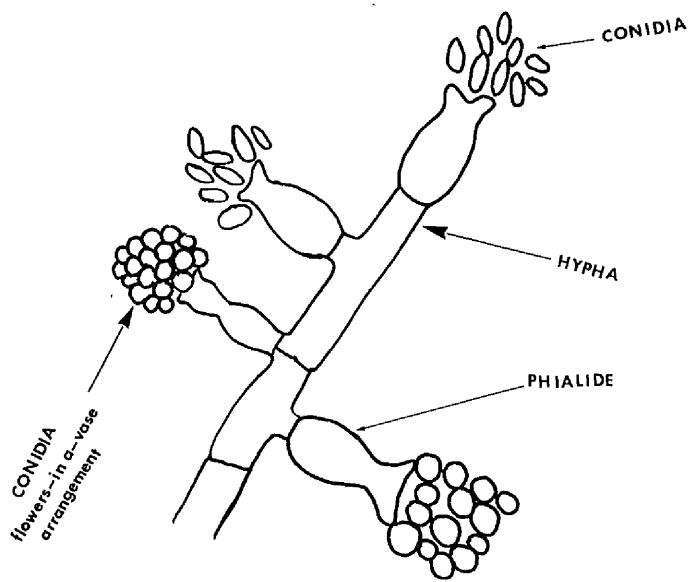


Fig. 38:

Rhinocladiella (Acrotheca) type of sporulation.

(After Rippon, 1974)

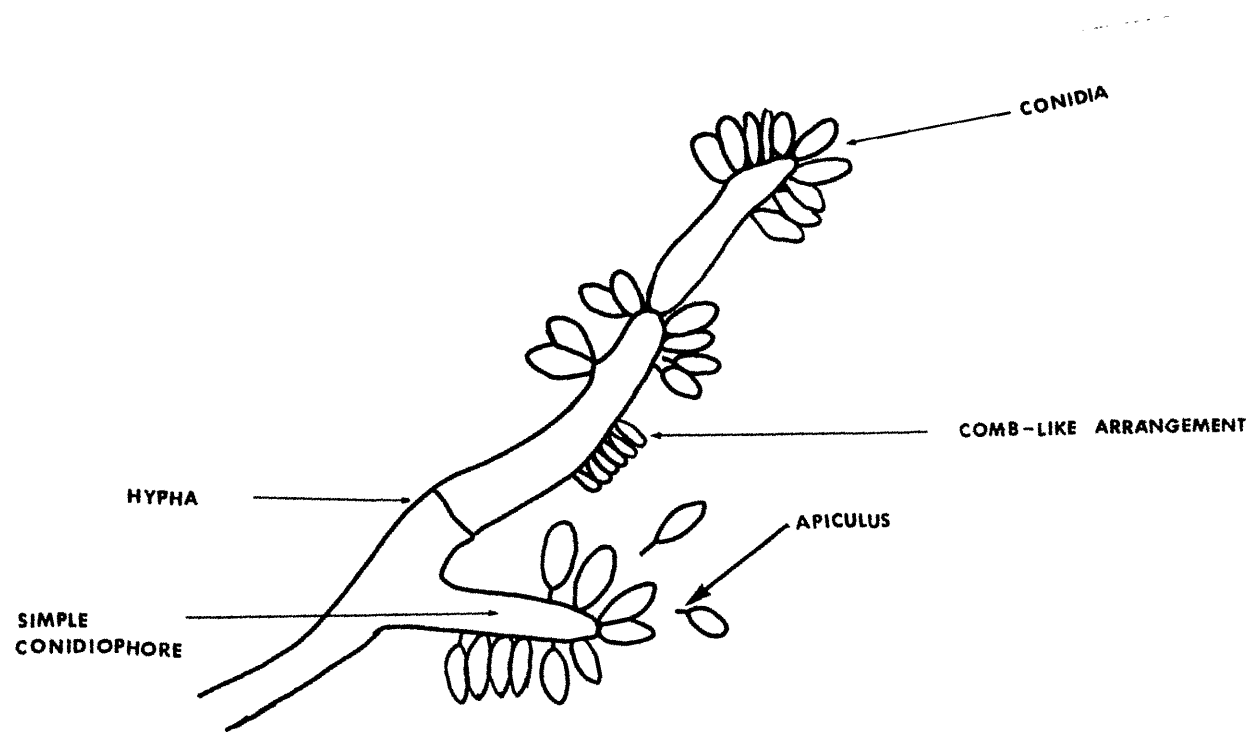
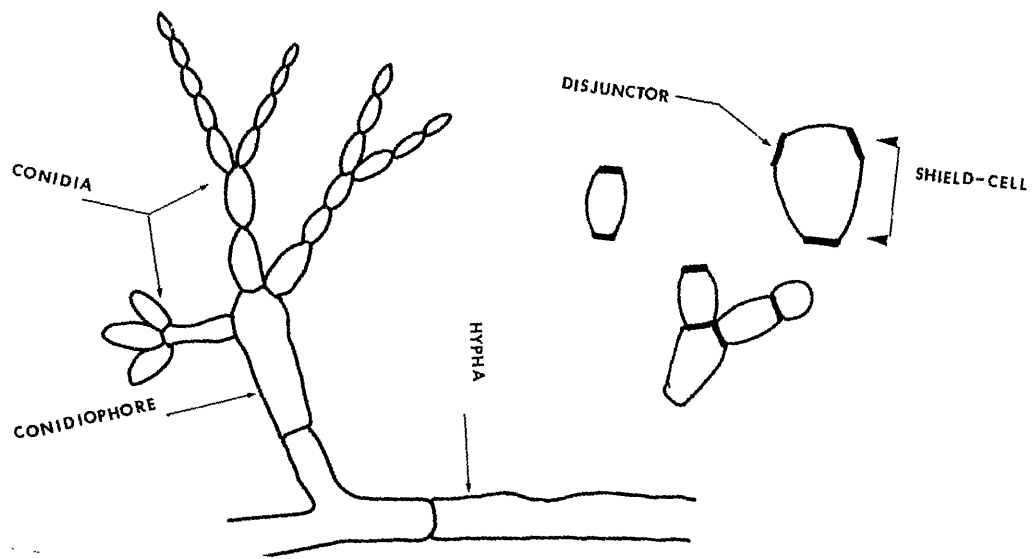


Fig. 39.

Cladosporium (Hormodendrum) type of sporulation.

(After Rippon, 1974)



Figs. 40-41: Colonial morphology of black yeast isolate V413

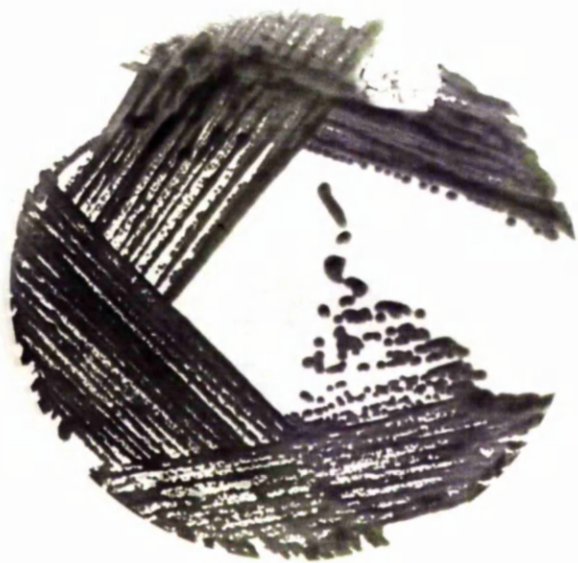
Fig. 40:

One-week-old streak culture on SDA at 28<sup>o</sup>C, showing a smooth yeast-like growth.

Fig. 41:

Four-weeks-old mycelial growth on PDA at 28<sup>o</sup>C, showing a circular, slightly raised radially furrowed colony, with a central mycelial growth and concentric zoning towards the periphery intermingled with yeast-like growth.





Figs. 42-44: Isolate V497

Fig. 42:

One-week-old yeast-like growth on SDA at 28°C, with black pigmentation.

Fig. 43:

Four-weeks-old mycelial growth, on PDA at 28°C, showing a circular colony with powdery texture and concentric zoning.

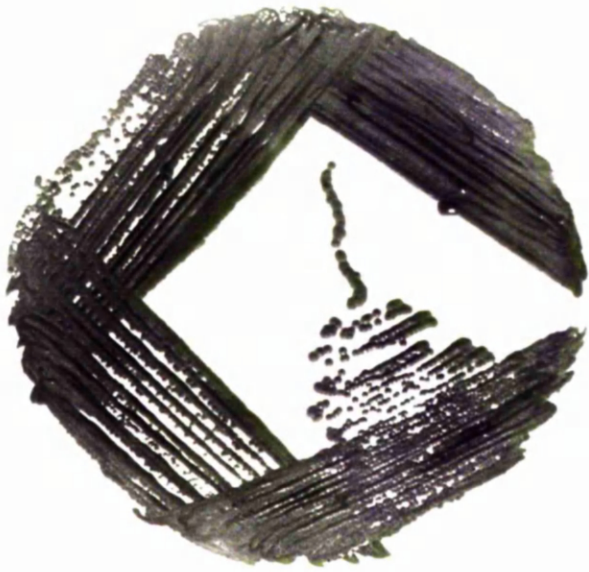


Fig. 44:

Wet mount from a 3-days-old culture, on SDA at 28<sup>o</sup> C,  
showing single, ovoid, thin-walled yeast cells and bud  
scars (arrows) on some cells.



Figs. 45-46: Types of sporulation in isolate V413.

Fig. 45:

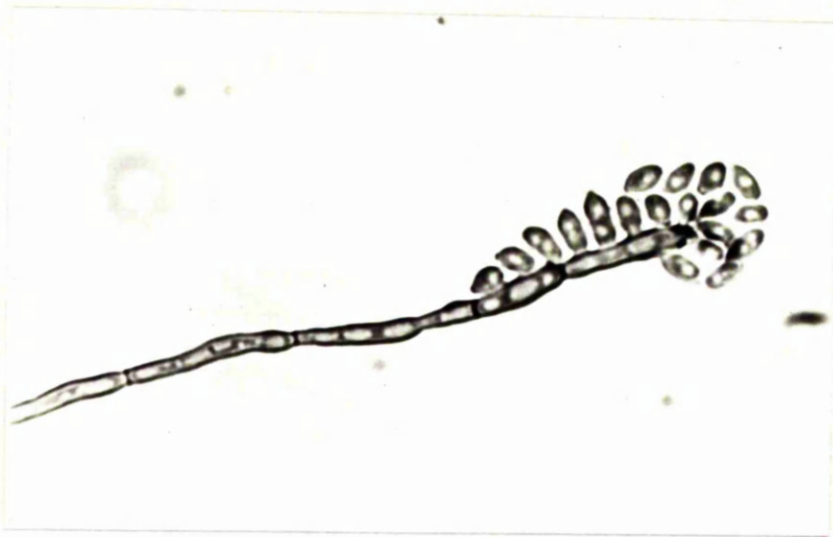
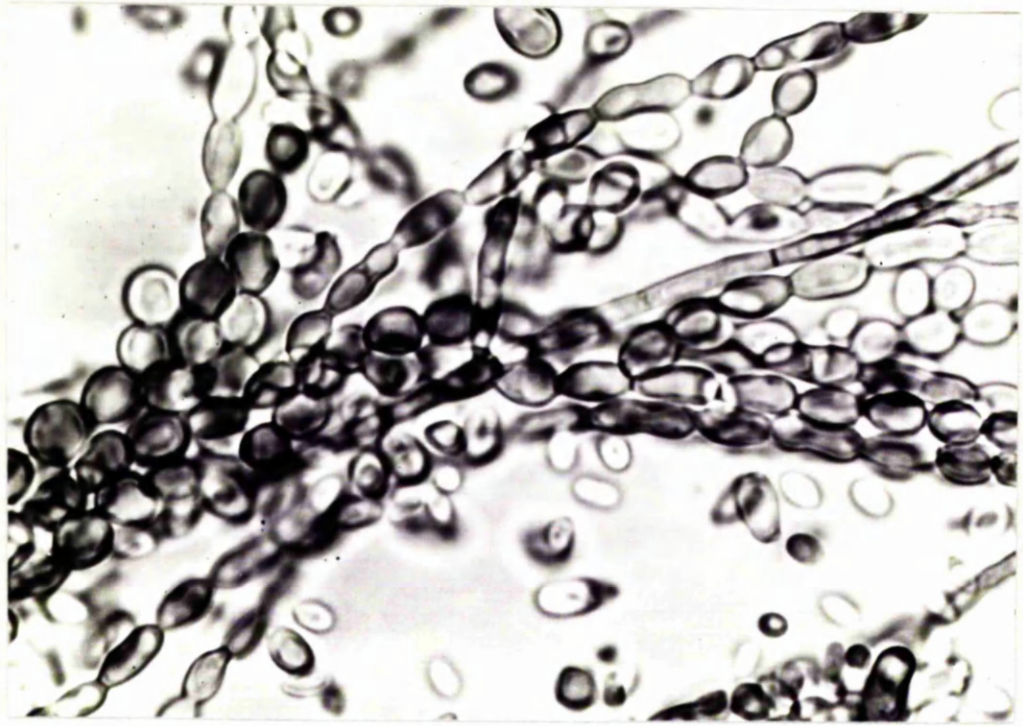
Long chains of spores in Cladosporium-like pattern, from  
a 6-weeks-old slide-culture on PDA at 28<sup>o</sup> C.

(Lactophenol, x 600).

Fig. 46:

Rhinocladiella-like arrangement of spores, in the same  
slide-culture in Fig. 45.

(x 600)



Figs. 47-48: Types of sporulation in isolate V497.

Fig. 47:

Cladosporium-like sporulation, in a teased preparation, after 8 weeks on SDA at 28<sup>o</sup>C. Disjunctors were visible on some cells (arrows).

(Lactophenol cotton blue, x 600)

Fig. 48:

Rhinocladiella-like pattern in a 6-weeks-old slide-culture on SDA at 28<sup>o</sup>C.

(Lactophenol, x 600)





Figs. 49-52: Isolate V436.

Fig. 49:

One-week-old yeast-like growth, on SDA at 28<sup>o</sup>C, with tar black pigmentation.

Fig. 50:

Four-weeks-old mycelial phase, on SDA at 28<sup>o</sup>C.

Colony was lobulate and sectored: an olive-grey membranous sector and a smooth black flat one.



Fig. 51:

Wet mount from a 3-days-old culture on SDA at 28<sup>o</sup>C,  
showing elongate hyaline cells; transverse septa were  
visible in some of the cells.

(x 600)

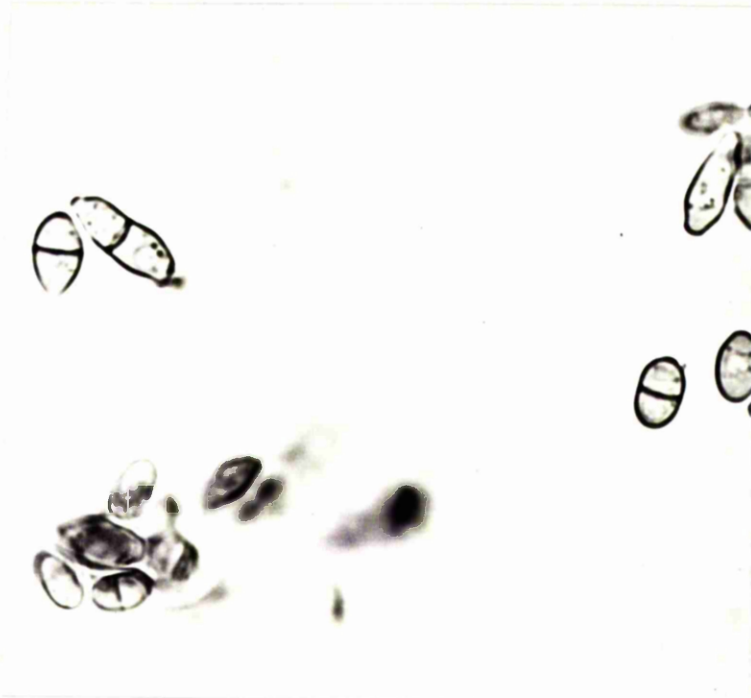


Fig. 52:

Cladosporium-type of sporulation, with long chains of thick-walled conidia; from a 6-weeks-old slide-culture on SDA at 28<sup>o</sup> C.

(Lactophenol, x 240)

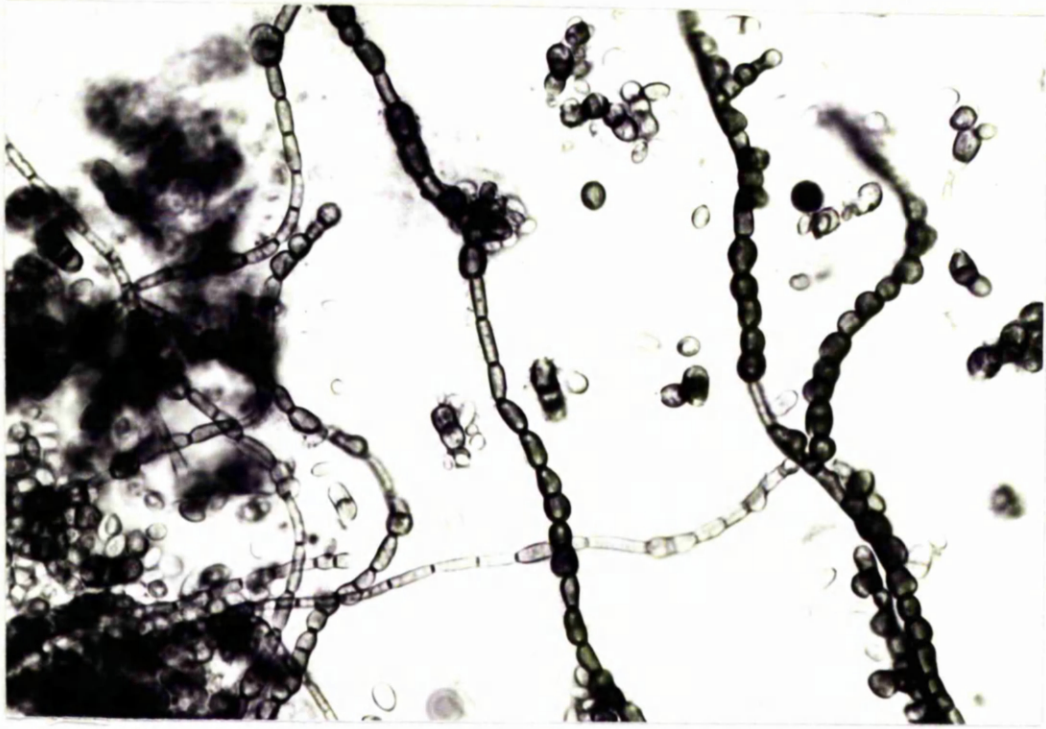


Fig. 53:

Subcutaneous granuloma in a mouse, 16 days after  
SC infection with isolate V413.





Fig. 54:

KOH touch preparation from the liver of a mouse infected intraperitoneally with isolate V413. The fungus was present in the form of short chains of rounded swollen cells (moniliform bodies).

(x 250)

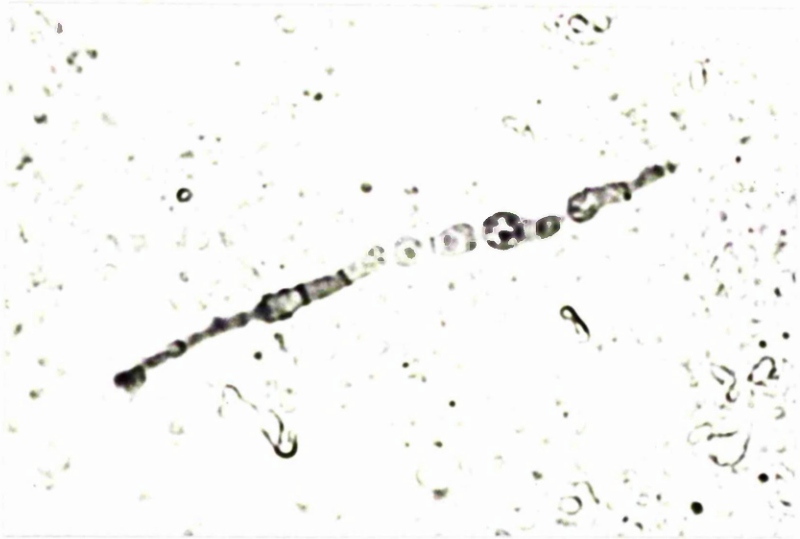


Fig. 55:

KOH squash preparation of brain from the same mouse in Fig. 54, showing abundant long branching slender hyphae and a few blastospores.

(x 250)



Fig. 56:

Section of brain from a mouse infected intraperitoneally with V413, Long branching hyphae were invading the brain.

(P.A.S., x 400)

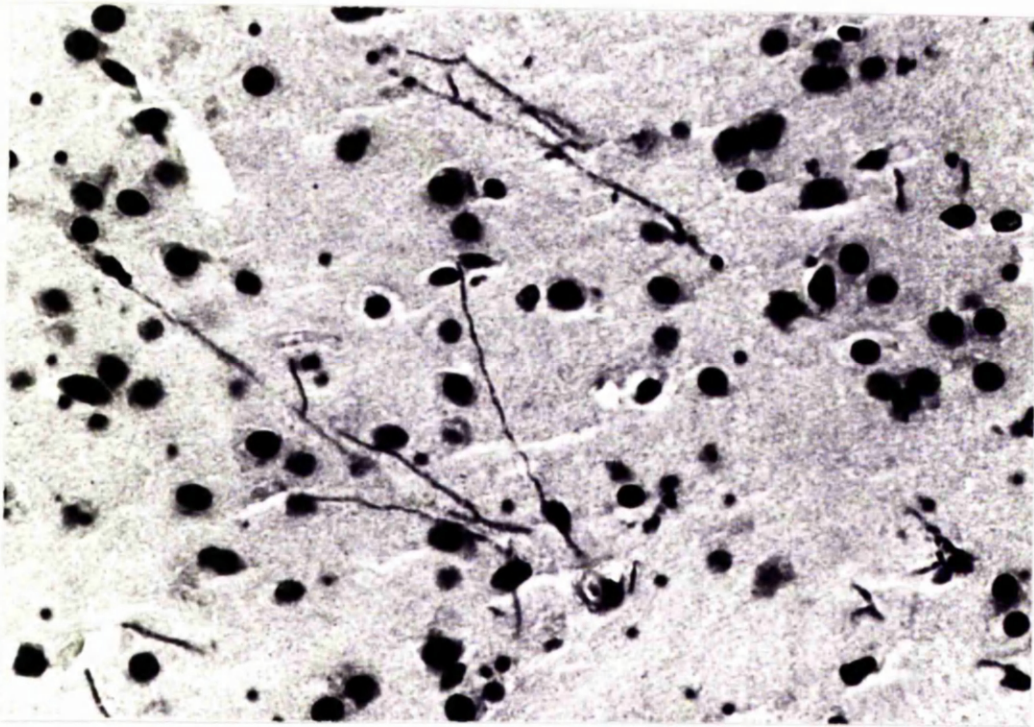


Fig. 57:

Hyphal invasion of mouse brain following SC inoculation  
with V413.

(P.A.S., x 400)



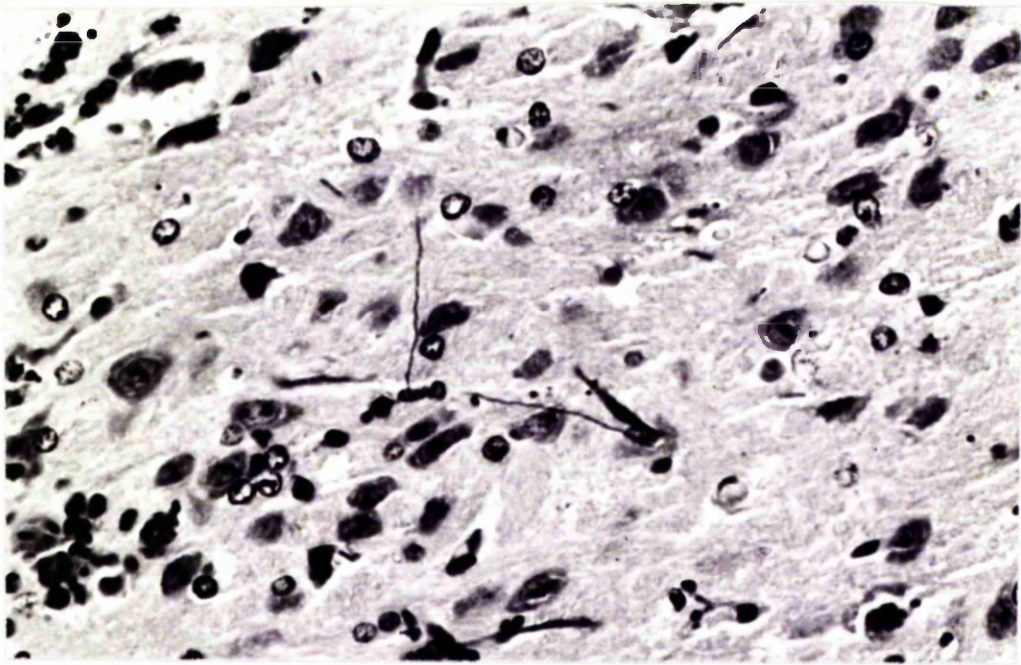


Fig. 58:

Hyphal invasion of mouse brain following SC inoculation  
with V497.

(P.A.S., x 400)

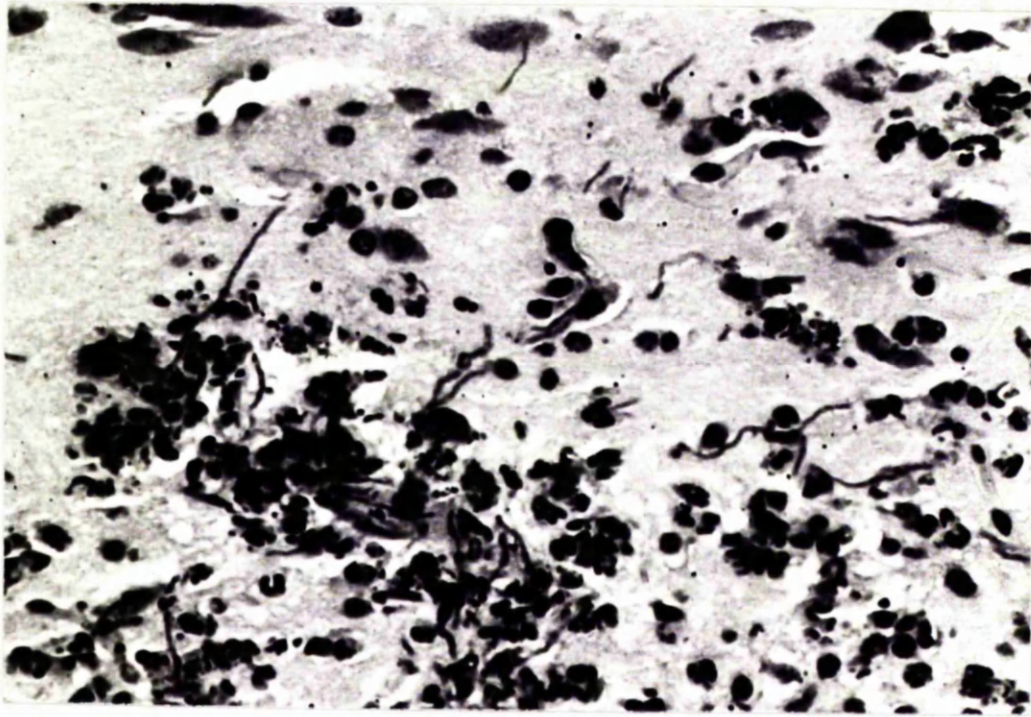


Fig. 59:

Many yeast cells, hyphae and moniliform-like hyphae (arrow) in the peritoneal connective tissue of a mouse infected intraperitoneally with V413.

(P.A.S., x 250)

Fig. 60:

High power view of Fig. 59.

(x 1000)



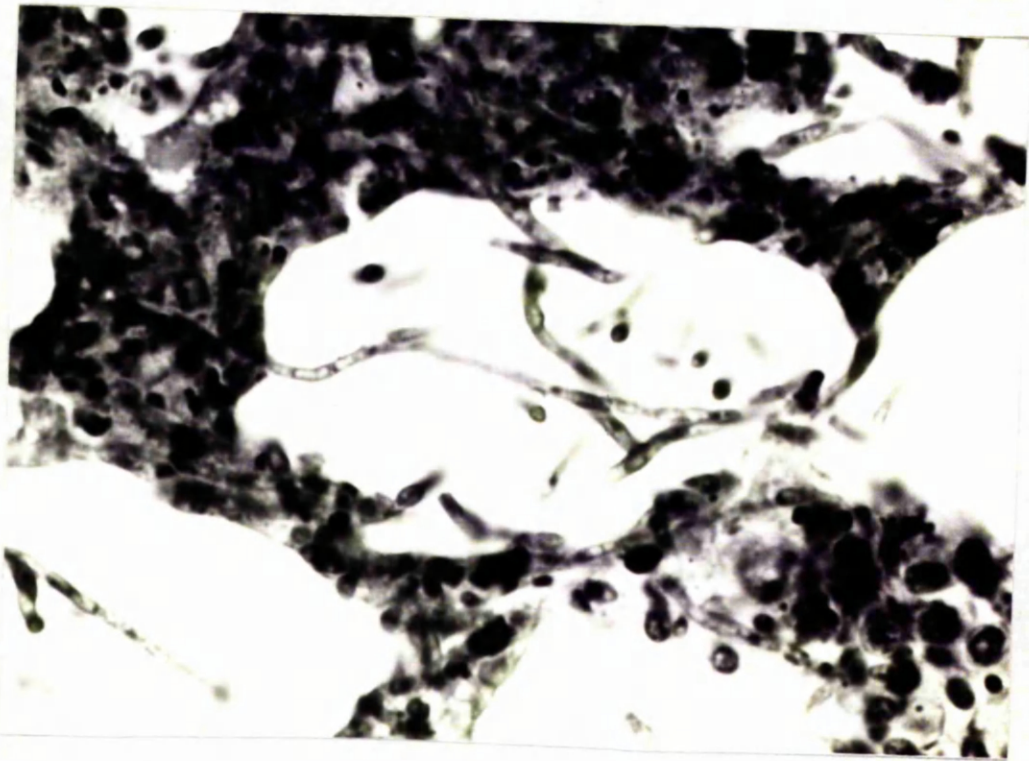
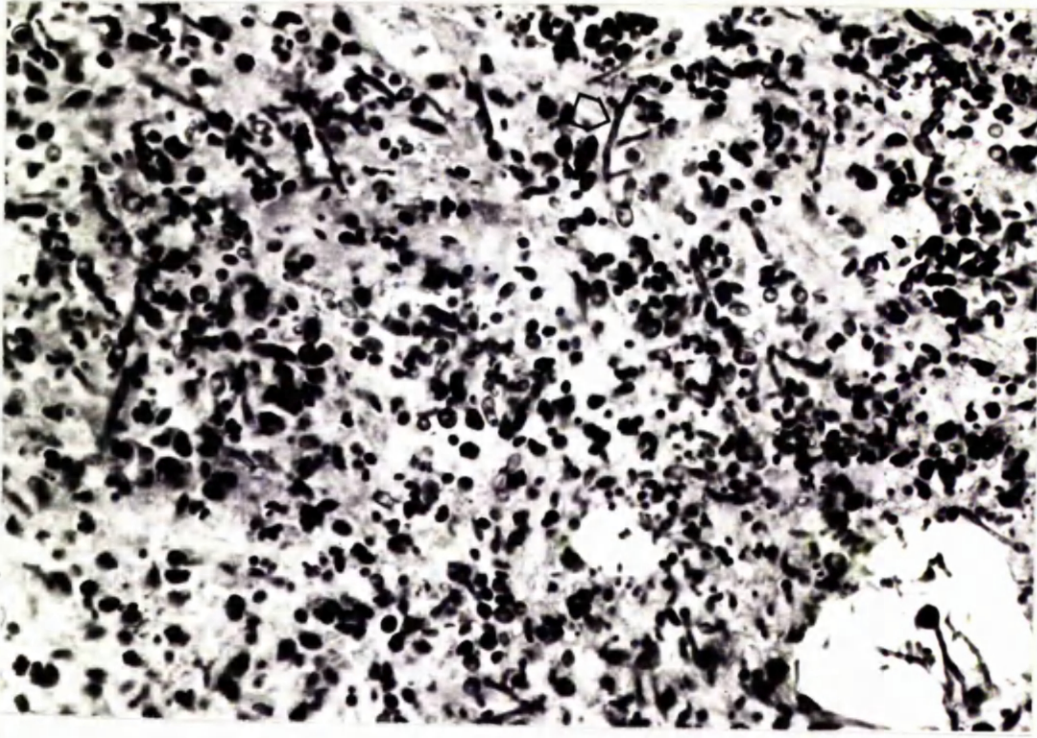


Fig. 61: Section through the SC granuloma in Fig. 53.

The fungus was present in the yeast-form, surrounded by an extensive inflammatory reaction.

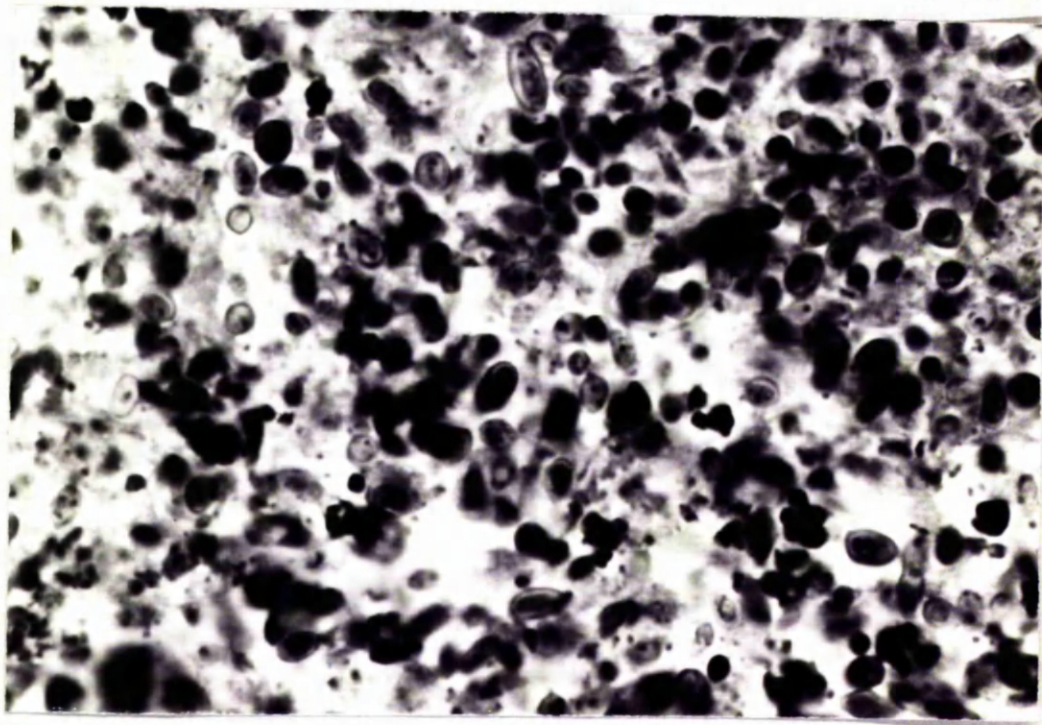
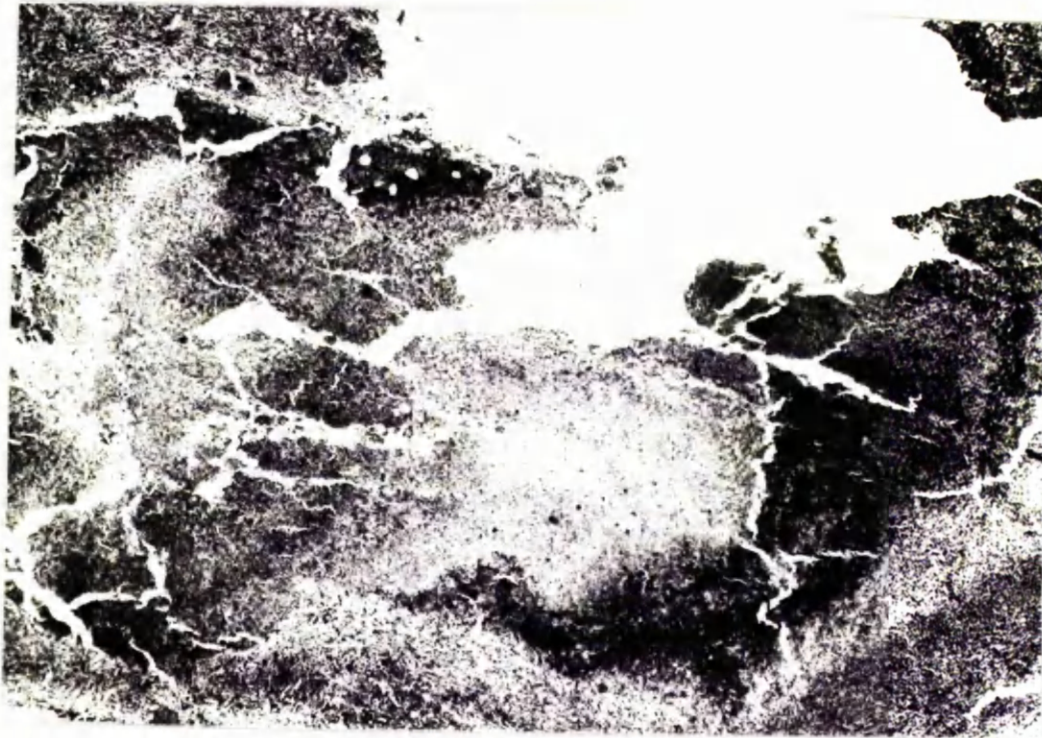
(P.A.S., x 30)

Fig. 62:

Higher magnification of Fig. 61, showing many yeast cells amid the necrotic debris.

( x 1000)





CONCLUSIONS



Investigation of pathological specimens has shown that animal diseases attributable to yeasts are not at all common in the area served by the Veterinary Hospital, Glasgow. Canine otitis externa associated with Pit. pachydermatis was the only disease condition encountered with any degree of frequency. Candida albicans was diagnosed as the cause of 2 cases of oral thrush in guinea-pigs. The disease was thought to be of a primary nature in both animals as no predisposing factors could be found. This yeast was also associated with cases of gastroenteritis in 3 puppies, in 2 of which prior antibiotic treatment might have caused proliferation of the yeast. A fatal gastroenteritis and systemic infection in a racing pigeon was believed to have been caused by Torulopsis pintolopesii which was isolated in heavy and profuse growth from liver and spleen as well as from the intestinal tract.

Black yeasts, isolated from samples taken at autopsy from 2 dogs and a pony, but not considered to be of aetiological significance in these cases, were identified and a pathogenicity study in mice undertaken. One Cladosporium sp. was non-pathogenic but 2 strains of Fonsecaea dermatitidis were highly virulent, showing marked neurotropic affinity. It is, therefore, suggested that black yeasts should be considered in differential diagnosis in animals with diseases of the nervous system.

One interesting result from the investigation of the yeast flora of canine skins was that Pit. pachydermatis was recovered from diseased skins 5 times more frequently than from healthy ones. It may be that pathological changes in the canine skin render it favourable for the proliferation of this yeast or it may be that this yeast is capable of causing skin infection in the dog.

The most frequently encountered yeasts on normal dog skin were T. candida, Rh. rubra and Tri. cutaneum and these might be regarded as commensals. Other species which occurred sporadically were assumed to be contaminants from the environment.

The yeast flora of the nasal cavity of dogs and cats was investigated by culture of turbinates. Although Cr. neoformans was not recovered, a number of saprophytic cryptococci were isolated. These were present more frequently in dogs than in cats. Species in the genera Candida, Rhodotorula and Torulopsis were common in dogs and cats.

Because 2 cases of oral thrush were diagnosed in guinea-pigs, the yeast flora of the mouth of normal guinea-pigs was investigated. The predominant species was Saccharomyces telluris, followed by C. albicans which was present in 6% of the animals. As far as I am aware, the isolation of C. clferrii from a guinea-pig represents the first record of the occurrence of this species in Scotland.

In the serological survey, precipitating antibody to Cr. neoformans was demonstrated in sera from dogs and cats by the CIE test, some canine sera were also positive by ID, but no serum gave a positive agglutination reaction.

The demonstration of antibody in apparently normal dogs and cats in conjunction with the lack of cases of cryptococcosis in this area suggests that either these exposed animals have a high innate resistance to infection by Cr. neoformans or that most of the strains of this yeast present in nature are of low virulence.

By using antigens prepared from each serotype, evidence suggesting that serotypes A, B, C and D must occur in nature in this country was

obtained. An interesting difference between positive feline and canine sera was noted. The most prevalent antibody in feline sera was that to serotype D whilst in canine sera antibody to serotype B predominated. No satisfactory explanation for this variation was found.

As might be expected, the proportion of animals with antibody was higher in rural than in urban populations.

In experimental studies, strains of Cr. neoformans, even those proved to be virulent for mice were, with one exception, non-pathogenic to cats. Only one of the 8 strains caused disease. None of the 16 cats challenged by the intranasal route developed overt infection, but 3 of the 8 intravenously infected cats developed clinical disease.

Predisposing factors appear to be important in enabling the disease to develop. One of the 3 diseased cats had feline leukaemia virus infection and another had calicivirus infection concurrently with cryptococcosis. No predisposing condition was recognised in the third infected cat, nor in a clinically normal cat killed as a control 17 days after intravenous inoculation with the same strains as the infected cats. At post mortem, this animal was found to have Cr. neoformans in the spleen, lungs, kidney, urine and nasal cavity. The clinical signs of disease shown by the cats varied markedly, confirming that feline cryptococcosis presents no well-defined clinical pattern.

Cryptococcus neoformans was not recovered in culture, at autopsy, from cats inoculated intravenously but which did not develop the disease. Cultures from cats infected by the intranasal route showed that Cr. neoformans remained viable within the nasal cavity for long periods,

suggesting that cats are unable to clear yeast cells from this site.

If such "carrier" cats become compromised, it seems possible that the disease might be activated.

Mycological methods of in vivo diagnosis were investigated. Apart from the first few days after intranasal challenge, cultures of nasal swabs were negative even from cats in which viable yeast was shown to be present in the nasal cavity. Retrospectively, it was realized that nasal washing would probably be of use in diagnosis. Negative results were also obtained from the culture of bronchial washings and faecal samples.

The yeast was, however, recovered in culture from an ocular discharge and from urine from 2 infected cats and from the control cat which was, I believe, developing cryptococcosis. It is therefore suggested that, as an aid to diagnosis, urine samples from cats with suspected cryptococcosis should be cultured.

As with the positive serum samples in the survey, sera from experimental cats were positive by CIE, giving one line of precipitation and all were negative at a 1:2 dilution. However, sera from 3 intranasally challenged cats were also positive by ID. Agglutinating antibody was not demonstrated in any serum from an experimental cat.

Precipitating antibody to Cr. neoformans developed in cats within 2 weeks of challenge. Antibody persisted for longer in cats infected intranasally than in those infected intravenously, probably because the yeast remained within the nasal cavity.

Sera from cats infected with individual serotypes were initially positive for antibody only to the infecting serotype, but later showed cross reactions with other serotypes. It was surprising that none of

the serum samples from the 15 cats infected with the 4 untyped strains gave a positive reaction with any serotype antigen. This seems to suggest that there may be another serotype of Cr. neoformans.

As a result of this work, it has been shown that the presence of antigen is the only definite serological proof of active cryptococcosis in the cat. Cryptococcal antigen was present in the sera of the 3 cats with clinical infection and in the control cat which was believed to be in the process of developing disease. It was shown that antibody could result from exposure to viable or killed cells and could persist for a long time, even in cats free from clinical disease. The presence of antibody cannot, therefore, be regarded as proof of active cryptococcosis. However, it could be a significant supplementary diagnostic factor in an animal with clinical signs suggestive of cryptococcosis.

In the course of this experimental study, 2 factors of significance in the epizootiology of cryptococcosis were noted. It was shown that cats with cryptococcosis can excrete the yeast in ocular discharges and in urine and that in quantity sufficient to affect normal in-contact cats. Control cats kept in the same room as infected cats developed antibody to Cr. neoformans within 4 weeks, proving that the yeast must have been present in the environment and that, to induce the production of antibody, yeast cells must have entered their bodies.

In view of this, I believe that cryptococcosis should be regarded as a contagious disease and that affected animals should be strictly isolated.

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