Aspergillomycosis in cultured tilapias

by

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Dedication

Dedicated to Adeola my wife, whose patience, encouragement and help eventually allowed the completion of this study; and to Jola, Fisayo and Bamidele my daughters, Ekunola my son, all of whom had to cope with my absence during the first year of this work, and later, my moments of depression occasioned by the rigours, frustrations and uncertainties of scientific research.

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I thank all the technical staff of the Institute for their tolerance, especially as regards my handling the potentially highly pathogenic human pathogen - Aspergillus - during the mycological aspects of my work. I thank especially Mary Alexander and Paul Thomson for accommodating me in the histology unit and for their advice with my sections and staining methods.

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CONTENTS

ABSTRACT

A study of aspergillomycosis in cultured tilapias was initiated following the observation that high mortalities associated with *Aspergillus* species occurred in an intensive tilapia culture system. Extensive virological and bacteriological examination of clinical specimens failed to reveal any evidence of any other pathogen; however, histopathological examination indicated the presence of a fungus. The organism was isolated and identified. *Aspergillus flavus* Link ex Fries; *A. niger* van Tieghen var phoenicis (Corda) Al-Musallam; *A. terreus* Thom; and *A. japonicus* Saito were isolated from such infected tilapia tissues as well as from tilapia pelleted rations; and *A. clavatus* Desmazieres; *A. chevalieri* Thom and Church; *A. repens* (Corda) Sacc; *A. candidus* Link ex Fries and *A. sejunctus* Bainier and Sartory were isolated from tilapia feeds but not the host fish. Experimental pathogenesis, established a definite cause-effect relationship of the various *Aspergillus* isolates. The infective *Aspergilli* were consistently re-isolated from the visceral organs of experimentally infected tilapias. The experiments provided positive support for the speculation that under aquaculture conditions, *Aspergillus* infections occur via contaminated food, subsequent to which systemic spread occurs Water temperature and fish confinement were important factors, among others, in the elucidation of the epizootiology of the disease in cultured tilapias.

Antibody production against *A. flavus*, *A. niger* and *A. japonicus* was detected by Ouchterlony gel diffusion-precipitation technique from tilapias infected with *A. flavus* organisms, but

was shown to be absent from uninfected tilapias. The indirect fluorescent antibody technique (FAT) gave a specific fluorescence with digested tissue sections from Aspergillus infected tilapias. Aspergillus antigens in formalin fixed tissues were suitable for such tests. Appropriate control measures include prevention by good husbandry practices and surveillance.

ASPERGILLOMYCOSIS IN CULTURED TILAPIAS

CHAPTER 1

1.1 HISTORICAL BACKGROUND

The genus of moulds, Aspergillus, has always been a factor in man's environment. Even before the development of the microscope, their colonies were recognised as the white, yellow, green, red or black moulds seen on foods, rotting vegetation etc, although serious attempts at interpretation as to the cause of the growths were impossible.

Micheli (1729) was the first to distinguish the stalks and spore heads peculiar to the genus. He noted that the spore chains radiated from a central structure to produce a pattern that suggested the aspergillum with which he, as a priest, was familiar. He therefore applied the name Aspergillus to the moulds he observed because of the similarity in appearance between the fruiting head of the mould and the brush aspergillum (L. rough head) used for sprinkling of holy water.

It was not until the middle of the nineteenth century that the Aspergilli began to be recognised as active agents in processes of decay, as fermenting agents capable of producing valuable metabolic products, and as causes of human and animal disease. With such cognizance came the first adequately described and illustrated species in the works of Fresenius, Van Tieghem, and De Bary, although Link (1809) had earlier introduced a few species names that are still recognised. The genus began to take definite form with the work of Wehmer (1901).

In 1926, Thom and Church attempted to put together all available material in the now obsolete monograph, The Aspergilli.

Continued study of the aspergilli showed some of their groupings to be inadequate and considerable new information became available as the genus became increasingly investigated. In 1945, Thom and Raper published A manual of the Aspergilli based on comparative study of thousands of strains of aspergilli in laboratory culture together with a few species recognised from published descriptions alone. In the years since 1945, many new species of Aspergillus have been described. Altogether, 132 species and 18 varieties are now recognised, in contrast to 77 species, and 8 varieties in the Manual of 1945. (Raper and Fennell, 1965).

Parallel with the dramatic increase in the size of the genus has been an even greater proliferation of the published literature relating to the aspergilli as agents of decomposition, as tools for physiological and genetic studies, as agents responsible for the production of a variety of products in industry and significantly, as primary or secondary pathogens of animals and man.

1.2 CLASSIFICATION

Considerable confusion exists as to the names which should be applied to members of the genus and not all mycologists agree with the current principles used in its classification. As originally used by Micheli (1729), the name was applied to the sexual stage of certain common species. Eighty years later, Link (1809) introduced the name Aspergillus glaucus for the conidial heads of a fungus he found on herbarium specimens, and not realizing their common origin, applied the name Eurotium herbariorum to the yellow cleistothecia formed by the same fungus. The common mycelial origin of the structures to which these names were given was clearly demonstrated by De Bary in 1854. Thom

and Church (1926) and Thom and Raper (1945) suggested that the generic name Aspergillus should be applied whether or not an ascosporic (sexual) stage was produced. Their suggestion was based on two principles viz: that finding and describing the sexual stage merely completed the characterisation of a fungus, usually already known and that adopting such a classification would avoid the unjustified separation of the limited number of aspergilli that develop a sexual stage from the majority of the species that do not. The precedent for this practice had been established by Fischer in his treatment of the aspergilli in Engler and Prantl's Naturliche Pflazen Familien (1897) (cited by Raper & Fennell, 1965), and by Wehmer in his monograph, Die Pilzgattung Aspergillus (1901). However, some recognised authorities have suggested other classifications, placing the aspergilli within both the ascomycetes and the Fungi imperfecti.

Thom and Raper (1945) emphasized the need for broadening the application of the name Aspergillus to include organisms whose structures point to close natural relationship. Careful consideration was given to the question as to whether the whole group should be retained as Aspergillus or divided into lesser entities such as Eurotium of Link (1809), Sterigmatocystis of Cramer (1859), Aspergillopsis of Spegazzini (1911), Diplostephanus of Langeron (1922), etc. The arguments for keeping the organisms in a single genus appear however to have outweighed any advantages that might have stemmed from subdividing them upon the basis of head size, uniseriate versus biseriate phialides (sterigmata) or the presence or absence of a sexual stage. Although a large body of biochemical information is now available, identification of Aspergillus has continued, as in the past, to be based on their morphological, developmental and cultural characterization, and

the morphology of the asexual states has continued as a primary characteristic for determination of placement within the genus.

Raper and Fennell (1965) recognised 132 species and 18 varieties in the genus. These they classified in 18 groups of one to several species having certain basic characteristics in common. These "groups" are not accredited the status of taxa but do serve as convenient slots for fast characterisation of strains. The characters utilized for delineation of groups include:

(i) Head colour - black, white, yellow, brown and various shades of green.

(ii) Head shape - globose, radiate, loosely or compactly columnar.

(iii) Phialides (sterigmata) in a single or double series developed directly from the vesicle or from metulae.

(iv) Vesicle shape - clavate, globose, subglobose, subclavate, turbinate, hemispherical, or terminally flattened and without or with constriction in the conidiophore immediately below the vesicle.

(v) Conidiophores - smooth or variously roughened, coloured or uncoloured.

(vi) Hülle cells - presence or absence and shape.

These major separating characters are usually clearly evident, and with this information alone, a group identification of an unknown isolate, can be made with nearly complete confidence. There are exceptions, of course, and an occasional species shows characters that appear to align it with more than one group. In these instances, species are assigned where the preponderance of their characters indicate.

1.3 THE PATHOGENIC ASPERGILLI

The aspergilli were among the first organisms to be recognised in pathological conditions in man and animals. Their role in a number of aetiological controversies appeared to have dominated much of nineteenth century medicine. Between the early observations of moulds or mucors, within living animal bodies during the years 1810 to 1820 and the experimental proof of the pathogenicity of the aspergilli in the 1880's, opinions varied from placing a causal significance on the presence of these fungi in lesions to dismissing them as common moulds which could be in no way involved in disease processes. The prevalence of the latter opinion and the lack of precise descriptions are the main problems in interpreting the early publications. In many cases these doubts exist largely because of difficulties with actual identification of the fungi and the diseases being described.

After 1860, the main pathogenic species of fungi appear to have been adequately described and the clarification enabled several investigators to use fungi in experimental work, thus contributing to their growing knowledge of epidemiology and pathology. By 1900, many forms of infection by the aspergilli had been described in domesticated animals, but with the rise in significance of the sciences of bacteriology and virology, these diseases and other systemic mycoses were subsequently neglected until recent years.

The first account of a fungus growing within a living animal was that of Montague (1813), who found a "mold or blue Mucor" within the thoracic airsac of a scaup duck (Aythya marila). Mayer and Emmert (1815) reported a similar condition in a jay

(Garrulus glandularis), and Jager (1816) saw a green fungus in the thoracic air sacs of two swans (Cygnus olor). The first recognition of the association of an Aspergillus sp. with such lesions appears to have been by Rayer and Montagne (1842), who identified A. candidus from the air sac of a bullfinch (Pyrrhula pyrrhula).

The first record of aspergillosis in a mammal was probably the mycotic lung lesions in an axis deer (Cervus axis) briefly mentioned by Rousseau and Serrurier (1841) incidental to their description of a similar infection in a parrakeet. The role of the aspergilli in human pulmonary disease was first stated in the classical work of Virchow in 1856.

From the second half of the nineteenth century, the occurrence of aspergilli in disease conditions was reported frequently, but their significance was differently interpreted. Inadequate descriptions prevented identification of the species of Aspergilli involved. A. niger was first reported by Cramer (1859) from ear infections (as Sterigmatocystis antacustica), and A. nidulans and A. flavus, also from otitis externa, were recorded by Siebenmann (1889). A. terreus appears to have been first obtained from a disease condition by Langeron in 1922, who named a culture isolated from a human ear in Brazil, S. hortai. A. restrictus has been shown by Marsalek et al (1960), to be capable of producing disease in man and animals.

From the time of its discovery in a Scaup duck by Montague in 1813 and in a jay by Mayer and Emmert in 1815, until the end of the nineteenth century, many papers appeared on aspergillosis. It has not been possible to ascertain precisely the priority of the authorities responsible for the recognition of aspergillosis

in the different species of domesticated animals because of the problems of identification.

1.4 AVIAN ASPERGILLOSIS

There are reports of outbreaks or individual cases of aspergillosis in such a large number of species of birds that a complete list cannot be attempted. The reviews of Verge (1927), Urbain and Guillot (1938), and Lesbouyries (1941) and the bibliography by Chute, O'Meara and Barden (1971) cite many records. The main groups of birds affected are:

1.4.1 Domesticated birds: fowl, turkey (Refai and Rieth, 1966; Hacking and Blandford, 1971), duck goose and pigeon (Taylor, 1966); especially young birds (Klimes and Rosa, 1964) and under conditions of intensive rearing (e.g. Asaj et al, 1965).

1.4.2 Captive wild birds: especially water birds e.g. penguins (Appleby, 1962; Kageruka, 1967; van den Sande, 1967). Reports of avian aspergillosis in zoological gardens include: Saez (1961), Paris; Dathe (1962), Berlin; Awad & Obeid (1962), Khartoum; Williamson, Tilden and Getty (1963), Chicago.

The clinical signs of aspergillosis are extremely varied because almost any organ of the avian body may be affected. They may be respiratory, digestive, or nervous and occur in birds of all ages. Acute aspergillosis typically occurs in very young birds while the chronic disease generally occurs sporadically and is the usual form to be described in adult birds.

Eye lesions have been noted in baby chicks by Hudson (1947) and in turkeys by Moore (1953), the latter author finding the

condition to be significantly more common in males than in females. Inflammatory lesions in the brain and meninges in turkey poults and in eider duckling (Somateria mollissima borealis) were reported by Hubben (1958).

Aspergillus fumigatus appears to be the commonest cause of avian aspergillosis but other species including A. flavus and A. nidulans have also been incriminated with varying frequency, (Ainsworth and Rewell, 1949). It has been established that infection usually follows the inhalation of fungus spores from mouldy litter, grain, meal or other sources. Chute and Barden (1964) demonstrated the abundance of aspergilli in the air of chick hatcheries. Although the quantitative aspects of natural infection are unknown, the main habitat of pathogenic aspergilli is in rotting plant and animal material especially damp hay, straw and grain which have heated up during storage.

Ross (1966) attributed an outbreak of aspergillosis in a broiler flock in Hawaii to the use of a sugar cane bagasse as litter and Szpakowski (1967) considered sawdust litter particularly dangerous source of aspergilli. While the source of infection for domestic fowls may often by traceable it is not so with the disease in free-living and captive wild birds. Davi s and McClung (1940) said that A. fumigatus developing in rotting seaweed was responsible for widespread deaths in herring gulls at Boston, U.S.A., whilst mouldy combine straw has been considered to have led to deaths of pheasants. In captive water birds the litter and food are generally regarded as the inoculum source, but Ainsworth and Rewell (1949) found advanced cases in birds which had been captured only two days previously so that infection must have been present in the wild.

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1.5 MAMMALIAN ASPERGILLOSIS

Except in man, aspergillosis is not a well-known disease of mammals, although it has been recognised with increasing frequency. It is probable that the most important aspect of mammalian aspergillosis is in connection with mycotic abortion in which infection of the placenta of cows is thought to be a sequel to subclinical pulmonary aspergillosis. The aspergilli may also contribute to allergic types of respiratory diseases such as pneumonitis and asthma, especially in young animals. Most of the reported cases of mammalian aspergillosis have been of the pulmonary disease, but systemic generalisation has been described. The clinical condition greatly resembles tuberculosis and is characterised by general wasting, usually followed by death.

Just as in the case of avian aspergillosis, a large number of the accounts of human disease are unsatisfactory both from the clinical and the mycological standpoints. The different forms of the disease in animals have not been studied sufficiently for a workable classification to be given. However, Rénon (1897) categorised the chronic, acute and the subacute disease in his review of mammalian aspergillosis which gave a comprehensive survey of the cases up to that period.

1.5.1 BOVINE ASPERGILLOSIS

Bournay (1895) reported aspergillosis in an eight year old cow. He noted signs which varied from a slight cough to laboured respiration, bloody and foetid diarrhoea. Lesions described consisted of cavitated sub-pleural nodules and inter lobular emphysema with inflammation of the small intestine. Typical pulmonary infection with nodule formation was also reported by

Pearson and Ravenel (1900) and further reports have been made at intervals (Bartolucci, 1903; Del Giudice, 1956; Eggert and Romberg, 1960; Mollelo and Busey, 1963; Griffin, 1969; and by Narayana et al, 1964 in a Murrah buffalo). Austwick (1962) studied the 'asteroid bodies' caused by Aspergillus fumigatus in the lungs of slaughtered dairy cows and discussed the significance of these bodies in relation to pulmonary aspergillosis and mycotic abortion. Bendixen (1926) described what appeared to have been a case of generalised aspergillosis in a cow with invasion of the lungs, liver, spleen and kidneys and another form of the disease was reported by Lucet (1897) in which generalised intestinal haemorrhages was a prominent feature and the lungs showed centres of hepatization without nodule formation.

1.5.2 OVINE ASPERGILLOSIS

There are relatively few reports of aspergillosis in sheep. The lesions in lambs consist of small (2-3 mm diam) bluish-grey nodules surrounded by a narrow haemorrhagic zone, and closely resemble those caused by the lungworm Muellerius capillaris (Andersen, 1927). Nobel and Shamir (1956) described a condition in which granulomatous lesions containing the hyphae of A. fumigatus were found in the lungs of a day-old lamb, and they considered the infection had taken place in utero.

1.5.3 EQUINE ASPERGILLOSIS

Foulerton (1899) described the lung of a horse with typical aspergillotic nodules. Additional cases were recorded by Nöller and Krause (1924), Tscherniak (1928), van der Gulden and Zuur (1961), Studaric (1965) and Long and Mitchell (1971). Meningeal involvement was reported by Romanov (1928). In the case

described by Thary and Lucet (1895) in a four-year-old mare, the disease developed rapidly and showed generalised interstitial haemorrhage with caseous nodules in the kidneys. Kardevan and Vetesi (1966) reported a generalised infection by A. fumigatus involving the intestine, liver and kidneys. Cook, Campbell and Dawson (1968) identified A. nidulans from four cases of equine guttural pouch mycosis but Björklund and Pålsson (1970) attributed their case to A. fumigatus.

1.5.4 PORCINE ASPERGILLOSIS

Records of aspergillosis in pigs are rare. Berg (1898-99) described the disease as a persistent cough associated with a lobar pneumonia and nodule formation. The spleen and kidney were enlarged and congested, and nodular lesions were found in the mesenteric lymph nodes. Berg did not obtain any isolates from the lesions but assumed that an Aspergillus was the cause of the disease.

1.5.5 ASPERGILLOSIS IN OTHER SPECIES OF DOMESTIC ANIMALS

Aspergillosis in a goat is recorded by Mori (1916) in Italy. Spontaneous aspergillosis in domestic rabbits has been reported by Schöppler (1919), Höppli (1923) and Marcato (1967), whilst Ainsworth and Austwick (1955) have reported the disease in guinea-pigs. Cases of respiratory aspergillosis have been found in the dog (Otto, 1970; Soltys and Sumner-Smith, 1971; Parker and Cunningham, 1971; Black and Nightingale, 1973; Lane, Clayton Jones, Thoday and Thomset, 1974; Lane and Warnock, 1977). Earlier records from this animal by Gotti (1871) and Stazzi (1905) are of nasal and auricular infection due to a variety of fungi. Cases in cats have been described by Sautter et al (1955); Pakes,

New and Benbrook (1967) and Soltys & Sumner-Smith, (1971).

1.6 ASPERGILLUS INDUCED DISEASES

Three types of disease are caused by the species of Aspergillus (Austwick, 1965). These are

1.6.1 Infection (Mycosis) - invasion of living tissue by the fungus. This invasion may be described as primary, when the fungus directly invades a healtny, susceptible organ of the body; or secondary, as a result of the growth of the fungus in active or arrested lesions of tuberculosis, histoplasmosis, carcinoma, etc, after wounding or after antibiotic and corticosteroid therapy of other diseases.

1.6.2 Allergy - associated with the regular inhalation of conidia (spores) or other contact with the fungus.

1.6.3 Toxicosis - arising from the ingestion of food containing toxic fungal metabolites.

The nomenclature of the diseases caused by the aspergilli has not been rationalised. It has usually been composed of a mixture of clinical, pathological and mycological terms used inconsistently and often on the basis of personal preference. The general term Aspergillosis embraces all infections caused by the aspergilli, but is usually restricted to the respiratory disease in man and animals. Pneumomycosis and bronchomycosis have often implied aspergillosis, but these terms are not specific and could be applied equally well to other lung mycoses unless they are qualified by the name of the causal organism. The

toxicoses caused by the ingestion of toxic metabolites are included in the mycotoxicoses, or more specifically for Aspergillus, aspergillotoxicosis. When individual toxins have been distinguished new terms have been coined - aflatoxicosis for the disease caused by "aflatoxin" produced by members of the A. flavus group. Such conditions are regularly recognised in cultured fishes vide infra. Aspergillomycosis has been used in the present study to denote systemic infections caused by species of Aspergillus regardless of the clinico-pathological picture. It embraces all such infections whether acute or chronic (Olufemi, Agius and Roberts, 1983).

1.7 MYCOTOXINS

Examination of recent scientific literature reveals an almost exponential increase in the number of publications relating to mycotoxins, the fungi which produce them, the conditions and substrates in which they are produced and the diseases associated with specific mycotoxins. The number of fungal metabolites now known to be toxic grows yearly, as also does the range of foods and feeding stuffs in which mycotoxins have been shown to occur.

Mycotoxins are important to man, as well as to his livestock, since in high doses many produce an acute disease syndrome whilst at lower doses they have carcinogenic, teratogenic or oestrogenic effects in experimental animals.

It is almost certain that clinical conditions due to mycotoxins have occurred sporadically in livestocks for centuries. In recent times intensification of farming, changes in storage conditions and the handling of cereals in bulk have made the problem potentially greater. (Harrison and Hacking, 1974).

Stored products which may be damaged by the growth of toxigenic fungi, range from cereals (e.g. wheat, barley, oats, maize, rice, sorghum, etc) and oilseeds (e.g. peanuts, hazel nuts, brazil nuts, pistachios, almonds, etc) to fruits and vegetables.

Toxic metabolites produced by certain species of Aspergillus affect man and animals in two ways. Firstly, there is evidence that some of the pathogenic species can produce both exotoxins and endotoxins when growing in living tissues, and these substances may influence their pathogenic potential. Secondly, saprophytic or pathogenic species growing on substrates later used as food for man and animals may produce toxic substances which contaminate the food and are absorbed during digestion and act on one or more body systems. It is the diseases produced by this type of activity which have received the name mycotoxicoses or more specifically aspergillotoxicoses (Forgacs, 1962; Forgacs and Carll, 1962). The range of metabolites identified from a single species of Aspergillus is often considerable (Miller, 1961); but only occasional ones have been shown to be toxic, and only a few of these have been chemically defined (Moss, 1977). The action of the toxins so far reported has varied from growth depression, as in undescribed metabolites of a species of the A. glaucus group (Richardson et al, 1962) to the lethality and carcinogenicity of the aflatoxin from A. flavus (Allcroft and Carnaghan, 1963; Lancaster et al, 1961).

1.8 AFLATOXIN

Research by many workers has shown that a powerful toxin is produced by the fungus Aspergillus flavus Link. This toxic metabolite was named "aflatoxin" (Spensley, 1963). The four

components of aflatoxin (B_1, B_2, G_1, and G_2) differ in their chemical structure, and differ in their toxicity and hepatocarcinogenicity to rainbow trout. The B_1 component is more carcinogenic than any of the others (Wales, 1970). Butler (1965) found aflatoxin B_1 to be 900 times more carcinogenic than para-dimethylamino-benzene and 75 more than dimethylnitrosamine in the induction of rat hepatoma, he concluded that aflatoxin is "the most active hepatocarcinogen known". A number of animal species are suceptible in varying degrees to aflatoxins (dog, pig, cow, duckling, turkey poult, pheasant, quail, chicken, guinea-pig, rabbit, rat, hamster, ferret, mink, monkey, rainbow trout and coho salmon). Many strains of A. flavus have been shown to produce toxic metabolites which induce a variety of clinical signs and pathological effects (Forgacs and Carll, 1962; Moss, 1977). Forgacs and Carll (1962) described mouldy corn disease of swine and a related disease of cattle termed Aspergillus toxicosis. Mouldy peanut disease has been recorded affecting guinea pigs and other domestic animals since 1951 (Paterson et al, 1962). Cereal feeds containing metabolites of Aspergillus species and other fungi, which thrive on these feedstuffs under favourable conditions of temperature, humidity and storage were shown to be a major source of these and other mycotoxins (Sargeant, Sheridan, O'Kelly and Carnaghan, 1961; Lancaster, Jenkins and Philp, 1961; Paterson, et al, 1962).

Aflatoxicosis has been recognised in fishes since 1963 when Wolf and Jackson showed that an unidentified hepatomagen occurred in the cottonseed meal fraction of a particular pelleted trout feed. Other lots of cottonseed meal fed to trout also induced hepatoma and were found contaminated with aflatoxins (Coates et al, 1967).

Table 1.1

Physical characteristics of aflatoxins*

Aflatoxin	Molecular formula	Molecular weight	Melting point	Ultraviolet (265 nm)	Absorption (326 nm)	Fluorescence (nm)
B_1	$C_{17}H_{12}O_6$	312	268-269	13,400	21,000	425
B_2	$C_{17}H_{14}O_6$	314	286-289	11,000	20,800	425
G_1	$C_{17}H_{12}O_7$	328	244-246	10,000	16,100	450
G_2	$C_{17}H_{14}O_7$	330	237-240	11,200	19,300	450

* Partially adapted from Schoental, R (1967) Aflatoxins. Ann. Rev. Pharmac. 7: 343-356.

The suspicion that the causes of such hepatomas derived from aflatoxin was proved in a number of experiments where crude and crystalline aflatoxins obtained from fungi cultured on shredded wheat media were fed at various dosages to trout over a period of five years. These mycotoxins consistently induced aflatoxicosis with a high incidence of hepatoma (Ashley et al, 1964; Halver, 1965; 1967; 1969).

1.8.1 AFLATOXIN INDUCED HEPATOMA IN RAINBOW TROUT

Hepatoma in trout has been known since 1933 when Haddow and Blake reported its occurrence in two fish in an English trout farm. During the period 1937-42, two epizootics of hepatoma occurred in trout hatcheries in California (Wales and Sinnhuber, 1966) and caused some local interest, but trout hepatoma did not become a widespread concern until 1960 when epizootics were reported in the United States and several European countries. Although it had generally been recognised that trout hepatoma was probably diet induced, the specific dietary component containing the carcinogen was not known until Wolf and Jackson (1963) demonstrated that it was the cottonseed meal component of their test diet which induced the hepatoma. It was later demonstrated that the cottonseed meal contained aflatoxin, and Sinnhuber et al (1968) produced hepatoma in trout by feeding aflatoxin contaminated cotton seed meal. It was subsequently shown that aflatoxin is the most potent of the several fungal metabolites to produce hepatoma. Some individual rainbow trout developed hepatoma after having been fed the control diet plus 20 ppm aflatoxin B_1 for a single day or having received a continuous feeding of levels as low as 0.4 ppb aflatoxin B_1 for six months. As the dosages increased in level or in the duration of administration, the incidence of hepatoma increased (Wales, 1970).

Usually certain easily recognisable liver abnormalities precede the neoplastic lesions (Lee et al, 1968). The parenchymal nuclei become enlarged and bizarre in shape, and the parenchymal cells themselves enlarge, probably due to some block in the normal process of cell division (Rogers and Newberne, 1967). The extent of these parenchymal cell abnormalities is generally indicative of the level of aflatoxin in the diet (Wales, 1967).

Another sign of aflatoxin injury in rainbow trout is bile duct hyperplasia, but this is less consistent than the abnormalities of the parenchymal cell nuclei. In advanced cases, only a small volume of normal liver tissue remains and it is assumed that some of the normal liver activities are performed by the neoplastic tissue as well as by the remnant of normal liver. To what extent death in trout is due to insufficient hepatic function, to the toxicity of necrotic tissue or to haemorrhage associated with neoplastic erosion of blood vessels is unknown.

An effective technique for the production of hepatomas in rainbow trout has been described by Wales (1979). This involves brief immersion of embryonated eggs in an aqueous solution of aflatoxin B_1 while maintaining the ambient water temperature. The procedure has since been modified in various ways to determine the optima for age of the embryo at time of treatment, the concentration of the toxin, the duration of the treatment and the temperature of the bath.

There is significant variation among "strains" of rainbow trout in their susceptibility to aflatoxin induced hepatoma. The wild stock has been found to be more sensitive to high level aflatoxicosis than the domesticated rainbow trout (Wales, 1970). The brook trout (Salvelinus fontinalis) is susceptible to aflatoxin

carcinogenesis, but tests indicate that it is less so than the domesticated rainbow trout. The five species of salmon (Oncorhynchus spp) in North America are relatively insensitive to the carcinogenicity of aflatoxin. Wolf and Jackson (1967) fed young coho salmon (O. kisutch) and young chinook salmon (O. tshawytscha) a diet containing aflatoxin for ten months but failed to induce hepatoma. Wales (1970), found sockeye salmon (O. nerka) to be susceptible, providing cyclopropene triglyceride was fed with the aflatoxin. Ashley (1970) described chronic and acute aflatoxicosis in aflatoxin-fed rainbow trout, coho salmon and channel catfish (Ictalurus punctatus) and found that coho salmon and channel catfish fed diet containing 320 ppb aflatoxin B_1 for two years had histologically normal livers but when force fed 10-15 mg aflatoxin B_1 per kg body weight, acute aflatoxicosis appeared in 21-28 days. Acute aflatoxicosis was characterised by generalised oedema of the gills often with marked hyperaemia of the branchial vessels. The liver pathology varied in extent, depending on the dose of aflatoxin, from slight hepatitis with scattered groups of hepatocytes having pycnotic or karyolytic nuclei or chromatin margination, to severe toxic responses with hepatic necrosis with or without hyperaemia and haemorrhagic patches. Chronic development from low and prolonged doses also results in significant incidence of hepatoma. Considerable displacement of adjacent visceral organs often occurs as the liver enlarges, their centres becoming avascular. Fibrosis, necrosis, degeneration and internal haemorrhages frequently reduces the abdominal cavity to a liquid mass of blood and tissue debris often with extensive fibrin adhesions. Some fish with massive hepatoma have nevertheless survived to five to six years of age (Halver, 1969).

1.9 PROBLEMS ASSOCIATED WITH STUDIES ON MYCOTIC DISEASES IN FISH

While mycotic diseases of fish are known to exist and have been documented in both fresh and salt water environments (Wolke, 1975; Richards, 1978) far less is known about these diseases than is generally known about diseases of bacterial or viral aetiology. The lack of knowledge probably relates to a significant degree to the consensus that fungi infecting fish are normally secondary invaders. This probably relates to the fact that the commonest most obvious and most frequently studied fungal pathogen of fish Saprolegnia parasitica appears to be the example of a secondary opportunistic invader par excellence. Although it is accepted that it may eventually result in mortality, it is generally assumed that it is traumatic or primary bacterial or viral disease which has allowed it to invade and which must be controlled. There are reports, however, that some piscine fungal disease outbreaks unassociated with other pathogenic agents have been epizootic in proportion and responsible for considerable mass mortality. Such epizootics have been recorded under both aquacultural and natural conditions (Wood et al, 1955; Carmichael, 1966; Fijan, 1969; Alderman, 1982; Chinabut, 1983 personal communication).

Any study of piscine mycoses may be complicated by two factors. First is the problem of identification. Scott (1964) noted that many of the organisms found in association with fish lesions have been incompletely or improperly identified. This has often led to the lumping of the freshwater fungi affecting fish under the term Saprolegnia. Confusion exists not only as to the species but also as to the genera involved. The only information which

may be gained from such reports is that a fungus was present in association with a pathogenic process or death. Questions of host and species specificity often remain unanswered. This lack of proper identification gives rise to the second complicating factor, the question of pathogenicity. This question can only be answered by proper gross and histopathological examination and description, isolation and identification of the organisms, reinfection of fish of the same species, and re-isolation and identification. Such investigations have been conducted for Saprolegnia by Tiffney (1939), Vishniac and Nigrelli (1957), and Hoshina, Sano and Sunayama (1960); but the whole discussion is complicated by the problem of the state of health, stress of handling and traumatic skin damage on the fish.

The pathologist faced with the diagnosis of a mammalian mycotic disease is often able to identify the pathogenic agent in microscopic tissue sections. He is backed by a corpus of knowledge regarding experimental reproduction of the disease and is able to reach a positive diagnosis with some certainty. The fish pathologist faced with similar problem has a limited body of experimental knowledge, and little histopathological lesion descriptions on which to rely for presumptive diagnosis. Mycological and pathological studies are therefore imperative for the isolation and identification of certain fungi associated with lesions and the careful descriptions of changes present in relation to the organism. Isolates must then be classified as saprophytes, primary pathogen, or opportunists by controlled experimentation.

In the account to follow, some associations of Aspergillus species and tilapias, which it is hoped may contribute towards the generation of such a corpus of information for at least one

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In the account to follow, some associations of Aspergillus species and tilapias, which it is hoped may contribute towards the generation of such a corpus of information for at least one

host/mycosis interelationship, are explored.

CHAPTER 2

INVESTIGATIONS OF AN OUTBREAK OF ASPERGILLOMYCOSIS, A DISEASE HITHERTO UNKNOWN IN TILAPIA

2.1 PRELIMINARY CONSIDERATION

Although systemic diseases caused by a range of different pathogenic fungi have been described under aquaculture conditions (Wolke, 1975; Richards, 1978), Aspergillomycoses in fish have not attracted any significant study before now.

The present study was initiated when it was observed that high mortalities were occurring in an intensive tilapia culture system in Mombasa, Kenya. The mortalities were thought to be connected with the toxin aflatoxin. Price (1981) analysed food-stuff from the farm for aflatoxin by thin layer chromatography. His results indicated that aflatoxin was barely detectable in the feeds. He concluded after several feed trials that the observed mortalities were not due to aflatoxicosis deriving from Aspergillus flavus contamination of the feed.

2.2 BAOBAB FISH FARM, MOMBASA, KENYA

Baobab fish farm is owned and operated by the Bamburi Portland Cement Company. The farm was created out of 1,700 acres of excavated limestone quarry, and developed as an adjunct to the decision to reclaim the derelict area of land. The farm has since developed under inspired management from virtual desert to a near nature reserve in the space of ten years!

At first, carp and tilapias were farmed in ponds, but the

water proved too saline for carp, so the company had turned to intensive tilapia culture since tilapias have the ability to adapt quickly and perform well in fresh or saline water. The water temperatures usually vary from 25-30°C with 14°/oo salinity and 90% oxygen saturation.

After spending three months in rectangular fry tanks, the tilapias are transferred to round intensive growing tanks, each one holding 5,000 fish. The fish grow quickly, reaching a marketable fish size 0.5 - 1 kg, by the time they are nine-months old. This allows two harvests in a year from each of the growing tanks. It also necessitates periodic reduction of stocking levels in the tanks. It is at these grading operations that fish not deemed to be growing fast enough are removed and fed to the farm's commercial crocodile enterprise. Twenty breeding crocodiles are kept to capitalize on the demand for their skins in a thoroughly ecologically acceptable fashion. Besides the fish farming operations on the farm, there is a flock of meat sheep and goats, and a broiler unit. Running water for the tilapia unit is pumped brackish water. Water seeping through the coral from the sea is pumped from underground collecting pits, this is a big advantage as this water has been filtered by the coral and also mixed with fresh water.

2.3 MATERIALS AND METHODS

2.3.1 THE DISEASE OUTBREAK

During the spring and early summer of 1981, losses of tilapia (Oreochromis species) were experienced on Baobab fish farm, Mombasa, Kenya. The condition was characterised by a sudden increase in mortality after any husbandry stress, particularly grading. Fish

were being fed a mixture of locally manufactured high protein pellets and locally available bulk meal low in protein. The steady mortality was associated with abdominal distension, darkening, lethargy and sudden death after handling. Incision of the abdominal cavity of distended fish resulted in the release of copious amounts of clear or blood stained abdominal fluids. Extensive liquefactive necrosis of the liver was evident and in the extreme cases only small amounts of liver tissue remained. Low level mortalities were still occurring by late autumn particularly among the larger fish (200-250 g). Total losses were of the order of 20% of total stock over the growing season.

2.3.2 LABORATORY INVESTIGATIONS

Virological, bacteriological and histopathological examinations of the affected fish were carried out at the Institute of Aquaculture, University of Stirling, upon the receipt of suitable specimens, from the fish farm in Kenya.

Virus isolations were attempted on fish cell cultures . It was hoped that any virus would grow and be subsequently identified. Direct detection of virus by electron microscopic examination of affected tilapia tissue was carried out in addition to virus isolation techniques.

2.3.2(i) Method employed for the detection of viruses

(a) Preparation of inocula: Gut and kidney tissues were sampled aseptically from 5 fish. Tissues were weighed and transferred into sterile mortar and pestle. Sterile sand was added and the preweighed sample of tissue was ground up while about 50 ml of Phosphate

bufferred saline (PBS) containing 100 units of penicilin per ml, 100 ug of streptomycin per ml and 0.5 mg of gentamicin per ml, was gradually added. The resulting homogenate was diluted with the PBS to contain 5 gm of tissue per 100 ml of tissue suspension. Two 10 ml aliquots of the homogenate were then centrifuged at 1,500 g for 15 minutes. The supernatant fluid was then aseptically filtered through a 0.45 um membrane.

(b) Innoculation of Cell Cultures with filtered samples: Since previous attempts at producing tilapia-cell lines have not been successful (Agius 1982, - personal communication) RTG-2 cell lines were used. The medium from the tissue culture flasks (RTG-2 indicator cells with good confluent growth) was tipped off. The cell layer was gently rinsed with about 10 ml of PBS. 0.5 ml of the filtered sample was aseptically innoculated into a 25 cm^2 flask of 70% confluent rinsed cell layers. 0.5 ml of PBS, instead of the sample, was added to another flask as a control. The flasks were incubated for 1 hr at room temperature (15°C), after which the sample fluids were drained off and the cell layers gently rinsed with PBS. 10 mls of Eagles, modified minimal essential medium (EMEM) containing 10% foetal bovine serum, 100 units of penicillin per ml, 100 ug of streptomycin per ml, and 0.5 mg of gentamicin per ml, was aseptically added to each flask, and incubated at 20°C. The flasks were examined daily for the presence of cytopathic effect (CPE) for 7 days. When no specific CPE appeared within this period, the cells were

disrupted by freeze thawing, three times, centrifuged and innoculated onto fresh cells with 0.5 ml of supernatant. The cells were again examined daily for 7 days at 20°C incubation temperature.

2.3.2(ii) Preparation of tilapia tissues for electron microscopy

Tissues from the liver, kidney, gut, heart, spleen and gills were dissected out and cut into approximately 1 mm^3 blocks (larger blocks generally resulted in inadequate fixation due to failure of the fixative to penetrate properly). The tissue blocks were fixed in 2.5% solution of glutaraldehyde in 0.2 M sodium cacodylate buffer of pH 7.2, for at least 2 hours at room temperature. They were then rinsed twice in the buffer solution, dripped dry on blotting paper and fixed for 1 hour in 1% osmium tetroxide at 4°C in a refrigerator. Tissues were dehydrated in graded alcohol as follows:

50% alcohol for 30 minutes
60% " " " "
70% " " " "
80% " " " "
90% " " " "
100% " " 15 "
100% (fresh alcohol for 30 minutes

and embedded as shown below - all steps carried out at room temperature.

75% propylene oxide : 25% resin for 1 hour
50% propylene oxide : 50% resin for 1 hour
25% propylene oxide : 75% resin for 1 hour
100% resin overnight.

The treated tissues were subsequently put in plastic capsules, covered with 100% resin and cured in an oven at 60°C for

24 hours. Epon mixture (Luft, 1961; Agius, 1979) or Emix resin mixture (EMscope Laboratories Ltd., U.K.) were both found suitable for embedding. Because of the time that elapsed between death of fish and sampling, tissue morphology was not suitable for examination but evidence of virus particles was still being sought.

(a) Sectioning and staining:

2 um thick resin sections were cut on an LKB pyramitone 11800 using glass knives, stained with 1% toluidine blue and viewed under the light microscope to select areas of tissue in the resin block. Tissues were trimmed off using a razor blade. Sections were cut from the trimmed tissue on an ultratome OM - 3 (Reichert) using glass or diamond knives. Sections in the gold region i.e. 90 nm (based on continuous interference colour and thickness scale for thin sections - SORVALL^{R} Connecticut) were collected from water onto Maxtaform copper grids (EMscope). Sections were stained by floating the grids on drops of saturated Uranyl acetate solution (Watson, 1958) for 30-40 minutes. After washing with distilled water they were floated for 30-40 minutes on drops of lead citrate solution (Venable and Coggeshall, 1965). Finally, they were washed with distilled water with an intermediate rinse in 0.02M sodium hydroxide and dried on pieces of filter paper.

(b) Viewing: The stained ultrathin sections were viewed in a JEOL JEM-100 C electron microscope.

2.3.2(iii) Bacteriological Examination

Specimens were examined bacteriologically from each of 10

clinically affected tilapias. The organs of choice were the heart and the kidneys, although impression smears were obtained from hepatic, and splenic tissues. These were stained by Gram's method and subjected to thorough microscopic examinations. Inocula for bacteriological culture were obtained by searing the exposed surface of the organ under investigation with a heated scalpel blade. Next, a sterile innoculating loop was inserted through the sterile area and the inoculum streaked on the medium. Trypticase soy agar (TSA) with a final concentration of 2% sodium chloride was used for the primary isolations. Incubation temperatures were at 20°C and at 25°C for 48-72 hours respectively.

2.3.2(iv) Histopathological Examination

Blocks of tissues from the spleen, heart, kidney, gills and liver were fixed in 10% neutral bufferred formalin, paraffin was - embedded, sectioned at 5 um and stained with haematoxylin and eosin (H & E). Selected sections were later stained with periodic acid Schiff's technique (PAS), and Grocott's modification of Gomori's methenamine silver technique for fungi. (Appendix B - D).

2.4 RESULTS

The absence of specific cytopathogenic effect (CPE) during the incubation period indicated negative results for virus infection. No virus was detected on electron microscopy.

There was no significant growth on the bacteriological plates. The extensive virological and bacteriological examinations failed

to reveal any evidence for a viral or bacterial pathogen. Significant results, however, emerged from the histopathological examinations.

The gills showed slight to severe oedematous changes with irregular denudation of the squamous epithelium of the primary and secondary gill lamellae. Large numbers of septate hyphae were present in the heart muscles, which also showed evidence of severe mycocarditis in association with areas showing the fungal hyphae. The walls of the actively growing vegetative fungal hyphae were basophilic with H&E but with PAS staining technique they were obvious with hyphae stained purple, while the other tissue polysaccharide materials stained purplish red; large numbers of the septate fungal hyphae stained light brown to black with Grocott's modification of Gomori's methenamine silver staining technique. The mycelium was branching and the hyphae were approximately 2 to 3 um in width.

The eye showed proliferation of fungal hyphae extending into the choroid and retina, the septate nature of the hyphae was clearly visible.

The main pathological feature in the kidney appeared to be irregularly scattered fungal lesions particularly in the haematopoietic tissue. The renal tubules showed evidence of oedema and renal tubular degeneration. Lesions were variably present in the spleen, essentially tissue necrosis and mainly peripheral in nature. The major lesions appeared in the liver. Extensive areas showed hepatic tissue necrosis in various stages and in some areas, complete destruction of the liver parenchyma had taken place, while a few areas still remained apparently normal. The principal reaction to the presence of fungal hyphae was the

development of typical granuloma. Massive tissue necrosis was associated with larger foci of fungus. Essentially, there were numerous organised granulomas, in various stages of development in some of which could be seen septate fungal materials, whereas in others, only amorphous debris was found. From the observed histopathological lesions, a tentative diagnosis of systemic mycosis was made, and from the morphology of the hyphal material, a tentative diagnosis of possible Aspergillus infection was made.

Fig. 2.1 Section of heart of affected tilapia showing extensive proliferation of fungal hyphae penetrating into the muscles. Grocott's stain X 375

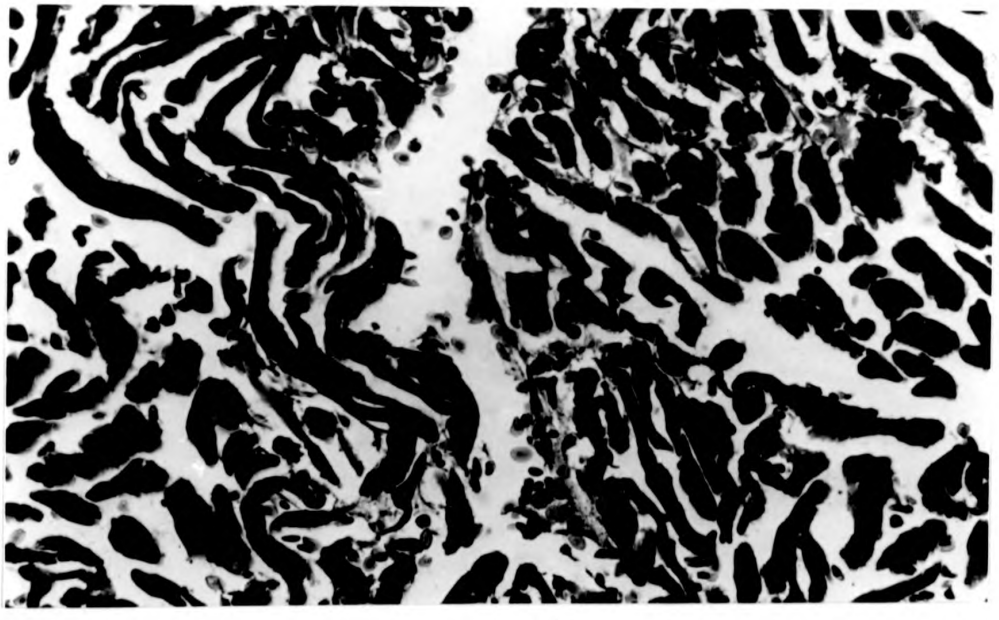

Fig. 2.2 Cellular infiltration of the myocardium associated with fungal proliferation. Grocott's stain X 600.

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Fig. 2.2 Cellular infiltration of the myocardium associated with fungal proliferation. Grocott's stain X 600.

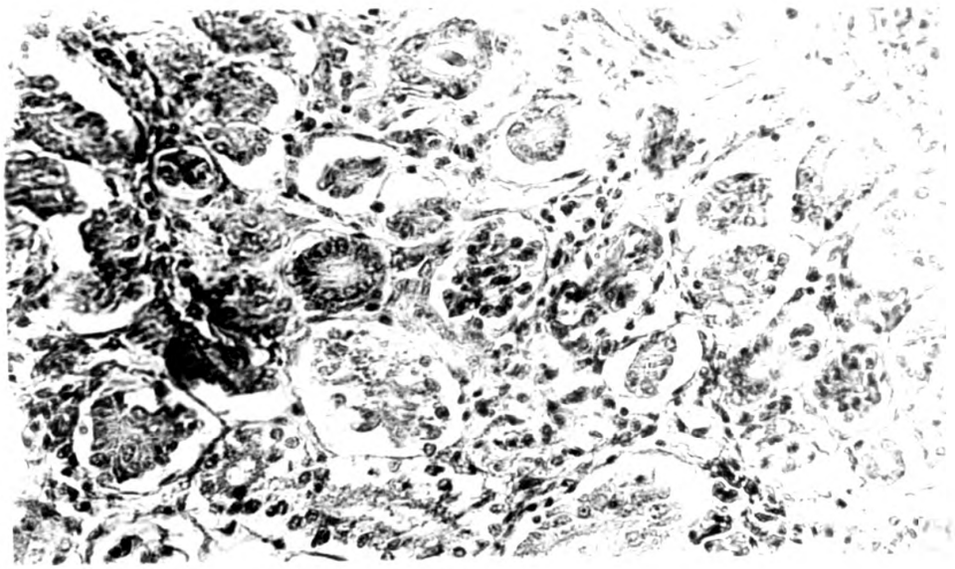


Fig. 2.3 Renal tubular degeneration and presence of fungal elements in the haematopoeitic tissue of the kidney H & E X 502.

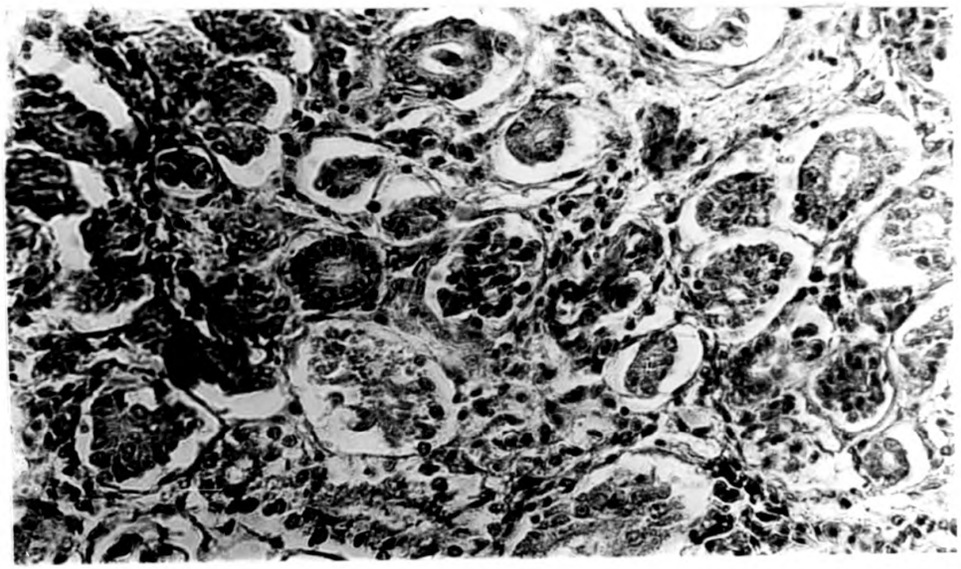


Fig. 2.3 Renal tubular degeneration and presence of fungal elements in the haematopoeitic tissue of the kidney H & E X 502.

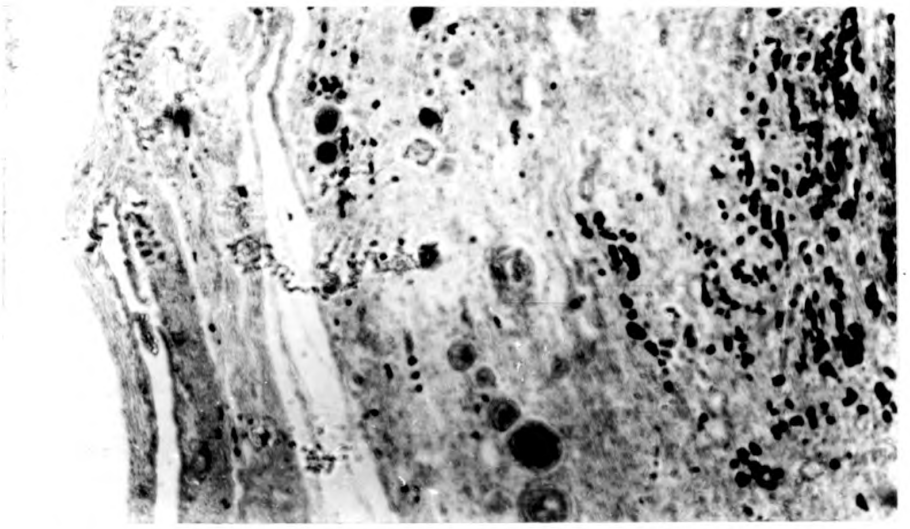


Fig. 2.4(a) Granulomas in the liver of affected tilapia. Note massive destruction of the liver parenchyma. Grocott's stain X 74.

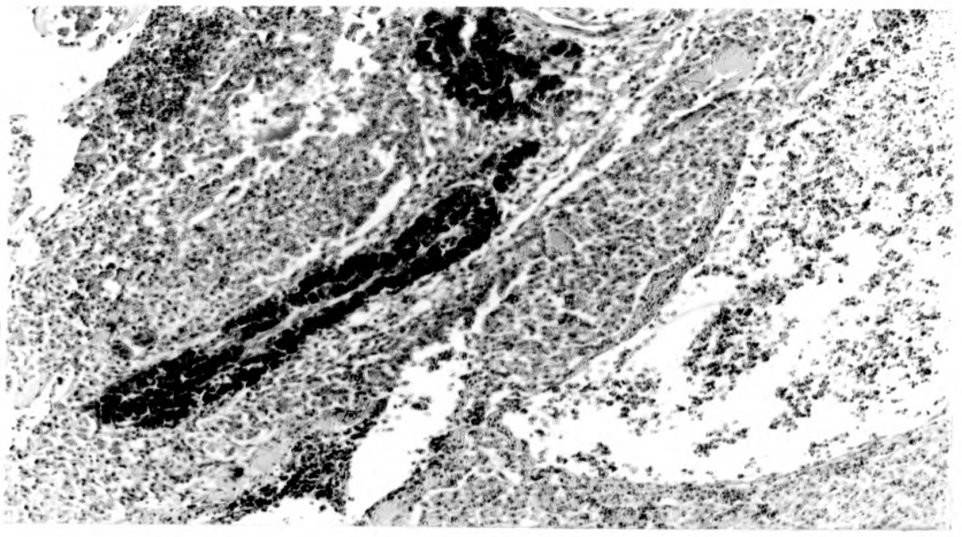
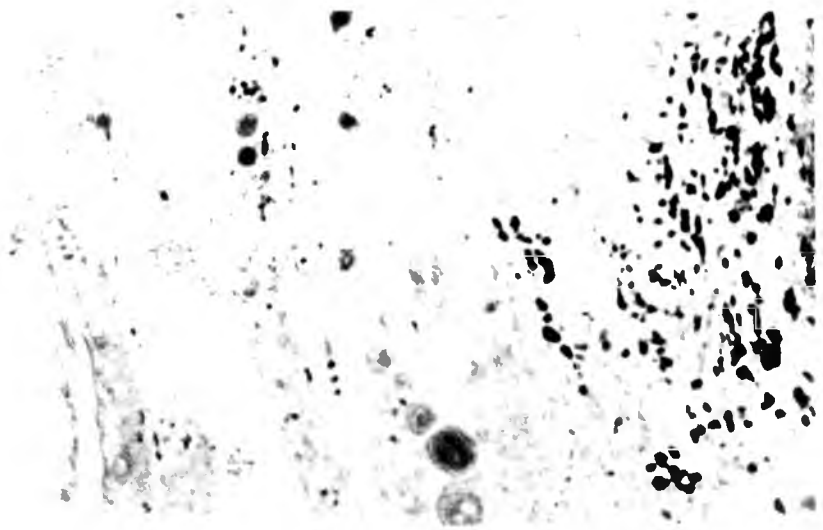


Fig. 2.4(b) Massive hepatic necrosis, H & E X 124.



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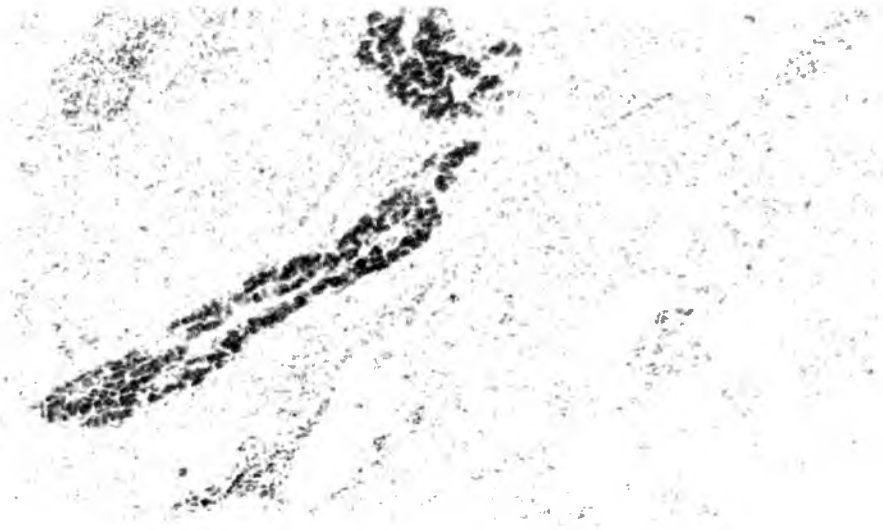

Fig. 2.4(b) Massive hepatic necrosis, H & E X 124.

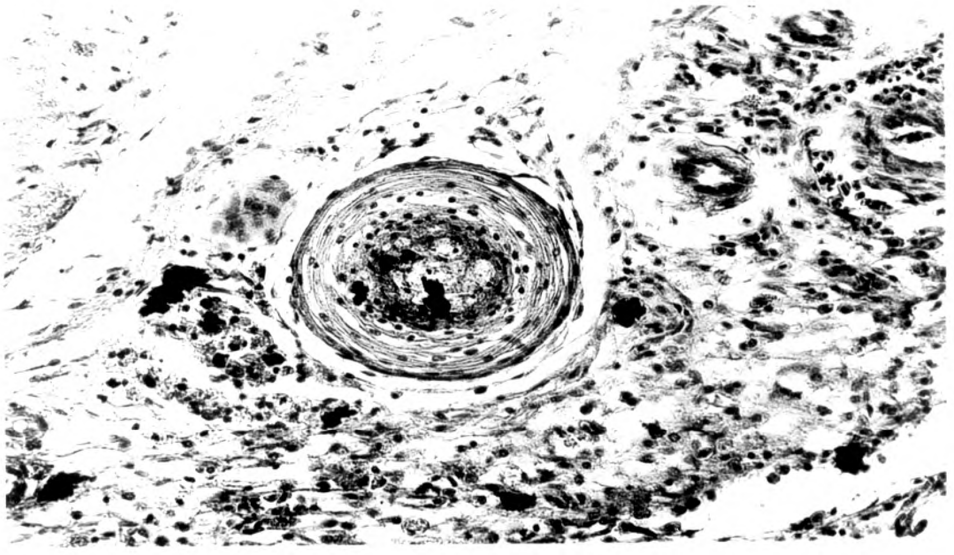

Fig. 2.5 One of the granulomas in the liver. H & E X 310.
Fungal material could be seen in many of the granulomas.

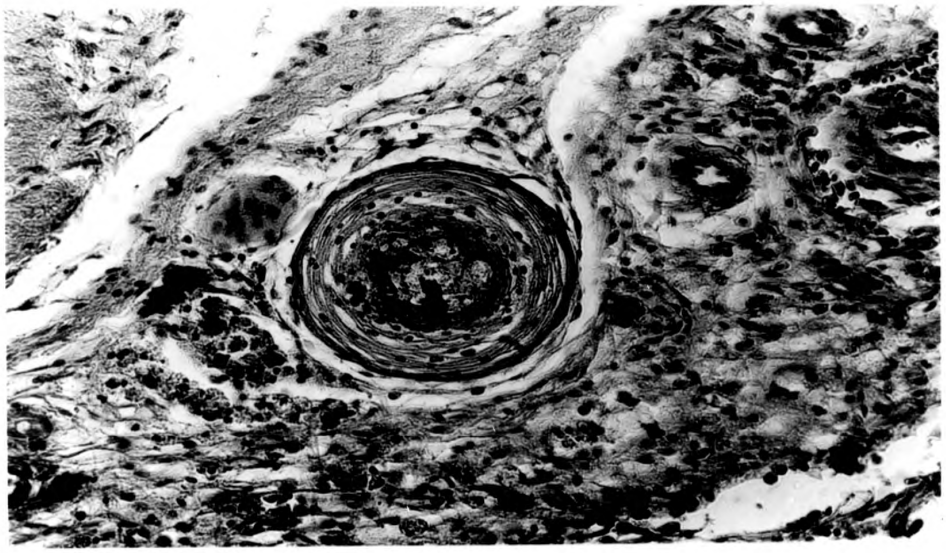

Fig. 2.5 One of the granulomas in the liver. H & E X 310.
Fungal material could be seen in many of the granulomas.

2.5 DISCUSSION

The results of the clinicopathological, and microbiological examinations indicated above, showed that this clinical material, forwarded from a clinical outbreak of high mortality among intensively cultured tilapias in Kenya, appeared to be infected by a fungus with septate hyphae, with no evidence of any other infectious agent to which it might be secondary.

In order to be able to ascertain the identity of the fungus and to demonstrate its pathogenicity, detailed isolation in pure culture and characterization studies coupled with experimental pathogenesis are essential. This is particularly in the case of fungal pathogens of fishes where so often the fungus is a secondary agent. There are already certain fungal conditions described in the literature in association with granulomata such as Ichthyophonus hoferi infection (Miyaki and Kubota, 1977; Chien, Miyaki and Kubota, 1979) and dematiaceous mould infections (Richards, Holliman and Helgasson, 1978; Blazer and Wolke, 1979). Ichthyophoniasis is normally a disease of marine fish and dematiaceous moulds are usually only associated with isolated outbreaks in a few fish. Neither is readily identifiable in tissue section although neither resemble the morphology observed in the present material. If these, or other fungal granuloma agents, such as Aspergillus species are to be identified they must be first cultured and then identified by formal mycological techniques.

CHAPTER 3

FUNGAL ISOLATION FROM CLINICAL MATERIAL

3.1 INTRODUCTION

In order to attempt to isolate the material observed in sections of the tilapias from Baobab farm, a series of culture studies was undertaken using a range of media. The isolations were made from fish tissues air freighted from Kenya on ice and also from samples of food materials obtained from the farm store, to determine if this could be a source of infection.

3.2 MATERIALS AND METHODS

3.2.1 Culture Media:

The primary aim of this part of the study was to obtain fungal growth from the clinical materials obtained from Kenya, and subsequently identify the various fungi isolated using known comparative cultural and morphological data. A number of commercial preparations were employed as cultural media. These include Sabouraud dextrose agar (SDA), corn meal agar (CMA), malt extract agar (MEA), Czapek's colution agar (CSA) and brain heart infusion (BHIB) broth, as an enrichment medium. (Appendix E - I).

3.2.2 Isolation method from fish tissue:

In many instances fungi were not readily apparent in smears or sections of the clinical specimens and culture or some other diagnostic technique was essential. For culture, once the

clinical specimens were examined macroscopically and microscopically they were ready to be processed and inoculated onto isolation media. If processing of the specimen had to be delayed, the specimen was refrigerated (not frozen). Delays in processing were avoided where possible because delay might contribute to decrease in yield of potential pathogens.

3.2.2(i) Primary Isolation technique:

About 1g of fish organ, whether gills, brain, liver, gastro-intestinal tract or kidney, was cut into small fragments using sterile scissors and forceps and homogenised in a sterile mortar and pestle. Usually all the livers, all the hearts or all the kidneys from one group of fish were pooled together so that up to eight livers etc would be pooled. Nine millilitres of freshly prepared sterile brain heart infusion broth was added, 2-3ml at a time. One millilitre (10 mg/ml) of gentamicin was added to give a final concentration of 1 mg/ml in the homogenate. Gentamicin was added to prevent bacterial contamination of the culture plates. After thorough mixing 0.1 to 0.2 ml of the homogenate was then inoculated onto the centre of fresh plates of Sabouraud's dextrose agar, corn meal agar, malt extract agar and Czapek's solution agar. The plates were then incubated at 25 to 30^{o}C in the dark. This method has been described elsewhere (Olufemi and Roberts, 1983).

3.2.2(ii) Technique for obtaining pure cultures:

Pure cultures are always mandatory for Aspergillus (and indeed other fungi) identification. Until it can be demonstrated to the contrary, all isolates from primary culture should be

considered contaminated. Since many fungi of veterinary or medical interest are polymorphic, it is important to know whether or not the various structures present on the primary isolation plates are produced by one fungus or several fungi. The correct conclusion can be made only when pure cultures are being studied. The isolates must also be free of bacterial contamination because some bacteria may interfere with the development of key morphological structures necessary for the identification of the fungus. In order to obtain pure cultures in the present case a single conidia structure, well separated from the others on the primary plate, was located with the aid of either a 10x hand lens or preferably, a binocular dissecting microscope, and removed using a sharply pointed inoculating needle (flame sterilised) and transferred to a suitable sterile medium in a petri dish; usually malt extract agar, or Czapek's colution agar. The inoculated plates were then incubated at 25-30^{o}C in the dark.

3.2.3 Isolation of *Aspergillus* species from fish pellets

The technique used for the isolation of *Aspergillus* species from fish food materials was basically similar to the method described for the isolation of *Aspergillus sp* from fish clinical material with the exception that fish pellets were substituted for fish tissue. The pellets were first ground using a sterile mortar and pestle. Since fungal spores are readily suspended in water and many are human pathogens, care was taken to avoid accidental spillage of the suspension onto the working bench. About 1 gm of the ground pellet material was weighed, and 9 volumes of brain heart infusion broth added and mixed thoroughly. Gentamicin was also added as a bacteriostat to give a final

concentration of 1 mg/ml, following which the suspension was inoculated onto sterile petri dishes containing 25 ml of the culture media, and incubated at 25-30^{o}C in the dark.

For routine purposes, plates that were quite satisfactory for examination were made by touching the sporulating surface of the stock culture with a flame-sterilized needle that had been cooled and consequently wetted in the sterile medium, and transferring the adherent conidia to selected positions on the solidified medium in the petri dish. Colony development was studied by placing three spots of conidia an equal distance apart on the medium surface in a petri dish containing 25 ml of the medium.

3.2.4 Slide Culture Technique

The spore or conidia bearing structures of aerial mycelium are often disrupted when samples are taken from a culture plate and mounted for microscopic examination. In this case the Aspergillus species were, for this purpose, grown on glass slides of media in a moist chamber, were then able to be mounted and examined with the added advantage of minimum disturbance. The form of sporulation could then be readily observed, undisturbed under the microscope.

3.2.4(i) Preparation of the slide culture system

In order to set up the slide culture system for this purpose, a single piece of filter paper was placed in a sterile 100 mm petri dish, the filter paper should just cover the bottom. On top of the filter paper was placed a glass rod that had been bent into the shape of a U. (A glass rod or a disposable 1 ml

glass pipette works very nicely.) A clean glass microscope slide was then placed on the U-shaped rod. Aseptic technique was strictly adhered to and each of the components of the set up had to be sterilized separately and then added aseptically to the sterile petri-dish. Once the entire system has been used, it can be autoclaved in its entirety and discarded (see slide culture set-up fig. 3.3.1).

3.2.4(ii) The slide culture

Using a Czapek's solution agar poured on a plate, the agar was cut with a sterile scapel into blocks approximately 10 mm square. One agar block was aseptically transferred with the sterile scapel onto the microscope slide in the slide culture set-up. With a long-handled inoculating needle, a small amount of the fungus colony was then transferred from the edge of the colony to each of the four sides of the agar block. The inoculated block was then aseptically covered with a sterile coverslip large enough to overlap each edge by 4 to 5 mm. (An 18 to 22 mm glass cover glass worked very well.) About 8 ml of sterile water was then added aseptically to the base of the slide culture. The moist filter paper was essential to maintain a humid atmosphere and prevent the agar from drying. The sterile culture set up was then ready for incubation, usually at 25^{o}C in the dark until sporulation occurred, usually after approximately 10 days.

3.2.4(iii) "Taking down" the slide culture

By "taking down" is meant the series of operations involved in the dismantling of the slide culture set up following sporulation,

for the subsequent microscopic examination. The glass slide was removed and all moisture on its bottom wiped off. It was then placed on the microscope to determine whether the conidia are mature. A drop of mounting medium (lactophenol) was placed on a microscope slide that has been cleaned with 70% ethanol. The drop was placed just off centre with a fine aperture pipette. (It is important to ensure that the drop of mounting medium is small to prevent excess of mounting fluid around the sides of the cover slip.) The cover slip was then carefully removed with a pair of forceps from the agar block. Care was essential to ensure that the agar block was not pushed off the coverslip as this would destroy part of the fungal growth on the cover glass. The cover glass was quickly passed once or twice through the blue portion of a bunsen flame to heat fix the fungus and its conidia in place. If the fungus was heat fixed in this manner there was usually no distortion of the diagnostic features. Generally, it did not seem to matter which side of the cover slip was exposed to the flame. It was however, critical to minimize the exposure time in the flame so as to prevent the resultant collapse of the hyphae and conidia or spores. The cover slip was finally placed at the edge of the small drop of the mounting medium and carefully lowered with a dissecting needle. As the coverslip was lowered, air bubbles and loose conidia were forced towards the opposite side of the coverslip. Any excess mounting fluid was blotted out using an appropriate tissue and if a permanent preparation was required the outer edge of the coverslip ringed with nail varnish to seal it and the slide labelled appropriately.

By removing the agar block from the microscope slide in the original slide culture, the slide is used as a second mount after heat fixing. The mounting fluid is placed on it followed by a

coverslip and then examined microscopically.

3.2.5 Subculturing *Aspergillus* Isolates

Owing to the potential danger to humans associated with *Aspergillus* species, a long-handled inoculating needle was always used in lieu of short-handled dissecting needles. Inocula from the colony were found to be more easily obtained by bending the tip of the inoculating needle at a right angle. Isolates could be subcultured in several ways. A simple procedure, however, was to carefully transfer a small portion of growth from the edge of the old colony to fresh media in a petri dish. Such inoculation was best taken from the edge of the colony where the fungus was mostly vegetatively active.

As *Aspergillus* species produce abundant numbers of conidia, preparation of conidia are readily obtained if the plates bearing colonies were first flooded with a few millilitres of sterile distilled water. A conidial suspension could then be aspirated with a sterile pasteur pipette. A drop or two of the suspension was then placed on the fresh medium. Aerosols were thus avoided and the danger of operative infection minimised, and contamination of other cultures avoided.

3.2.6 Storage Technique for the Isolates

Many methods have been proposed and are indeed, used for maintaining culture collections. The more common techniques include dispersal in sterile soil, sterile mineral overlay, deep-freezing, ultralow freezing, lyophilization etc. Some of these techniques involve substantial amounts of time and expensive equipments. The following simple and inexpensive technique was

found to be the most useful in the laboratory in this study.

3.2.6(i) Water culture technique

About 2 ml of sterile distilled water was aseptically added to an actively sporulating culture on any suitable medium. Conidia were dislodged with a sterile long-handled inoculating wire without digging into the medium. The resultant suspension was removed with a sterile capillary pipette and transferred into a sterile vial appropriately labelled. Additional sterile distilled water was added to ensure that water was not less than approximately 5 ml in volume. The cap of the vial was then screwed down tightly and then stored at room temperature or in the refrigerator at +4°C. Additional sterile distilled water was added at anytime if drying out was thought to be taking place. Most isolates stored in sterile water should remain viable for years (McGinnis, 1980). Certainly, they were fully viable over the timespan of the present study.

3.2.7 Procedure for preparing subcultures from water cultures

The neck and cap of the vial were first wiped off with 70% ethanol. The fungus was then resuspended in its water by careful and gentle shaking, the vial opened and the mouth flamed, and 0.2 - 0.5 ml of the suspension aseptically transferred with a pipette to a plate containing any suitable medium. The plate was appropriately labelled. Meanwhile, the cap on the vial on the water culture was tightened and then returned to storage at room temperature. The culture plate on the other hand was incubated at 25-30°C until growth appeared. A slide culture was then prepared or subcultured as necessary to confirm the

identity of the isolate.

3.3 RESULTS

In positive cases, fungal growth was usually readily observed after incubation for 48 to 72 hours depending on the species and/or strain of the Aspergillus present. Growth on Sabouraud's dextrose agar was usually dense and profuse and was therefore not very useful for species identification using colony characteristics. Growth on corn meal agar was generally scanty but single colonies were readily picked out for subculturing for pure isolates. In most cases, cultural characteristics on plates were better observed with malt extract agar and best studies were made on Czapek's solution agar, on which medium, morphological and morphometric details of the various species isolated were used for identification.

3.3.1 Fungi isolated from tilapia tissues and pelleted diets

The following fungi were isolated from tilapia tissues, and were also isolated from different batches of tilapia pelleted diets.

3.3.1(i) Aspergillus flavus Link ex Fries

Colonies on Czepek's solution agar (CSA) were variable, and ranged from rapidly growing 6 to 7 cm in diameter, to slowly growing 3 to 4 cm in diameter in 10 days at 25 $\pm$ 1^{o}C. The mycelium was rather thin and close textured and usually remained submerged in the marginal 1.0 to 1.5 cm of the medium. Colonies were commonly plane but occasionally were radially furrowed or wrinkled. Abundant conidial structures were generally produced

often in yellow shades but quickly shading to bright to dark yellow-green shades; the reverse of the plates were commonly uncoloured but sometimes appeared pinkish. Sclerotia were produced in variable form.

Conidial heads were typically radiate, splitting into several poorly defined columns rarely exceeding 500 to 600 u in diameter. Conidiophores were heavy walled, uncoloured, but coarsely roughened and were usually less than 1 mm in length but sometimes more, stalk diameter immediately below the vesicles ranged between 10 and 20 u. Vesicles were usually elongate when young but in older cultures, were subglobose or globose and varied from 10 to 65 u in diameter. The phialides were uniseriate or biseriate but both rarely occurred in the same head. The primaries were between 6 to 10 u by 4.0 to 5.5 u and occasionally longer. Sometimes they were elliptical when first formed.

3.3.1(ii) Aspergillus niger van Tieghem var phoenicis (Corda) Al-Musallam

Colonies on CSA were more restricted in growth reaching 2.5 to 3.0 cm in diameter in 10 days to two weeks at 25 $\pm$ 1^{o}C.

Conidial structures were typically black to deep brownish black, covering the entire colony except for the narrow growing margin. The reverse side of the plate was usually colourless. Conidia heads were large, black and globose at first, but becoming radiate or, with age, often splitting into two or more loose to reasonably defined columns, commonly reaching 700 to 800 u in diameter.

The conidiophores were variable, commonly 1.5 to 3.0 mm by

15 to 20 u with smooth walls which were colourless or brownish and were generally up to 2.0 to 2.5 u thick. The vesicles were globose or nearly so, commonly 45 to 75 u in diameter, or smaller.

The phialides were in two series, brownish in colour. The primary phialides varied with age of the conidial heads usually 20 to 30 u by 5 to 6 u at the onset of sporulation but may reach 60 to 70 u by 8 to 10 u and sometimes septate at maturity. Secondary phialides were more uniform usually 7 to 10 u by 3.0 to 3.5 u with comparatively heavy and irregularly roughened walls.

3.3.1(iii) Aspergillus terreus Thom

Growth on CSA in 10 days at 25 $\pm$ 1^{o}C varied in diameter from 3.5 to 5 cm. They were either flat or marked by shallow radial furrows. Colonies appeared velvety at first becoming floccose or tufted, with thin or irregular margins. Colour of colonies was generally brown and the reverse of the plate usually appeared dull yellow to brown.

Conidial heads were long, columnar, compact and of uniform diameter throughout their length, commonly ranging from 30 to 50 u in diameter and 150 to 500 u or more in length at maturity. Conidiophores were smooth, colourless, and ranged from 100 to 250 u by 4.5 to 6.0 u. They were approximately uniform in diameter throughout their length. Vesicles were hemispherical and dome-like, commonly 10 to 16 u in diameter and merged almost imperceptibly into the supporting conidiophore. The phialides were in two series, the primaries of which were usually crowded and parallel and were 5.0 to 7.0 u by 2.0 to 2.5 u in size. The secondaries were closely packed and were 5.5 to 7.5 u by 1.5 to 2.0 u. The

conidia were globose to slightly elliptical, smooth and commonly 1.8 u to 2.4 u in diameter.

3.3.1(iv) Aspergillus japonicus Saito

Colonies on CSA grew rapidly reaching a diameter of 5.0 to 6.0 cm in 10 days at 25 ± 1°C. Conidial structures in purple brown or purple black. The reverse of the plate appeared usually uncoloured at first but later became dull purple, occasionally with a slight yellow-green tinge. Conidial heads were variable, generally small, radiate or split into few indistinct columns, rarely exceeded 300 u in diameter in 10 days, but when aged, sometimes became distinctly columnar and usually up to 600 to 700 u in length.

The conidiophores were smooth with a limited surface granulation. They were colourless or slightly pigmented especially below the vesicles - mostly 500 to 1000 u by 5 to 10 u. The vesicles were coloured in brownish shades, often somewhat elongate but in older cultures or larger heads, appeared more nearly globose, mostly 20 to 30 u by 25 to 35 u but ranged from less than 15 u to 45 u in diameter. The phialides were uniseriate, 5.5 to 8.0 u by 3.0 to 4.5 u rarely swollen to double their normal size.

Conidia were mostly globose, but were occasionally subglobose, strongly echinulate with echines discrete and regularly shaped, commonly 0.5 u long, but occasionally longer, and the spore bodies were mostly 3.0 to 3.5 u. Abundant sclerotia were produced which were often white to cream in colour and somewhat globose in shape approximately 500 u in diameter.

3.3.1 (v) Fungi other than *Aspergillus* species isolated and identified from tilapia tissues as indicated in table 3.1

The following fungi were also isolated and identified:

Penicillium citrinum Thom,

Penicillium waksmanii Zaleski

Cladosporium cladosporioides (Fres) de Vries

Cladosporium oxysporum Berk and Curt

Trichoderma harzianum Rifai

3.3.2 *Aspergillus species* isolated from different batches of tilapia pelleted ration:

Aspergillus species isolated from tilapia tissue as in 3.3.1 were also on different occasions isolated from batches of tilapia pelleted rations. Others included the following:

3.3.2(i) *Aspergillus candidus* Link ex Fries

Colonies on CSA were usually comparatively, slow growing, reaching 1.5 to 3.0 cm in diameter in two weeks at 25 $\pm$ 1^{o}C. They were usually thin with a submerged vegetative mycelium. The surface growth usually consisted of fruiting bodies borne directly from the substrate, or from a scanty aerial mycelium. They were persistently white becoming cream to yellowish cream. The sclerotia were purple to black. Where colonial growth was scanty the reverse of the plate was uncoloured but with dense growth and a compact basal mycelium it was very pale yellow to faintly pink.

The white to cream conidial heads were globose in young cultures, with spore chains, but later they became adherent in loose divergent columns, reaching diameters of 600 to 800 u.

Conidiophores varied from less than 500 u to 1000 u or longer, and from 5 u to 10 or 20 u in diameter. They were generally thick walled, smooth and occasionally septate, although in older cultures they often lost their colour or became slightly yellow. The vesicles were globose to subglobose and ranged from 40 u or more in diameter to less than 10 u in small heads. Small vesicles often supported only a limited number of phialides which appeared almost penicillate. The phialides were usually in two series, but occasionally were uniseriate, they were characteristically wedge shaped and ranged from 5.0 to 8.0 u by 2.5 to 3.5 u to 25 to 30 u by 10 to 12 u in dimensions, a few were septate. The secondary phialides were usually more uniform in dimension from 5.0 to 8.0 u by 2.0 to 2.5 or 3.0 u.

The conidia were globose or subglobose in most strains. They were often thin walled, smooth and colourless and were generally 2.5 to 3.5 u. Sclerotia were white when freshly produced but quickly became reddish to purple black and consisted of thick walled parenchyma-like cells.

3.3.2(ii) Aspergillus chevalieri Thom and Church

Colonies on CSA grew somewhat restrictedly reaching 2.5 to 3.0 cm in diameter in 2 weeks at 25 $\pm$ 1°C. The growth was thin and bluish grey in central areas with age, and with the development of conidial heads. Cleistothecia were produced but were confined to the marginal areas. The reverse of the plate was yellow-orange to maroon in colour. Conidial heads were abundant and appeared to radiate from divergent conidial chains and were mostly 125 to 175 u in diameter.

Conidiophores were mostly 700 to 800 u in length, enlarging

to an almost globose vesicular apex 25 to 35 u in diameter. The phialides were in a single series (uniseriate) closely packed and range from 5 to 7 u by 3.0 to 3.5 u. Conidia were ovate to elliptical with ends often flattened. The cleistothecia were closely enmeshed in orange-red with encrusted hyphae, mostly 100 to 140 u, occasionally up to 150 u, globose to subglobose, yellow to orange. Asci were 9 to 10 u in size.

The ascospores appeared lenticular 4.6 to 5.0 u by 3 to 4 u with smooth or very faintly roughened walls and with a prominent equitorial crest, thin and often recurved and with furrow consisting more of a trough between parallel crests than an equatorial depression in the spore body.

3.3.2(iii) Aspergillus clavatus Desmazieres

Colonies on CSA reached 3.0 to 3.5 cm in diameter in 10 days at 25 $\pm$ 1^{o}C. The conidiophores were abundant, were mostly erect and were up to 3.0 mm in length, with large, blue-green, clavate conidial heads arranged in well-defined zones. They ranged from 300 to 400 u by 150 to 200 u in size. The reverse of the plates were usually uncoloured, but with age became brownish.

Conidiophores were 1.5 to 3.0 mm in length, and 20 to 30 u in diameter, thin walled, smooth and colourless. They were enlarged at their apices to form clavate vesicles fertile over an area up to 200 to 250 u in length and 40 to 60 u wide.

The phialides were in a single series and varied in size from 2.5 to 3.5 u by 2.0 to 3.0 u at the base of the vesicle ranging to 7 or 8 and occasionally 10 u by 2.5 to 3.0 u at its apex. The conidia were elliptical, with comparatively heavy wall.

They were smooth and generally 3.0 to 4.5 u by 2.5 to 3.5 u in size.

3.3.2 (iv) Aspergillus repens (Corda) Sacc

Colonies on CSA attained a diameter of 5 to 6 cm in 2 weeks at 25 ± 1°C. They were often smooth or slightly wrinkled and were commonly characterised by broad zones or patches of dull green to grey-green due to the colour of the conidial heads. The reverse of the plates varied from yellow-orange to deep maroon in colour due to the diffusion of pigment into the agar.

Conidial heads were abundant, radiate to very loosely columnar and varied from 125 to 200 u in diameter. Typically they consisted of diverging chains of conidia radiating from the hemispherical vesicular apex of the conidiophore. The conidiophores were smooth and mostly colourless, measured 500 to 600 u in length, broadened at the apex to a vesicular area about 25 to 40 u in diameter, and were occasionally branched. The phialides were uniseriate and measured 7 to 10 u by 3.5 to 4.5 u. The conidia were ovate to subglobose or globose, spinulose and varied in size from 4.5 to 7 or 8 u but mostly 5.0 to 6.5 u.

The cleistothecia were abundant and were borne on loose networks of yellow to orange-red hyphae. They were themselves yellow and typically spherical to subspherical, ranging from 75 u to 100 u. Asci varied from 10 to 12 u and the lenticular ascospores were usually 4.8 to 5.6 u by 3 to 4.5 u. They were smooth walled with an equatorial area which was rounded or somewhat flattened and were occasionally indented to show the trace of a furrow. They were however without crests or ridges.

3.3.2(v) Aspergillus sejunctus Bainier and Sartory

Colonies on CSA were usually flat or somewhat raised, yellow orange or orange-brown to red-brown in colour, and reached a diameter of 6 to 7 cm in 2 weeks at 25 $\pm$ 1°C. The reverse of the plates took on shades of orange-red to dark red-brown after prolonged growth.

Cleistothecia were very abundant and largely enmeshed in a felt at the agar surface. They were yellow to orange-red, spherical to subspherical and ranged between 80 to 120 u in diameter. Conidial heads commonly projected above the felt. They were pale grey-green, and were generally crowded near the centre or scattered randomly over the colony. They were radiate, 150 to 250 u in diameter.

Conidiophores were smooth, colourless to orange-brown with absorption of pigment, and averaged 500 to 750 u in length. They were 14 to 16 u broad except in the terminal subglobose vesicular area where they were nearer to 25 to 35 u in diameter. The phialides were typically uniseriate and measured 7 to 9 u by 4 to 5 u. The conidia were elliptical, ovate, subglobose or globose, closely spinulose and mostly 5 to 7.5 u in diameter. The asci were comparatively large, 12 to 15 u in diameter. Ascospores however, were lenticular, 5.0 to 6.0 u by 4 to 5 u, with a furrow generally evident as a broad shallow depression around the spore equator; ridges were low and often inconspicuous. The walls were generally smooth except for a minute roughness disposed along the equatorial ridges.

3.3.2(vi) Fungi other than *Aspergillus* species isolated and identified from batches of tilapia pelleted ration

The following fungi were also isolated and identified:

Penicilium cyclopium Wrestling

Penicillium citrinum Thom

Cladosporium cladosporioides (Fres) de Vries

Clasdosporium sphaerospermum Penz

Both species of *Cladosporium* are common saprophytes

The identity of all of the fungi isolated and described here were confirmed by Dr A H S Onions, Dr D W Minter and Dr B L Brady all of the Commonwealth Mycological Institute, Surrey, England.

Table 3.1 Fungal isolations from tilapia tissues

Source	Organ	Fungus Isolated
Fish from Ex-arena 2 (Breeding stock) (8)	Liver	*A. japonicus* Saito
		A. flavus Link ex Fries
	Kidney	*A. flavus* Link ex Fries
	Mouth lesion	*Trichoderma harzianum* Rifai
		Cladosporium oxysporum Berk and Curt
Fish from Raceways (6)	Liver	*A. flavus* Link ex Fries
		A. niger Van Tieghem var. phoenicus (Corda) Al-Musallam
	Kidney	*A. niger* Van Tieghem var. phoenicus (Corda) Al-Musallam
Fish from tank 6 (*O. niloticus* hybrid) (5)	Heart	*A. terreus* Thom
	Liver	*A. flavus* Link ex Fries
		A. japonicus Saito
	Kidney	*A. candidus* Link ex Fries
	Body lesion	*Penicillium waksmanni* Zaleski
	Tail & fin	*Cladosporium cladosporiades* (Fres.) de Vries

Table 3.2 Fungal isolations from tilapia feed

Feed Batch	Fungi isolated
240400	1. A. Clavatus Desmazieres 2. A. chevalieri Thom and Church 3. A. repens (Corda) Sacc 4. Penicillium cyclopium Westling
485	1. A. candidus Link ex Fries 2. A. flavus Link ex Fries 3. Cladosporium sphaerospermum Penz 4. Penicillium citrinum Thom
2027	1. A. chevalieri Thom and Church 2. Cladosporium cladosporioides (Fres) de Vries 3. Cladosporium sphaerospermum Penz
124520	1. A. flavus Link ex Fries 2. A. repens (Corda) Sacc 3. Penicillium citrinum Thom
051341	1. A. flavus Link ex Fries 2. A. niger Van Tieghem var. phoenicus (Corda) Al-Musallam 3. A. repens (Corda) Sacc 4. A. sejunctus Bairier and Sartory
F	1. A. japonicus Saito 2. A. terreus Thom
G	1. A. flavus Link ex Fries 2. A. niger Van Tieghem var phoenicus (Corda) Al-Musallam 3. A. chevalieri Thom and Church 4. Cladosporium oxysporum Berk and Curt
H	1. A. clavatus Desmaziere 2. A. chevalieri Thom and Church 3. Penicillium cyclopium Westling

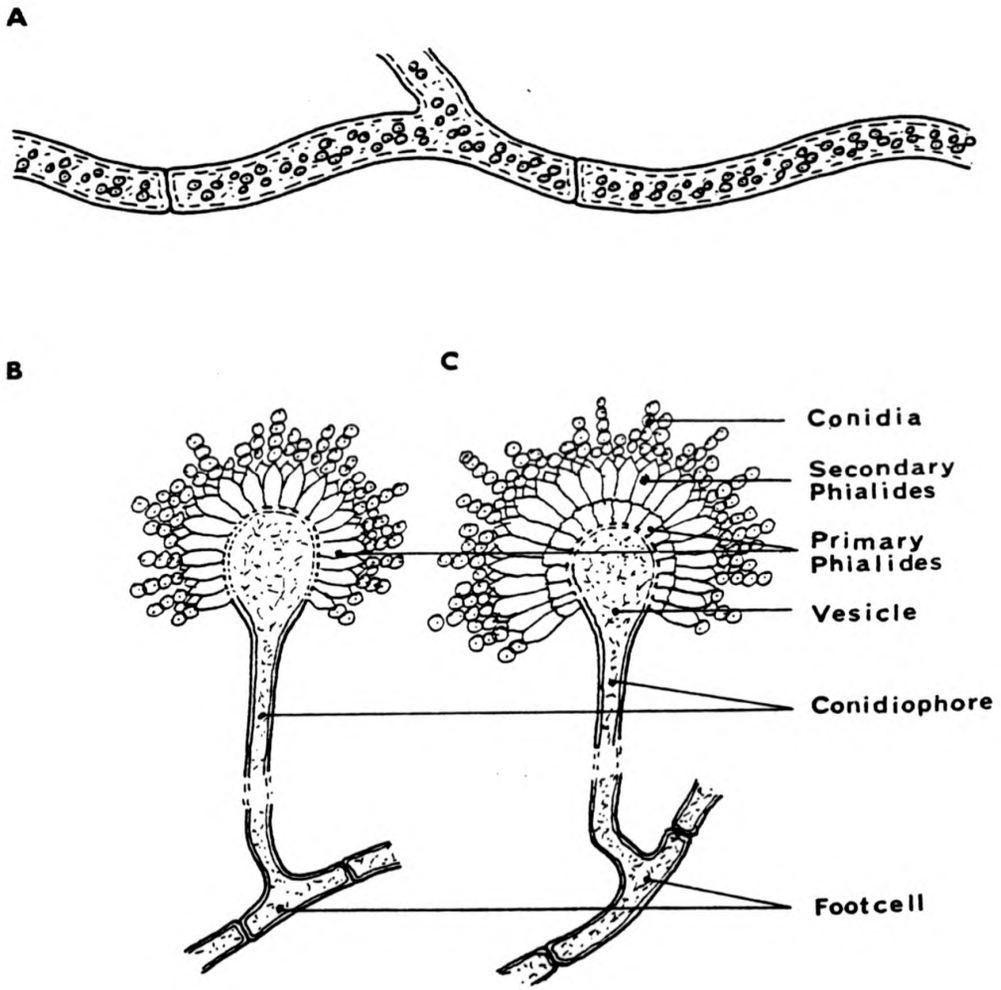

Fig.3.1.1 CONIDIOPHORES AND HYPHA OF ASPERGILLUS

A = Hypha showing multinucleate condition

B = Conidiophore with one row of phialides

C = Conidiophore with two rows of phialides

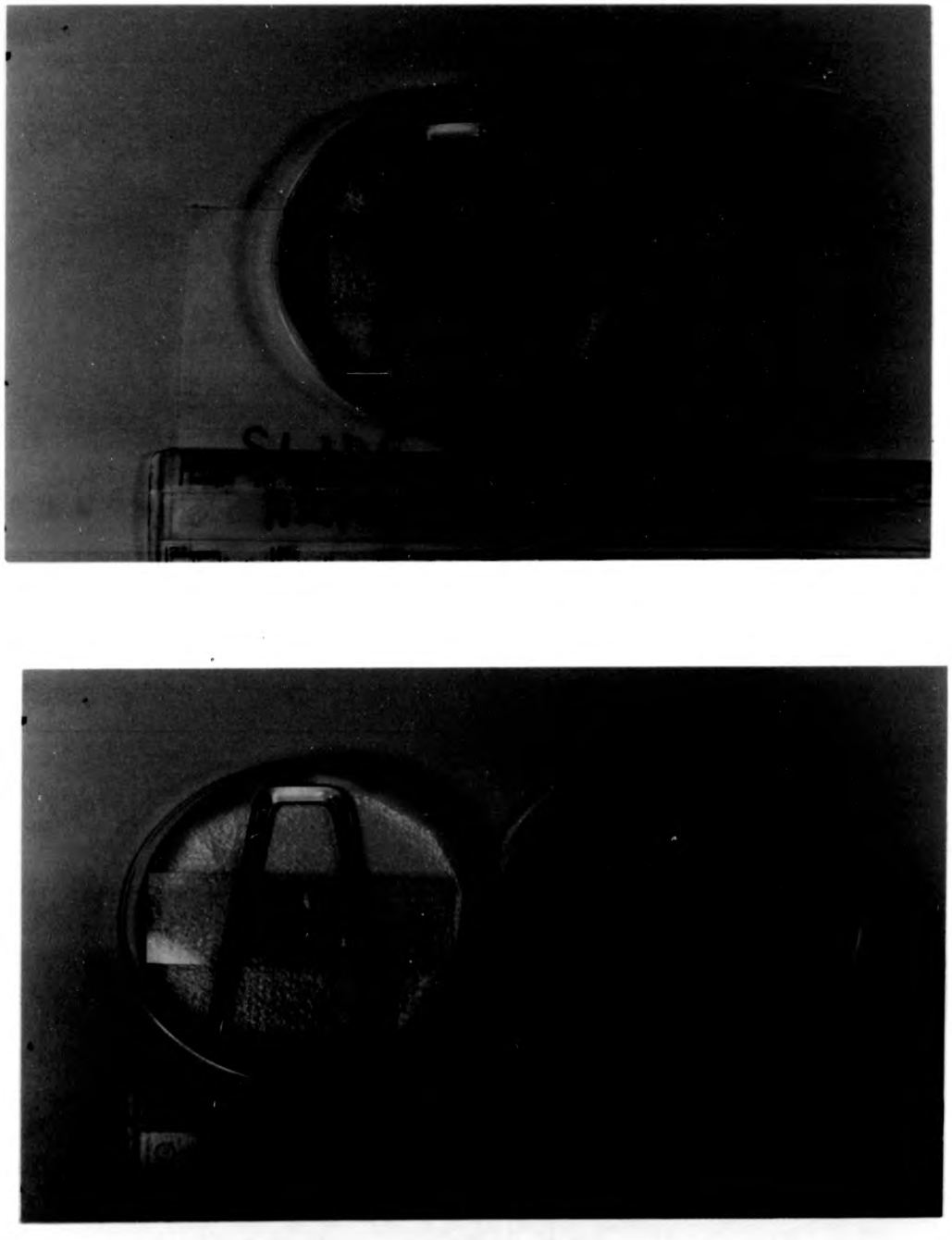

Fig. 3.3.1 Slide Culture arrangement.

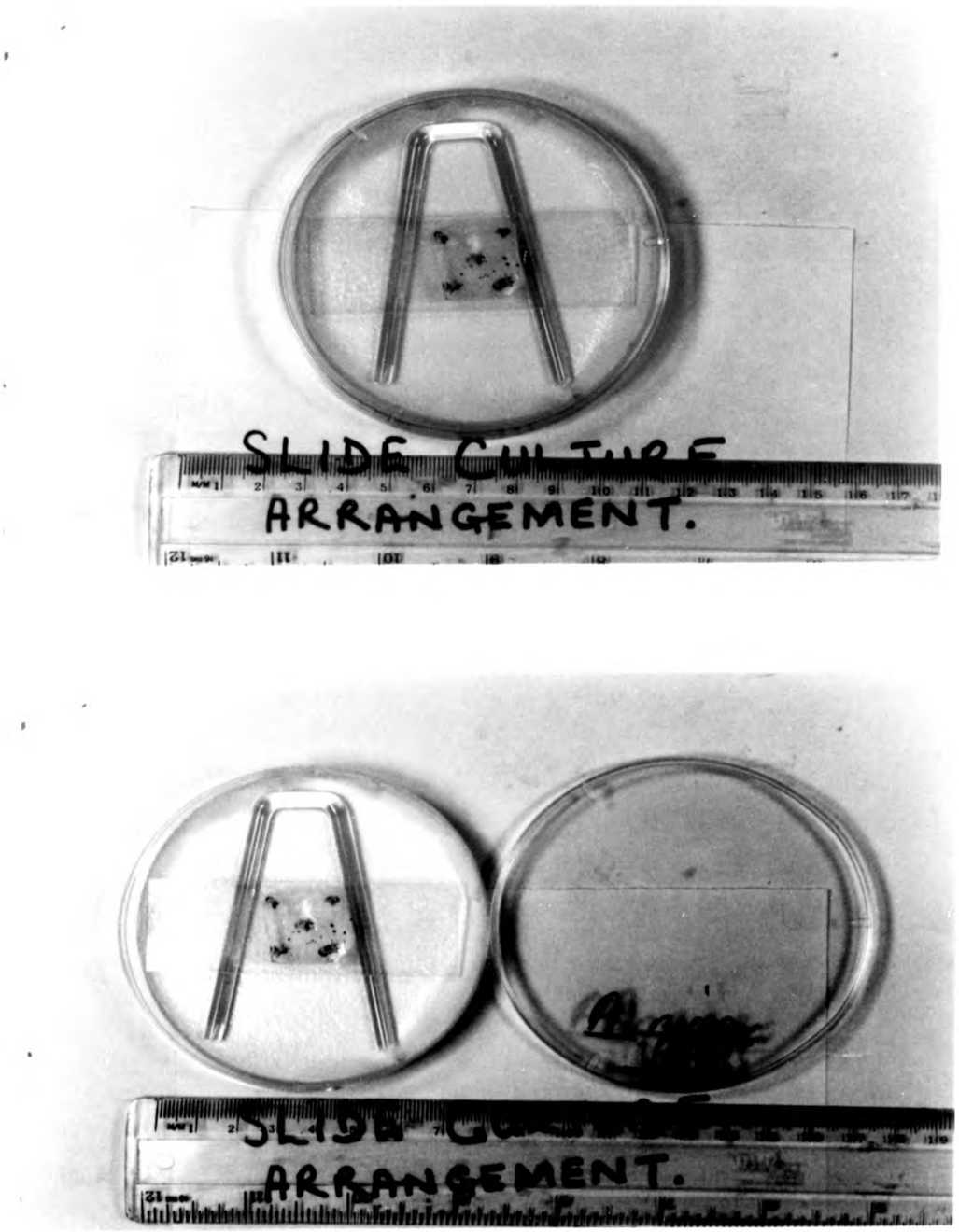

Fig. 3.3.1 Slide Culture arrangement.

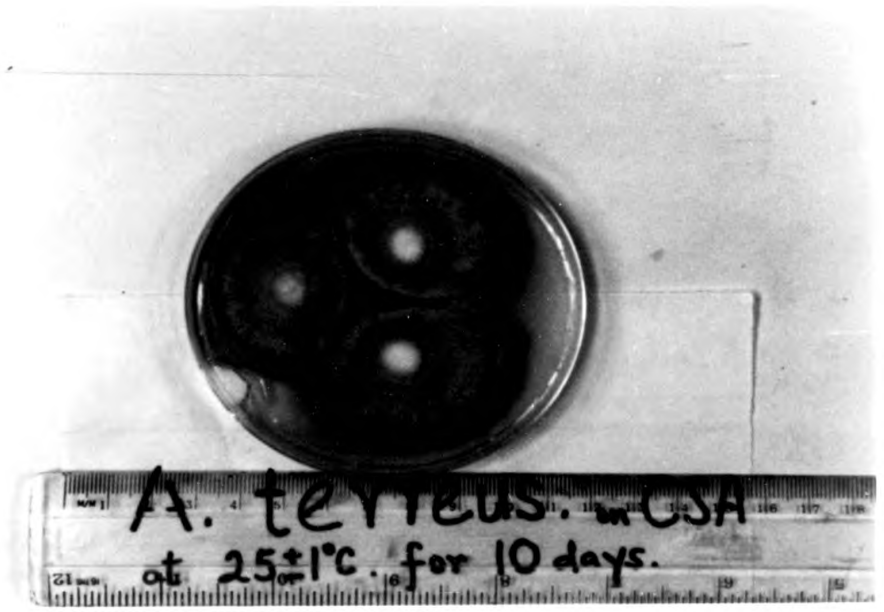

Fig. 3.3.2 A. terreus on CSA at 25 ± 1°C for 10 days.

Fig. 3.3.3 A. terreus on CSA, plate underside after 10 days incubation.

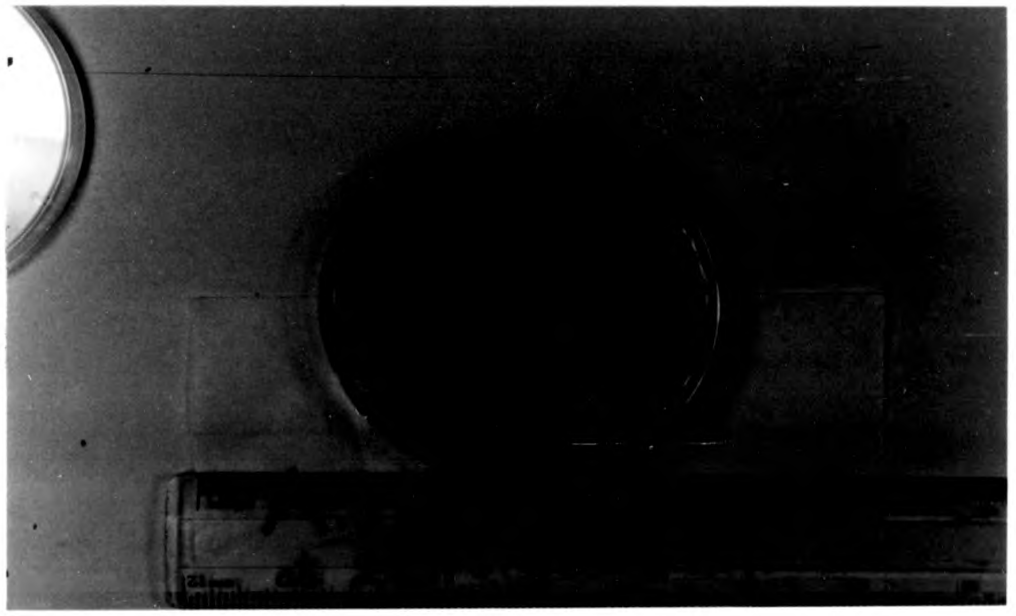

Fig. 3.3.2 A. terreus on CSA at 25 ± 1°C for 10 days.

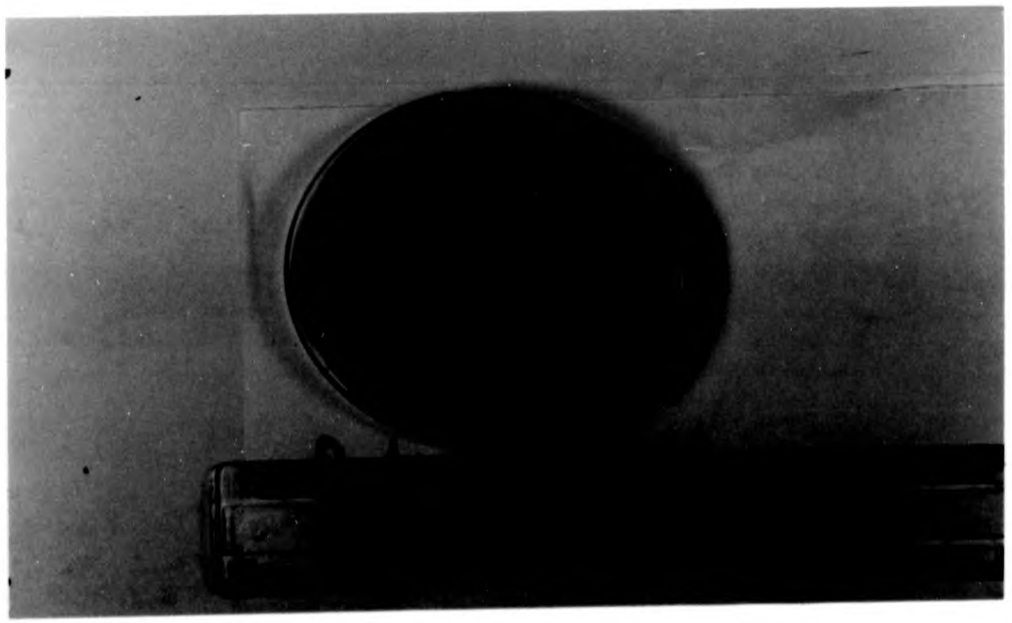

Fig. 3.3.3 A. terreus on CSA, plate underside after 10 days incubation.

Fig. 3.3.4 A. terreus - showing columnar arrangement of the conidia on the heads X 128

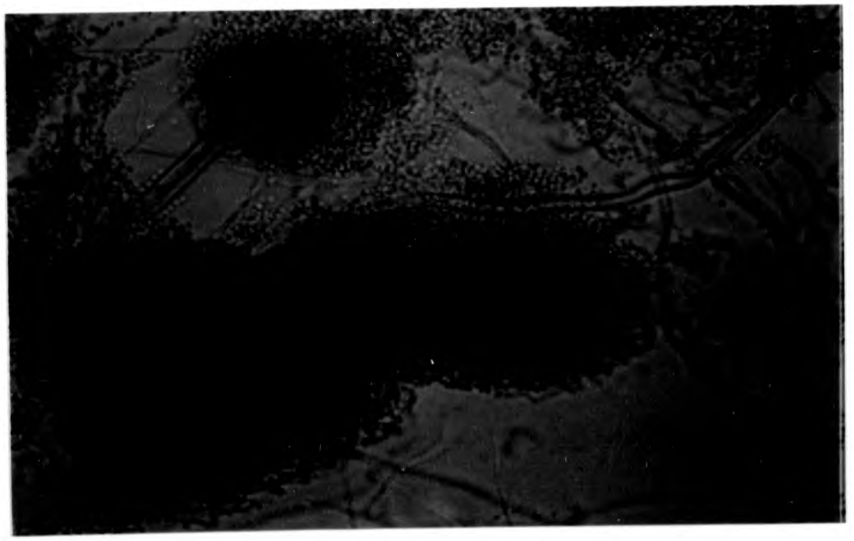

Fig. 3.3.5 A. terreus showing the hemispheral and domelike vesicles X 128

Fig. 3.3.4 A. terreus - showing columnar arrangement of the conidia on the heads X 128

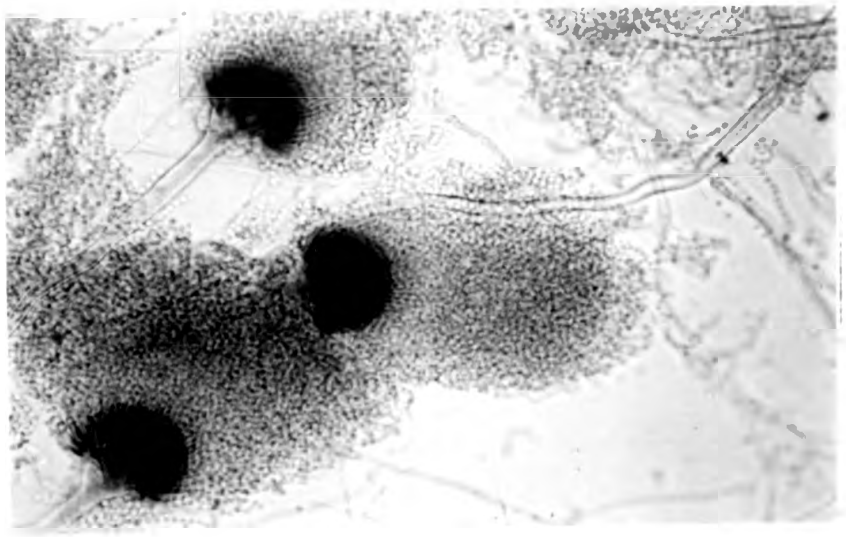

Fig. 3.3.5 A. terreus showing the hemispheral and domelike vesicles X 128

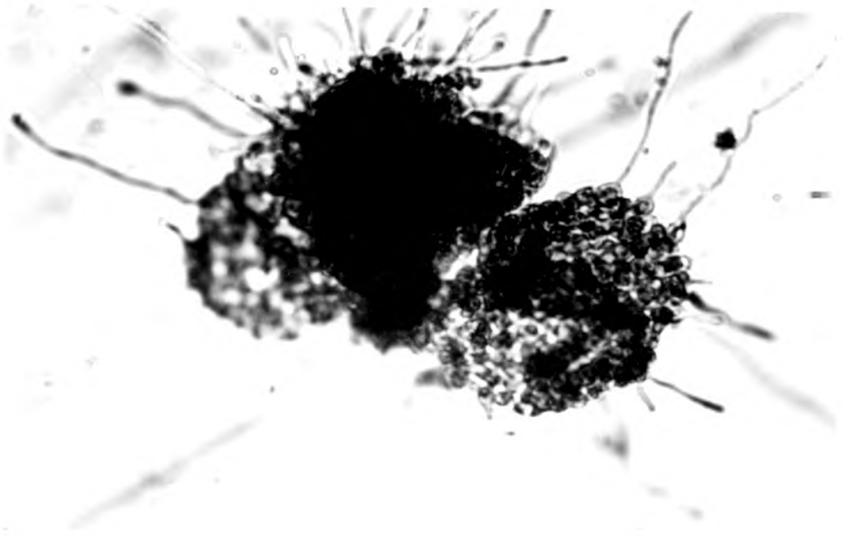

Fig. 3.3.6 A. flavus showing germinating sclerotium X 256

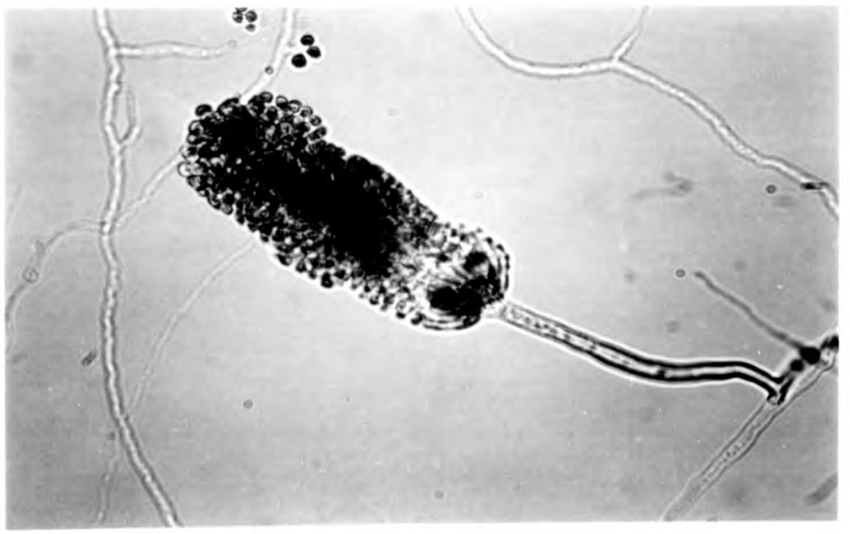

Fig. 3.3.7 Typical head, conidia, conidiophore and the footcell of A. flavus X 160

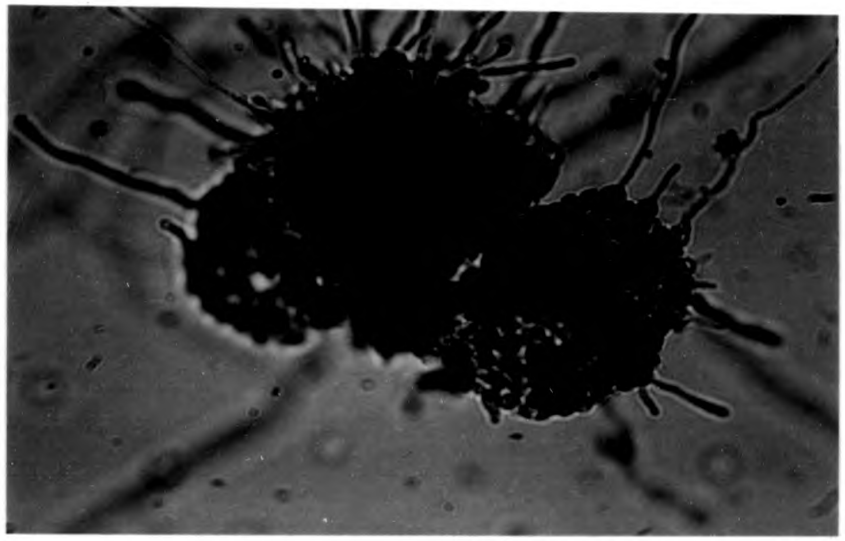

Fig. 3.3.6 A. flavus showing germinating sclerotium X 256

Fig. 3.3.7 Typical head, conidia, conidiophore and the footcell of A. flavus X 160

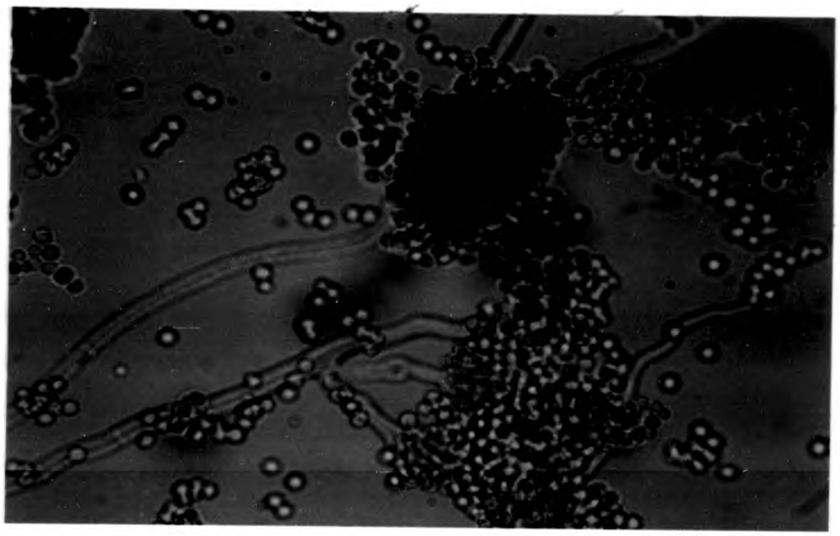

Fig. 3.3.8 Typical smooth conidia of A. flavus X 256

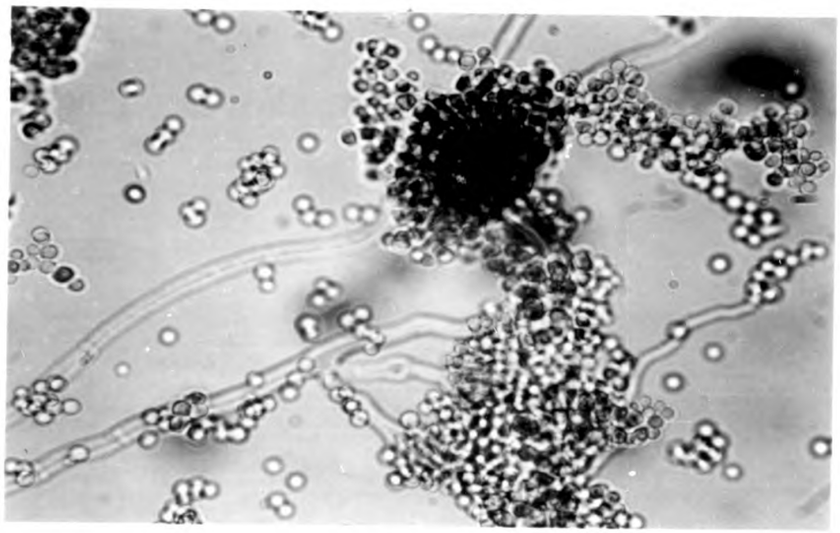

Fig. 3.3.8 Typical smooth conidia of A. flavus X 256

3.4 DISCUSSION

Several formulations and types of media are in common use for the isolation of pathogenic fungi. Bothast and Fennell (1974) described a medium for the rapid identification and enumeration of A. flavus and related organisms. It is imperative that cultures of Aspergillus for identification or description be grown on a variety of media so that their complete development can be observed and recorded. Czapek's solution agar (CSA) always has to be included since it is on this medium that most species produce the characteristic colonies, pigmentation and conidial heads upon which most descriptions are based. From the point of view of Aspergillus species pathogenic to fish, malt extract agar (MEA) and CSA gave consistently good results in this study for both growth and identification. It appeared that the depth of the agar in the plates significantly influenced the character of the colonies developed. In order to ensure that adequate comparisons could therefore be made, approximately 25 ml of medium, in 8.5 cm diameter sterile petri dishes, giving a depth of 0.5 cm of medium, was routinely used and found to be adequate for diagnostic study.

Homogenisation of the clinical material appeared to provide the greatest opportunity for infectious agents to come into contact with the medium surface and hence provided the maximum recovery of fungi present in the specimens. The brain heart infusion broth (BHI) essentially enhanced the growth of Aspergillus species where they were present. Isolations of Aspergillus species from fish clinical material were sometimes difficult, probably because of the small numbers of spores or fungal material present or the unsuitability of the media without the incorporation

of the brain heart infusion broth. The addition of the BHI broth consistently improved the growth.

Colony age, exposure to light, incubation temperature and medium composition have to be held constant when identifying species of Aspergillus. The uniseriate, uniseriate and biseriate, or biseriate arrangement of the phialides was the major characteristic used in distinguishing and identifying the Aspergillus species isolated both from the clinical fish material and from the pellets. Although a hand lens is frequently used in mycology laboratories, in the present study, it was found to be much more satisfactory to use a simple dissecting microscope and to focus on the edge of a row of phialides or to gently open up the heads and determine whether or not the phialides were attached to metulae. The dissection microscope facilitated viewing of the arrangement of the conidial chains, and bridged the gap between gross colony characteristics and microscopic observations.

There are always difficulties in specifically relating particular species of Aspergillus to actual clinical disease outbreaks because of the ubiquity of the organism. Mere isolation without direct association with clinical histopathological damage is not satisfactory. This has led to confusion in the animal literature which is further compounded by the way in which many medical mycologists have used species epithets and Aspergillus group names interchangeably. For example, A. glaucus is occasionally reported in the literature as an aetiological agent of aspergillosis. Aspergillus glaucus was originally described in vague terms and was subsequently used for a number of different Aspergillus species. The name A. glaucus has no real value since it does not represent any particular species of

Aspergillus.

Although isolation techniques were used in this stage of the study, which allowed a wide range of organisms to grow, Aspergillus species predominated throughout, and in view of the clinical evidence of Aspergillus within smears of affected tissues, growing within lesions of affected fish and the same species being associated with the fish and the relevant feed, it was concluded that the Aspergillus isolates obtained from the clinically affected fish stock could well have arisen from food contamination.

It is difficult to ever be certain with outbreaks of apparent clinical Aspergillus infection, as to whether the infection was primary or the Aspergillus species was simply taking opportunistic advantage of a pre-existing condition. Thus, it was considered, as a result of the consistent isolation particularly of A. flavus and A. niger from both sources, fish and feedstuff, that a detailed study involving attempted infection of known healthy fishes held under optimum conditions with the fungi in the absence of any other pathogen was necessary.

CHAPTER 4

EXPERIMENTAL ASPERGILLOMYCOSIS IN CULTURED TILAPIAS

4.1 Infection by intra-peritoneal injection of fungal material into Oreochromis niloticus

4.1.1 MATERIALS AND METHODS

4.1.1(i) Aspergillus species used for this study

Pure cultures of Aspergillus flavus Link ex Fries and Aspergillus niger van Tieghem var phoenicis (Corda) Al-Mussallam isolated from the outbreak described in Chapter 2 were used in this part of the study.

4.1.1(ii) Experimental design

Eight experimental tanks measuring 46 x 20 x 20 cm were used, each holding between 15 and 21 Oreochromis niloticus. The fish, derived from common parental stocks and weighing 5 - 10 g were randomly distributed in the tanks. The water in four tanks (tanks 1 - 4) was maintained at 26°C while the other four tanks (tanks 5 - 8) were maintained at 18°C. A box filter was introduced into each tank to aerate as well as filter the water. Fish were fed on trout pellets ad libitum and allowed to acclimatise for four days.

4.1.1(iii) Preparation of the fungal inoculum

The inoculum was prepared by the introduction of 5 ml of sterile phosphate buffered saline in aliquots of 1 - 2 ml into

the plates containing pure 7 - 10 day old cultures of A. flavus and A. niger. The harvested fungal materials were aseptically transferred into sterile bottles. A third sample containing a mixture of A. flavus and A. niger (1:1) was prepared in a similar fashion.

4.1.1(iv) Conidial count using a haemocytometer

The object of this exercise was to make an estimation of the number of conidia (spores) per mm^3 of the inoculum. A known quantity of the inoculum prepared in 4.1.1(iii) was taken and was diluted to a further known volume with a diluting fluid - 1% Tween 80. The number of conidia in a very small known volume of diluted fungal inoculum was counted and the number multiplied to give the number of conidia present in a mm^3. The Improved Neubauer haemocytometer slide is divided into three parts. The surface of the smaller rectangular area in the centre of the slide is 0.1 mm lower than the other two parts so that when a cover slip was placed over the centre, a chamber of depth 0.1 mm was formed. The upper surface of the central region bears a set of finely etched squares. When examining the slide, a microscope was used to locate this ruled area. Each of the smallest squares has a side of length 1/20 mm and thus an area of 1/400 mm^2. In the case of the inoculum spore counts, the pipette used to dilute the suspension of the inoculum was graduated at 0.5, 1.0 and 101 units.

The prepared slide containing exactly 1/4000 mm^3 of suspension per square was placed on the microscope stage and the low power was used to focus on the counting chamber. Under high power, when counting conidial suspensions it was most convenient to use

a X 40 objective and to count in the central, triple-ruled area of the haemocytometer.

Conidia in 80 of the small squares occupying five of the larger squares across the centre of the counting area were counted. All the spores in each square were counted while any which touch the bottom or right side of the square were disregarded. In this way counting the same conidia twice was avoided.

To find the total number of conidia in 1 mm^3 of inoculum the following formula was applied

Let n = the number of conidia counted over 80 squares

80 squares represents a volume of 80 x 1/4000 mm^3 = 1/50 mm^3.

Therefore there are n conidia in 1/50 mm^3 of diluted suspension, i.e. there are 50 n conidia in 1 mm^3 of the diluted inoculum. And, the number of conidia in 1 mm^3 of undiluted inoculum =

50 n x 200 = 10,000 n or n x 10^4 conidia.

The exercise was carried out four times and mean of the counts was used to estimate the approximate number of conidia in the suspension of the inoculum. The A. flavus inoculum contained approximately 9 x 10^4 conidia per mm^3 and A. niger contained approximately 2.7 x 10^5 conidia per mm^3.

4.1.1(v) Intraperitoneal injections of the prepared inoculum

All fish were anaesthetised using benzocaine dissolved in alcohol prior to inoculation. Each fish was inoculated via the intraperitoneal route with 0.2 ml of the fungal suspension.

Fish in tanks 1 and 7 were inoculated with A. niger. Fish

in tanks 2 and 6 were inoculated with a 1:1 mixture of A. flavus and A. niger while those in tanks 3 and 5 were inoculated with A. flavus only. Fish in tanks 4 and 8 acted as controls. Thus one group from each treatment was held at 26°C and one from each held at 18°C. The dose rate of the spores per inoculum per fish was approximately 18,000 spores for the A. flavus injected fish and 54,000 spores for the A. niger injected fish.

4.1.1(vi) Histopathology

Blocks of liver, kidney, gut, heart, gills and muscle were dissected out of fish from experimentally infected and control batches and fixed in 10% neutral buffered formalin, paraffin wax-embedded, cut at 5 um and stained with haematoxylin and eosin (H & E), periodic acid - Schiff's technique (PAS) and Grocott's modification of Gomori's methenamine silver technique for fungi. (Appendix B - D).

4.1.1(vii) Fungal re-isolation and re-identification

Blocks of liver, kidney, gut, heart and gills were dissected out of fish from the experimentally infected batches and fungus re-isolation carried out as described in Chapter 3. The fungi recovered were re-identified.

4.1.2 RESULTS

It can be seen from Tables 4.1.1 and 4.1.2 that both species of Aspergillus used for this pathogenicity study were significantly pathogenic. There was a marked difference in the time taken to cause mortality at the two differing temperatures, but even more remarkable was the consistent finding that A. niger was

completely non pathogenic at 26°C, whilst at 18°C, although still not as pathogenic as A. flavus, it did produce the typical mycotic syndrome after a relatively longer period (approximately 20 days).

All fish, except for the control and the fish injected with Aspergillus niger at 26°C, eventually succumbed. Table 4.1.3 indicates the differences between the acute pathogenicity of the different species expressed as level of mortality associated with fungal infection after 4 days exposure.

Re-isolation from moribund or dead fish produced pure cultures of the A. flavus or A. niger fungus which had been inoculated and so Koch's postulates were fulfilled. Where a mixed culture was inoculated, a mixed growth was also recovered on re-isolation. In the case of A. niger tested at 26°C, although there was no mortality over the length of the study, when they were sacrificed at day 17, A. niger could be readily re-isolated from the liver and kidney of the test fish.

Clinical observations of the fish during such exposures showed that several days before they succumbed, they showed darkening of the skin, lack of mobility and in some cases exophthalmia. Most of them refused to feed, and by the end of the study, even the group injected with A. niger, which did not show any actual mortality were nevertheless inactive, dark in colour and inappetent.

Gross pathology: When moribund fish were necropsied, there was generally a significant volume of straw coloured, opaque fluid in the abdominal cavity. In fish such as those infected with A. flavus at 26°C, where death took place very soon after

injection, there was extensive focal or generalized necrosis of the liver with often only a limited area of normal tissue remaining. In all fish, whether dying immediately or after prolonged cachexia, there was pallor of the gills and kidney, and the heart was generally paler than normal.

Histopathology: The organs principally affected in all fish, were the liver, pancreas, heart, eye and gastro-intestinal tract. In the case of acute mortalities, there was generalized fungal growth throughout the necrotic hepatic tissue, with hyphae extending into hepatic vessels and the biliary tract. It seemed likely that in many such cases, the hepatic necrosis was related to obstructive infarction of the vascular system by hyphal proliferation. Occasionally conidia were seen in close association with septate hyphae as they penetrated the tissue. The degree of liver necrosis seemed to be directly correlated with acute mortality. Those fish which died in the first few days after exposure all showed severe hepatic necrosis as the main presenting sign. In such cases, splenic infarction was also seen, and splenic necrosis usually accompanied the more severe infection.

The gills of affected fish were pale and oedematous with intra vascular conidia observed in a few cases. The effect on the gill however was generally considered to be secondary to the internal lesions. The gut was oedematous in acute infections. In the more chronic cases especially with A. niger at 18°C, there was a generalized chronic granulomatoses of the gut submucosa, with epitheliod cells enclosing foci of necrotic fungal material (Fig. 4.1.7). The kidney and liver of such chronic cases also showed evidence of granulomatous reaction to the activity of the

fungi but the fish succumbed or were sacrificed before fibrosis of the granulomata could develop properly.

Occasionally, in all cases, a few fish showed extension of the fungus into the skeletal muscle. This appeared to have been by direct extension from the peritoneal focus, but in some cases may have resulted from tracking back of the fungus along the route of injection. The presence of exophthalmia was not directly related to the actual presence of fungal hyphae in the eye. In many cases, it appeared to be bilateral and related probably to the oedematous response due to lack of blood proteins following severe chronic liver damage. In many cases of monolateral and occasionally bilateral exophthalmia there were fungal hyphae accompanied by a chronic granulomatous inflammatory response in the rete of the choroid behind the eye or else the anterior and/or posterior chamber was itself infected, with exuberant growth of the fungus occurring without any obvious inflammatory effect other than a retinitis with inflammatory under-running of the retinal layer.

Table 4.1.1. (A) Experimental intraperitoneal inoculation of *Oreochromis niloticus* maintained at 26°C. Injection of *Aspergillus flavus*

Total Number of fish used	Days after injection	Mortality with evidence of Aspergillomycosis	Other Mortalities
16 treated	1	0	4
(16 control)	2	0	0
	3	7	0
	4	2	0
	5	2	0
	6	1	0

N.B. All fish dying after two days had evidence of generalised proliferation of hyphal elements in the visceral organs. The injection dose consisted of 0.2 ml of a suspension of the inocula (4.1.1.iv). No control fish died

Table 4.1.1 (B) Experimental intraperitoneal inoculation of *Oreochromis niloticus* maintained at 26°C. Injection of *Aspergillus niger*

Total Number of fish used	Days after injection	Mortality with evidence of Aspergillomycosis	Other Mortalities
16 treated	1	0	1
(16 control)	30	0	0

N.B. All fish dying after two days had evidence of generalised proliferation of hyphal elements in the visceral organs. The injection dose consisted of 0.2 ml of a suspension of the inocula (4.1.1.iv). No control fish died

Table 4.1.1 (C) Experimental intraperitoneal inoculation of *Oreochromis niloticus* maintained at 26°C

Injection of *Aspergillus flavus*/*Aspergillus niger* (1:1) mixture

Total Number of fish used	Days after injection	Mortality with evidence of Aspergillomycosis	Other Mortalities
15 treated	0	0	4
(16 control)	1	0	5
	2	2	0
	3	2	0
	4	2	0

N.B. All fish dying after two days had evidence of generalised proliferation of hyphal elements in the visceral organs. The injection dose consisted of 0.2 ml of a suspension of the inocula (4.1.1.iv). No control fish died

Table 4.1.2 (A) Experimental intraperitoneal inoculation of *Oreochromis niloticus* maintained at 18°C

Injection of *Aspergillus flavus*

Total Number of fish used	Days after injection	Mortality with evidence of Aspergillomycosis	Other Mortalities
21 treated	1	0	2
(20 control)	6	5	0
	7	3	0
	8	1	0
	10	3	0
	11	2	0
	12	3	0
	19	1	0

N.B. All fish dying after day four had evidence of proliferation of hyphal elements in the visceral organs.

Table 4.1.2 (B) Experimental intraperitoneal inoculation of *Oreochromis niloticus* maintained at 18°C

Injection of *Aspergillus niger*

Total Number of fish used	Days after injection	Mortality with evidence of Aspergillomycosis	Other Mortalities
15 treated	1	0	1
(20 control)	4	0	1
	8	1	0
	11	1	0
	13	1	0
	16	1	0
	19	1	0
	22	1	0
	25	2	0
	28	1	0
	29	1	0
	30	2	0

N.B. All fish dying after day four had evidence of proliferation of hyphal elements in the visceral organs.

Table 4.1.2 (C) Experimental intraperitoneal inoculation of *Oreochromis niloticus* maintained at 18°C
Injection of *Aspergillus flavus*/*Aspergillus niger* (1:1) mixture

Total Number of fish used	Days after injection	Mortality with evidence of Aspergillomycosis	Other Mortalities
15 treated	0	0	1
(20 control)	1	0	2
	9	1	0
	11	1	0
	12	1	0
	13	1	0
	15	1	0
	17	6	0

N.B. All fish dying after day four had evidence of proliferation of hyphal elements in the visceral organs.

Table 4.1.3 Comparative acute mortality after four days of Oreochromis niloticus injected intraperitoneally with with A. flavus, A. niger or a mixture of both at 26°C and 18°C

Aspergillus species injected	Percentage mortality associated with fungal infection after four days at	
	26°C	18°C
A. flavus	56.25	0
A. niger	0	0
A. flavus/A. niger (1:1)	39.99	0

The dose, fish origin and maintenance regime for all groups was identical.

The above data applies for comparative purposes, to the period four days after injection. All experimental fish, except the group injected with A. niger at 26°C succumbed with typical clinical signs by the 30th day post exposure.

Fig. 4.1.1 Tilapia showing typical acute mycotic syndrome with abdominal distention and darkening. Control fish - (below)

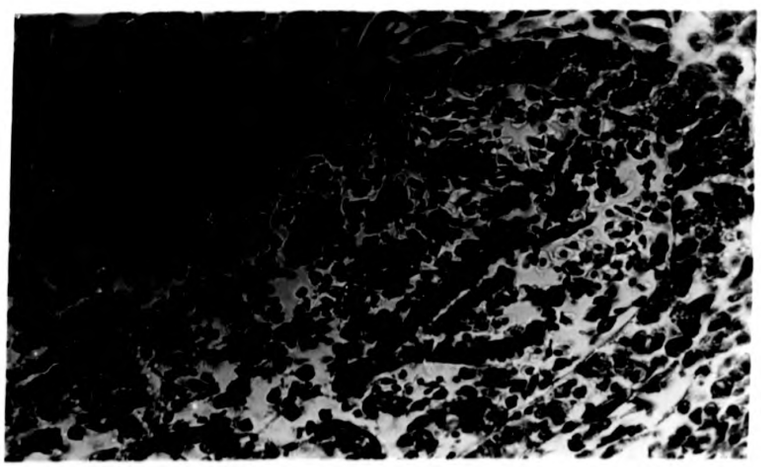

Fig. 4.1.2(a) Fungal hyphae within the hepatic vessel and hepatopancreas H & E X 398

Fig. 4.1.1 Tilapia showing typical acute mycotic syndrome with abdominal distention and darkening. Control fish - (below)

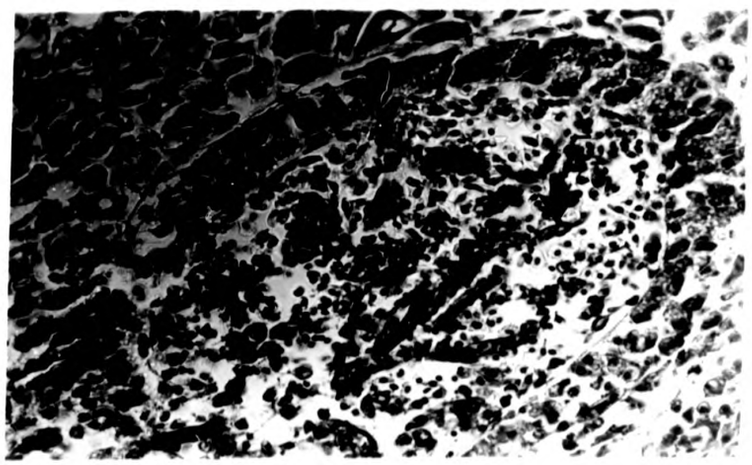

Fig. 4.1.2(a) Fungal hyphae within the hepatic vessel and hepatopancreas H & E X 398

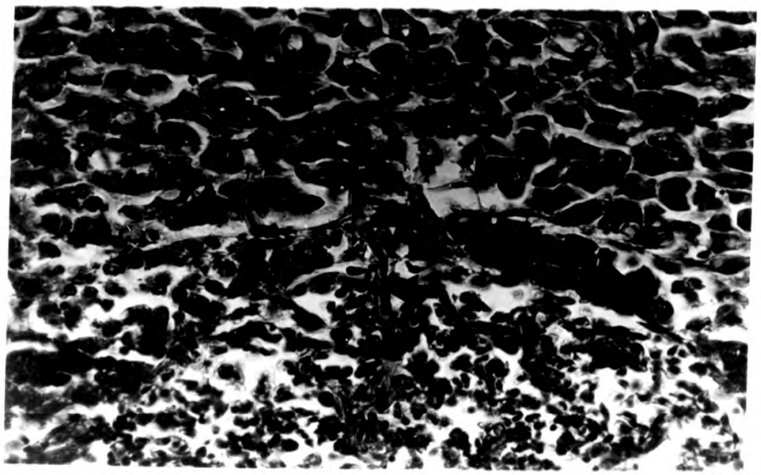

Fig. 4.1.2(b) Fungal hyphae extending into liver parenchyma
H & E X 398

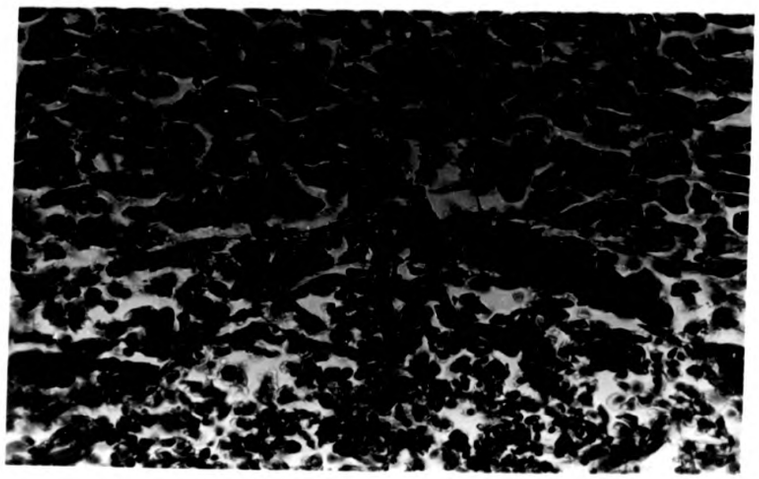

Fig. 4.1.2(b) Fungal hyphae extending into liver parenchyma
H & E X 398

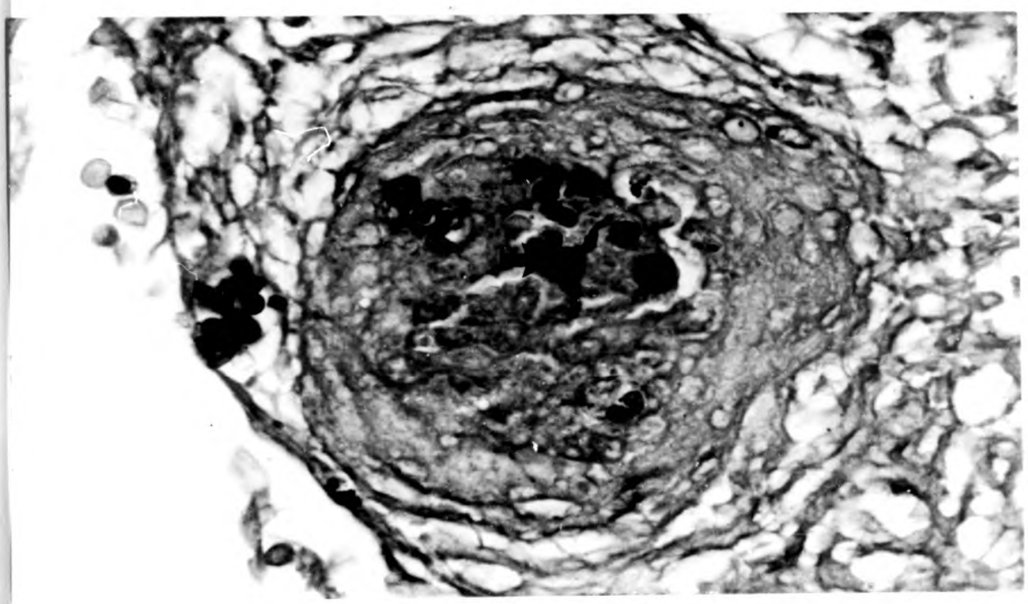

Fig. 4.1.4 Globose or oval-celled hyphae and spores located within a liver lesion. Grocott's stain X 600. A germinated conidium and its primary hypha is arrowed.

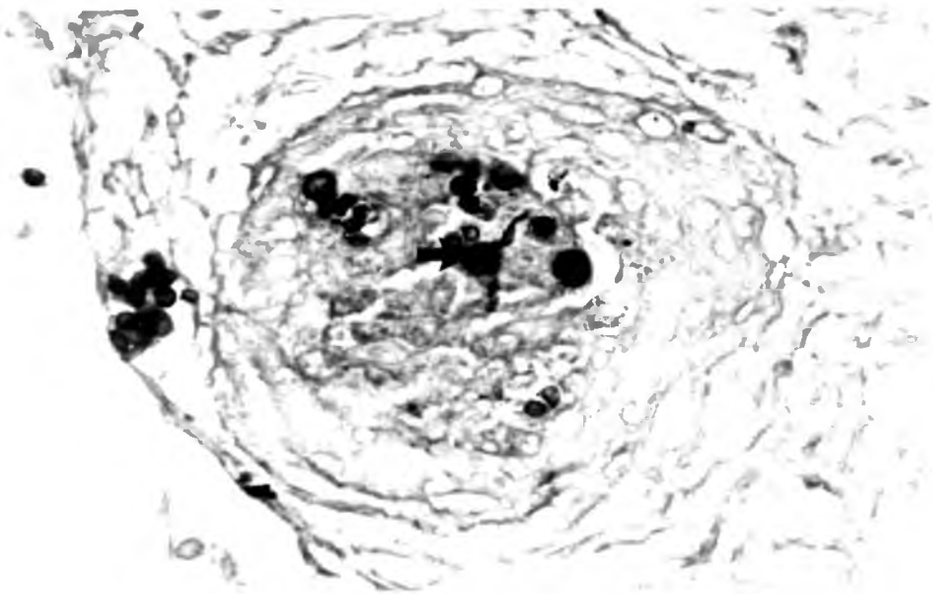

Fig. 4.1.4 Globose or oval-celled hyphae and spores located within a liver lesion. Grocott's stain X 600. A germinated conidium and its primary hypha is arrowed.

Fig. 4.1.3 Unstained fresh preparation from acute lesion in the liver of Oreochromis niloticus X 600.

Note: Direct microscopic examination was carried out by mounting small pieces of lesion in 20% sodium hydroxide solution on a slide, dissecting them with mounted needles, applying a cover slip and warming gently over a low flame. Slight pressure on the cover slip tended to spread the cleared and softened tissue and the refractive hyphae were more easily seen.

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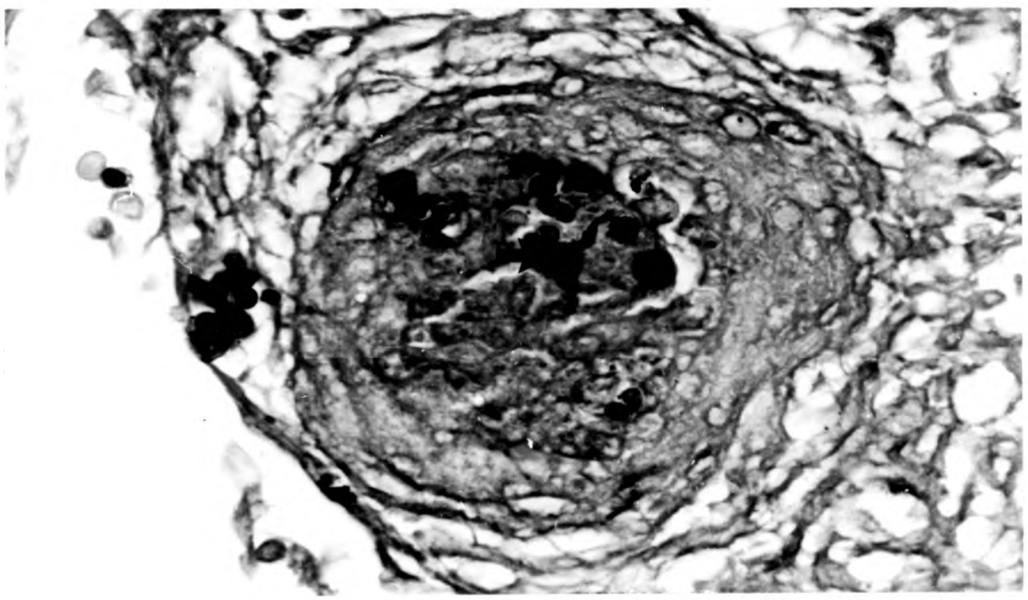

Fig. 4.1.4 Globose or oval-celled hyphae and spores located within a liver lesion. Grocott's stain X 600. A germinated conidium and its primary hypha is arrowed.

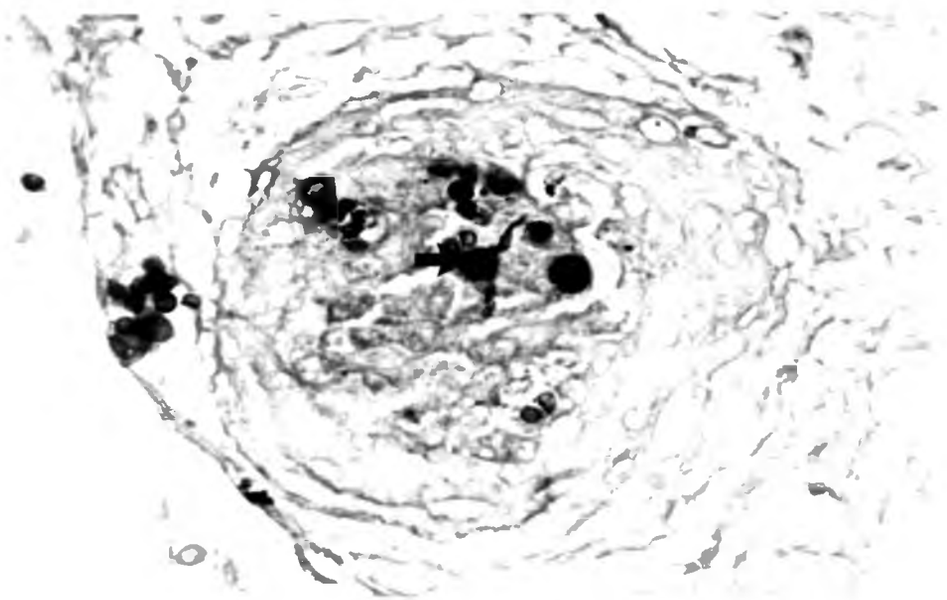

Fig. 4.1.4 Globose or oval-celled hyphae and spores located within a liver lesion. Grocott's stain X 600. A germinated conidium and its primary hypha is arrowed.

Fig. 4.1.5 Straight or spiral hyphae some of which are septate, some nonseptate. They are 2 to 3 u in diameter and appear to be spreading through hepatic parenchyma from a primary focus. Grocott's stain X 150.

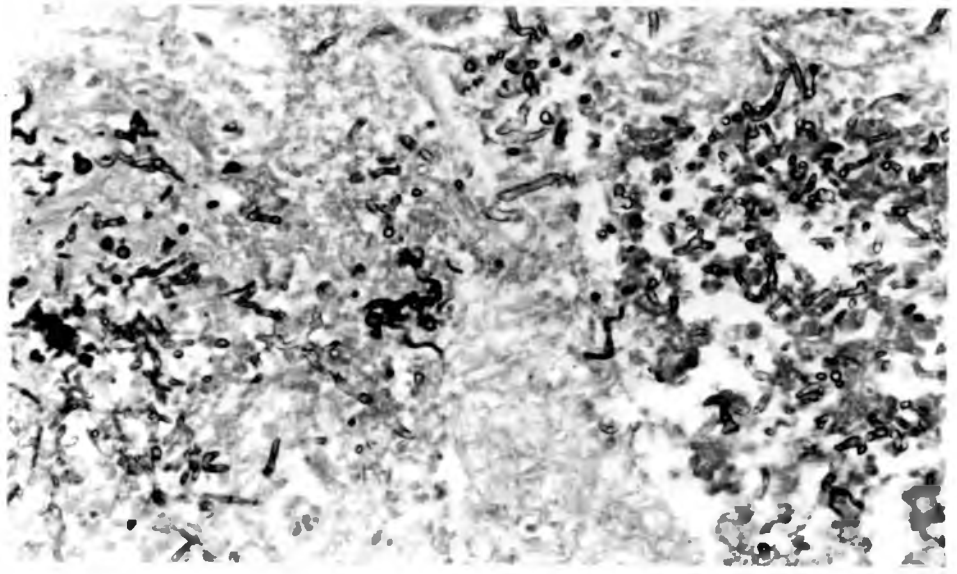

Fig. 4.1.5 Straight or spiral hyphae some of which are septate, some nonseptate. They are 2 to 3 u in diameter and appear to be spreading through hepatic parenchyma from a primary focus. Grocott's stain X 150.

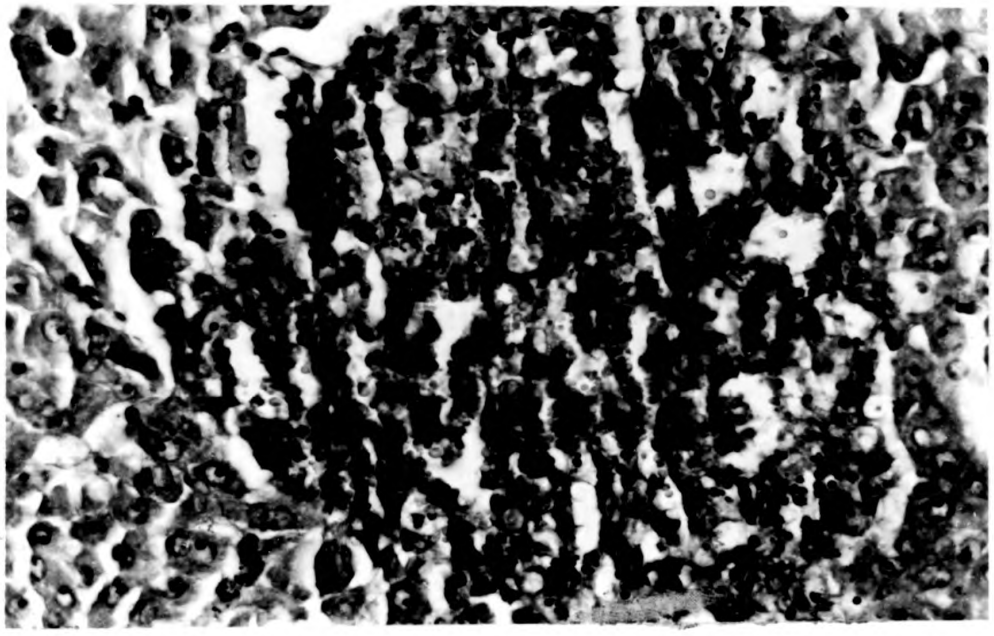

Fig. 4.1.6 Focal cellular infiltrations of the liver 10 days post exposure. H & E X 600.

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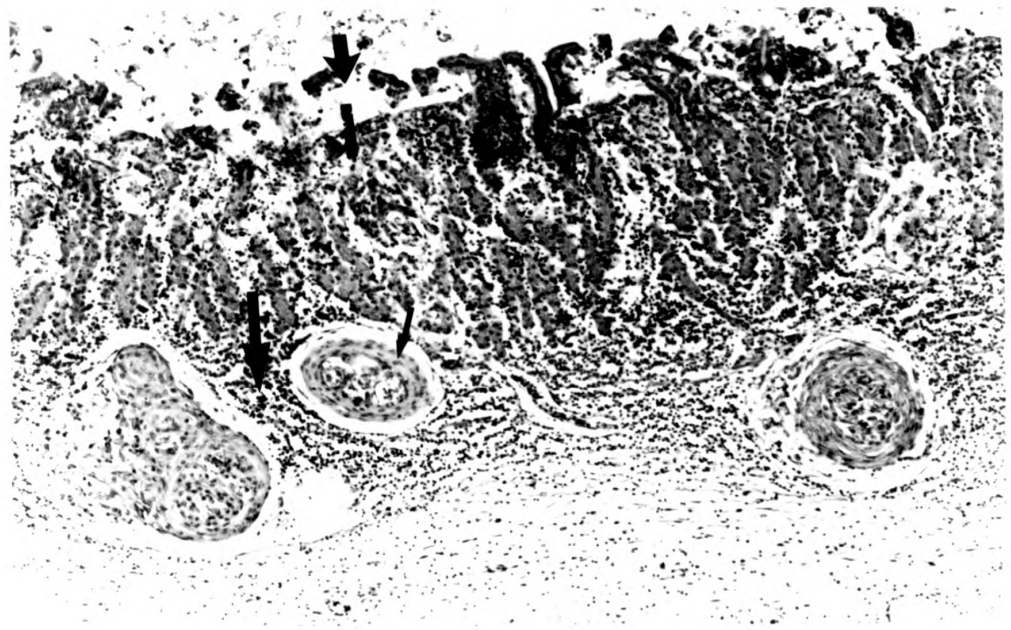


Fig. 4.1.7 Granulomatoses of the submucosa of the gut of a moribund Oreochromis niloticus. Epithelioid cells enclosing foci of necrotic fungal elements (arrowed). There is a generalised inflammatory cellular infiltrate of the submucosa and degeneration and sloughing of the mucosal epithelium. This is a characteristic picture of the late stages of infection and suggests that the gut is a likely source of release of infective material for infecting other fish (H & E X 150).

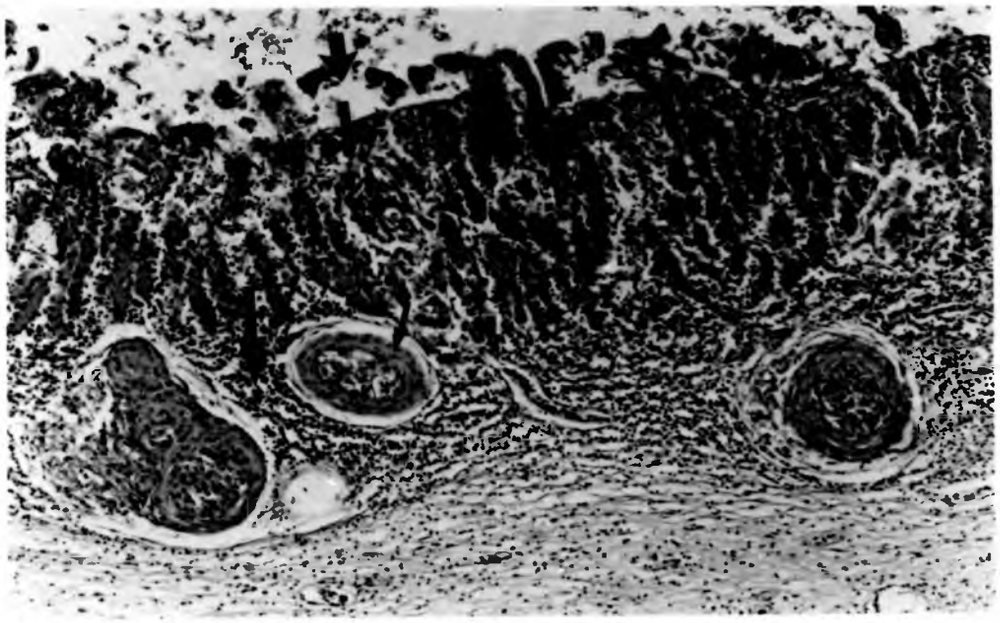


Fig. 4.1.7 Granulomatoses of the submucosa of the gut of a moribund Oreochromis niloticus. Epithelioid cells enclosing foci of necrotic fungal elements (arrowed). There is a generalised inflammatory cellular infiltrate of the submucosa and degeneration and sloughing of the mucosal epithelium. This is a characteristic picture of the late stages of infection and suggests that the gut is a likely source of release of infective material for infecting other fish (H & E X 150).

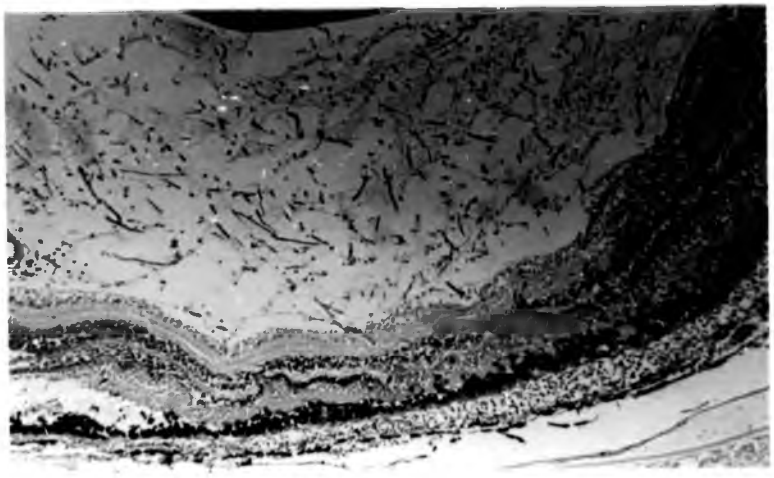


Fig. 4.1.8 Section of the eye showing acute non-granulomatous ophthalmitis with fungal strands in the retina, choroid, iris and the anterior chamber of the eye H & E X 64.

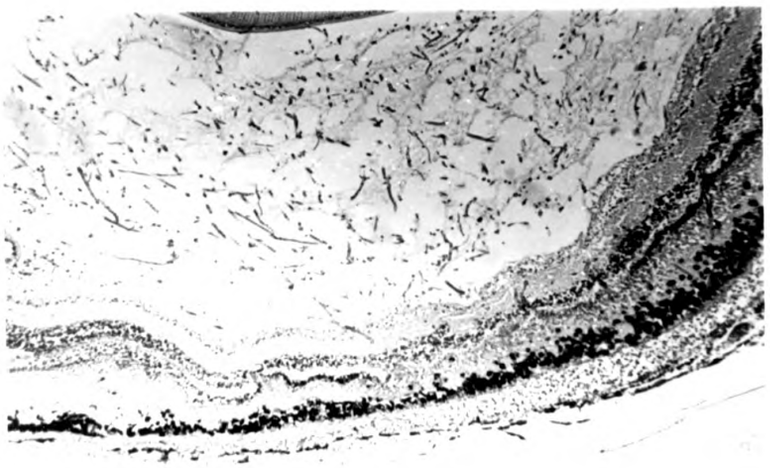


Fig. 4.1.8 Section of the eye showing acute non-granulomatous ophthalmitis with fungal strands in the retina, choroid, iris and the anterior chamber of the eye H & E X 64.

4.2 Oral infection with pure cultures of A. terreus and A. japonicus

Having defined the pathogenic capability of Aspergillus organisms for tilapias by intra peritoneal injection, it was considered important to try to define the route of natural infection. The most likely route, given the level of contaminants found in tilapia feeds was considered to be the oral route, and, so the following experiments were designed. Aspergillus terreus and Aspergillus japonicus were used for the investigation of the oral route of infection because these were the strains found to be regularly associated with tilapia feed pellets, and had also been isolated from fish tissue from clinically affected farm fish (Chapter 3).

4.2.1 MATERIALS AND METHODS

The fast growing hybrids between female Oreochromis aureus and Oreochromis niloticus male were used for this experiment. Fish weighing between 20-50 g were randomly placed in each of four tanks, measuring 60 x 30 x 30 cm, 20 fish per tank.

Two tanks were used for A. terreus infection, one containing freshwater maintained at 25 $\pm$ 1^{o}C and the other containing freshwater maintained at 17 $\pm$ 1^{o}C. The remaining two tanks were similarly arranged and used for A. japonicus infection.

Into each tank was inserted a box filter to aerate as well as filter the water. The system was left for four days for observation after which stabilization was established. Subsequently, the tanks were washed and replaced with clean fresh water at the respective temperatures, once weekly. Ten day old pure cultures of A. terreus and A. japonicus with confluent growth on Czapek's

solution agar (CSA) were scored and cut into small lumps similar in size to the trout pellets (0.5 - 1 mm) upon which the fish normally fed. Prior to feeding, fish were deprived of food for 48 hours following which the small lumps containing the fungal materials were introduced into the respective tanks; as a result of their deprivation the fish fed voraciously on this material. All fish were fed fungal materials twice, two days apart. It was not possible to determine how much of the dose was consumed by each individual fish, so no measure of actual amounts of fungus taken up is possible.

4.2.1(i) Histopathology

Two fish were sampled on the 7th, 14th and 18th day from each of the tanks. On each occasion fish with significant clinical signs were used. Blocks of gills, heart, kidney, liver and gut were dissected out, fixed in 10% neutral buffered formalin, paraffin wax-embedded, cut at 5 um and stained with haematoxylin and eosin (H & E), periodic acid-Schiff's technique (PAS) and the Grocott's modification of Gomori's methanamine silver technique for fungi.

4.2.1(ii) Fungal re-isolation and re-identification

Following the feeding of fungal spores, it was confirmed that viable spores had been ingested, because isolation could be achieved from expressed faeces of the experimental fish one week after feeding. Fungal re-isolations were also carried out from the brain, gills, heart, kidney and gut of the two experimental fish from each group eight weeks post-ingestion, using the techniques described in Chapter 3. All fungal isolates were

re-identified.

4.2.2 RESULTS

The mortality pattern in the fish exposed per os was much less dramatic than in the case of those exposed to a heavy parenteral dose earlier. Two fish died from the A. terreus fed tanks kept at 17 ± 1°C on day 7 after ingestion. Nine days later another death was recorded from the same tank. Although no deaths were recorded from each of the other tanks within the eight week period, swimming movements and feeding were reduced when compared with fish in the control tanks. Only A. terreus was re-isolated from the dead fish and the moribund sampled fish in A. terreus infected tanks, and A. japonicus was re-isolated in pure culture from those fish fed that particular fungus. The respective fungus was also re-isolated from the faeces of fish from the experimental tanks. No fungi was re-isolated from fish in the control tank.

Histopathology:

One week post ingestion, A. terreus at 17 ± 1°C produced haemorrhages in the liver and the haemopoietic tissue of the kidney accompanied by a varying degree of degeneration of the renal tubules. In all, oedema of the gill lamellae and in a few cases, with resultant desquamation of the primary lamellae epithelium was a constant feature. Early organisation of mycotic granulomas occurred along the glandular region of the gut, and especially at one week, there was an extensive degeneration of the gut endothelium and oedema of the muscles of the intestinal wall.

By the second week, typical mycotic granulomas appeared in the kidney and in the glandular region of the stomach.

There were large amounts of mucous secretion essentially from the lamellae of the gills. The mucus in many areas appeared to have attracted fungal materials in the water during respiration, which then stained black with Grocott's methenamine silver stain technique. The mucous secretion appeared less dense at eight weeks in all cases. The eyes showed no granulomas but there was evidence of fungal components especially frequently in the anterior chamber, extending into the choroid and the retina of the eye.

Generally, the histopathological lesions observed, were similar in all cases, except that the lesions in fish kept at $25 \pm 1^{\circ}C$ appeared comparatively less marked than in those kept at $17 \pm 1^{\circ}C$.

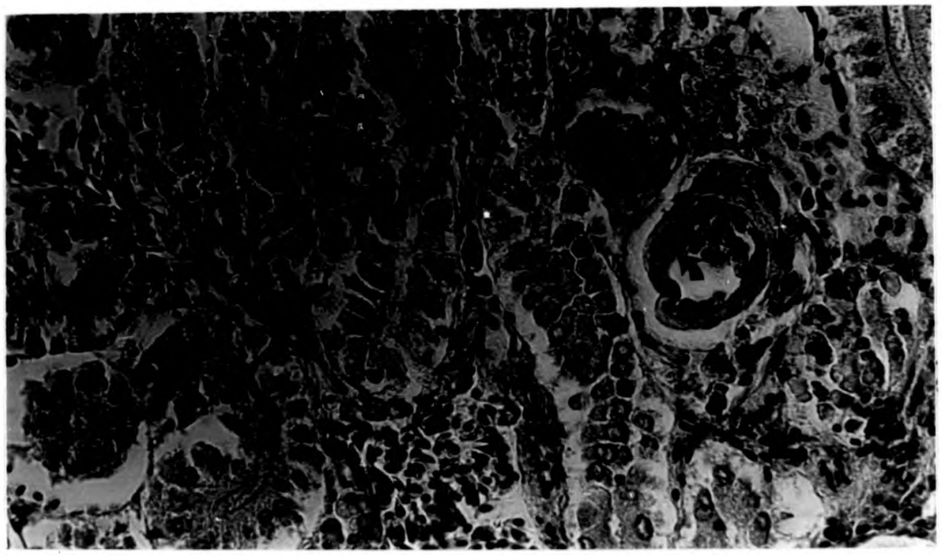


Fig. 4.2.1(a) A. terreus infection one week post ingestion at 17 ± 1°C. Note early organisation of epithelioid cells and a few giant cells PAS X 504

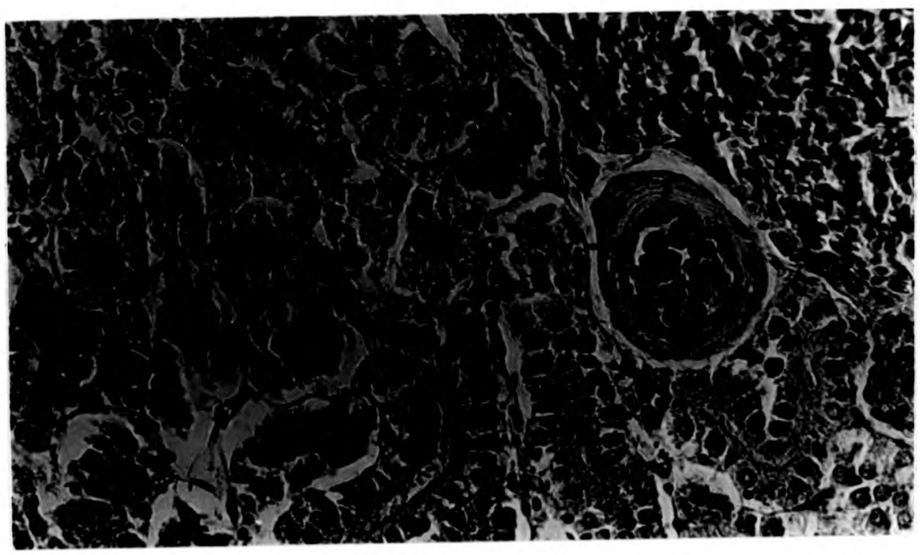
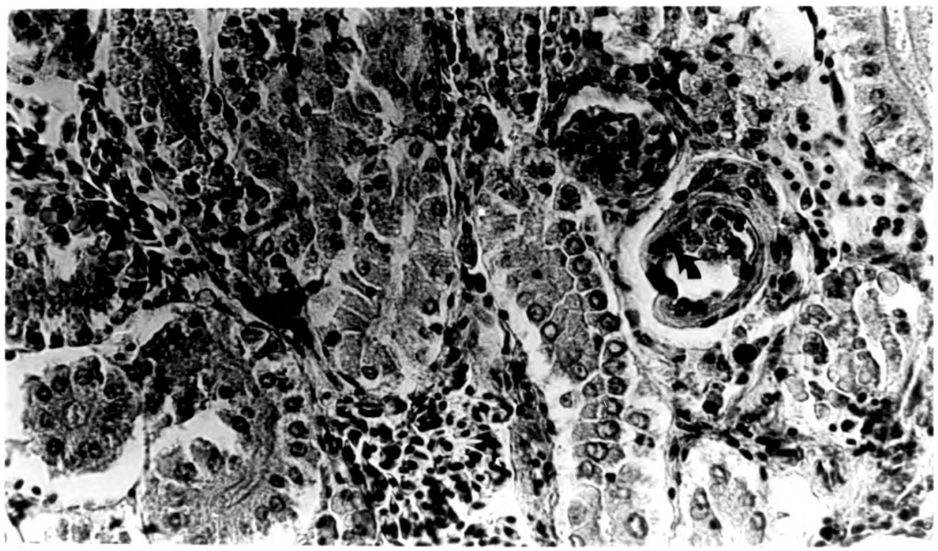


Fig. 4.2.1(b) A. terreus at 17 ± 1°C - one week post ingestion Granuloma and macrophage infiltration PAS X 494



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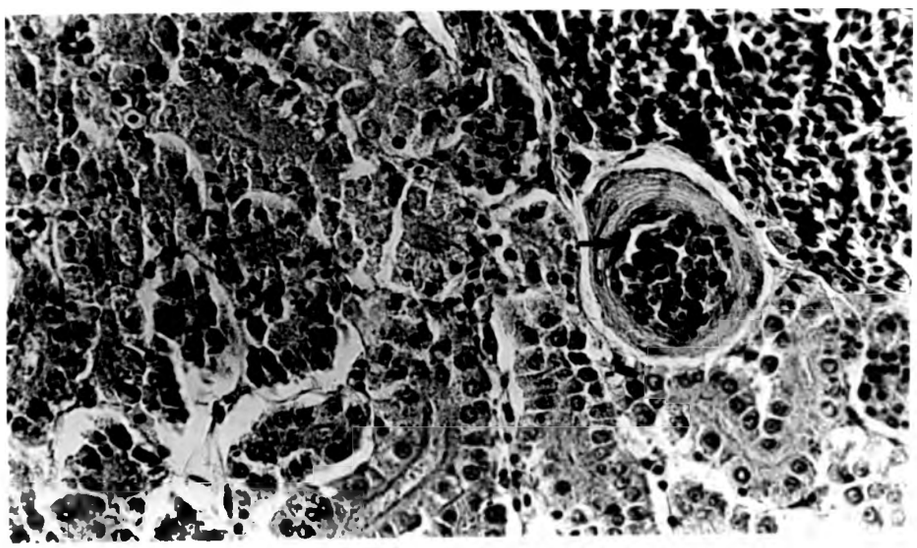
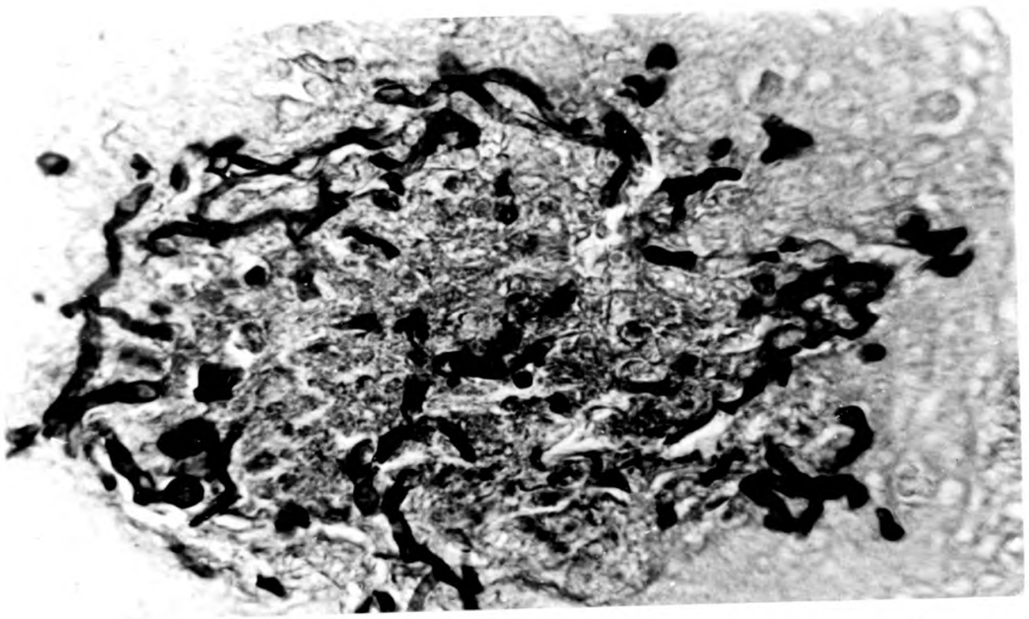


Fig. 4.2.1(b) A. terreus at 17 ± 1°C - one week post ingestion Granuloma and macrophage infiltration PAS X 494

Fig. 4.2.2 Densely branching septate hyphae located in an organised granuloma in the liver. Note hepatic necrosis. Grocott's X 600



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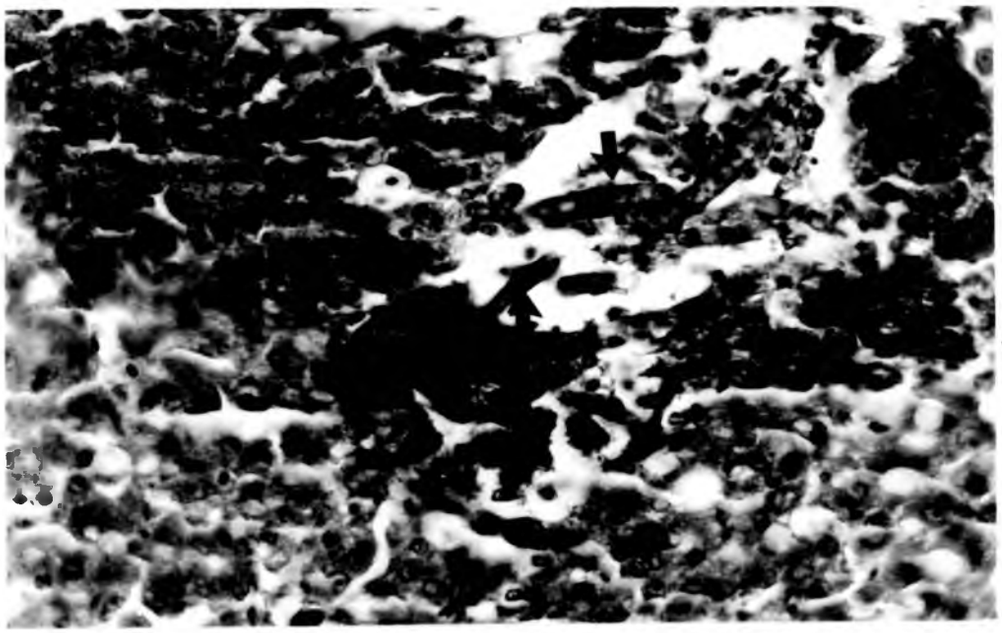


Fig. 4.2.3 Fungal hyphae (arrowed) in the pancreatic acini and biliary tract of Oreochromis hybrid. H & E X 600

A. terreus infection at 17 ± 1°C, 4 weeks post ingestion. Note also hepatic necrosis probably related to obstructive infarction of the vascular system by hyphal proliferation.

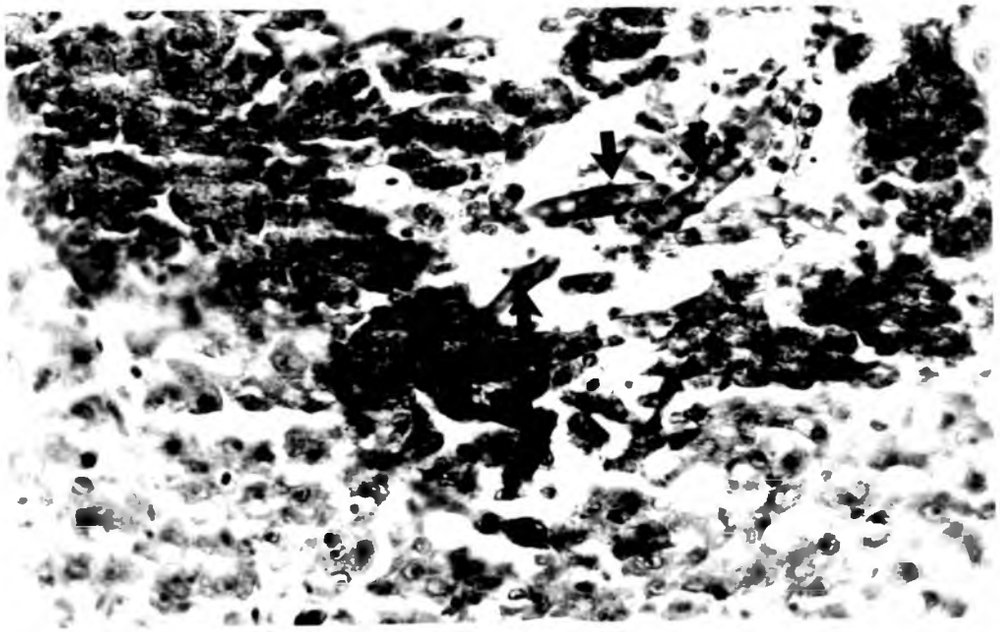


Fig. 4.2.3 Fungal hyphae (arrowed) in the pancreatic acini and biliary tract of Oreochromis hybrid. H & E X 600

A. terreus infection at 17 ± 1°C, 4 weeks post ingestion. Note also hepatic necrosis probably related to obstructive infarction of the vascular system by hyphal proliferation.

Fig. 4.2.4 A. japonicus at 17 ± 1°C - 4 weeks post ingestion
Granulomas in the submucosa of the glandular stomach.
Grocott's stain X 85

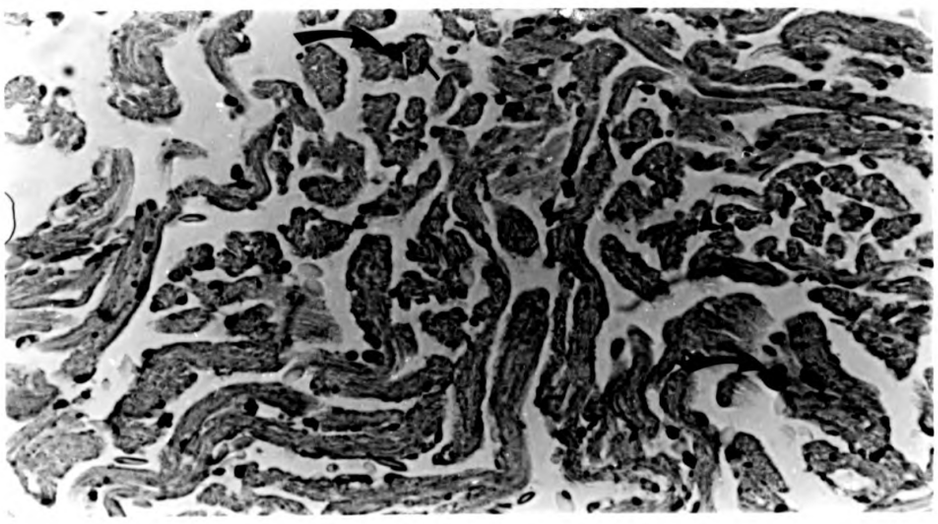


Fig. 4.2.5 Fungal elements in the ventricular endothelium
A. terreus at 17 ± 1°C 4 weeks post ingestion.
Grocott's stain X 502

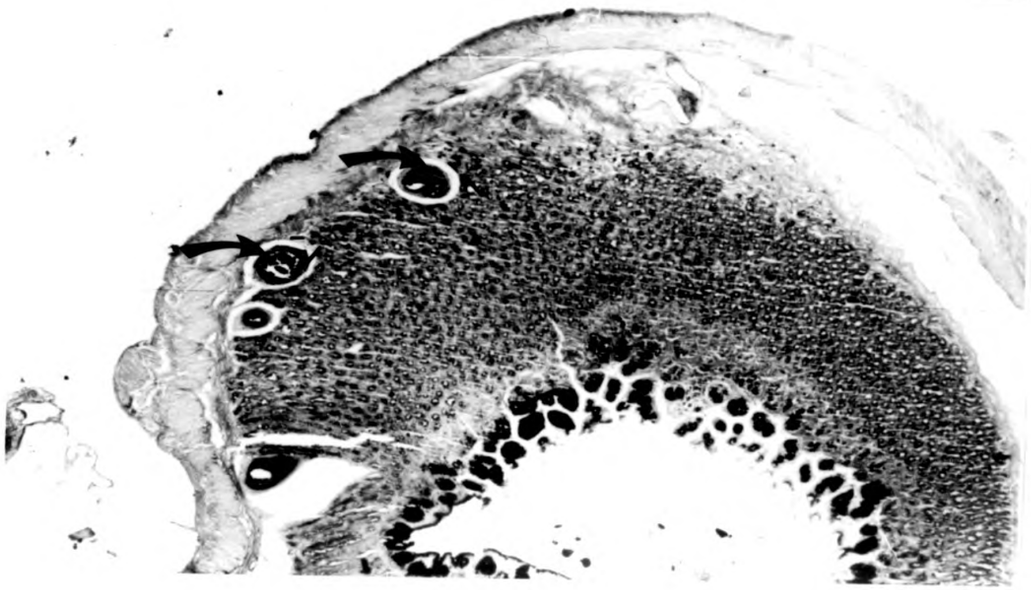
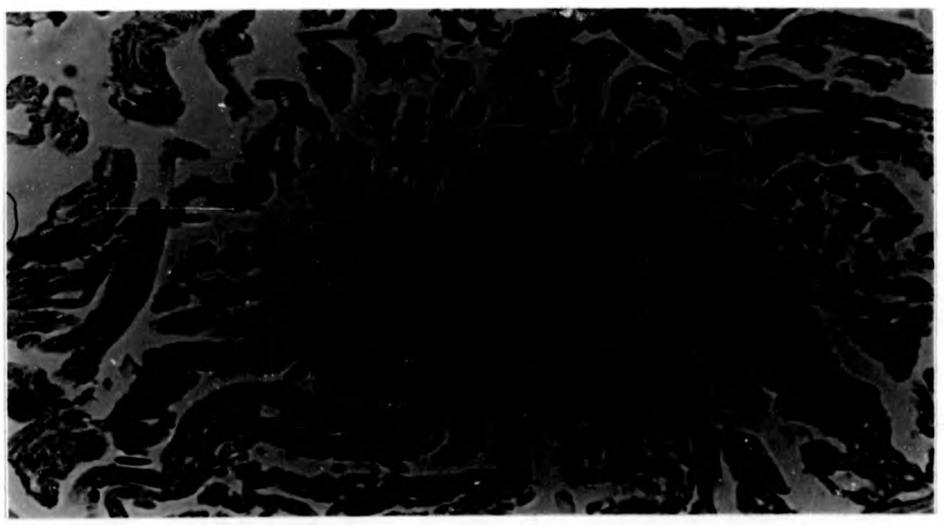


Fig. 4.2.4 A. japonicus at 17 ± 1°C - 4 weeks post ingestion
Granulomas in the submucosa of the glandular stomach.
Grocott's stain X 85



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Grocott's stain X 502

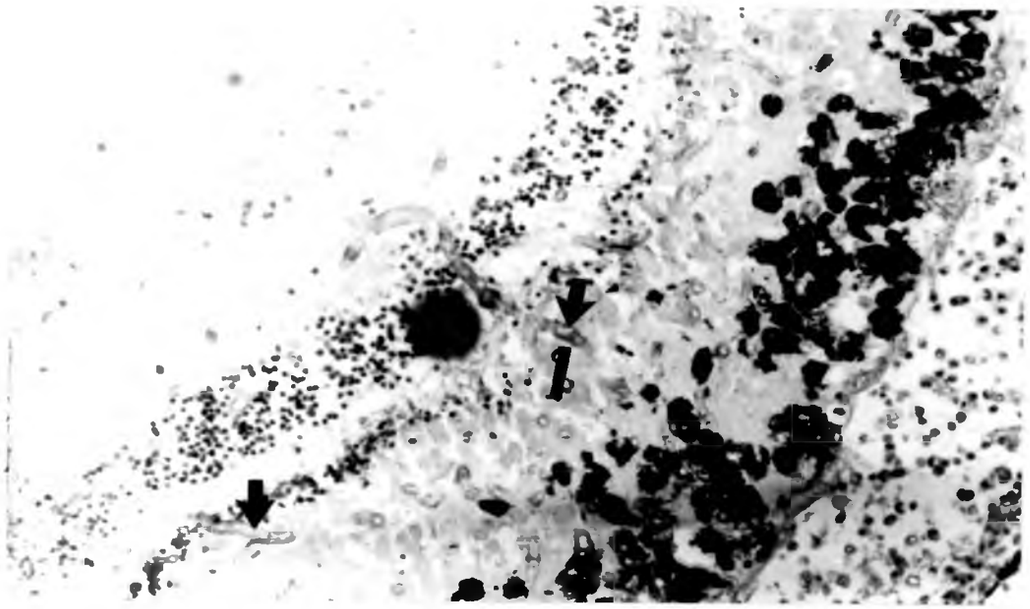


Fig. 4.2.6 A. japonicus at 25 ± 1°C - 4 weeks post ingestion. Fungal components in the eyes showing evidence of endophthalmitis H & E X 535

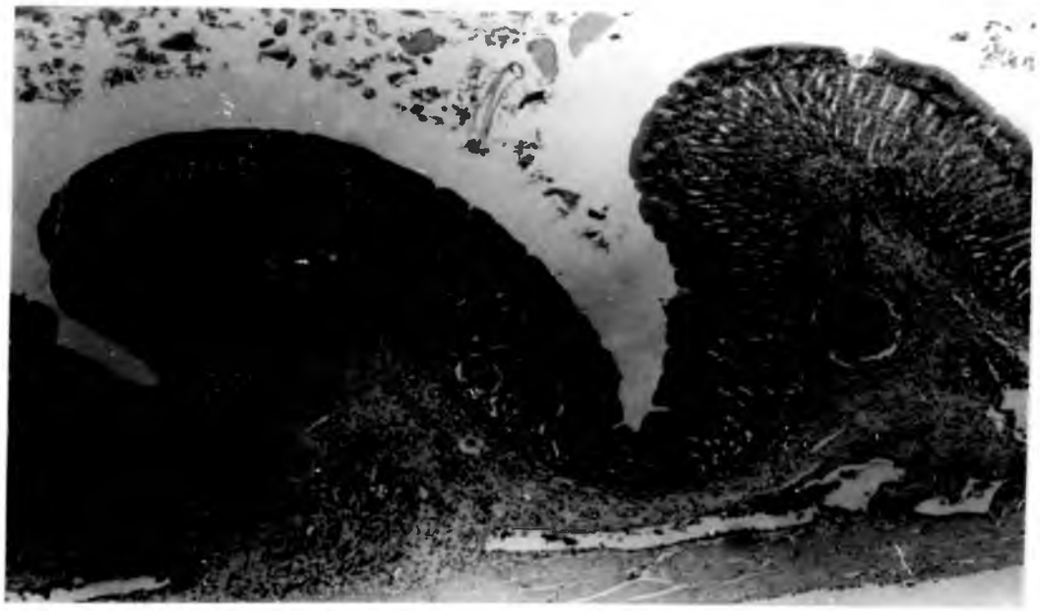


Fig. 4.2.7 Typical granulomas in the submucosa of the gut H & E X 85

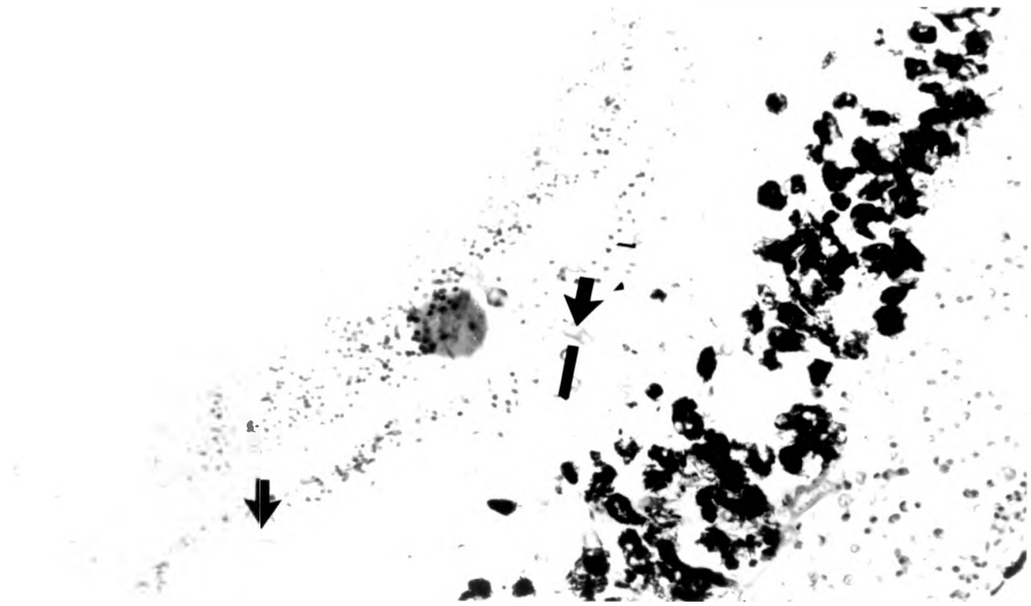


Fig. 4.2.6 A. japonicus at 25 ± 1°C - 4 weeks post ingestion. Fungal components in the eyes showing evidence of endophthalmitis H & E X 535

Fig. 4.2.7 Typical granulomas in the submucosa of the gut H & E X 85

Fig. 4.2.8 Focus of necrosis and inflammatory oedema of the subglandular mucosa of the stomach. A. terreus infection - 4 weeks post ingestion. H & E X 150

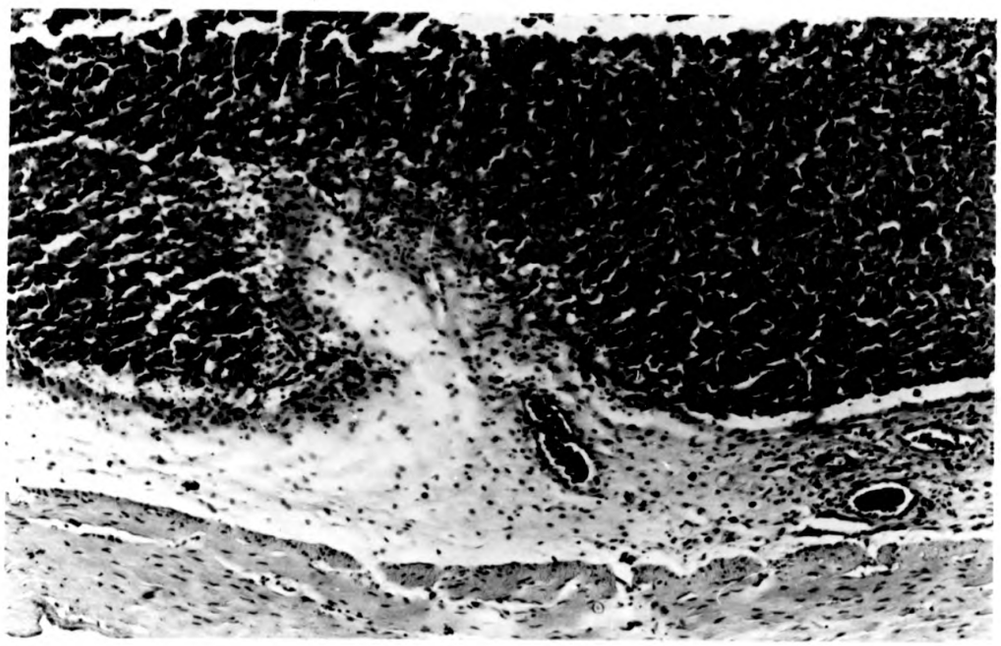


Fig. 4.2.8 Focus of necrosis and inflammatory oedema of the subglandular mucosa of the stomach. A. terreus infection - 4 weeks post ingestion. H & E X 150

Table 4.2.1 Results of fungal re-isolation eight weeks post ingestion

Fungus ingested and re-isolated	Brain	Gills	Heart	Kidney	Gut
A. japonicus from fish at 17 ± 1°C	-ve	+ve	-ve	-ve	+ve
A. japonicus from fish at 25 ± 1°C	-ve	+ve	-ve	+ve	+ve
A. terreus from fish at 17 ± 1°C	-ve	+ve	+ve	-ve	+ve
A. terreus from fish at 25 ± 1°C	-ve	-ve	-ve	-ve	+ve

+ve = growth of respective fungi from organs indicated

-ve = no growth of respective fungi from organis indicated

N.B.: Two fish were randomly sampled from each of the tanks at the end of eight weeks and their organs individually cultured.

4.3 Experimental Infection of *Oreochromis niloticus* by ingestion of fish pellets mixed with pure cultures of *A. flavus*

As a result of the definitive cause - effect pathogenicity relationship which was successfully ascribed to isolates of *Aspergillus* species by intra-peritoneal inoculation, and the subsequent systemic infection observed in tilapia following ingestion of pure cultures of *A. terreus* and *A. japonicus* isolates, an association between aspergillomycosis and diets containing the infective fungi was speculated. Consequently, another experiment was designed to study the effects of feeding a single species of *Aspergillus* mixed with tilapia diets, using contaminant fungus, under conditions most closely simulating field situations.

4.3.1 MATERIALS AND METHODS

Oreochromis niloticus weighing between 120-150 g were used. Fish were randomly distributed into six 220 litre glass aquaria, 10 fish per tank. Into each tank was introduced an Eheim pump filter and a box filter to aerate as well as filter the water. Water temperatures were maintained at 25 $\pm$ 1^{o}C in three tanks and 17 $\pm$ 1^{o}C in the other three tanks. The system was left for four days for observation after which stabilization was established. Subsequently the tanks were cleaned and replaced with clean freshwater at the respective temperatures once weekly.

Surface cultures of *A. flavus* strain previously isolated from tilapia tissues (Chapter 3) were grown at 25 $\pm$ 1^{o}C in 500ml flat bottom flasks containing about 200 ml to 250 ml of Czapek's solution medium. The medium was inoculated with a suspension

of spores from the A. flavus strain, and grown for two weeks. After this period, tilapia pellets (size no 4) were introduced into the flasks, covered and sealed, and thoroughly mixed together with the fungal materials by gentle shaking. The flasks were then emptied and the mixture of fungal materials and pellets was subsequently used in feeding the tilapias.

The remaining medium in the flask was autoclaved and discarded. The prepared mixture was kept in sterile plates, and sealed off until use. Plates were not kept for more than 48 hours, because of the resultant humidity which tended to attach the pellets to the plates, when kept for a longer period. Fish in four of the tanks (two tanks at 25 $\pm$ 1^{o}C and two at 17 $\pm$ 1^{o}C) were fed with the prepared pellets plus the fungal materials and fish in the other two tanks (one at 25 $\pm$ 1^{o}C and the other at 17 $\pm$ 1^{o}C) were fed with uncontaminated pellets, and were the control groups. Feeding was ad lib twice daily.

Although fish in the experimental tanks fed at first introduction of the mixture, feeding rate subsequently declined during the second week. Feeding of the contaminated pellets was stopped during the fourth week when the fish in the experimental tanks began to show typical mycotic syndrome and 2 - 3 per experimental tank were moribund.

4.3.1(i) Histopathology:

Two fish were sampled from each tank in the fifth week after the initial feeding. Blocks of gills, heart, kidney, liver and intestine were separately dissected out, fixed in 10% neutral buffered formalin, paraffin was embedded cut at 5 um and stained with haematoxylin and eosin (H & E), periodic acid - Schiff's

technique (PAS) and Grocott's modification of Gomori's methenamine silver technique for fungi.

4.3.1(ii) Fungal re-isolation and re-identification

Fungal re-isolation from fish in the tanks was carried out. Organs from fish were dissected out and fungal isolation carried out as described in Chapter 3. The fungal isolates were re-identified.

4.3.2 RESULTS

Fish in the experimental tanks had started dying at four weeks from initial feeding of contaminated feed. All of the fish fed the contaminated diet had shown progressive loss of activity with reduced swimming movements. The moribund fish, 2-3 per tank, showed darkening of the colour of the skin, corneal opacity and bilateral exophthalmia (Fig. 4.3.1). Exophthalmia was obvious when infected fish were compared with fish in the control tanks.

Usually the dorsal fins of the infected tilapias appeared in fairly extended positions, the mouths remaining opened most of the time. A few severely affected ones showed haemorrhages on the skin, mainly in the lateral region (Fig. 4.3.2). Fish at lower temperatures appeared most severely affected. The gills were variably pale and showed lots of mucous secretion. Grossly, the intestine was markedly oedematous and some areas of intestinal mucosa appeared completely absent, resulting in large areas of ulcerations. The liver in some areas, showed the parenchyma in various stages of necrosis, while in some areas, complete dissolution of the liver parenchyma had taken place. In other

areas, it appeared apparently normal. The gall bladder was usually distended, containing greenish coloured fluid. The posterior kidney appeared haemorrhagic, with the haemorrhage usually more marked in fish kept at lower temperatures (17 ± 1°C). Fish in the control tanks were normal and fed normally.

4.3.2(i) Histopathology:

The walls of the gut were oedematous often with accompanying massive desquamation of the mucosal lining. Typical granulomas appeared predominantly along the sub-mucosa of the glandular region of the gut, although there were often a few granulomas at the lowest levels of the mucosa, sometimes close to the basement membrane. The granulomas varied in shapes and sizes (Fig. 4.3.3) but the majority were spherical to oval and measured approximately 100 x 80 um. The centre of the granulomas usually contained amorphous materials which were PAS and Grocott's methenamine silver stain positive. Where the intestinal lining was still fairly intact, there were numerous mucus containing cells, the secretions of which always seemed to have attracted fungal materials and were invariably PAS and Grocott's silver stain positive. Comparatively more mycotic granulomas were observed in the glandular area of the gut of fish maintained at 25 ± 1°C than in fish maintained at 17 ± 1°C, however, the mucous cell secretions were less dense, suggesting less copious secretion than in fish at 17 ± 1°C. The oedema of the gut wall was also comparatively less marked than in fish at 17 ± 1°C.

Tilapia liver parenchymatous cells usually possess irregularly shaped nuclei which are normally uniform in size. The parenchyma also may contain vacuoles resulting from glycogen or lipid

storage. The small and relatively uniform size of the nuclei appeared to be a constant feature of normal liver cells. However, in the liver of tilapia experimentally infected with A. flavus by ingestion of contaminated diet, the parenchyma nuclei usually appeared to have become fairly enlarged and varied in size at five weeks post infection. Some of the cells were necrotic, especially at the peripheral areas or margins. Fungal materials staining positive with PAS and Grocott's techniques were often seen in the liver, particularly in the hepatic vessels, with variable extension into the liver parenchyma. In more advanced cases, the breakdown of normal liver structure appeared accentuated and granulomas also developed.

The heart showed evidence of ventricular myopathic degeneration and slight oedema with evidence of inflammatory reaction in areas associated with fungal proliferation. Fungal mycelia was often picked up by the differential Grocott's stain (Fig. 4.3.7).

Peripheral splenic tissue necrosis was common with fungal components usually in aggregates in the melano-macrophage centres.

The eyes showed evidence of oedema in the retinal and choroid layers, frequently with fungal mycelial material present in these layers and the anterior chamber of the grossly exophthalmic eyes.

4.3.2(ii) Fungal re-isolation and re-identification

A. flavus was re-isolated from the gills, eyes, heart, intestine, kidney and liver but not from the brain of the infected fish at five weeks sampling period.

Fig. 4.3.1(a) Tilapia showing bilateral exophthalmia

Fig. 4.3.1(b) Exophthalmia shown from fish from the inside of experimental tank.

Fig. 4.3.1(a) Tilapia showing bilateral exophthalmia

Fig. 4.3.1(b) Exophthalmia shown from fish from the inside of experimental tank.

Fig. 4.3.2 A. flavus infected tilapia - showing haemorrhages on the body

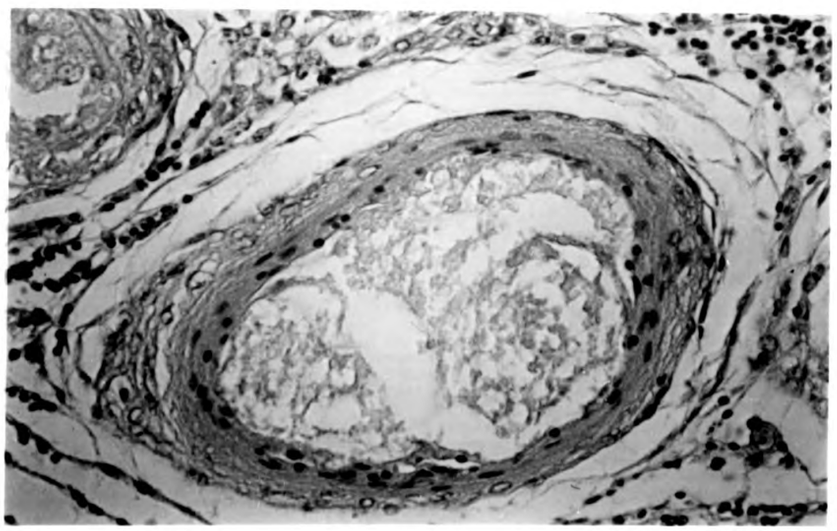


Fig. 4.3.3 Typical granuloma in the gut, containing amorphous debri PAS X 384

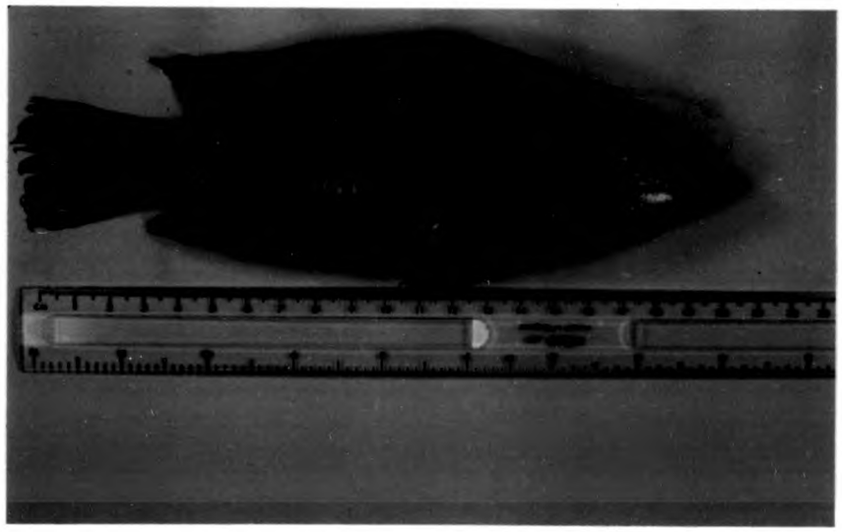


Fig. 4.3.2 A. flavus infected tilapia - showing haemorrhages on the body

Fig. 4.3.3 Typical granuloma in the gut, containing amorphous debri PAS X 384

Fig. 4.3.4 Primary gill lamellae showing mucous cells secretion which have attracted fungal components Grocott's stain X 128

Fig. 4.3.5 Renal pathological changes
(i) Aggregation of macrophages usually in areas of (ii)
(ii) Focal granuloma
(iii) variable tubular atrophy H & E X 320

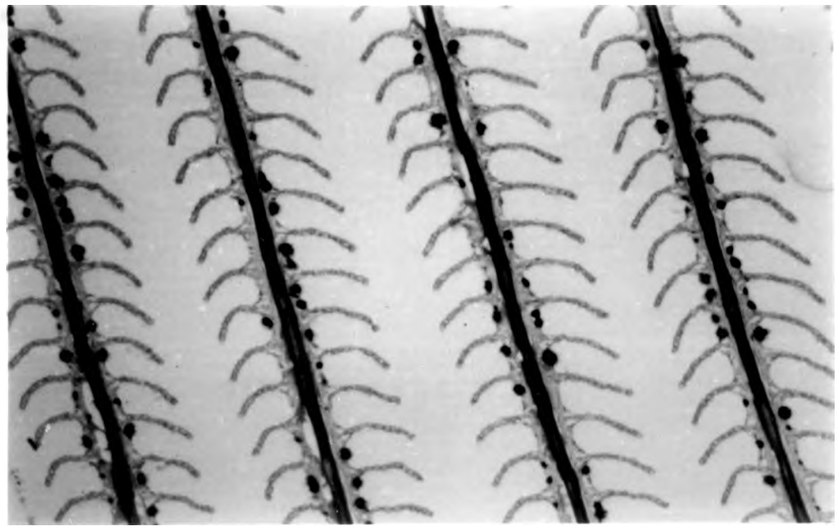


Fig. 4.3.4 Primary gill lamellae showing mucous cells secretion which have attracted fungal components Grocott's stain X 128

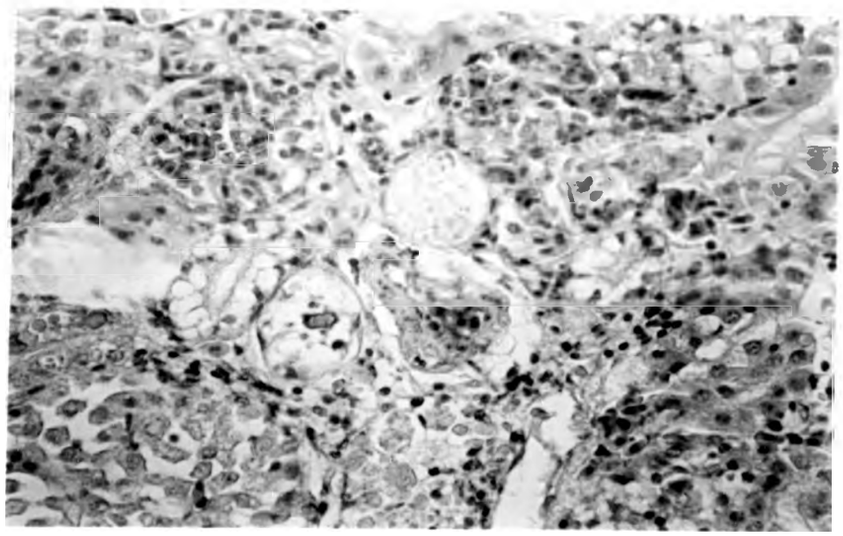


Fig. 4.3.5 Renal pathological changes
(i) Aggregation of macrophages usually in areas of (ii)
(ii) Focal granuloma
(iii) variable tubular atrophy H & E X 320

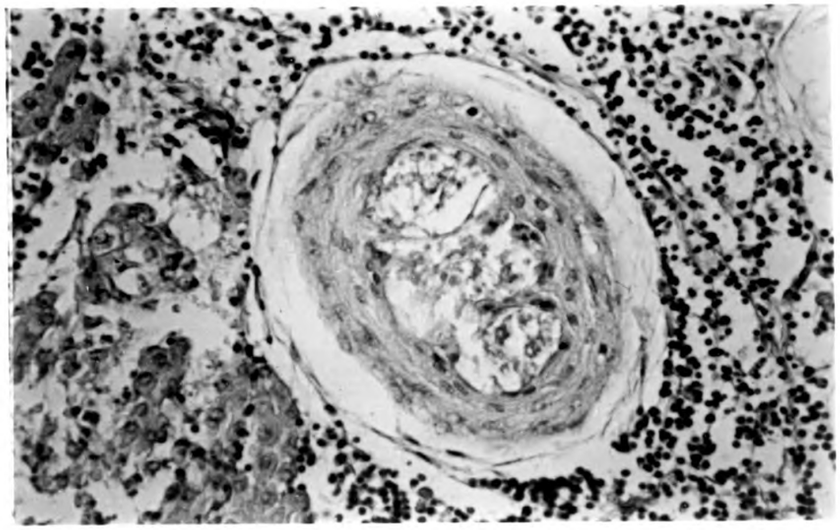


Fig. 4.3.6 Typical liver granuloma PAS X 256

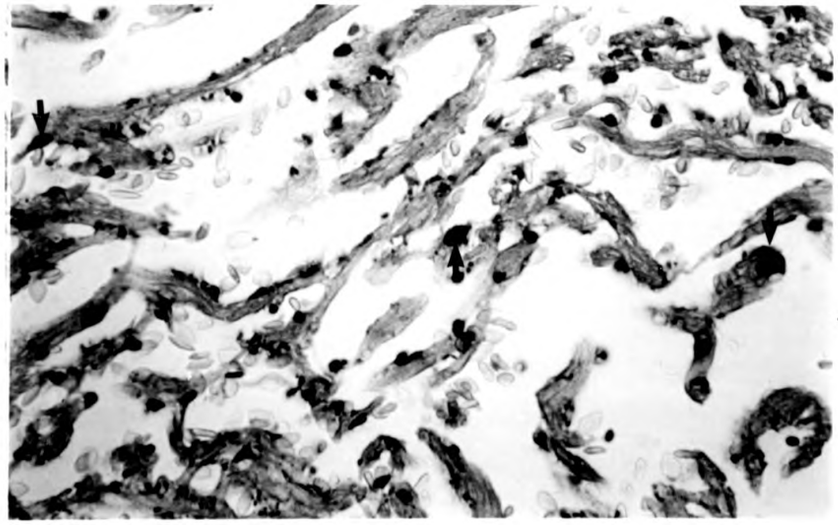


Fig. 4.3.7 Fungal elements in the ventricular muscles with cellular infiltration Grocott's stain X 240

Fig. 4.3.6 Typical liver granuloma PAS X 256

Fig. 4.3.7 Fungal elements in the ventricular muscles with cellular infiltration Grocott's stain X 240

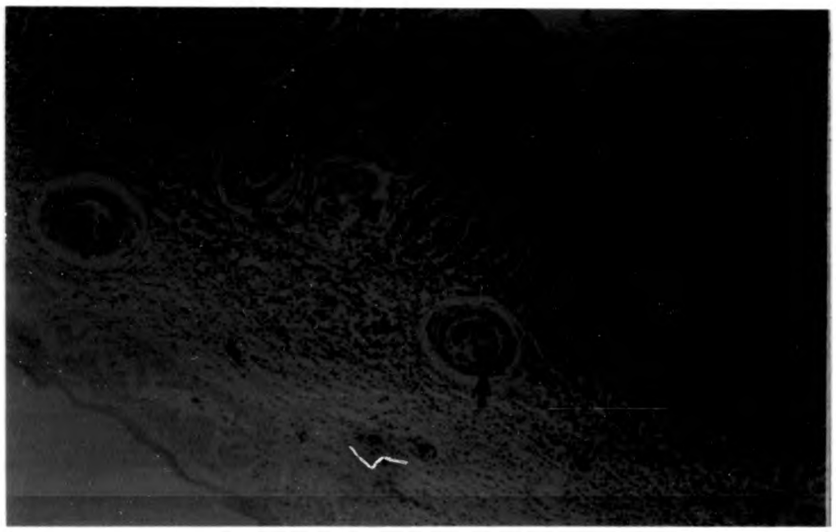


Fig. 4.3.8 Typical granulomas in the sub mucosa of the small intestine PAS X 128

Fig. 4.3.9 One of the granulomas PAS X 240

Fig. 4.3.8 Typical granulomas in the sub mucosa of the small intestine PAS X 128

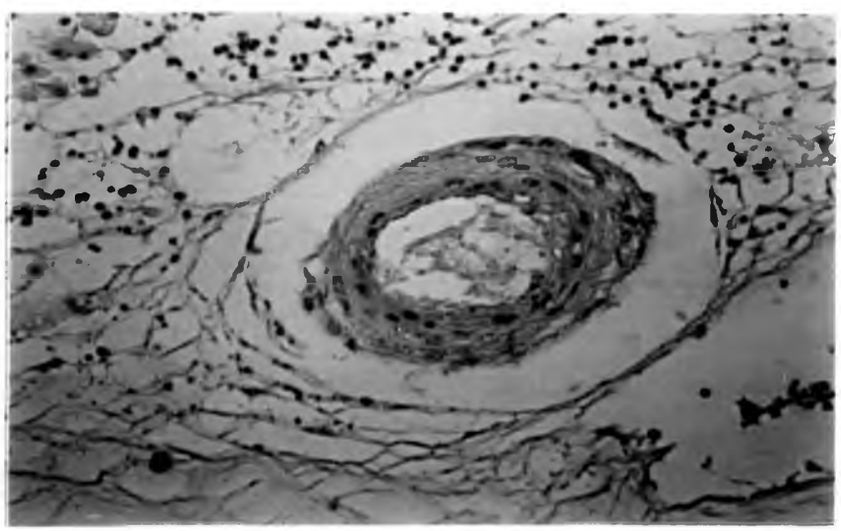


Fig. 4.3.9 One of the granulomas PAS X 240

4.4 DISCUSSION

The experimental pathogenesis of aspergillomycosis in tilapias described in 4.1, 4.2 and 4.3 shows clearly that tilapias are susceptible to members of the genus Aspergillus. Deductions from the experiments described suggest that there is variability in the pathogenicity of the various species.

In experiment 4.1, A. flavus appeared more pathogenic to fish than A. niger. The combination of the two species produced a more serious disease than the mono-specific infection. This may well explain the serious nature of the clinical outbreaks described in Chapter 2. Most natural disease conditions quite possibly result from infection by more than one Aspergillus species - conditions which may be termed poly-specific infections.

The pathogenicity of Aspergillus species could be attributed to its ability to grow under the environmental conditions provided by the host, water temperature appearing to play a significant role in this regard. In experiment 4.1, at 26°C, A. flavus was about twice as pathogenic to Oreochromis niloticus than at 18°C. Generally, increased temperatures favour the development, transmission and pathogenicity of many infectious diseases of fish. A. flavus infection appears to be no exception. A. niger on the other hand, was less pathogenic at 26°C although it could be re-isolated, A. flavus was able to produce mortalities at each of the experimental thermal regime, whereas A. niger was able to initiate the disease when the temperature was low (18°C). The stress experienced by the experimental fish at low water temperature and lowered metabolic rate of its defensive systems probably contributed to the susceptibility of Oreochromis to Aspergillus species.

From experiment 4.2, it is appropriate to suggest that both A. terreus and A.japonicus are each capable of producing acute, sub-acute or chronic wasting disease under varying conditions, including the infective dose, the duration of the ingestion, the size of fish, the fish environment and other husbandry practices. Typical systemic mycotic granulomatous lesions were produced by feeding pure cultures of A. terreus and A. japonicus separately. Besides confirming the oral route of infection, the isolation of the ingested fungi from the faeces of experimental fish recovered from the water, shows that it is more probable that fish howbeit infected with Aspergillus species could spread infective fungus spores into the water through the faeces, thereby transmitting the pathogen to other fish. Confinement of fish population as is of necessity the case in intensive aquacultural practices is an important factor to be considered in this regard. Such transfer of a water borne pathogen is the basis of the usual "contact method" of infection.

Until the present study there has been no report of Aspergillus fungus itself invading the tissues of a host fish and inducing a systemic aspergillosis (aspergillomycosis).

Experiments 4.2 and 4.3 have confirmed the clinical evidence reported in Chapter 2 that this can in fact occur in tilapias, and the evidence also suggests strongly that the main portal of entry is the digestive tract subsequent to which haematogenous spread allows dissemination throughout the tissues. These features observed by histopathological examination have been confirmed by the re-isolation of pure cultures of the infective agent in each case from heart, liver, kidney and gut of experimentally infected tilapias.

Internal fungus infections of freshwater fish seem to be relatively rare. Only a few such cases have been reported. Wood et al (1955) described a mycosis-like granuloma and reviewed earlier American literature on systemic mycoses in fish. Scott (1964) summarised his experiences and work on external mycoses which are more common than the internal ones. The present account shows that A. niger, A. flavus, A. terreus and A. japonicus should be considered to be of primary significance as causal agents of disease in cultured tilapias. The occurrence of two or more species of fungus may very well exacerbate the observed clinical picture, especially under field and/or aquacultural conditions (cf Chapter 2). Polyspecific infection is only one of the factors affecting the development and severity of such infections. It was observed during the course of the study that smaller fish were the more vulnerable but it is also probable that the particular species of Aspergillus and its infective dose, the nutritional status of the fish, the water quality and the type of husbandry will all affect the degree of infection of a population. Several factors therefore stand clearly important in the elucidation of the epidemiology of aspergillomycosis in cultured tilapias.

The pathological picture which developed in aspergillomycosis is of considerable interest. The presence of the various fungi in the myocardial endothelium may well explain the extension of fungal hyphae into the ventricular muscles as observed in the field outbreak (Chapter 2). However, the absence of typical mycotic granulomas in such heavy infections of the heart is more difficult to explain. The fine structure of the teleost heart has been described by Kisch and Philpott (1963) for the goldfish (Carassius auratus), and for the plaice (Pleuronectes platessa)

by Santer and Cobb (1972). The atrium is composed of numerous endocardial-lined trabeculae, presenting a mesh-like appearance and has therefore a large surface area suitable for endocytosis, Ferguson (1975) investigated cardiac phagocytosis in the plaice, using a variety of different circulating particles including yeasts, he found that whereas the atrium had a very active role, the liver played virtually no part in blood clearance, but the spleen and kidney were responsible for the clearance of most of the injected materials. Ferguson pointed out the vulnerability of the heart of teleost fish which has no lymph nodes and pathogens could therefore reach the heart or any other major organ without first coming into contact with reticular tissue. The present work indicates that the cardiac andothelium in tilapias is probably similarly involved in phagocytosis. This active process may leave little or no opportunity for the typical organisation into granulomas, but may occasionally allow extension of fungal materials from the endocardium into the myocardium during the disease process.

The ophthalmic pathology of the acute aspergillomycosis is also of importance. Although a great deal is known about ophthalmic pathology in mammals, endogenous intra-ocular fungus infection is a relatively rare diagnosis in fish disease. In spite of its availability for examination, the eye has all too frequently been neglected during disease investigations. In fish, exophthalmos (pop-eye) and cataract (white or opaque eye) appear to be the most commonly reported eye conditions and specific pathological descriptions are lacking in most cases. In humans, fungus infections may occur in the eye following injuries and operations or during the course of other fungal infections, such as actinomycosis of the lungs, sporotrichosis, mucormycosis of

the sinuses or aspergillosis. Similar mycotic infections have been reported in the eyes of patients who showed no other evidence of systemic disease and in whom the portal of entry remained obscure (Hogan and Zimmerman, 1962).

Cogan (1949) made a thorough search of the intra-ocular fungus infections of humans reported in the literature and added one of his own. In these cases, the causative organism was blastomyces in three cases, an actinomycete-like organism in three cases, and a cylindrical branching fungus thought to be Aspergillus species in six cases. Birge (1952) in his comprehensive review of ocular aspects of mycotic infection, stated that Aspergillus is a large group of fungi often isolated, A. fumigatus being one of the few pathogenic varieties. He recognised two types of ocular mycotic infections: (1) infection by fungi which produce superficial lesions only, and (2) infection by fungi which produce both deep systemic and ocular lesions.

The pathology of acute aspergillomycosis in tilapias included essentially, an endophthalmitis with associated inflammatory processes primarily involving the anterior and posterior chambers of the eye from where the fungus frequently extended to adjoining retina and adjacent choroidal tissues. The re-isolation of cultures of the fungus from the eye suggests that the fungal materials are still viable and their growth may therefore lead to a progressive worsening of the fulminating endogenous intra-ocular aspergillomycosis, from the initial endophthalmitis to total or complete blindness. Although the portal of entry into the eyes may be obscure, it would be safe to assume that the circulation must be, at least in part, by way of the blood or lymph flow and the most likely portal of entry into the eye would be by way

of the choroid. Fungal fragments were seen in this site in the present study. The circulation of the intra-ocular fluids would also have carried the fungal materials into the anterior chamber of the eye where they often settled.

The isolation of fungi from fish clinical material has been regularly carried out as part of the present series of experimental infection studies using the method developed in the course of this work (Chapter 3). It was therefore readily possible to re-isolate and re-identify the fungal species from the various fish organs. The organs of choice in the event of an indicated isolation trial are the kidney and the liver. Better isolation rates were obtained from clinical materials by using freshly prepared medium and by incubating the plates at 25 to 30^{o}C.

CHAPTER 5

SEROLOGICAL ASPECTS OF ASPERGILLOMYCOSIS IN CULTURED TILAPIAS

5.1 INTRODUCTION

In recent years, species-specific antigen-antibody reactions have been recognised for many fungal pathogens. This has allowed a further element to enter into the study of fungal taxonomy (Fukui and Yasuda, 1961). Hitherto fungi had been classified solely on the basis of morphological criteria.

The role of serological reactions in the diagnosis of fungal diseases has been principally applied to the diagnosis of fungal allergies (Pepys, Riddell, Citron, Clayton and Short, 1959), and the use of immunological tests to indicate the presence of actual fungal infection has been relatively less developed. There are however, some conditions for example, pulmonary aspergillosis in humans (Longbottom and Pepys, 1964), detection of precipitins to Aspergillus fumigatus infection in dogs (Lane and Warnock, 1977; Richardson, Warnock and Bovey, 1982), where they do have some diagnostic value. Detection of precipitating antibodies by the gel diffusion technique has been used in other fungal infections including several aspergilloses (Pepys, 1960).

In order to determine the feasibility of utilizing immunological tests for diagnosis of fungal infection in tilapias, serological studies were carried out with extracts of Aspergillus species as antigens. To investigate if the particular strain of A. flavus under study was highly antigenic and to obtain a stock rabbit anti A. flavus serum, rabbits were exposed parenterally

to pure cultures of the fungus. Serum samples from clinically affected fish were tested for the presence of precipitating antibody by means of Ouchterlony gel-diffusion technique, using the rabbit antiserum as positive control.

5.2 MATERIALS AND METHODS

5.2.1 Collection of normal rabbit serum

Rabbit: Two male, New Zealand white breed, category IV (categories of Accredited Animals, 1974) and about 10 weeks old, were obtained from HACKING AND CHURCHILL, Wyton, Huntingdon. They were fed on a pelleted diet known as SG.1 supplied by "OXOID", manufactured by Herbert C Styles (Bewdley) Ltd., and kept in metal cages with grid floors and drop trays containing wood chips. The woodchips were changed twice weekly, while the grids were changed every week.

After the initial period of two weeks observation of the rabbits, about 10-15 ml of blood was collected from the ear veins of each of the rabbits into sterile bottles and allowed to stand and clot overnight at $4^{\circ}C$ to express the serum. The serum was aseptically collected and stored at $-20^{\circ}C$ until required.

5.2.2 Raising of rabbit antiserum

5.2.2(i) Antigen preparation for rabbit inoculation

Ten day old pure cultures of Aspergillus flavus Link obtained from a clinical outbreak and grown on Czapek solution agar (CSA) was used for the preparation of rabbit inocula.

The fungus was harvested with about 10-15 ml of sterile

distilled water, transferred into a sterile polythene bag and homogenised using the STOMACHER 80 Lab blender. The homogenate was then emulsified (1:1) with Freund's incomplete adjuvant until a stable water-in-oil emulsion was obtained. (This was checked by gently placing one drop of the emulsion onto saline. If the emulsion is stable the first or second drop will not disperse.)

5.2.2(ii) Inoculations into New Zealand white rabbits, and collection of serum

Following the initial two week period of observation, freshly prepared emulsion was intramuscularly injected into each of the hindquarters of the rabbits, 1 ml at a time. Two weeks later, the injections were repeated. After two further weeks, the rabbits were anaesthetised with thiopentone sodium and bled from the heart. Blood was kept in sterile glass bottles and allowed to clot at room temperature for about 1 hour. The clot was loosened from the glass bottles using a sterile glass rod to aid retraction, subsequent to which they were stored at 4°C overnight to express the serum. The serum was thereafter collected using sterile techniques and stored in aliquots of about 5 ml in bijou bottles at -20°C until required.

5.2.3 Preparation of Fungal Antigenic Extracts (A. flavus; A. niger and A. japonicus)

Fungal antigenic extracts were prepared from pure cultures of Aspergillus flavus, and later A. niger and A. japonicus, grown on synthetic CSA media. Each of the fungi was separately grown (one species at a time) at 27°C in 500 ml conical flat

bottom flasks, into which had been introduced approximately 200 ml of the medium. Media were inoculated with a suspension of spores from previously isolated strains of Aspergilli (Chapter 3). The same strains of A. flavus, A. niger and A. japonicus were used throughout the investigation. Ten day old cultures were used in each case. About 20 ml of sterile distilled water was used to collect fungi materials from the culture flask, transferred into sterile flasks and frozen at -70°C, the frozen material was allowed to thaw at room temperature. Freezing and thawing in this fashion was done three to four times repeatedly. It was then centrifuged at 1000 g for 15 minutes. Two ml each of the supernatant was introduced into sterile bijou bottles and subsequently frozen at -70°C for about 1 hour, and freeze dried for 12-18 hours. The freeze-dried extracts were later reconstituted in phosphate buffer saline, pH 7.4 at 30-40 mg per ml.

5.2.4 Serum samples from A. flavus infected, and non-infected tilapias (Oreochromis niloticus)

Fish sera samples were obtained for use in this investigation using a special technique involving puncture of the posterior aorta. The fish was anaesthetised with Benzocaine dissolved in alcohol and held 'belly-up'. A disposable sterile needle was inserted midway between the posterior base of the anal fin and the caudal fin. When the needle was stopped by the backbone, the posterior aorta was penetrated and the blood then flowed into the attached syringe. On occasions, the same area was reached by insertion of the needle from points along the side of the fish, but the former technique was the more reliable.

Three groups of tilapias (Oreochromis niloticus) were bled

and the blood from fish in each of the groups had to be pooled together for serum collection due to the size of fish. One group comprised fish kept in water at 25 ± 1°C and infected with A. flavus by oral ingestion of pellets plus fungi material. The second group comprised of fish held in water at 17 ± 1°C and simultaneously infected with A. flavus by ingestion of pellets and fungi materials (Experiment 4.3). The third group of fish were from the control lots kept at 25 ± 1°C and had been fed on uncontaminated pellets only.

Blood from fish in each group was pooled together as only 1 - 2 ml of blood could be obtained as described from each fish. The bloods were allowed to clot at room temperatures, and stored at 4°C overnight to express the serum. Serum was subsequently collected and stored in aliquots of about 2 ml in sterile bijou bottles at -20°C until required.

Whereas the experimental fish had typical lesions and were positive for the presence of Aspergillus organisms, the control group were free from any such lesions and were found to be free from the infective Aspergillus following examination.

5.2.5 Ammonium sulphate precipitation of rabbit globulins from serum obtained from A. flavus inoculated rabbits

1000 g of ammonium sulphate was dissolved in 1 litre of distilled water at 50°C and allowed to stand overnight at room temperature. The pH was adjusted to 7.2 with dilute ammonia solution and sulphuric acid. The rabbit serum obtained from A. flavus inoculated rabbits was diluted 1:2 with saline and the prepared saturated ammonium sulphate solution added to give a

final concentration of 45% (V/V). This was stirred at room temperature for 3 minutes, and the precipitate spun off at 1000 g for 15 minutes, at 4°C. The precipitate was washed with 45% saturated ammonium sulphate and re-centrifuged. It was redissolved in the same volume of phosphate buffer saline (PBS pH 7.2) as the original serum. Any insoluble material was removed by centrifugation. The globulin was reprecipitated with a final concentration of 40% saturated ammonium sulphate, centrifuged and washed with 40% saturated ammonium sulphate. After centrifuging the washed precipitate, it was re-dissolved in a minimum of PBS and dialysed against about 5 litres of PBS at 4°C overnight. Any precipitate was centrifuged off and the gamma-globulin fraction kept at 4°C until required.

5.2.6 Preparation of Agar-gel plates

One per cent Noble agar in 0.9 per cent saline containing 0.1 per cent sodium azide as preservative was prepared. The agar was stored at 4°C until required or dispensed directly into sterile plates, 25 ml in each plate. After the agar had set, a gel punch was used to cut a pattern which provided a large central well for the test sera, 12.5 mm diameter, surrounded at a distance of 6 mm by six peripheral wells, 4 mm diameter, for the antigen. The agar plugs were removed with a blunt Pasteur pipette.

5.2.7 Preparation of agar-coated slides

Glass slides were pre-coated with a weak solution to hold the final agar gel in place, by dissolving 0.5 g of Noble agar in 100 ml of 0.9 per cent saline. The agar was pipetted onto clean,

dry slides, adding enough just to cover one surface of the slide. The slides were then dried and stored at room temperature until required.

Before use, the 1% Noble agar in 0.9 per cent saline with 0.1 per cent sodium azide previously prepared was melted in a boiling water bath and gently layered onto the precoated slide on a levelled surface and allowed to set. After the agar had set, a gel punch, 4 mm in diameter was used to cut about eight pairs of wells per slide. Each pair being 2-3 mm apart. The agar plugs were carefully removed with a blunt Pasteur pipette.

5.2.8 Agar-gel precipitin tests

Double-diffusion tests (Ouchterlony, 1953) were performed in the 1% Noble agar in 0.9 per cent saline containing 0.1 per cent sodium azide as preservative. Tests were allowed to develop for up to 10 days at 28°C for the tests in plastic plates, during which time precipitation lines developed in positive cases.

Pari-pasu with the plate tests, slide double diffusion tests were also carried out with the rabbit anti-A. flavus serum fractionated with saturated ammonium sulphate and the three antigenic extracts. In this case, precipitation lines developed in positive cases between 18-24 hours, with agar-gel on coated slides. Altogether, the following sera were tested with the different Aspergillus antigenic extracts.

Normal (or control) serum obtained from the rabbits prior to inoculation of A. flavus.

Normal (or control) serum samples from apparently healthy Oreochromis niloticus.

Serum samples from rabbit inoculated with an emulsion of A. flavus and incomplete Freund's adjuvant.

Serum samples from Oreochromis niloticus previously infected with A. flavus and kept in water at 25 ± 1°C and another group kept in water at 17 ± 1°C.

Three different antigenic extracts were used for the various tests, these were A. flavus, A. niger and A. japonicus. Table 5.1 shows the summary of the precipitin tests and the results.

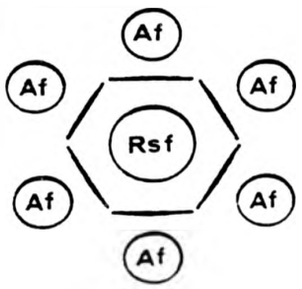
5.3 RESULTS

Antibody production specifically against A. flavus was detected by Ouchterlony gel-diffusion precipitation technique from rabbits injected with A. flavus. Figure 5.3.1 shows the pattern of precipitation lines produced.

It was found in practice that the use of a large central serum well (12.5 mm diameter) surrounded at a distance of 6 mm by six peripheral wells, 4 mm in diameter, for the antigen, gave the greatest number of positive reactions when immune sera were tested with the antigens. The arrangement also prevented solubilization of precipitates in cases of probable antigen excess. Although tests were allowed to develop for up to 10 days at 28°C, for the tests in plastic plates, precipitation lines were visible after three days and became clearer at four to five days in tests that were positive. With the slide tests however, precipitation lines developed in positive cases between 18-24 hours. The normal (or control) serum obtained from rabbits prior to inoculation with A. flavus always gave a negative result (Fig. 5.3.1).

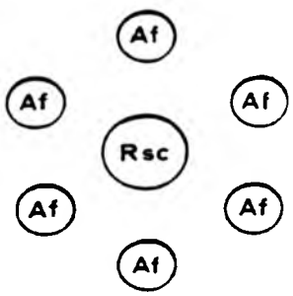
Serum samples from Oreochromis niloticus previously infected with A. flavus and kept in water at 17 + 1°C and the second group kept in water at 25 ± 1°C both reacted and produced precipitation lines with A. flavus antigen extracts (Fig. 5.3.3). The precipitation lines of positive serum of fish kept in water at 17 ± 1°C appeared clearer, more distinct or dense than the serum of fish kept in water at 25 ± 1°C, suggesting that infected fish at lower temperatures produced more antibody or else probably more avid to A. flavus. Tests also showed that antibody against A. flavus was absent from apparently healthy and uninfected tilapias.

The findings demonstrated that the agar-coated slide technique was a particularly useful method for the rapid detection of precipitins to Aspergillus organisms, results being readable from unstained gels between 1-24 hours. No false-positive or false-negative reactions occurred in these tests.



Rsf = rabbit anti A. flavus serum

Af = A. flavus extract

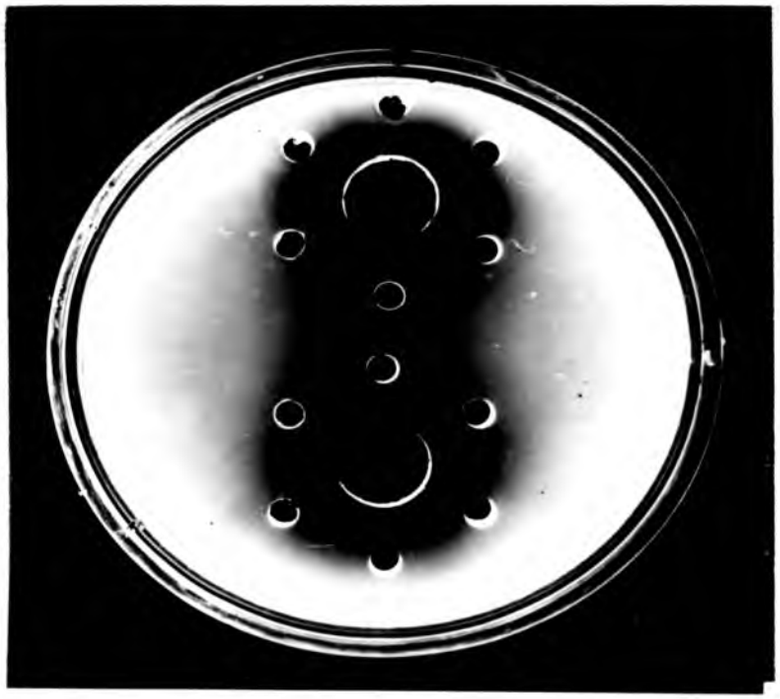


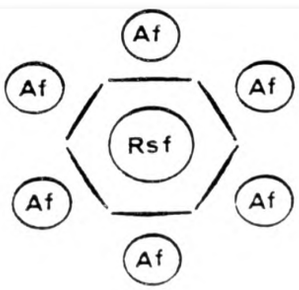
Rsc = Pre-immune rabbit serum (rabbit control serum)

Fig. 5.3.1

a) rabbit A. flavus immune serum and A. flavus extract

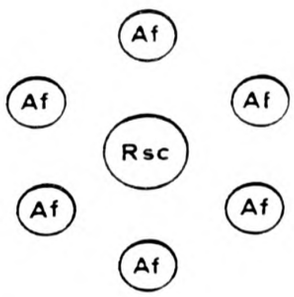
b) pre-immunised rabbit serum (control) and A. flavus extract





Rsf = rabbit anti A. flavus serum

Af = A. flavus extract



Rsc = Pre-immune rabbit serum (rabbit control serum)

Fig. 5.3.1

a) rabbit A. flavus immune serum and A. flavus extract

b) pre-immunised rabbit serum (control) and A. flavus extract

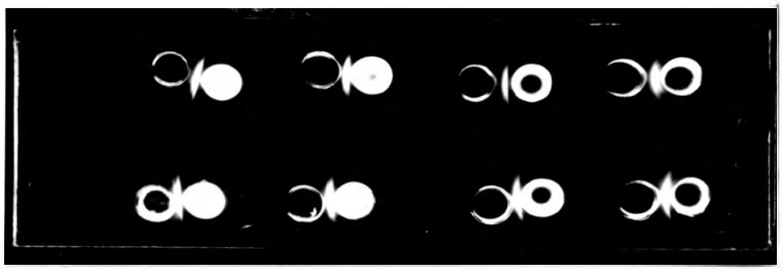
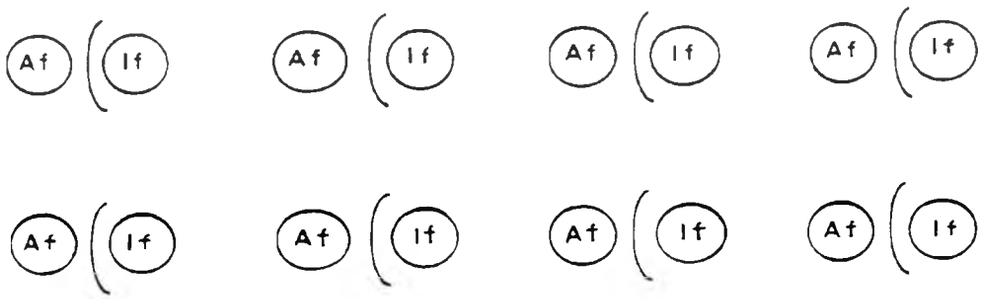


Fig. 5.3.2 Glass slide technique using precipitated Immunoglobulin from rabbit injected with A. flavus and antigenic extract of A. flavus.



If = Immunoglobulin from rabbit anti A. flavus serum.

Af = Aspergillus flavus extract

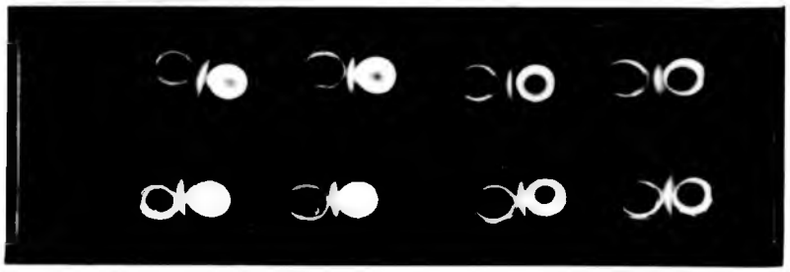
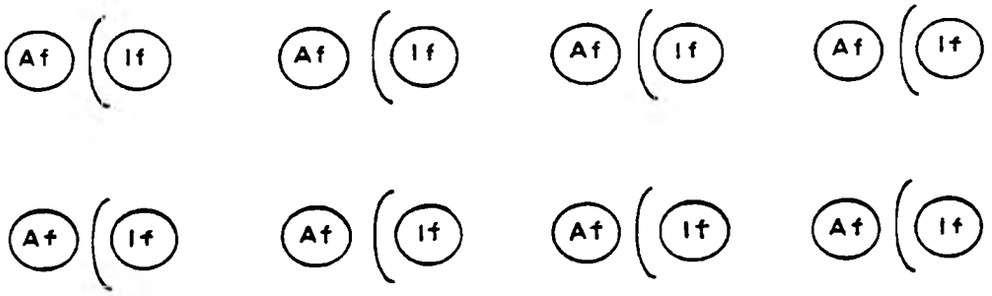
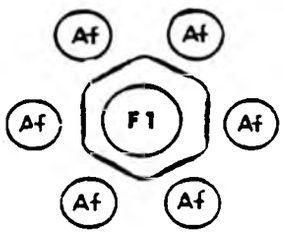


Fig . 5.3.2 Glass slide technique using precipitated Immunoglobulin from rabbit injected with A. flavus and antigenic extract of A. flavus.



If – Immunoglobulin from rabbit anti A. flavus serum.

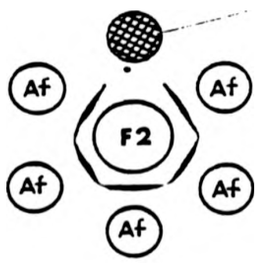
Af – Aspergillus flavus extract



F1 = Fish serum at 25±°1C

Af = A. flavus extract

Agar plug unremoved.



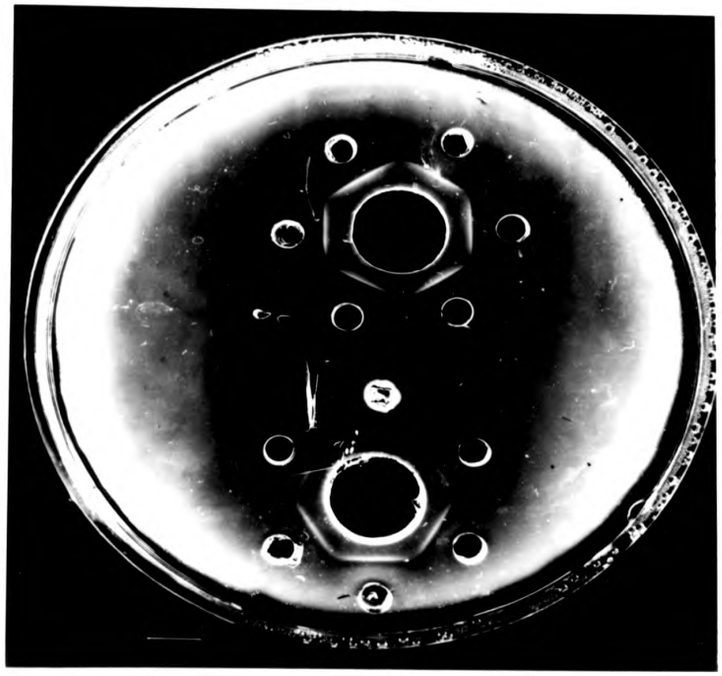
F2 = Fish serum at 17±1°C

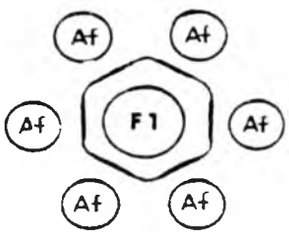
Af = A. flavus extract

Fig. 5.3.3

(i) Serum from fish maintained at 25±1°C (F1) and A. flavus extract

(ii) Serum from fish maintained at 17±1°C (F2) and A. flavus extract

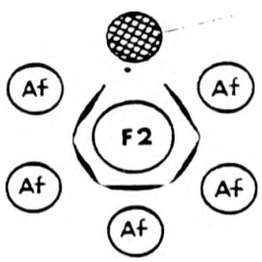




F1 = Fish serum at 25±°1C

Af = A. flavus extract

Agar plug unremoved.



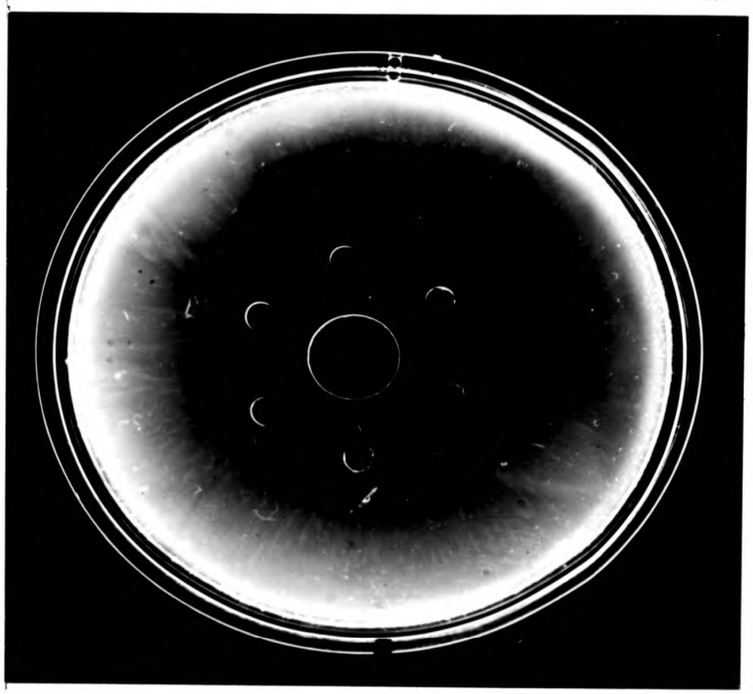
F2 = Fish serum at 17±1°C

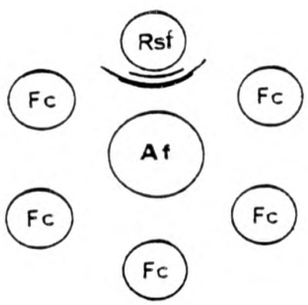
Af = A. flavus extract

Fig. 5.3.3

(i) Serum from fish maintained at 25±1°C (F1) and A. flavus extract

(ii) Serum from fish maintained at 17±1°C (F2) and A. flavus extract





Rsf = rabbit anti A. flavus serum

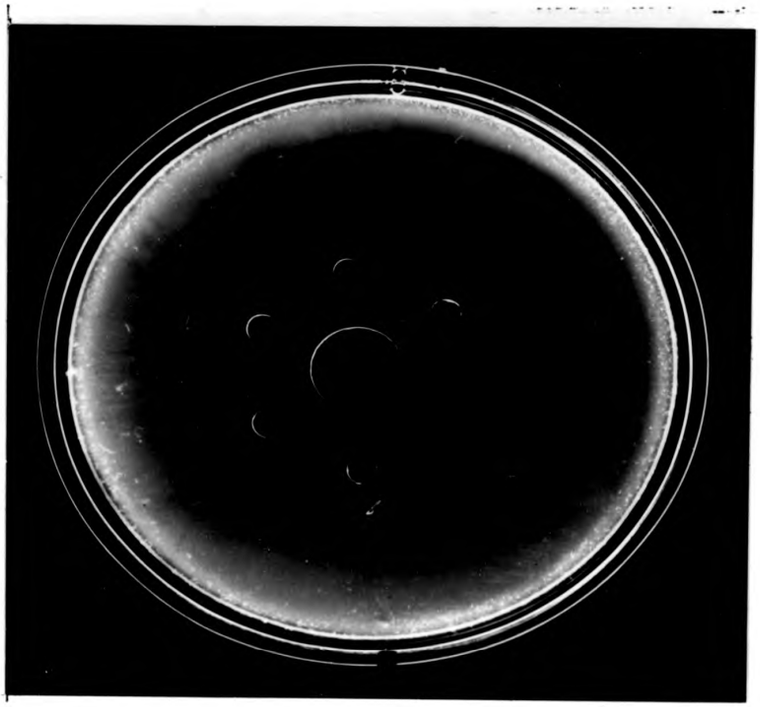
Fc = serum from control fish

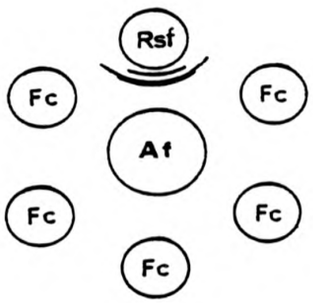
Af = A. flavus antigenic extract

Fig. 5.3.4

(i) A. flavus antigenic extract in the central well & rabbit anti A. flavus serum in the top peripheral well.

(ii) Uninfected (control) fish serum in five peripheral walls (fc) and A. flavus antigenic extract in the central well.





Rsf = rabbit anti A. flavus serum

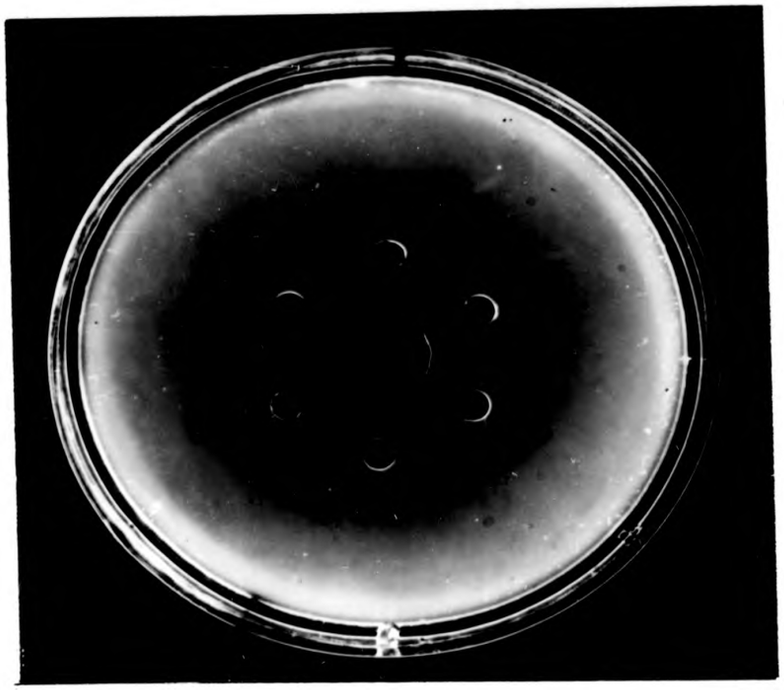
Fc = serum from control fish

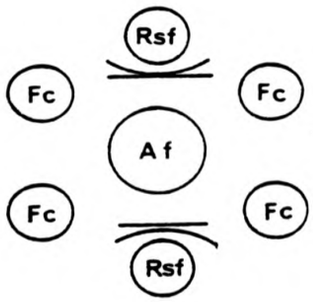
Af = A. flavus antigenic extract

Fig. 5.3.4

(i) A. flavus antigenic extract in the central well & rabbit anti A. flavus serum in the top peripheral well.

(ii) Uninfected (control) fish serum in five peripheral walls (fc) and A. flavus antigenic extract in the central well.





Af=A flavus antigenic extract

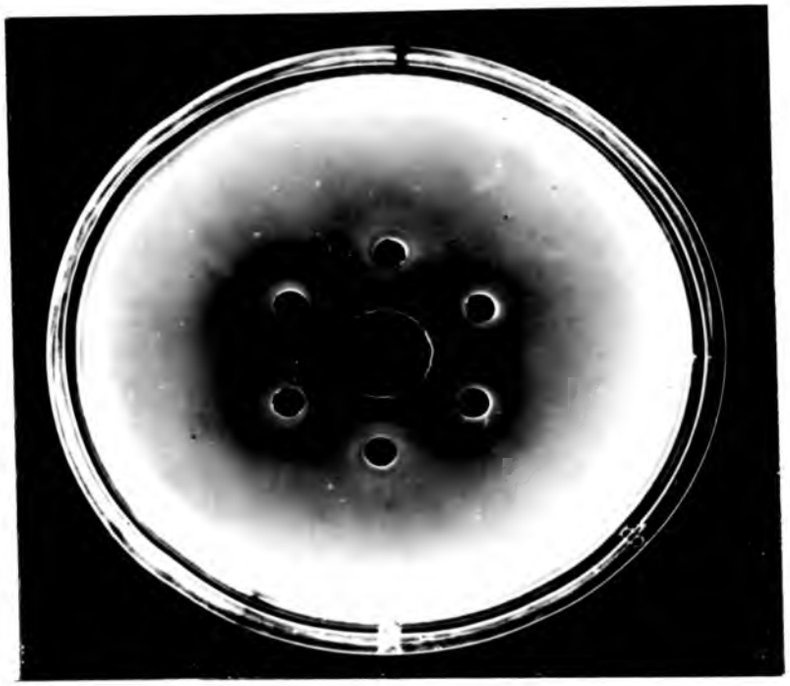
Fc = serum from control fish

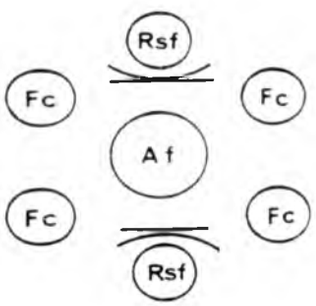
Rsf = rabbit anti A. flavus serum

Fig 5.3.5

(i) Uninfected (control) fish serum A. flavus extract in the central well

(ii) rabbit anti A. flavus serum and A. flavus antigenic extract in the central well.





Af = **A flavus** antigenic extract

Fc = serum from control fish

Rsf = rabbit anti A. flavus serum

Fig 5.3.5

(i) Uninfected (control) fish serum A. flavus extract in the central well

(ii) rabbit anti A. flavus serum and A. flavus antigenic extract in the central well.

Table 5.1 shows the summary of the results of the precipitin tests.

Table 5.1 Summary of results of Agar-gel precipitin tests

Serum samples	Antigenic extracts of		
	A. flavus	A. niger	A. japonicus
Rabbit anti A. flavus serum	+ ve	+ ve	+ ve
Fish serum at 25 ± 1°C) infected with	+ ve	+ ve	+ ve
Fish serum at 17 ± 1°C) A. flavus	+ ve	+ ve	+ ve
Normal (control) pre-immune rabbit serum	- ve	- ve	- ve
Normal (control) serum from apparently healthy tilapias	- ve	- ve	- ve

N.B. Tests were carried out in quadruplicates in each case for both the plates and the agar coated slides.

5.4 DISCUSSION

Investigations by classical serological methods have resulted in a better understanding of antigenic relationships among many genera and species of fungi, such as Candida albicans, Candida stellatoidea, Candida tropicalis and related species (Hasenclever, Mitchell and Loewe, 1961; Tsuchiya, Fukazawa and Kawakita, 1961), the genera Torulopsis (Hasenclever and Mitchell, 1960; Tsuchiya et al, 1961), Saccharomyces (Tsuchiya et al, 1961), Trichosporon (Seeliger and Schroter, 1963), and Geotrichum (Cheek and Barrett, 1962; Torheim, 1963). A variety of immunological, clinical and pathological phenomenon is encountered in aspergillosis in man. Antibody responses comparable with those in hyperimmunized animals have been shown to be present in the sera of patients with pulmonary aspergilloma due to A. fumigatus (Longbottom, 1964). Specific reactions to other species have been obtained in occasional patients in whom the aspergilloma was due to other species, such as A. nidulans, A. niger and A. flavus (Longbottom, Pepys and Temple-Clive, 1964). The detection of antibodies to A. fumigatus infection by the double-diffusion test has been employed by Coleman and Kaufman (1972). The test has also been used to demonstrate A. fumigatus antibodies in cases of bovine mycotic abortion (Corbel, 1972) and mycotic abortion in sheep (Corbel, Pepin and Millar, 1973; Thurston, Cysewski, Pier and Richard, 1972). Chandler (1975) employed the same serological test to confirm a diagnosis of intranasal A fumigatus infection in a boxer dog after swab tests had produced false negative results. The increased application of new and highly refined test procedures have enabled the serologically oriented mycologist not only to improve the diagnosis of fungus infection in man, but

also to determine the antigenic structure of fungi. The alleged short-comings of these tests were due in part to the crudeness of the antigens rather than the tests per se (Campbell, 1960).

In the classical precipitin test and by means of chemical methods, purified polysaccharides of fungi usually behave serologically and chemically as whole entities. The numerous cross reactions observed have been thought to be due to various kinds of sterical arrangements and to the presence of special sidechains adhering to the molecule of the antigen thereby conferring serological specificity. However, by use of agar-gel diffusion tests many more antigenic components have been revealed in those preparations. Their separation is attained due to different diffusion in gels, by their varying migration rate and by their subsequent reaction with corresponding factors of the globulin fractions of the antisera.

To assess the accuracy of serologic diagnosis, the specificity of the antigen, the type and stage of an infection from which a serum is taken must be considered. In addition, there is the matter of viability of the fungus and the host's ability to eradicate it. When micro-organisms are able to be readily phagocytized by mobile defence cells, they are probably not only rendered non-viable but may well be rapidly eliminated from the host, without proper stimulation of the immune system. Other organisms, like Aspergillus for example however, may escape the mobile agents of defence and become "trapped" by the host cells they have parasitized. In response, the host's granulomatous tissues are produced, and if successful may lead to encapsulation or slow disintegration of the fungus. However, as long as the fungus or fragments of it are present in these tissues, even

though nonviable and culturally impossible to recover, it is probable that they continue to stimulate antibodies to some of the several antigenic components they contain, and these antibodies residual as they may be, may persist for very long periods, depending upon the extent and site of the lesion, the state of the fish's immune mechanism, and a host of other factors. The findings in the serologic investigation of aspergillomycosis in tilapias suggest that this is probably the case as far as this disease is concerned. The cross reactivity tests also showed that antigenic components are commonly shared by A. flavus, A. niger and A. japonicus, and no false-positive or false-negative results were obtained from tilapias.

The quality and complexity of antibodies produced in response to fungal infection have been reported to be to a considerable extent dependent on the age and maturity of the inoculum (Campbell, 1960). Antigens occuring in different phases of growth (conidia and mycelia) may differ as to their immunogenicity. For example, reports by Kong, Levine and Smith (1963) and Levine, Cobb and Smith (1960) indicate that the endospores of non-disrupted spherules of Coccidiodes immitis have lesser immunogenic properties than the purified spherule walls. A variety of cultural conditions, such as surface or shake cultures on liquid or solid media of different types and at different temperatures appropriate to particular fungi, have also been used. In the present study, a synthetic solid medium of Czapek-solution agar provided a good source of both conidia and mycelia from which soluble antigen were extracted, by repeated freezing and thawing, by freeze-drying and reconstitution suggesting that each sample was at a similar stage of development. Reproducible results in antigenic content were consistently

obtained. The ready solubility of the fungal antigens made extraction with sterile distilled water satisfactory for the purposes of the double diffusion tests.

Because of cross-reaction, it seemed from the study that although a serological diagnosis of aspergillomycosis was readily feasible, the actual specific infecting fungus species could not be defined.

Antibodies against the antigens in cultures of Aspergillus were shown to be uniformly present in the serum of tilapia with evidence of the fungal infection. The reactions to the aspergillus antigens have been shown to occur with the gamma-globulin of the sera. The finding that sera of infected fish produced precipitin lines with extracts of other members of Aspergillus species is of significance.

The clinical picture of aspergillomycosis in tilapias may not always be highly suggestive of the diagnosis, an unequivocal diagnosis depends upon the isolation and identification of the causative agent. While the isolation and identification of Aspergillus species from fish clinical material may no longer pose a difficult problem, the procedures involved may be time consuming. It is also important to note that although the rapid method for making a presumptive diagnosis of a fungus disease, by means of direct microscopic examination of clinical materials is the best method of diagnosis, fungal elements are infrequently detected in clinical materials, particularly in late or very mild infections. Serological methods employed in diagnosis may thus offer a simple and rapid approach. At present, serological methods in diagnosis of any type of infection in fish have not

yet been given a great deal of attention. It appears from the foregoing that the double diffusion technique, a procedure that has been found to be useful for the rapid detection and identification of a wide range of micro-organisms, represents a potentially valuable tool for the probability of positive diagnosis of aspergillomycosis in tilapias. The encouraging results in the present study have led to further evaluation of the practicality and reliability of the fluorescent antibody technique which also depends for its action on the presence of serum antibodies for the rapid diagnosis of the disease (Chapter 6).

CHAPTER 6

APPLICATION OF THE FLUORESCENT ANTIBODY TECHNIQUE (FAT) TO THE DEMONSTRATION OF *ASPERGILLUS* ORGANISMS IN FORMALIN - FIXED TISSUES OF TILAPIAS

6.1 INTRODUCTION

The work of Coons, Creech, Jones and Berliner in 1942 brought a new serological tool for the rapid detection and identification of micro-organisms - the fluorescent antibody technique (FAT). Basically the FAT is an immunological staining procedure which allows microscopic visualization of an antigen - antibody reaction. The visualisation is accomplished through the use of antibody coupled to a fluorochrome, this complex is usually referred to as the labelled antibody or conjugate. The combination of the labelled antibody and its homologous antigen results in a complex that fluoresces when excited by light of proper wavelength. This fluorescence is observed by the use of a fluorescent microscope.

The fluorochromes in frequent use have been derivatives of fluorescein, a dye which fluoresces with a yellow-green colour. Coons *et al* (1942) and other early workers (Goldman and Carver, 1957) used fluorescein isocyanate as a labelling compound. However, this was unstable in the presence of moisture and thus presented certain practical difficulties. This disadvantage was overcome by Riggs, Seiwald, Burkhalter, Downs and Metcalf (1958) who synthesized fluorescein isothiocyanate (FITC) - a more stable fluorescein derivative. This fluorochrome is at

present the most widely used labelling agent. Fluorochromes of other colours such as rhodamine and its derivatives, which fluoresce with a reddish colour have also been employed as labelling compounds. In this study, a commercially available conjugate was employed DAKOPATT's^{R} fluorescein - conjugated (FITC) antisera; the chromatographically purified immunoglobulin fraction of antiserum being in this case conjugated with fluorescein isothiocyanate isomer 1 (FITC). After conjugation, unreacted FITC is completely removed by gel-filtration on Sephadex G25, following which further purification is carried out by ion-exchange chromatography. The conjugate therefore consists of optimally labelled antibody molecules and has the added advantage of requiring no absorption with tissue powders prior to use.

The simplest FAT procedure involves the application of solutions of labelled antibody directly to dried smears of antigens - the direct method. The commonly employed modification of the basic procedure is the indirect method. The indirect method which was used in this study is the most widely used because of its greater sensitivity and the need for only one fluorescent conjugate, it involves the use of two different antigen - antibody systems, the last of which is labelled with FITC. In the first step, the antigen is combined with the unlabelled antibody. In the second step, the combined antibody reacts with the labelled antiglobulin specific for the globulin of the animal species in which the unlabelled antibody of the first step was prepared.

The value of the fluorescent antibody technique (FAT) as a diagnostic and research tool has been amply documented. Subsequent to the introduction of the technique by Coons et al (1942) microbiologists applied the technique to the rapid diagnosis

of bacterial (Thomason, Moody and Goldman, 1956) viral (Goldwasser and Kissling, 1958) and protozoon diseases (Goldman, 1954). Generally, the results have been satisfactory and in some systems have had practical application. Its advantages over conventional diagnostic methods have been discussed by Kaplan and Kaufman (1961). These include the fact that fluorescing antigen-bearing cells with distinct morphological characteristics can usually be observed; the fluorescent cells even when few in number or in mixture with other organisms can be easily detected against a dark background; antigen bearing organisms need not be viable for detection; and the advantage of its rapidity, and very high specificity which eliminates the time required for carrying out conventional isolation, purification and identification procedures.

A number of workers (Berge and Kaplan, 1967; Eveland, Marshall, Silverstein, Johnson, Inversion and Winslow, 1957; Hotchi, 1967; Kase and Marshall, 1960; Marshall, Inversion and Eveland, 1959; 1961, Metzger, Kase and Smith, 1962; Yamaguchi, Andriano and Braunstein, 1963), have recognised the potential value of immunofluorescence and have investigated the feasibility of using the technique to demonstrate fungi in routine formalin-fixed, paraffin-embedded tissue sections. These workers have reported that in tissue sections, it is possible to stain with FAT such diverse fungi as Blastomyces dermatitidis (Kaplan and Kraft, 1969), Coccidioides immitis (Kaplan and Clifford, 1964), Cryptococcus neoformans (Eveland et al, 1957), Histoplasma capsulatum (Yamaguchi, Andriano and Brainstein, 1963) and Sporotrichum schencki (Kaplan and Ivens, 1960). Apparently, fixation with formalin, storage of the fixed tissue, embedding in paraffin, and deparaffinization of sections do not cause any observable reduction in antigenicity of these fungi. This is

a particular advantage compared to most bacterial and viral antigens which are destroyed by such processing and have to be prepared for immunofluorescence by cryopreservation and cryostat sectioning.

Fungus diseases are normally identified by a combination of examination of wet microscopical preparations, culture and histopathology. Such diagnostic procedures are normally highly accurate but may take some considerable time to complete. However in light infections, or where material is not suitable for culture and/or histology, then it may be difficult to arrive at a definitive diagnosis. Also in research terms, studies on the pathogenesis of a fungal organism often requires some form of marker to allow detection of the movement of the pathogen through tissues. In such circumstances it would be highly advantageous to use the techniques of immunofluorescence and the purpose of the present work was to investigate the suitability of the immunofluorescence technique for adaptation to the diagnosis and study of aspergillomycosis in tilapias.

6.2 MATERIALS AND METHODS

6.2.1 Case material: Formalin-fixed, paraffin-embedded tissue from Aspergillus experimentally infected fish (Chapter 4.2 and 4.3) were used in this study. Sections were cut 5 um in thickness, deparaffinized in the usual manner by passage through two changes of xylol and then hydrated by passage through alcohol and methanol into buffered phosphate saline pH 7.2.

6.2.2 Digestion of sections: It was necessary to digest the tissue sections to enhance the staining of the mycotic disease agent by fluorescent antibodies. It was of great value particularly, when the organisms were few in number and small in size or were located in dense tissue. The enhancement of the specificity of the staining results appeared to be principally the result of the removal of extraneous protein, which interferes with the staining of the fungus by the labelled antibodies. The digestant used was trypsin. A 2.5 per cent in Hank's balanced salt solution was used as the stock solution. The stock solution stored at -20°C was allowed to thaw at room temperature and diluted with phosphate-buffered saline (PBS) pH 7.2, to obtain the 1% desired working concentration of trypsin and adjusted to pH 8.0 with sodium bicarbonate buffer, pH 9.0. Tissue sections were digested in the 1% trypsin solution for 1 hour. The digestion of the tissue sections was performed in Coplin jars at 37°C in a water bath. Following this treatment each section was heat-fixed, while still wet, to inactivate residual trypsin as well as to enhance adhesion of the tissue to the glass slide, it was then stained by the fluorescent antibody technique (FAT).

6.2.3 Fluorescent antibody technique (FAT)

The indirect method was employed. This involves the use of two different antigen-antibody systems, only the last of which is labelled with fluorochrome, in this case, fluorescein isothiocyanate isomer 1 (FITC).

In the first step the antigen is combined with the unlabelled rabbit A. flavus antiserum (Chapter 5.2.2).

In the second step the combined antibody reacts with the labelled swine anti-rabbit immunoglobulin FITC (Dakopatts, Denmark).

The slide after heat fixing was placed cell side uppermost and supported in a moist box. Drops of rabbit A. flavus anti-serum (Chapter 5.2.2) were carefully spread on the slide to cover the tissue. It was then incubated at 37°C for 1 hour. Next the slides were arranged in a rack and washed in phosphate buffered saline (PBS) pH 7.2 for 15 minutes with occasional agitation and three changes of the buffer. Slides were air dried and replaced into the moist box. The slides were then covered with the labelled swine anti-rabbit immunoglobulins in the dilution of 1:20 to which dimethyl sulphoxide (DMSO) was added in the ratio 1:11. Slides were then incubated again at 37°C for 1 hour. The slides were finally washed for 15 minutes using three changes of phosphate buffered saline with agitation, air dried and mounted using the non-fluorescent mountant Fluolorite®.

The slides were then examined using a fluorescent microscope. Fluorescent microscopy was performed using a Leitz ORTHOPLAN microscope fitted with a fluorescence vertical illuminator according to Ploem. An HBO 200W super-pressure mercury lamp was used as incident light source and a fluorescence-exciting beam of 495 nm was obtained using a BG 38 exciting filter and BG 12 interference filter combination in the lamp housing. The appropriate built-in dichroic beam-splitting mirror and suppression filter was selected to provide maximum reflection of exciting light at 495 nm.

6.3 RESULTS

The indirect FAT gave a specific fluorescence with digested tissue sections from all the Aspergillus infected tilapia, with the rabbit anti-A. flavus serum and the swine anti-rabbit labelled immunoglobulin. Aspergillus antigens in formalin fixed tissues were stained brightly greenish by the fluorescein (FITC) labelled swine anti-rabbit globulins. Even where relatively few antigenic elements were present in these sections, they were stained brightly and stood out in sharp contrast to the surrounding tissue, and were therefore very readily observed.

The addition of DMSO (Dimethyl sulphoxide) to the conjugates improved the FAT staining results in the digested tissue sections. The tissues became subdued and darker, which improved the contrast with the stained fungal antigenic materials.

There was no specific fluorescence, when pre-immunisation serum (Chapter 5.2.1) was substituted for rabbit anti-A. flavus serum. Similarly, the antiglobulin conjugate did not stain replicate sections of normal and infected tilapia tissues when the procedure was repeated on them.

Figs. 6.3.1 - 6.3.4 demonstrate the specific fluorescence of the Aspergillus fungal antigens in the various organs of experimentally infected tilapias.

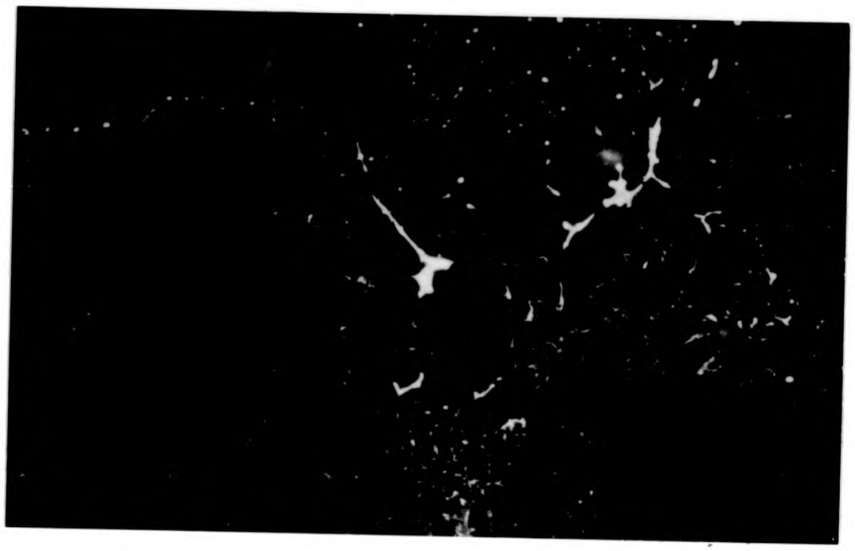


Fig. 6.3.1 Liver parenchyma of Oreochromis niloticus showing fluorescence of fungal elements X 80, fluorescing yellow/green against a dark background.

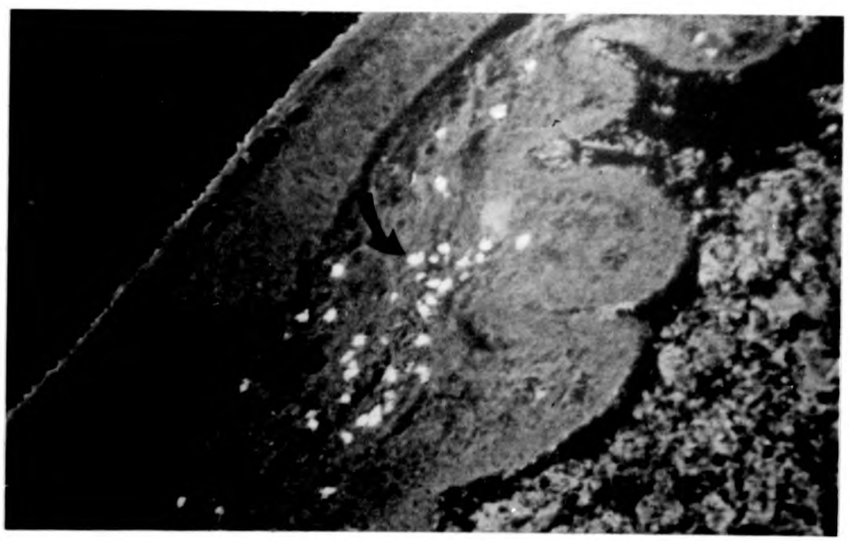


Fig. 6.3.2 Fluorescence of fungal elements in the sub-mucosa of the cut X 80

Fig. 6.3.1 Liver parenchyma of Oreochromis niloticus showing fluorescence of fungal elements X 80, fluorescing yellow/green against a dark background.

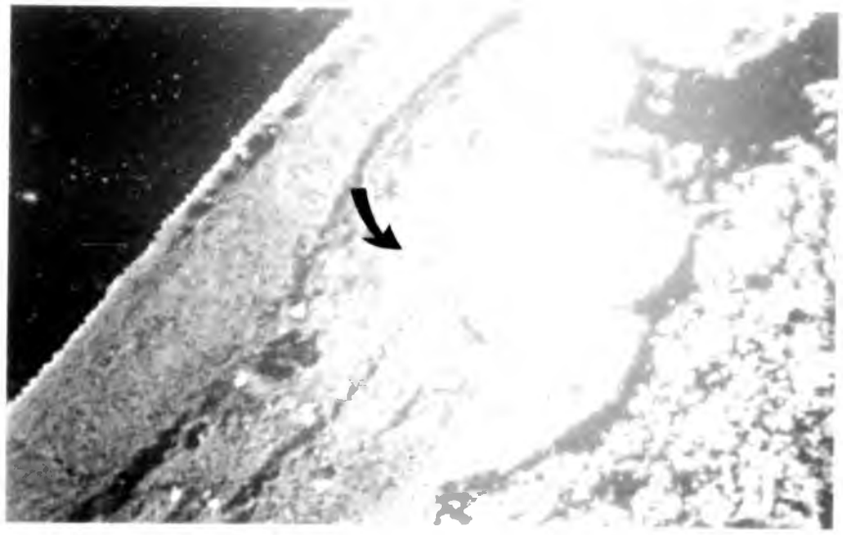


Fig. 6.3.2 Fluorescence of fungal elements in the sub-mucosa of the cut X 80

Fig. 6.3.3 Gut wall from "control" tilapia tissue: preparation X 80. NOTE: no fluorescence

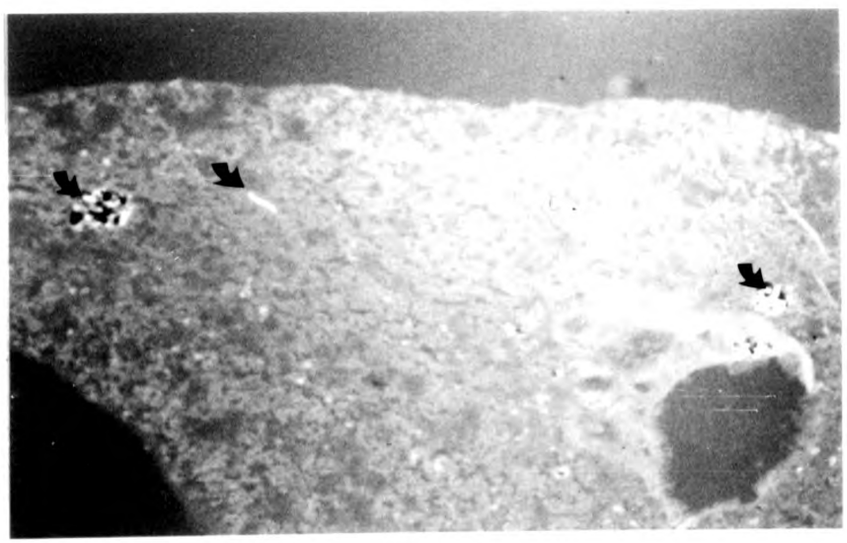


Fig. 6.3.4 Fungal elements scattered in the splenic tissue but note aggregation in the melanomacrophage centres X 80

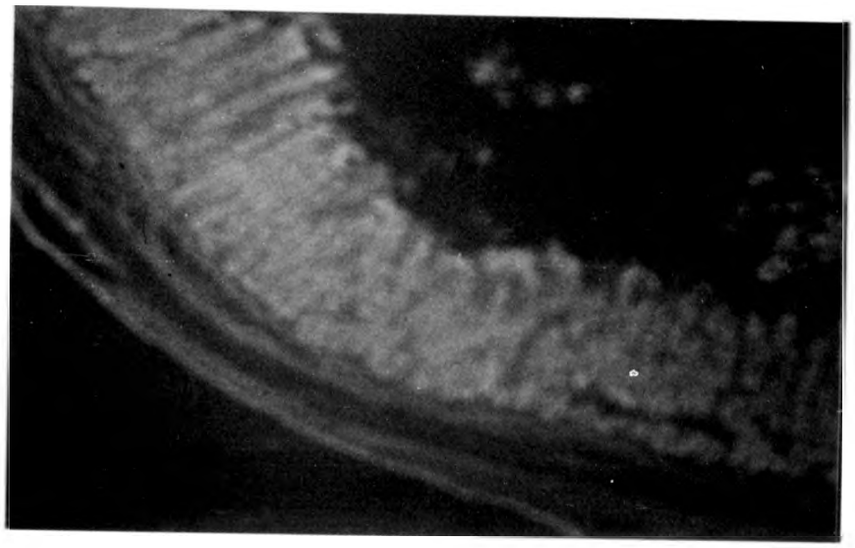


Fig. 6.3.3 Gut wall from "control" tilapia tissue: preparation X 80. NOTE: no fluorescence

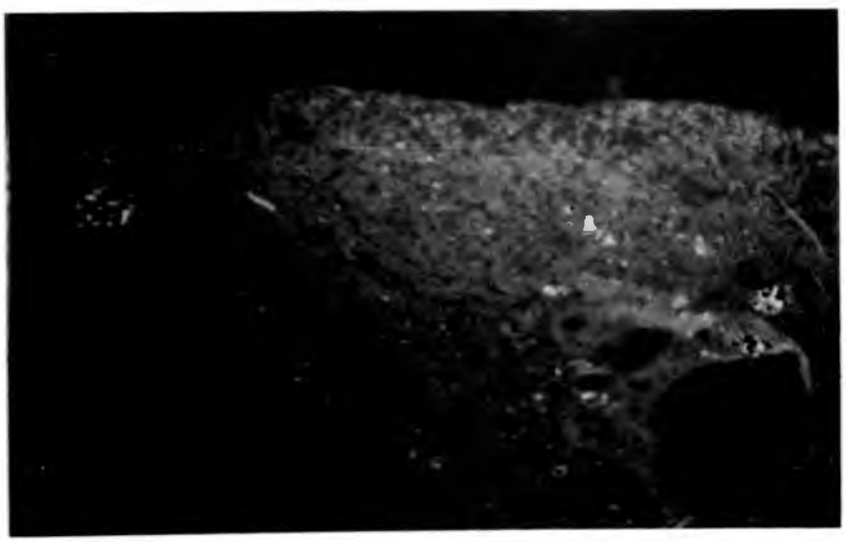


Fig. 6.3.4 Fungal elements scattered in the splenic tissue but note aggregation in the melanomacrophage centres X 80

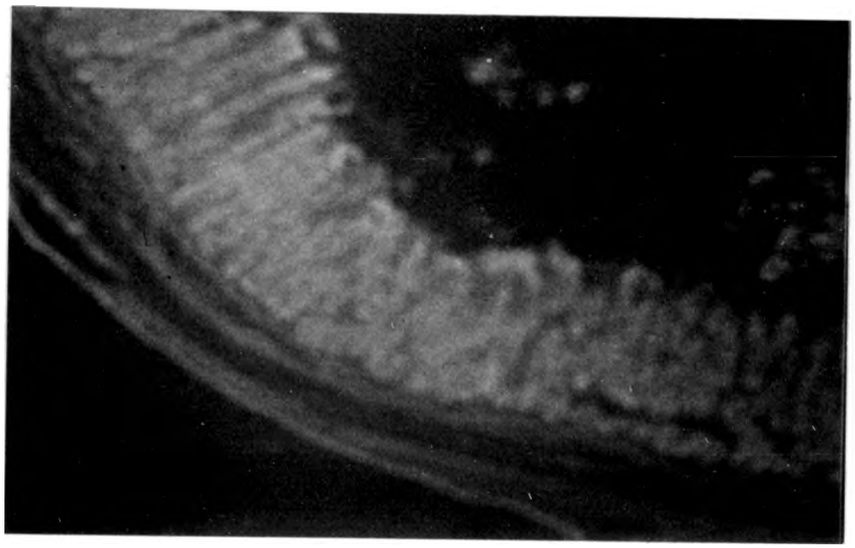


Fig. 6.3.3 Gut wall from "control" tilapia tissue: preparation X 80. NOTE: no fluorescence

Fig. 6.3.4 Fungal elements scattered in the splenic tissue but note aggregation in the melanomacrophage centres X 80

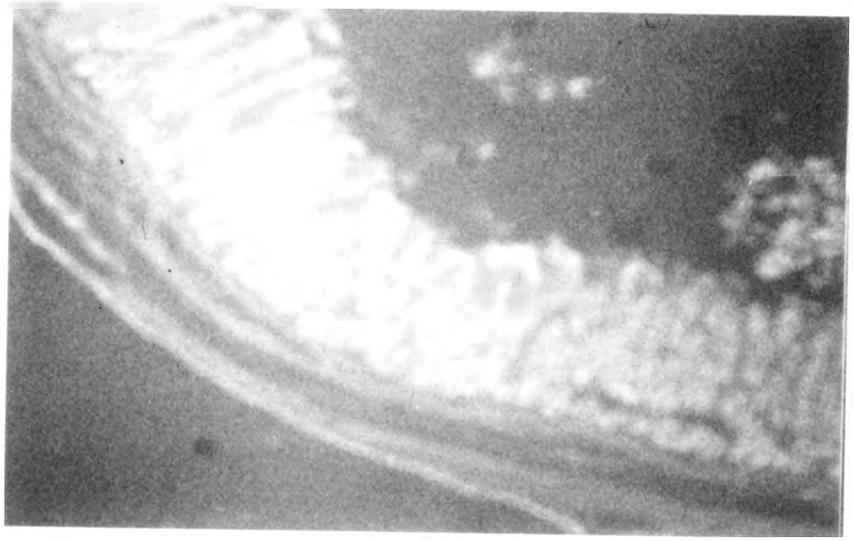


Fig. 6.3.3 Gut wall from "control" tilapia tissue: preparation X 80. NOTE: no fluorescence

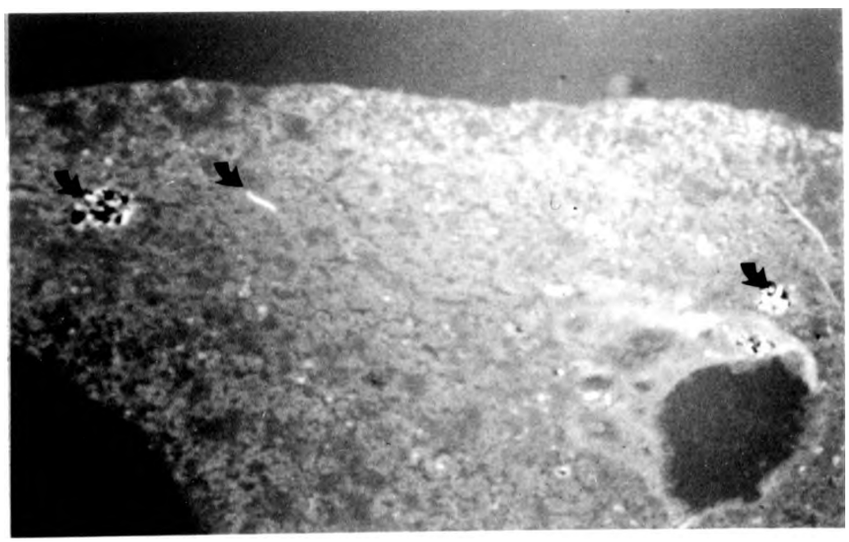


Fig. 6.3.4 Fungal elements scattered in the splenic tissue but note aggregation in the melanomacrophage centres X 80

6.4 DISCUSSION

The results of the study of the applicability of the fluorescent antibody technique (FAT) to aspergillomycosis in tilapias showed this is a most valuable research and possibly diagnostic tool, which in this instance is actually more versatile than the technique has proved to be in relation to other fish diseases e.g. Aeromonas salmonicida, Klontz and Anderson (1968) or Egtved virus disease, Jorgenson (1972) because it was readily applicable even to formalin fixed tissues. This is something which lends much greater flexibility to the technique since for use in the other types of microbial infection vide supra, fresh cryostat frozen sections were essential to a satisfactory diagnosis whereas in the case of aspergillomycosis, stored, fixed and embedded material was viable for FAT examinations many months after sampling. As indicated earlier, this is probably because fixation with formalin, storage of the fixed tissue, embedding in paraffin and deparaffinization of sections do not cause any observable effect on the antigenicity of the Aspergillus organisms.

This is the first instance of the use of FAT to study a fish mycosis, and in view of its complete success in demonstrating the presence of organisms even in lightly infected, or old lesions, it appears to be most suitable for development for further use in diagnostic laboratories. It is likely that it could also be used for young cultures which are too immature for morphological identification. Such cultures could possibly be rapidly identified by immunofluorescence tests as could small numbers of viable or non-viable cells or cells mixed with other types of cells as in contaminated cultures. It could also have great potential as a screening technique in this and possibly in other fungal diseases such as Ichthyophonus infection or branchiomycoses.

CHAPTER 7

GENERAL DISCUSSION

Studies on intensively cultured and wild tilapias have shown that although they appear to be more resistant to diseases than many other species, nevertheless, a wide range of disease problems can occur in members of these genera under certain circumstances. Roberts and Somerville (1982) have reviewed current knowledge of the diseases of tilapias which occur in culture systems or those of wild fishes which have a clinical manifestation or potential significance for culture. To their list may now be added aspergillomycosis, which is a systemic fungus disease caused by members of the genus Aspergillus.

In describing this fungus pathogen for the tilapias, it has been necessary to give two different proofs. The first of these was the demonstration of pathogenicity (Chapter 4) and the second, that the pathogen is indeed a fungus, the identity of which has been confirmed by isolation from tilapia infected tissues and by subsequent identification to the species level (Chapter 3).

Most of the species assigned to groups of aquatic fungi have zoospores or gametes or both, which bear flagella (Kole, 1965). The geofungi or the soil fungi to which Aspergillus belongs, are without special organs of dissemination (flagella) capable of searching out a special food supply, or without special organs on the spores to aid in achieving the disseminules or to resist the movement of water. Members of the genus Aspergillus however, produce many conidia which are disseminated throughout

their environment in great multitudes. It may therefore be accidental as far as the individual spore is concerned that it falls on suitable substratum, but such success is rendered much more likely by the very large number of opportunities created by the very high level of spore production. As the disseminules of these species reach the water of any pond, lake or stream, especially if a relatively rich nutrient solution formed by organic pollutants is present, growth may take place and the fungus may be added to the biota of that body of water. A number of filamentous geofungi which have been isolated from organically enriched streams include members of the genus Aspergillus - A. candidus, A. chevalieri, A. clavatus, A. flavus, A. niger, A. terreus, A. fumigatus and other genera (Cook, 1976).

Besides the ubiquitous nature of the Aspergillus species pathogen, other stress factors can directly or indirectly play a significant part in the pathogenesis of aspergillomycosis in cultured tilapias. Various types of environmental "stress" tend to lower the resistance of fishes to infectious diseases (Mackie and Menzies, 1938; Fish and Rucker, 1943; Bisset, 1946; Snieszko, 1957, 1958; Rucker, 1958; Bullock, 1964; Haley, Davies and Hyde, 1967). Examples of stress-mediated fish diseases are frequently encountered in hatchery practice. Potential fish pathogens common in water seldom cause disease problems unless fish are stressed by excessive handling or crowding (Bullock, 1964). Thus bacterial gill disease, caused by myxobacteria, is ubiquitous under high population densities. However, if the population density is reduced, fish having bacterial gill disease often recover in the same water supply without additional treatment (Snieszko, 1962). Snieszko (1957)

has shown that the incidence of systemic bacterial infections is considerably reduced if carp are not subjected to handling stress in the early spring when water temperatures are rising. Bissett (1948) showed that goldfish which are carriers of a wide variety of saprophytic water bacteria show no signs of disease if the water temperature remains below 15°C. However, when the temperature is increased above this level, septicaemia ensues with a resulting mortality. Presumably, metabolic changes in both the host and the parasite are involved in diseases mediated by temperature changes. Dubos (1955) expressed the role of stress (predisposing factors) in the host response to infectious diseases as follows: "There are many situations in which the microbe is a constant and ubiquitous component of the environment but causes diseases only when some weakening of the patient by another factor allows infection to proceed unrestrained, at least for a while. Theories of disease must account for the surprising fact that, in any community, a large percentage of healthy and normal individuals continually harbour potentially pathogenic microbes without suffering any symptoms or lesions". Thus, the process "infection into disease" is a strong function of the physiological state of the host and is the sum of a complex series of metabolic interactions between the invading organisms and the host tissue. Several factors then, will influence the epizootiology of aspergillomycosis. Some of these factors are the consequences of environmental changes which may or may not be directly associated with husbandry practices.

Temperature: Relationships between Aspergillus species and water temperatures have been observed under experimental conditions. Generally, increased temperatures favour the development,

transmission and pathogenicity of many infectious diseases of fish. The effects of water temperature on Aspergillus pathogenesis among young tilapias was recognised almost immediately following intra-peritoneal injection of the fungal materials (4.1). Additional investigations soon established the probability of the existence of different species of Aspergillus that killed young tilapias at lower temperatures (17 ± 1°C). Although A. flavus appeared to be the most pathogenic of the Aspergillus species isolated, A. terreus and A. flavus produced mortalities at each of the experimental thermal regimes whereas a relatively low-virulence species (A. niger) was able to initiate infection when the thermal regime was low (17 ± 1°C). A slowly progressing chronic infection was produced at higher temperature regime (25 ± 1°C) by A. japonicus which appeared to be a relatively low-virulence species. Although A. flavus initiated infection and produced the disease at low water temperature (17 ± 1°C), fish mortality rates became progressively slower at this lower temperature while at higher temperature (25 ± 1°C) fish quickly succumbed or were relatively rapidly killed. The observed differences in the degree of virulence may well be unrelated to the species of Aspergillus concerned, but may rather be temperature dependent.

Fish confinement: Fish infected with Aspergillus species could spread infective materials into the water through their faeces, thereby transmitting the pathogen to other fish. Tilapias are particularly vulnerable as substrate feeders to such faecal reinfection.

Such transfer of a water borne pathogen is the basis of the

usual "contact method" of infection during experimental studies, which involve dispersal of cultured fungal spores in a container of water and placing test fish in the medium. That confinement of fish is important in implementing outbreaks of aspergillomycosis is indicated from reports of the disease problem from Kenya, where heavy mortalities occurred, following infection with Aspergillus organisms, among fish held in holding ponds and during farm operations such as grading. High concentration especially of adult tilapias in tanks or holdings not only exposes them to liberated Aspergillus spores, but will lower their resistance to disease by exposing them to reduced oxygen levels, accumulation of metabolic wastes, mechanical injury and other stresses. Confinement of fish therefore is certainly likely to increase infection and dissemination of the disease and deaths of already infected fish.

Water quality: The effect of water quality on survival of Aspergillus conidia released from fish or introduced from feed is not well known. It is probable that waters of different hardness and alkalinity, pH, and organic content will influence the viability of Aspergillus conidia. This is an area that would require further investigation. At present, no data is available for the prevalence of aspergillomycosis among fish raised under intensive conditions, in relation to their water quality. However, the disease can certainly be of economic importance if proper husbandry is not practised.

The histopathological lesions associated with aspergillomycosis may best be described as a systemic necrotising inflammation characterised by the formation of granulomas. External lesions include a varying degree of emaciation and exophthalmia. The

disease may either occur as an acute fulminating or a chronic proliferative form. In the former, large areas of organs, especially the liver undergo necrosis. Histologically there is usually diffuse distribution of macrophages within a stroma of necrotic tissue and fungal hyphae. The chronic form is probably more common under aquacultural conditions and is characterised by the production of granulomas. The granulomas generally have two zones - a central necrotic zone surrounded by a second zone of epitheliod cells. Giant cells - especially of the Langhan's type are rare. In some cases, fungal hyphae are easily observed in tissue sections stained by periodic acid schiff method (PAS) or Grocott's methenamine silver stain.

The production of a toxin or toxins by the Aspergillus may be involved in the virulence of the fungus, its infectivity and pathological effects especially in the acute state. Toxin production by Aspergillus species, resulting in the condition of aflatoxicosis has already been reported by several workers (Forgacs, 1962; Forgacs and Carll, 1962). The range of metabolites identified from single species of aspergilli is often considerable (Miller, 1961); and some of these have been chemically defined (Moss, 1977). The action of the toxins so far reported has varied from growth depression, as in undescribed metabolites of a species of A. glaucus group (Richardson et al, 1962), to the lethality and carcinogenicity of the aflatoxin from A. flavus (Allcroft and Carnaghan, 1963; Lancaster et al, 1961).

A presumptive diagnosis of aspergillomycosis can be made from recently dead or moribund fish. A positive diagnosis must rely on the isolation of the organism with histopathologic

examination of the lesions. If isolations are not made, and histopathologic diagnosis is the sole recourse, then the advantages of the FAT as discussed above, used in conjunction with histopathology are very obvious. It is particularly valuable that such analyses are possible on formalin fixed materials since in the tropics this is often the only material that can be supplied. Porter, Comfort, Menges, Habermann and Smith (1965) considered that only the fluorescent antibody technique and the cultural and histopathological examinations are reliable in identifying infections of blastomycosis and histoplasmosis. The present study would suggest that aspergillomycosis is similar in this respect.

Immunological techniques are widely used in human and animal medicine for the detection of the disease organism or for testing for the carrier state. The amount of immunity conferred by previous exposure or vaccination can usually be measured by the quantity of specific antibody present in that animal. The use of such techniques for the diagnosis of disease exposure or infection in fish has not been utilized a great deal. This is often because of difficulties in obtaining adequate amounts of blood, but there are also major problems relating to environmental effects on antibody production. This study indicates that by using the described Ouchterlony immunodiffusion test, it is possible to detect and to identify antigens of the Aspergillus species fungi in fish with overt disease. Consequently the technique may have some value in the diagnosis of aspergillomycosis as it appears reliable and definitive.

The application of the fluorescent antibody staining technique

to formalin-fixed tissue was initiated by Eveland et al (1957) as an experimental procedure for studying cryptococcosis. Organisms in a known specimen of human cryptococcal pneumonia reacted positively with specific anticryptococcal rabbit serum which had been conjugated with fluorescein isocyanate. Since 1957, the technique has been utilised extensively on such formalized tissue as a screening method. The specificity of the technique depends upon two essential factors: first, a reliable antiserum must be produced and this must react with the variety of different strains; second, the antigen to be stained must remain intact in the diseased tissue as it is carried through the various techniques preparatory to routine histologic sectioning. Good results have been obtained during routine use of the fluorescent antibody staining technique in experimental infections.

The practical use of the fluorescent antibody technique as a diagnostic aid in the routine pathology laboratory is dependent on the volume of material the laboratory staff screens. One of the difficulties in aspergillomycosis as in other infections, is the production of an antiserum of high enough titer to be reliable in repeated tests. Once the antiserum has been evaluated, the stain is simple to apply and allows quick examination of a variety of samples of formalin-fixed, infected or noninfected tissues. In those laboratories in which the expected application would be rare, the simpler Grocott's methenamine silver stain may be used to provide at least morphological evidence of the likelihood of Aspergillus species organism in fish tissue. The fluorescent antibody test shows the organism brightly and very specifically and is not impractical if the volume of diagnostic material is large enough to warrant

the expense and the time necessary for producing and maintaining the stock antisera.

The knowledge of the epidemiology of any disease provides the possibility of an approach to its control. Although until recently, aspergillomycosis had not been described in fishes, there is an extensive literature on aflatoxicosis which is intoxication leading to hepatoma production in a wide range of fish species (Butler, 1965; Wolf and Jackson, 1963; Ashley et al, 1964; Halver, 1965, 1967, 1969; Wales and Sinnhuber, 1966; Wales, 1970, 1979), but particularly in the rainbow trout (Majeed, Jolly and Gopinath, 1984). Aflatoxicosis results from the elaboration of aflatoxins, as metabolic products by aflatogenic species of Aspergillus flavus as it grows on feed stuff prior to feeding. Undoubtedly one way to prevent fungal contamination of feeds and subsequent mycotoxin formation or aspergillomycosis developing is the maintenance of good husbandry and the adequate and rapid drying of feeds to a water content at which fungal growth cannot occur. Since problems may arise due to moisture migration during storage most fresh fish feeds are subject to fungus spoilage if stored for any excessive periods of time and/or under adverse conditions. Consignments of nuts or cereals rejected for human use should not be used for fish feed compounding. Although aflatoxin residues and infective Aspergillus spores may be removed during oil refining (Dollear, 1969) the meal will retain residual toxicity. Growth of fungi on oilseeds may also produce undesirable chemical changes in the lipids so that oil extracted from fungus-damaged nuts may be of lower quality than required (Eggins and Coursey, 1968). Some ingredients, especially oil seed meals such as peanut and

cottonseed meals, used in the formulation of modern pelleted fish feeds are closely recognised as the foods most frequently contaminated by aflatoxins or Aspergillus spores (Wolf and Jackson, 1963; Sinnhuber et al, 1968).

Fish feed manufacturers especially those producing pellets for tilapias, will inevitably include increasing levels of vegetable proteins in rations as fish meal prices rise, with limits imposed by the necessity of providing sufficient dietary essential amino acids. Such ingredients must, therefore, be very carefully checked for the presence of pathogenic (or toxin producing) fungi such as the Aspergilli.

Numerous methods have been evaluated for the destruction of Aspergillus organism and aflatoxin in agricultural produce. Although aflatoxins are relatively stable to heat (Dollear, 1969), studies with cottonseed meal and cereal grains have demonstrated the feasibility of decontamination by very high temperature treatment in the presence of ammonia (Campbell, 1972; Goldblatt, 1973). The ammonia treated product may subsequently be used for animal feed. Treatment of agricultural products with ethylene oxide not only kills insects and fungal contaminants but destroys fungi and aflatoxins. These modes of treatments or decontamination warrant further investigation as far as fish pellets are concerned, although it is postulated that there may be problems due to possible toxic reaction products of the ethylene oxide (Mayr, 1973).

The problem of contamination of equipments, buildings - walls, ceilings, and other structures on the fish farm should not be overlooked. Aspergillus spores are readily air-borne and may

travel considerable distances. Cleanliness, of course, will contribute significantly to retardation of growth of fungi. In addition, fungistatic paints may be used on walls, ceilings and all structures likely to be subject to fungal growth. Commonly used in such paints are copper-containing compounds. Care should, however, be taken to prevent pollution of water by the copper-compounds as these are toxic to fish above certain levels. Investment in husbandry and storage improvements will therefore appear to be more productive than the continuous expenditure on chemotherapy which as far as aspergillomycosis is concerned, may be at best palliative. Consequently, the only sound approach to the disease in tilapias is prevention. Care should be taken in the storing, handling, shipping and processing of high-fungus hazard products so that they cannot become invaded by Aspergillus species. Together with this must go a relatively foolproof system of surveillance - sampling and testing to make sure that no products significantly contaminated with the fungus escape detection. In the developed countries this is, if not exactly easy, at least feasible, in the less developed countries it may not be, and in some of those countries while aspergillomycosis in cultured tilapia may now be recognised, it is likely to remain a problem for a long time to come.

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APPENDIX A

10% Neutral buffered formalin

Sodium dihydrogen phosphate (anhydrate)	4.0 g
Disodium hydrogen phosphate (anhydrate)	6.5 g
Formaldehyde	100 ml
Distilled or tap water	900 ml

APPENDIX B

Haematoxylin and eosin (H & E) staining technique

1. Xylene - 5 minutes
2. Absolute alcohol I - 2 minutes
3. Methylalcohol - 1½ minutes
4. Running water - 1 minute
5. Haematoxylin - 12 minutes
6. Wash in tap water
7. Acid alcohol - 4 quick dips
8. Wash in tap water
9. Scott's tap water - 1 minute
10. Check under microscope
11. Wash well in tap water
12. Eosin - 5 minutes
13. Methyl alcohol - 30 seconds
14. Absolute alcohol II - 2 minutes
15. Absolute alcohol I - 1½ minutes
16. Xylene - 5 minutes
17. Mount using a synthetic resin medium

N.B. Do not leave out of xylene for a long time before mounting as dehydration occurs rapidly

SCOTT'S TAP WATER SUBSTITUTE

Sodium bicarbonate ..... 3.5 g

Magnesium sulphate ..... 20.0 g

Tap water ..... 1 litre

Dissolve by gentle heating if necessary

Add several crystals of thymol as preservative

APPENDIX C

The periodic acid-Schiff (PAS)

Preparation

Schiff's reagent

Boil 200 cm^3 of distilled water and add 1 g of basic fuchsin. When dissolved, cool and filter. Bubble SO_2 gas slowly through the solution from a syphon shaking occasionally, until it becomes a clear transparent red colour. Stand the stoppered flask in a dark cupboard overnight. If the solution is pale straw-coloured or colourless next morning it is ready for use. If some residual red colour remains decolourize with 1 g of activated charcoal; shake and filter. Store in the refrigerator in a dark bottle and discard if a pink colour develops.

Technique

1. De-wax and take sections to water
2. Oxidize in 1% aqueous periodic acid for 10 minutes
3. Wash in running tap water for five minutes and rinse in distilled water
4. Place in Schiff's reagent for 20 minutes
5. Wash for ten minutes in running water
6. Stain nuclei with iron haematoxylin (5 minutes). Do not use Ehrlich's haematoxylin which will also stain some PAS positive compounds
7. Differentiate in Acid alcohol - 2 dips
8. Blue in Scott's tap water substitute
9. Wash in running water
10. Methyl alcohol - 30 seconds
11. Absolute alcohol - 1 minute
12. 0.3% tartrazine in cellusolve - 5 minutes
13. Absolute alcohol - 30 seconds
14. Clear in Xylene - 5 minutes
15. Mount in a synthetic resin medium

Results

Fungi hyphae in lesions are stained purple.

Other polysaccharides stained purplish red.

Background counterstaining is green.

APPENDIX D

Grocott's Modification of Gomori's Methanamine Silver

This technique is for the demonstration of fungi in tissue sections.

Methenamine silver nitrate solution

Stock Solutions

1. 5% borax (photographic grade) in distilled water
2(a) 5% silver nitrate in distilled water, 5 ml
2(b) 3% hexamine (Methenamine) in distilled water, 100 ml

A white precipitate will form but this dissolves on shaking. Must be freshly prepared or clear solutions will keep for several months at +4°C in the refrigerator.

Working solution:

Borax 5% solution	3 ml
Distilled water	25 ml

Mix and add 25 ml of the hexamine-silver nitrate solution.

TECHNIQUE

1. De-wax and take sections to distilled water
2. Oxidize in 5% chromic acid for 1 hour
3. Wash in running tap water for a few seconds
4. Rinse briefly in 1% sodium bisulphite to remove residual chromic acid
5. Wash in tap water for 5 minutes.
6. Wash with three or four changes of distilled water
7. Place in pre-heated working hexamine-silver nitrate solution in the oven at 56°C for 30 - 60 minutes. The section should be yellowish-brown. The time required varies with the fixative of the tissue
8. Rinse in six changes of distilled water
9. Tone in 0.1% gold chloride solution for 2 - 5 minutes
10. Rinse in distilled water.
11. Place in 2% sodium thiosulphate ('hypo') solution for 2 - 5 minutes to remove the unreduced silver

12. Wash thoroughly in water
13. Counterstain with light green solution (light green S.F.O. 2 g glacial acetic acid 0.2 ml, distilled water 500 ml) for 30 seconds
14. Dehydrate in methylated spirit - 30 seconds
15. Clear in absolute alcohol, 2 minutes, and Xylene, 5 minutes
16. Mount in a synthetic resin medium - usually Dipex

RESULTS

Fungi - sharply outlined in black

Mucin - is grey and
background is pale green or green

APPENDIX E

SABOURAUD DEXTROSE AGAR (Gibco, Europe)

Formula

Ingredients per litre of sterile distilled water.

Peptone 180 (Animal Tissue - Casein polypeptone)	10 g
Dextrose	40 g
Agar	15 g

Preparation of Medium

65 grams of the medium is dissolved in 1000 ml of distilled water. After thorough mixing, it is heated to boiling with frequent agitation to completely dissolve the medium. It is then sterilised by autoclaving at 121^{o}C (15 psi) for 15 minutes. Prolonged boiling, or over-autoclaving results in a soft medium which should be avoided. In practice, boiling to completely dissolve is better done in a water bath. The final pH is 5.6 $\pm$ 0.2 at 25^{o}C. Any deviation from this preparation may alter the biological characteristics of the medium. About 25 ml of the sterilized medium is dispensed into sterile disposable plates, or other appropriate sterile containers.

APPENDIX F

CORN MEAL AGAR (Difco)

Formula

Corn Meal Infusion from	50 g
Bacto-Agar	15 g

Medium preparation

17 grams of the medium is suspended in 1000 ml cold distilled water and heated to boiling, usually in a water-bath, to dissolve the medium completely. It is then sterilized in the autoclave for 15 minutes at 15 psi (121^{o}C), and dispensed, 25 ml into sterile plates, or appropriate sterile containers.

Final pH is 6.0 at 25^{o}C.

APPENDIX G

MALT EXTRACT AGAR (Difco)

Formula

Ingredients per litre

Maltose, Technical	12.75 g
Dextrin, Difco	2.75 g
Glycerol	2.35 g
Bacto-Peptone	0.78 g
Bacto-Agar	15.00 g

Medium Preparation

33.6 grams of the medium is suspended in 1000 ml cold distilled water and heated to boiling to dissolve the medium completely. It is then sterilized in the autoclave for 15 minutes at 15 psi (121^{o}C).

The medium has an acid reaction and hence care should always be taken to avoid overheating which results in a softer medium. The final pH at 25^{o}C is 4.6

About 25 ml of the sterilized medium is dispensed aseptically into sterile plates.

APPENDIX H

CZAPEK'S SOLUTION AGAR (CSA) (DIFCO)

Formula

Ingredients per litre

Saccharose, Difco	30 g
Sodium Nitrate	2 g
Dipotassium Phosphate	1 g
Magnesium Sulphate	0.5 g
Potassium chloride	0.5 g
Ferrous sulphate	0.01 g
Bacto-Agar	15 g

Medium Preparation

49 grams of the medium is suspended in 1000 ml distilled water. It is then heated to boiling in a water bath to dissolve the medium completely with frequent agitation. After this, it is sterilized in the autoclave for 15 minutes at 15 pounds pressure (121^{o}C) and subsequently dispensed into sterile plates, 25 ml into each plate. Other suitable containers may be safely used. The final pH at 25^{o}C is 7.3.

APPENDIX I

BRAIN HEART INFUSION BROTH (Gibco, Europe)

This medium is used freshly prepared, however, if the medium is not used the same day it is prepared, it is not stored under anaerobic conditions. It is heated in a boiling water bath for 10 minutes to drive off the absorbed oxygen and cooled quickly without agitation prior to use.

Formula

Ingredients per litre of distilled water

Calf Brain	-
Beef Heart Infusion	3.5 g
Peptone 70 (Pancreatic Digest of Animal tissue)	15.0 g
Peptone 140 (Pancreatic digest of Casein)	9.0 g
Dextrose	2.0 g
Sodium Chloride	5.0 g
Sodium phosphate dibasic	2.5 g
Sodium citrate	1.0 g

Medium Preparation

38 grams of the medium is suspended in 1000 ml of distilled water, after thorough mixing, it is heated to boiling in a water bath, with frequent agitation to completely dissolve the medium. It is then dispensed into appropriate containers and sterilised by autoclaving at 121^{o}C (15 psi) for 15 minutes. The final pH at 25^{o}C is 7.4 $\pm$ 0.2.