EVALUATION OF THE ROLE OF FUNGI AND BACTERIA IN CAUSING EPIZOOTIC ULCERATIVE SYNDROME IN FISHES

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This is to certify that Sri Subhas Chandra Routh, M.Sc., B.Ed. has worked under my supervision in the Department of Zoology, University of North Bengal for Ph. D. Thesis entitled **"Evaluation of the Role of Fungi and Bacteria in Causing Epizootic Ulcerative Syndrome in Fishes"** I am forwarding his thesis for the Ph.D. degree (Science) of the University of North Bengal.

I recommend that he has fulfilled all requirements according to the rules of the University of North Bengal regarding the works embodied in his thesis.

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Contents

Introduction

From the inception of human civilization people used and exploit the water bodies in different ways. Water was being used not for drinking purpose and water ways but became a spot of hunting as there lived consumable aquatic animals. Man learned that the edible lives in water bodies are very cheap and started to exploit the same.

Fish have long been regarded as an important source of protein for human and consumed all over the world. It is consumed for its wide variety of tastes and nutritive qualities. There is ever increasing demand for more fish protein. Recently efforts have been made to increase fish production by adopting intensive and semi-intensive method of production to meet the ever increasing demand for fish. Intensive rearing through high stocking densities, artificial manuring and feed use has created the condition leading to physiological stress and increased the risk of disease out break (Pillay, 1996; McLean 1996).

Vast and varied water bodies of India are potentially one of the richest in the world. There are big and famous rivers, their tributaries, large creeks, canals and a long coastal line. The dug up impounded water bodies like ponds, tanks and lakes are also potential areas for pisciculture. Oxbow lakes locally known as beels and baors are also highly productive.

Recently, inland fish production of India has gone up through adoption of improved technology and culture method. India produced 6.3 million tones of fish during 2004-05 out of which 3.2 million tones of fish and other aquatic products came from the inland sector (Vass, 2006). Fish culture is also important from ecological point of view because fish is the most efficient among farm animals in converting feed into nutritious food.

The state West Bengal has rich fishery resources and inland fish production is quite substantial. The state has largest brackish water fishery resources in the India. The river of the state harbour twenty four species of economically important fishes (Jain, 1990). The state of West Bengal has more than 150 beels with an estimated water spread area of 42,000 hectares

and twenty four endemic fish species have so far bean recorded from beels of West Bengal (Chauhan, 2006). The large agricultural field also hurbour a large number of edible fishes which are not only rich in protein percentage but also cheap.

The outbreak of fish disease in India till 1988 had not been alarming. The most common diseases of fresh water fish in India were haemorrhagic septicemia, dropsy, unlcerative disease, columnaries disease, microsporidiasis, dactylogyrosis, gyrodactylosis, ligulosis, argulosis and saprolegniosis (Kumar and Dey, 1992; Das and Das 1995). The epizootic ulcerative syndrome the most destructive of all fish diseases ever witnessed by India broke out for the first time in May, 1988 in various states of north-eastern India such as Tripura, Magalaya, and Assam (Das, 1988). In October 1988 the disease spread to northern districts of West Bengal such as Jalpaiguri, Cooch Behar, West Dinajpur, Maldah and the plains of Darjeeling district. (Pal and Prodhan, 1990). The incidence of the disease was also reported from sourthern part of West Bengal such as Murshidabad, Nadia, North and South 24-Parganas and Midnapore (Pradhan and Pal, 1990). The disease affected almost all the district of West Bengal except Purulia by 1989 and spread to other states of India like Orissa, Bihar, Uttar Pradesh by 1990 (Jhigram and Das, 1990). Almost all the states of India except Gujarat, Punjab, Himachal Pradesh and Jammu & Kashmir were affected by 1993 (Das and Das, 1993).

The disease caused panic among the common people as a result the rate of fish consumption declined sharply. The fish farmers, fish traders and fishermen suffered heavy economic losses. The situation became so alarming that the Government of West Bengal and Ministry of Agriculture, Government of India jointly organized a "National Workshop on ulcerative disease syndrome in fish" 1990 at Calcutta, West Bengal.

The name epizootic ulcerative syndrome (EUS) was adopted in 1988 at the consultation of Experts meeting on ulcerative fish disease in Bangkok (FAO, 1986). It was accepted that the condition was primarily an infectious

disease and a complex condition involving more than one etiological factors like bacteria, fungus and viruses.

Various pathogenic organisms e.g. viruses (Frerichs *et al.* 1986; Ahne *et al.*, 1988; Lio-Po *et al.*, 2001) bacteria (Llobrera and Gacutan, 1987; Boonyratpallin, 1989; Callinam and keep, 1987; Pal and Pradhan, 1990; Subassinghe *et al.* 1990; Jhingran, 1990; Chakraborty and Dastidar, 1991; Lio po *et al.* 1992; 1998; Yadav *et al.* 1992; Saha and Pal, 2000) and fugus (Frazer *et al.* 1992; Roberts *et al.* 1993; Chinabut *et al.* 1995; Willoughby *et al.* 1999) have been isolated from affected fishes. Opinion also prevailed that some environmental factors such as lowering of water temperature, low alkalinity and pH fluctuation might act as potential predisposing facrtors (Bondad-Reantoso, 1992, Das and Das 1993; Callinan *et al.* 1995; Lumanlan-Mayo *et al.*, 1997; Roy and Pal 2005).

Epizootic ulcerative syndrome (EUS) was redefined at a DFID Regional Seminar in Bangkok in 1994 as "a seasonal epizootic conditions of fresh water and estuarine warm water fish of complex infectious etiology characterized by the presence of invasive *Aphanomyces* infection and narcotizing ulcerative lesion typically leading to a granulomatous response" (Robersts *et al.*, 1994a).

Heterogenicity of viral isolation e.g. snake head virus (Saitanu *et al.*, 1986), Rhabdoviruses (Frerichs *et al.* 1986, 1989; Roberts *et al.* 1993, Lio-po *et al.*, 2001) a retrovirus (Freirichs *et al.* 1991) and reovirus (Frerichs 1995) with low recovery rate led same worker to conclude that viruses are adventitious agent (Frerichs, 1995).

But bacteria have been claimed to play a key role in causing EUS (Llobrera and Gocutan, 1987; Boonyaratpalin, 1989, Costa and Wijeyaratne, 1989; Subasinghe *et al.* 1990; Jhingran and Das, 1990; Pal and Pradhan, 1990; Chakraborty and Dastidar, 1991, Lio-Po *et.al.*, 1992; Saha and Pal, 2000).

Pal and Pradhan (1990) isolated two fluorescent Pseudomads (R_1 and R_2) and one Aeromonad (R_3) and one coccus (c) from the ulcer of air breathing fishes during the initial phase of out break of the disease in North Bengal. R_1 R_2 and R_3 were found to be pathogenic in healthy fishes. Mohan and Sankar (1995) reported detection of aseptate highly invasive fungus in the histological section of EUS affected fish. Only a few Indian worker reported presence of fungus in EUS affected fish (Karunasagar *et al.* 1994; Quereshi *et al.*, 1995; Pal, 1996; Viswanath *et al.* 1997, 1998; Saha, 1998; Mohan *et al.* 1999). Very little work has been carried out on the experimental induction of EUS with fungal isolates in India.

At present there is a declining trend of EUS in India. Outbreak of EUS is reported every year from various regions of North Bengal. So it appears that the primary source of the pathogen or the transmission factors are not yet controlled.

Under this circumstance it was considered worth while to study about the role of fungus and bacteria as causative agents of epizootic ulcerative syndrome.

The main objectives of this study are: (1) Isolation and characterization of fungus from EUS affected fishes (2) Isolation and characterization of bacteria from EUS affected fishes. (3) Induction of ulcer by fungal isolates (4) Induction of ulcer by bacterial isolates. (5) Induction of ulcer by fungus and bacteria in mixed condition (6) Histopathological studies of ulcer tissue, liver, kidney and spleen of naturally infected fishes and experimentally infected fishes by fungus in pure condition and bacteria in pure and mixed condition.

Review of Literature

The epizootic ulcerative syndrome (EUS) is one of the dreaded diseases of fish of all kinds, like fresh water, brakish water, esturine water, farmed, wild, sports and ornamental fishes. Ever since the emergence of EUS no single aetiological agent has been conclusively identified as causative agent of the disease. Disease results from a complex interaction between host, pathogen and environment. Studies on various aspects of biology of the host and the pathogen and interactions between them help to understand the disease process. The main purpose of this review is to present briefly the important observations of the previous workers as far as possible to highlight the present state of understanding of epizootic ulcerative syndrome. The different aspects of this review are:

- History of the disease.
- Fish species affected.
- Socio-economic impact of the disease
- Aetiology of the disease
- Environmental factors.
- Clinical signs
- Histopathology
- Haematological studies.
- Control measures

History of the disease

It is more than thirty five years different fish, cultured and wild have been affected by an ulcerative disease in different parts of Asia and Asia – Pacific region. The disease has been given various names viz. mycotic granulomatosis (MG) in Japan, red spot disease (RSD) in Australia and epizootic ulcerative syndrome (EUS) in Southeast and South Asia.

The above mentioned diseases have been described separately as distinct conditions in the past but the recent findings have shown that the some pathogenic *Aphanomyces* fungus is involved in each case .Epizootic

ulcerative syndrome is now recognized to be synonymous with mycotic granulomatosis and red spot disease (Chinabut and Roberts ,1999).

Egusa and Masuda (1971) reported an ulcerative conditon in farmed in ayu (*Plaecoglossus altivellis)* in Japan in the year 1971. The disease was named mycotic granulomatosis (Myazaki and Egusa, 1972). Various types of fish such as ayu , goldfish, bluegill and some wild fishes were affected by the disease (Miyazaki and Egusa, 1972, 1973 a. b. c). An epizootic characterized by shallow hemorrhagic ulcers named red spot disease (RSD) broke out affecting estuarine fish particularly grey mullet in Queens land , Australia in the year 1972 (Mckenzie and Hall, 1976) with recurrence in subsequent years (Rodgers and Burke, 1977;1981). The disease afterwards affected fresh water and estuarine fish in coastal rivers in New South Wales (Callinan *et al.,* 1989), Northern Territory (Pearce, 1990) and western Australia (Callinan, 1994a). After the out break of mycotic granulomatosis and red spot disease the fish disease characterized by demal ulcer with large scale mortalities was reported in fresh water and estuarine fishes in different countries of the Asia-Pacific region. The disease is called epizootic ulcerative syndrome, EUS (FAO, 1986). Papua New Guinea witnessed a disease similar to red spot disease characterised by dermal ulcers in the rivers of South during 1975-76 (Haines, 1983) and North during 1982-83 (Coates *et. al.* 1984). Sulawesi and Kalimantan Jothy (1981) reported the out break of the ulcerative disease in December, 1980 in rice-field fish in northern Malaysia . The affected fish had red or necrotic areas of ulcers all over their bodies and was called "Weback kudes". Shariff and Law (1980) reported high mortality rates in fish in southern peninsular Malaysia in 1979. Shariff and Saldin (1994) described the status of the epizootic ulcerative syndrome after 1986 in Thailand. The EUS outbreak occurred for the first time in 1981and the second (1982-83) and third (1983-84) outbreaks affected the intensive fish culture systems of Thailand causing devastating effects (Ulcerative fish Disease committee 1983; Tanguthai 1985).

The disease entered Myanmar, Lao PDR and Combodia during 1983-84 via Malaysia and Thailand (Roberts *et al.*, 1986; Lilley *et al.*, 1992) A severe out break of EUS in December, 1985 was reported from Laguna de Bay in the Philippines affecting snakeheads, gobies, gouramies, cat fish, crucian carp etc . (Llobrera and Gacutan 1987). In Philippines the disease later extended to whole country affecting wild fish in Lakes, rice-fields, swamps and pond cultured fish (Bondad-Reantaso, 1992; Bondad-Reantaso *et al.*, 1994).

Reports about the outbreak of EUS also came from China (Lian, 1990; Guizhen, 1990). The first occurance of EUS in Vietnam came from Mekong delta in 1983 (Xuan, 1990). Wilson and Lo (1992) reported EUS on snakeheads (*Channa maculata*) in late summer in Hong Kong since 1988. The outbreak of EUS was first reported in fresh water and estuarine fish in western Srilanka in December, 1987 (Costa and Wijeyaratne, 1989). It was suspected that the spread of the disease was from infected ornamental angel fish (*Pterophyllum scalare)* imported from south-east Asia (Balasuriya ,1994). In February, 1988 the disease extended across Myanmar into Chandpur district of Bangladesh (Kar and Dey , 1990; Roberts *et al*, 1992; Hossain *et el.*, 1992 Ahmed and Rab 1995; Ahmed and Hoque, 1998). Rahim *et al.*, (1985) reported ulcerative disease in five species of brackish water fish of Bangladesh prior to the out break of EUS in February , 1988.

Epizootic ulcerative syndrome broke out for the first time in May, 1988 in some North eastern states of India such as Tripura, Maghalaya and Assam (Das 1988). In Assam severe outbreak of EUS was reported from Borak Valley districts of Cachar and Karimganj. The disease had also been reported frim Nalbari, Jorhat, Kamrup, Naogao and Lakhkimpur of Assam. The villages adjacent to Bangladesh border were seriously affected. The northern, southern and western part of Tripura were seriously affected by EUS. In Meghalaya the ulcerative disease was reported from the districts of West Garo Hills, East and West Khasi Hills, Jaintia Hills and also from Borak Valley districts of Jowai (Jhingran and Das, 1990, Kumar *et al.*, 1991). In October,

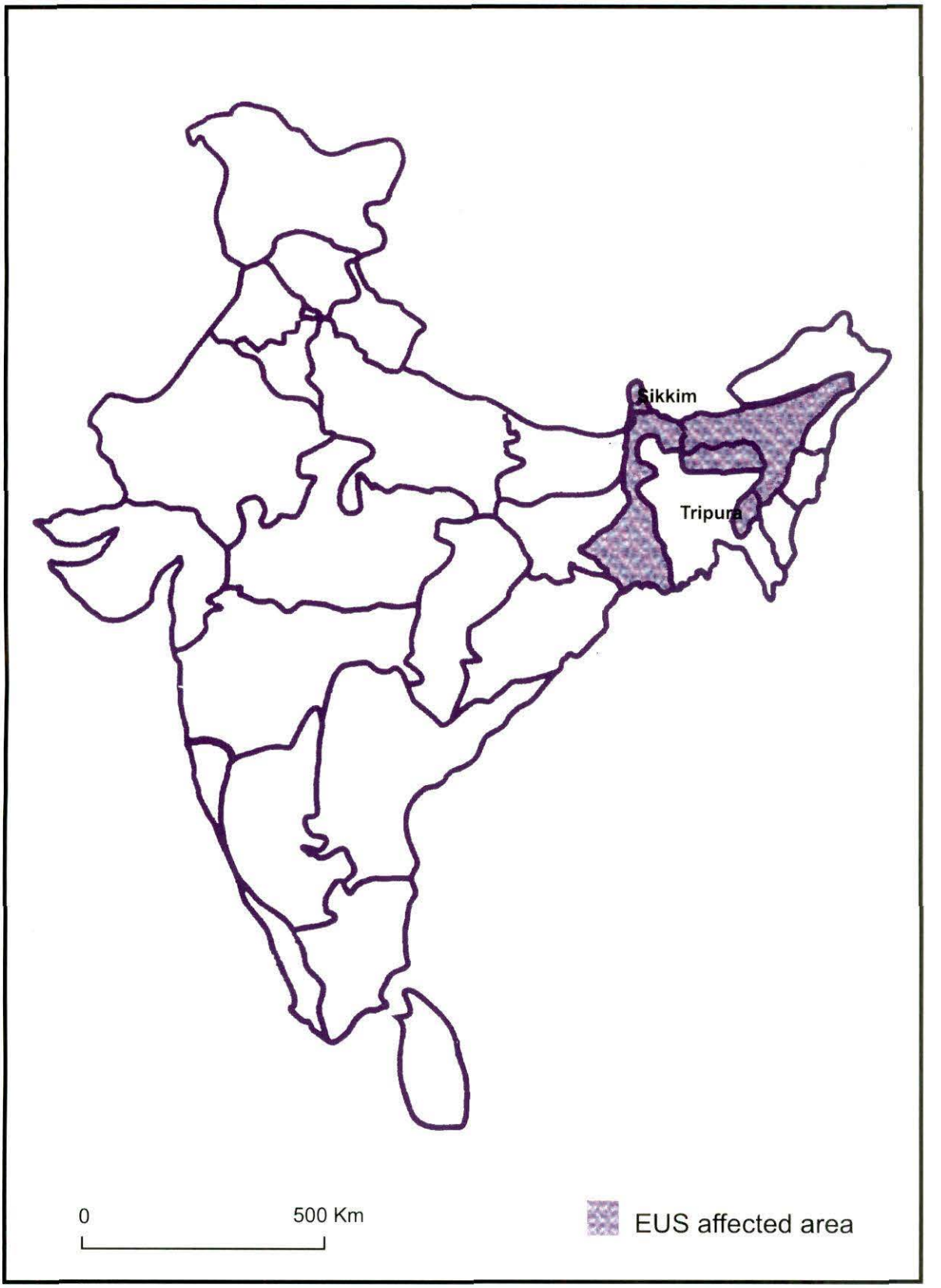


Fig. 1: Spread of EUS in India during 1988

Fig. 2: Spread of EUS in India during 1993

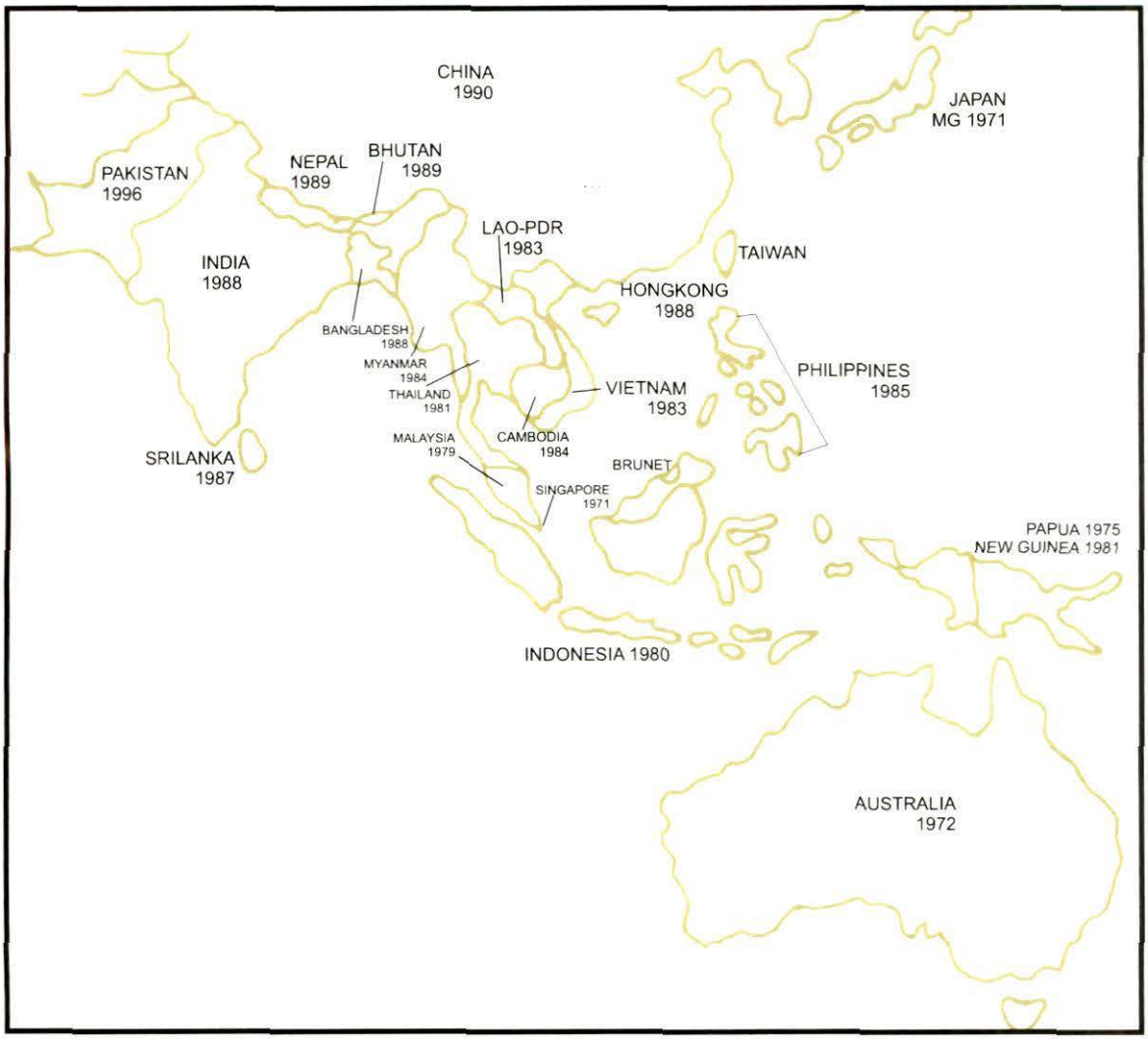


Fig. 3: Map showing the countries affected by EUS across the Asia-Pacific region.

1988 severe out breaks of the ulcerative fish disease occurred in northern districts of West Bengal such as Coochbehar, Jalpaiguri, West Dinajpur and plains of Derjeeling District (Pal and Pradhan, 1990). In the same year the disease spread to some southern Districts of West Bengal such as Murshidabad ,Nadia and 24-parganas and Midnapore (Pradhan and Pal, 1990) (Fig.1). By the year 1989, the disease had spread to almost all the districts of West Bengal except Purulia (Jain, 1990). In Bihar, Katihar and Kisanganj districts boardering the West Bengal was affected during April and May ,1989 (Prasad and Sinha, 1990; Kumar *et al.,* 1991). In Orissa the disease spread to the northern districts adjoining to West Bengal namely Balasore and Mayurbhanj (Prusty and Nayak, 1990). The disease also spread to other states of India in 1989 viz. Mizoram, Arunachal Pradesh, Manipur, Uttar Pradesh and within 1993 almost all the states of India were affected except Jammu & Kashmir , Punjab, Himachal Predesh and Gujarat (Das and Das, 1993) (Fig.2). Sardesai (1996) reported that the disease first broke out in Goa during August, 1993. EUS first broke out in the Cauvery river and its tributary Kabini river in T. Narasipura Taluk of Mysore district of Karnatak in August . 1991 and then spread to other part of the state (Abdul Hamed, 1996).

Bhutan and the eastern Terai of Nepal were first affected in 1989 and by 1993 it spread to Himalayan Valley regions (Phillips , 1989; Shresta , 1990;1994).EUS entered Pakistan in 1996 where EUS was confirmed in snakeheads from Punjab province in April 1996 and in *Cirrhinus mrigala* from Sindh province in January , 1998 (DFID, 1998) (Fig.3).

At present a declining trend of the outbreak of EUS is observed in different states of India. In the districts of North Bengal the disease has been reported every year since its first outbreak in 1988 (Pal and Pradhan, 1990; Pradhan and Pal, 1990; Pradhan, 1992; Pal 1996, 1997; Saha, 1999; Saha and Pal, 2000; Roy and Pal, 2005).

Fish species affected

The epizootic ulcerative syndrome affected more than 100 species of both fresh water and brackish water fish (Lilley *et al.*, 1992). Das and Das (1993) reported that the occurrence of EUS was highest in the genera *Channa* (5-100%) and *Puntius* (20-100%). Besides these *Mystus, Mastocembelus ,Glossogobius, Anabas, Clarias* and *Heteropneustes* were highly susceptible to EUS. The percentage of incidence was low in case of carps. Mohan and Shankar (1994) reported that EUS first attacked the bottom dwelling snakeheads (*Channa* sp.), and than attacked the catfish (*Mystus* sp. and *Wallago* sp.) minor carps (*Puntius* sp.) featherbacks (*Notopterus* sp.) etc. The Indian major carps were mostly unaffected in the state of Kamataka. The other major fish species affected in India are *Ctenopharyngodon idella, Hypophthalmichthys molitrix, Nandus nandus, Rasbora* sp. *Ompak* sp. *Mugil cephalus, Mugil parsia, Sactophagus* sp. *Epinephelus* sp. *Catla catta. Labeo rohita, Labeo calbasu, Cirrhinus mrigala* (Jhingran and Das, 1990; Pal and Pradhan, 1990; Kumar *et al.*, 1991; Abdul Hameed, 1996; Mukherjee, 1996; 1997) In India about 46 fish species are affected (Table 1).

In Thailand snakehead fish (*Ophicephalus striatus*), serpent fish (*Channa micropeltis)*, sand goby (*Oxyeleotris marmoratus*), three spoted gourami (*Trichogaster trichopterus*), striped croaking gourami (*Trichopsis vittatus*) Siamese fighting fish (*Betta splendens*) and wrestling half beak (*Dermogenus pustillus*) were the most affected fishes (Saitanu *et al.*, 1996).

In philppines the affected fish species were snakehead (*O. striatus),* catfish (*C.batrachus*) gourami (*Trichogaster pectoralis)* goby (*Glossogobius girus*), crucian carp (*Carassius carassius*), Manila sea catfish (*Anus manilensis*) and silvery theraponid (*Therapon plumbius)* (Llobrera,1987). During the out break of 1990,the estuarine fish species such as mullet (*Mugil sp*) flatfishes (*Platycephalus* sp.and *Psethodes* sp.*),* goat fish (*Vpeneus bensasi)* croaker (*Johnius* sp.) and spadefish (*Scaptophagus* sp.) were affected (Bondad-Reantaso, 1991).

Table 1: Fish species affected by EUS in India

FRESH WATER	
Wild	**Cultured**
Anabas testudines	*Catla catla*
	Cyprinus carpio
Acrossocheilus hexagonolepsis	
Ambasis ranga	*Cirrhinus mrigala*
Amblypharyngodon mola	*Ctenopharyngodon idelle*
Amphipnous cuchia	*Hypophthalmichthys molirix*
Ailia coila	*Labeo rohita*
Clarias batrachus	*L bata*
Channa punctatus	*L calbasu*
C. gachua	*Puntus javanicus*
C.striatus	*P..sarana*
Callichrous pabda	
Chanda chanda	
Colisa fasciata	
Gadusia chapra	
Glossogobius giuris	
Heteropneustes fossils	
Mystus sp .	
Macrognathus aculeatus	
Mastocambelus sp.	
Monopterus cuchia	
Nondus nandus	
Mystus cabasius	
Notopterus sp	
Ompak sp	
Puntius sophore	
Rhinomugil corsula	
Rasbora sp	
Salmostoma bacila	
Trichogaster sp	
Wallago sp. '	
BRACKISH WATER	
Epinephelus sp.	*Mugil parsia*
Etroplus sp.	*M. cephalus*
Platycephalus sp	*M. subviridis*
Scatophagus sp	*Sillago* sp.

In Australia yellow fin bream (*Acanthopagrus australis*) and striped mullet (*Mugil cephalus)* were among the affected species (*Callinan et al.,* 1995a). Thai silver barb, *Puntius gonionotus (*Bleeker) was among the most susceptible species in Bangladesh (Ahmed and Rab, 1995). Different snakeheads and *Cirrhinus mrigala* were reported to be affected by EUS in Pakistan (*Callinan et al.,* 1997; DFID, 1998). In Srilanka, *O. striatus, O. punctatus*, *H. fossilis* were the commonly affected fish (Subhasinghe *et al.,* 1990).

However, some fishes are unaffected e.g., milk fish (*Chamos chamos*), Nile telapia (*Oreochromis niloticus*), striped cat fish (*Pangasius sutchi*) and big head carp (*Aristichthys nobilis*), (Flores, 1986).

Socio –economic impact

The social and economic impact of epizootic ulcerative syndrome are obviously severe. Scientists of the different countries have tried to estimate the economic values of the fish losses due to EUS but there are very few accurate estimate of losses.

Tonguthai (1985) reported the economic loss of Thailand during 1982-83 was of 200 million Thailand currency and the loss was of about 3600 million Thailand currency within 1983-93 (Chinabut, 1994).

It was reported that the first outbreak of EUS in the Philippines affected 15,000 lakeshore families in Laguna Lake with a 30% decrease in average daily income of fishermen (Llobrera, 1987). In Pangasina Province of Philippines 75,000 people dependent on the Mangabol swamp of 5000 ha suffered over 50 and 40% loses during the 1989 and 1990 outbreaks respectively (Bondad-Reantaso *et. al.,* 1994). They also reported about great panic created among the consumers and farmers in the Philippines. In 1988, in some communities, a wide spread but unfounded fear of disease transmission among consumers led to a drastic decrease in market demand for all food fish in Bangladesh. The concurrent deaths of ducks, cattle and

other animals related to paddy fields increased the fear more. Though, there was no scientific evidence that the disease itself caused any human or animal illness, but it has badly affected the socio-economic status of the fish farmers and fish traders (Rahaman *et al.*, 1988). In Bangladesh fish traders suffered heavy losses during 1988 and 1989 due to price reduction of fish upto 75%.The total economic loss were about 118million and 88.2 million Taka in Bangladesh in the year 1988 and 1989 respectively (Barua, 1990). In Srilanka the fisherman and fish traders faced a heavy economic loss due to EUS outbreak. During 1988-89 the economic loss was of Rs 1 million (ADB /NACA, 1991) but upto 1993 the economic loss increased to 20-40 million Srilankan Rupees (Balasuriya 1994). About 15-20% of total fish production was lost in Napal during initial outbreaks. During 1989-90 the total economic loss in eastern Napal was about of 30 million (ADB/NACA, 1991). Pakistan also faced a heavy economic loss during the first outbreak of EUS in 1996, the total economic loss in Pakistan was about of Rs 15 million (AAHRI,ACIAR, IOA and NACA,1997).

In India economic loss due to EUS outbreak , was not estimated accurately from different parts of the country. The assumption that economic loss was obviously great during the initial outbreaks of EUS in different states of India. Panic was created among the people of the affected areas and fish consumption declined sharply (Das, 1988, 1997; Pal, 1996; Sardesai ,1996).

After the initial outbreak investigations carried out in five districts of West Bengal showed 73% aquaculture units were adversely affected by EUS, The fish consumption rate went down by 28.7%, 23.3% and 20.5%in urban, sub-urban and rural areas respectively. (Bhowmick *et, al.,* 1991). It was also reported that 73% of the culture ponds of West Bengal were affected by EUS outbreaks during 1988-89 and 30-40% of the stocked fishes were lost. The loss of fish costing about Rs 48.0 lakhs was reported during initial outbreaks of 1989-90 in Bihar (Prasad and Sinha, 1990). Orissa suffered loss of Rs 30.0 lakshs during 1989-91 (Das, 1994). EUS completely paralised the fish market in Kerala and the fisherman (both, men and women) had to seek

alternative employment to live (Sanjeevaghosh, 1992). Only during 1991-92 the economic loss of Kerala was about Rs 20 millilon (Das 1994). Sardesai (1996) reported that in Goa farmers sustained losses 20 metric tones fish amounting to Rs 8.0 laks in 1993. The fish losses were 30 metric tones mounting to Rs 12.0 lakh and 15 metric tones amounting to Rs 6.0 lakh in 1994 and 1995 respectively. Abdul Hameed (1996) reported that about 25 tones of fishes valued at approximately Rs 5.00 lakhs in about 20 tanks in three taluks in Dharwad district of Karnataka during February, 1994 was destroyed by EUS. The demand even for healthy fish also declined. Study in Assam showed that the total loss of fish due to EUS had been estimated 10,625 metric tones affecting 81,400 numbers of fish farmers (Das,1996). Das (1997) reported that about 42.19% of the aquaculturists suffered 31 to 40% loss of fish in their culture ponds , the pecuniary loss faced by 50% aquaculturists was in the range of Rs 1,001`to Rs 5,000while 19.73%aquaculturists suffered a greater loss ranging from Rs 5,001 to 10,000. A section of the farmers had to search for alternate jobs and 88.9% fish traders also suffered losses to some extent during the affected period.

Signs of the disease

After the initial outbreak of EUS in India Jhingran and Das (1990) reported that the symptoms and other characteristics of EUS were conspicuously different from the low level ulcerative conditions reported earlier. Fish in the river as well as in confined waters exhibited abnormal swimming behaviour. In the initial stage of the disease the infection usually occurred in the from of multiple inflammatory red spots on the body causing haemorrhage. In carps these appeared within scale pockets. In advanced stage of infection the ulcer spread to a larger area with sloughing of scales and degeneration of epidermal tissue. With further advancement of the disease the ulcers became deep, haemorrhagic and necrotic often with a black melanistic rim . In advanced stage of the disease large and deep ulcers were very commonly seen in all parts of the fish especially in the head abdomen and peduncle. Pal and Pradhan (1990) collected 129 *Anabas*

testudineus,16 *Heterioneustes fossilis* and 11 *Clarias batrachus* from different affected areas of North Bengal. They observed that in case of fish without scales the symptoms of the disease first appeared as a red spot. Gradually the red spot increased in size and ulcer developed in the affected area. Ultimately the underlying muscle layer became affected and occasionally the ulcers became deep and necrotic. In scaly fish red spot appeared in some regions of the body. Ultimately the scales were sloughed and the ulcer became deep and necrotic. In both cases fins were also affected.

Kumar et al (1991) found that the distribution of severe ulcerative skin lesion varied species to species. In murrels the ulceration were more pronounced and occurred mostly in the head and caudal areas. In severe cases the tail lesion could affect the area to such an extent that there was total loss of peduncle portion and sometimes even the erosion extended upto the posterior abdominal cavity. In *Puntius* sp. there had been usually a dark red hemorrhagic but superficial ulcer on the body side. In Indian major carps, long striped haemorrhagic lesions were found in the region of the caudal peduncle. Pradhan et , al (1991) reported infection of different stages of development in *Catla catla, Lebeo rohita and Cirrhinus mrigala.* In some fishes infections were at the primary stages with single or multiple haemorrhagic red sopts on the body.. Some fishes showed abnormal swimming behaviour and occasional jumping out the water in a pond.

Pradhan (1992) noticed that in severe infection the lesion eroded the total peduncle portion of a *Clarias batrachus.* Das and Das (1993) reported that in acute cases total loss of caudal region took place and in the head region the cranium was destroyed exposing the brain.

Viswanath et, al (1997) classified the lesions into three distinct type. Type I lesion appeared as tiny red spots on the body surface with no noticeable haemorrhages and ulcerations. This lesions were of pin head size. Skin tissues around the red spot were of normal colour and there was no discolouration of the skin.

Type II lesions were large (2-4 c.m.)and appeared as a dark raised, circular, discoloured areas on the body surface. Skin and scales were relatively intact in these lesions.

Type III lesions occurred as a circular to oval open dermal ulcers extending in to skeletal musculature. These advanced lesions were characterised with haemorrhagic and necrotic open ulcers on the body surface and were devoid of epidermis and scales.

Chinabut and Roberts (1999) classified the disease into five types of clinical patterns :

Type I. In this case the affected fishes had highly distinctive dark red to brown lesion on one or both flanks and float on the surface grasping for air some time before expiring.

Type II. This type of infections were with chronic and extensive lesions. This type were mostly found in the top predator air breathing fishes, such as the snakeheads.

Type III. This type of lesion depended on the infection and the size of the fish. In this case the ulcers were chronic and extensive and in earliest lesion a small red rosacea or ulcerated scale bed were found. This type was also founds in the snakeheads.

Type IV. The gray mullet (*Mugil cephalus*) showed this type of lesion. In this type the important characteristic feature was small necrotic erosions of one or more scale beds which gradually extend to produce a shallow ulcer with a red centrums, white rim and surrounding black edge.

Type V. This type was found in some very large carps. In this case small, often single, area of haemorrhagic ulceration on fin or back was found. This type was also found in some fishes, which were resistant to EUS as Tilapia.

Aetiology of the disease

Epizootic ulcerative syndrome has been accepted primarily as an infectious disease and it is a complex condition involving certainly fungal and bacterial elements in its later stages and probably one or more viruses (Chinabut, 1995). Aetiological studies have been the subject of major research in the affected countries. The FAO Consultation recommended, that further research should take place, because of the complexity of its aetiolog in different fields viz.. virology, bacteriology and mycology associated with different outbreaks. The investigations carried out by various workers on the aetiology of the desease will be discussed in the following headings:(i) fungus (ii) bacteria (iii) virus (iv) animal parasites.

(i) Fungus

Fungus was suspected to be involved in the aetiology of epizootic ulcerative syndrome when "severe chronic granulomatous mycosis" was found in histological sections of affected fishes in Thailand (Limsuwan and Chinabut, 1983). A wide range of saprolegniaceae, including *Achlya* sp. *Saprolegnia* sp. were identified on the surface of EUS lesions (Pichyangkura and Bodhalamik 1983; Limusuwan and Chinabut, 1983). These were afterwards recognized as secondary agents (Tonguthai, 1985). Roberts *et al.*, (1993) isolated a slow growing and thermo-labile fungi, *Aphanomyces* from the affected fishes of Thailand. When a mycelium from these strains was placed below the dermis of healthy fishes, it caused an inflammatory response and proceed to migrate down into the tissues of the fish, inducing severe myonecrosis with chronic epithelial reaction.

Chinabut *et al.* (1995) reported that spore suspension of the specific pathogenic *Aphanomyces* induced histopathological changes in the muscle of injected fishes kept at three different temperatures, 19, 26 and 31^{0}C. The mortalities and myonecrosis were considerably higher in the fish kept at 19^{0}C than in the fish kept at 26 and 31^{0}C Willoughby *et al.*, (1995) named the fungus *Aphanomyces invaderis*. Miyazaki and Egusa (1972, 1973a, 1973b,

1973c) were the first to isolate a specific fungus from mycotic granulomatosis affected fishes in Japan. As their publication were entirely in Japanese the efforts did not draw the attention of wider community of scientists (Chinabut, 1995). Hatai *et al.* (1977) reported isolation of a fungus from fish, *Plecoglossus altiveliss* in Sliga Prefecture, Japan and it was named *Aphanomyces piscicida* (Hatai, 1980). Association of *Aphanomyces* sp. was reported from RSD outbreask in Australia in 1989 (Fraser *et al.* 1992). Paclibare *et al.* (1994) reported isolation of fungus from EUS affected fish in Philippines. Callinan *et al.*, (1995a,b) reported isolation of *Aphanomyces* sp. from EUS in Philippines and RSD affected fish in Australia. *Aphanomyces* sps. were also isolated from EUS affected fish from the Philippines, Indonesia, Bangladesh (Lilley and Roberts, 1997).

The *Aphanomyces* sp. from various countries have been compared and shown by means of protein banding profiles (Callinan *et al.*, 1995b, Lilley *et al.*, 1997b), growth characteristics (Lilley and Roberts, 1997) and chemical susceptibility (Lilley and Inglis, 1997) to belong to the same species. Genetic fingerprinting techniques have also been used to show that the various isolates were all very similar (Lilley *et al.*, 1997). The pathogenic *Aphanomyces* sp. has been reported under various names such as *Aphanomyces piscicids* (Hatai, 1980), *Aphanomyces invadaris* (Willoughby *et al.*, 1994) and EUS related *Aphanomyces,* ERS (Lumanlan-Mayo *et al.*, 1997). *Aphanomyces invadaris* was renamed to *Aphanomyces invadaris* (David and Kirk, 1997). Willoughby (1999) suggested that *Aphanomyces invadans* could not produce zoospores on the fish itself, through which they can transmit from one fish to another, and the fish become infected from the spores produced in the environment. Shahen *et al.*, (1999) reported that the EUS like ulcerated disease was caused by *Aphanomyces* sp. in Egypt. Vogelbein *et al.*, (2001) suggested from histological evaluation of skin ulcers in over 200 wild menhaden from Virginia and Maryland portions of the Chesapeaks Bay and the Pamlico Estuary, North Carolina, that all ulcers harboured a deeply invasive, highly pathogenic fungus now known to be

Aphanomyces piscicidans. Kurata *et al.*, (2002) recently purified galactose-binding protein (GBP) from *Aphanomyces invadans* and the molecular weight of this GBP is 40KD. They observed that this 40 KD GBP is closely associated with *Aphanomyces* infections such as mycotic granulomatosis, epizootic ulcerative syndrome, red spot disease and ulcerative mycosis.

Mycosis due to *Saprolequia parasitica* was recorded in Indian major carps by Lal *et al.* (1990). Kumar *et al.* (1991) reported presence of *Aspergillus* sp. from EUS affected fishes. *Saprolegnia parasitica* was detected by Mohanta and Patra (1992) in the infected specimens of *Anabas testudineus* in India. Karunasagar *et al.*, (1994) observed the fungi in the deep musculature underneath the EUS effected ulcers in both fresh water and estuarine fishes. However, the fungi was not found in the early stages of the lesions before the development of ulcers. *Aphanomyces* and *Saprolegnia* were the common fungi associated with the ulcerative condition. The fungal isolates could not infect uninjured EUS susceptible fishes in the laboratory. But if placed under the scale by damaging the intact skin lesion developed which subsequently healed without treatment. They suggested that EUS in the natural environment is of complex etiology involving more than one pathogen.

Mohan and Shankar (1995) conducted histopathological studies of EUS affected fresh water and brakish water fishes of Karnataka and observed that numerous nonseptate, highly invasive fungal hyphae associated with massive tissue necrosis and therefore suggested that fungus is one of the etiological agent of EUS.

Qureshi *et al.*, (1995a) after conducting mycological examination of affected fish *Mystus cavasius* collected from Bhopal, suggested the presence of aseptate hyphae within and around the lesions. In most of the cases the hyphae of different species of fungi were found but in some cases zoosporangia of *Saprolegnia* were also seen. They isolated seven species of fungi belonging to the genera *Saprolegnia, Achlya* and *Aphanomyces* from the lesions of EUS affected fishes.

Pal (1996, 1997) found different types of fungi within the ulcer of EUS affected fishes such as *Saprolegnia* sp. *Dictyuchus* sp. and *Aspergillus* sp. in *C. punctatus, A. testudineus* and *C. catla.* He also reported the presence of highly branched and aseptate slender fungus most probably *Aphanomyces* on the operculum of an infected *Anabas testudineus* and no fungus was detected at the initial stages of the ulcer formation. Viswanath *et al.*, (1998) hypothesized that EUS specific fungus capable of entering the fish, only following primary damage to the skin. Mohan *et al.*, (1999) has suggested that the invasive fungus *Aphanomyces invadans* associated with EUS is a primary pathogen.

(ii) Bacteria

Available evidences have suggested that bacterial pathogen have important roles in the outbreak of EUS. Various types of pathogenic bacteria were isolated by different workers from the ulcers as well as from internal organs of the EUS infected fishes. Though the bacteria isolated from different fishes varies from species to species but *Aeromonas* sp. were most predominant pathogenic bacteria. *Aeromonas hydrophila* was consistently isolated by Llobrera and Gacutan (1987) from the ulcers of *O. striatus. C. batrachus, G. giurus* in Laguna de Bay, Philippines from December, 1985 to February, 1986. Boonyaratpalin (1989) reported that primarily *A. hydrophila* and occassionaly *Pseudomonas* sp. were associated with the outbreak of EUS in Burma, Indonesia, Lao People Democratic Republic, Malaysia, Singapore and Thiland. Costa and Wijeyaratne (1989) reported association of *A. hydrophila* with EUS affected fish in Srilanka. Subasinghe *et. al.*, (1990) reported association of *A. hydrophila* with EUS affected 19 species of fish from Sri Lanka such as *O. striatus, O. punctatus, H. fossilis* and *M. armatus. P. fluorescens* and some unidentified bacteria were also found occasionally.

Jhingran and Das (1990) isolated *Micrococcus* sp. from the infected fishes. Kar *et. al.* (1990) isolated *Pseudomonas aeruginosa* from the lesions and gill tissues. Pal and Pradhan (1990) isolated four types of bacteria, two fluorescent pseudomonads (R1 and R2), one aeromonad (R3) and one

Micrococcus sp. (C) from the ulcer of air breathing fishes. R1 resembled with *Pseudomonas fluorescens,* R2 resembled with *P. aeruginosa* and R3 was found to be *Aeromonas caviae* (Pradhan, 1992). This was the only report of the isolation of *A. caviae* from the ulcer of EVS affected fishes. The bacterial culture in mixed condition induced severe ulcer in healthy *A. testudineus.* Pure culture of each of two pseudomonads and an aeromonad induced superficial ulcers. Pradhan *et. al.,* (1991) also isolated two Pseudomonads (R1, R2) which resembled with *Pseudomonas fluorescens, one Aeromonad* (R3) and another coccus (C1) from the Indian major carp *Cirrhinus mirgala.* Ali and Tamuli (1991) isolated *Vibrio* sp. *Aeromonas* sp. and *Micrococcus* sp. from the ulcers of *L. rohita, C. batrachus, C. punctatus* and *A. testudineus* from Assam. Mukherjee *et. al.,* (1991) isolated five distinct strains of *A. hydrophila* from 182 EUS affected fresh water and brackish water fishes. Chakraborty and Dastidar (1991) reported isolation of chemoautotropic nocardioform (CAN) bacteria from different types of skin lesion of EUS affected fish as the major or only pathogenic agent.

McGarey *et. al.,* (1991) isolated *A. hydrophila* and *A. sobria,* from the EUS affected fish. It was thought that these played an important role in this disease. Lio-Po *et. al.,* (1992) isolated *A. hydrophila* from the EUS affected fish and showed that EUS like lesions could be induced in *O. striatus* and *C. batrachus.* Torres *et al.* (1993) isolated 54 strians of *Aeromonas* sp. and among these strains *A. hydrophila* was highly pathogenic. Lio-Po *et al.* (1998) also isolated four types of bacteria such as *Aquaspirilum* sp. *Pseudomonas* sp. *Streptococcus,* sp. and *A. hydrophila* from the ulcers of EUS affected fishes of Philippines. After inoculation of those bacteria to healthy *C. batrachus* and *O. striatus* they observed that *A. hydrophila* was highly pathogenic and *Aquaspirillum* sp. and *Pseudomonas* sp. were slightly and *Streptococcus* sp. was moderately pathogenic to *C. batrachus. A. hydrophila* also induced severe ulcer in *O. straitus* and *Streptococcus* sp. induced slight ulcer which healed rapidly.

Qureshi *et al.* (1995b) isolated nine types of bacteria, from EUS affected fishes, of which three are Pseudomonads (*P. fluorescens; P. aeruginosa* and *Pseudomonas* sp.), two Aeromonads *(A. hydrophila* and *Aeromonas* sp.)., one Cytophage sp. and three cocci (*Micrococcus varians, Streptococcus* sp. and *Staphylococcus* sp.) Out of these bacteria aeromonads and pseudomonads were highly pathogenic while micrococcans and cytophagans were less pathogenic. Rest of the bacteria were non-pathogenic Karunasagar *et al.* (1995) isolated *A. sobria* and *A. hydrophila* from the ulcer of *Puntius* sp. in Karnataka, India.

Saha and Pal (2000) isolated 16 strains of bacteria from *C. punctatus, Puntius* sp. and *Mystus* sp. belonging to the genus *Pseudomonas, Aeromonas, Micrococcus, Bacillus, Vibrio* and *Moraxella.* They showed that four Aeromonads out of sixteen strains induced ulcers in healthy *A. testudineus* when injected intramuscularly. (Saha and Pal, 2002).

(iii) Virus

Virus was considered as the causative agent of EUS when virus like particles were demonstrated in various tissues of affected fish during 1982-83 outbreaks in Thailand (Rattanaphani et al , 1983;Wattanavjam et al , 1983 a, b; 1984). Subsequently these workers isolated snakehead rhabdovirus (SHRV) and this virus was shown to be serologically distinct from other fish rhabdoviruses (Ahne *et al.,* 1988; Kasornchandra *et al.,* 1992). Isolation of birna virus from cultured sand goby (*Oxyeleotris marmoratus*) was reported by Hedrick *et al.,* (1986). Frerichs *et al.,* (1986,1989) isolated rhabdoviruses from diseased fishes in south-east and south Asia and suggested this could be the initiating factor in the outbreak of EUS. Though this virus was not isolated from more that 5% of the diseased fishes examined and could not be shown experimentally to induce the disease in healthy fish. Saitanu *et al.,* (1986) isolated a new virus named as snakehead fish virus (SHV) from various infected fish such as *O. striatus, C. micropeltes, O. marmoratus, T. trichopterus, T. vittatus, B. splendens* and *Dermogenus pustillus.* This virus was not affected by ether or chloroform and was resistant at 60^{0}C for

30minutes. SHV produced cytopathic effects, rounded cells and complete destruction of cell sheet on BB, BF2and FHM cells. Intraperitoneal injections of this virus resulted in scale damage in 80% of small snakeheads but not all in larger fish. In early 1990's one birna virus in Singapore (Subramaniam et al, 1993), one rhabdovirus and reovirus like agent in Thailand (Roberts et al, 1994) were isolated from diseased fish. Rhadoviruses were also isolated from the diseased fish collected from Thailand, Myanmar, Australia (Roberts et al , 1989,Roberts et al , 1994, Lilley and Frerichs, 1994) and these were named ulcerative disease rhabdovirus (UDRV).

No virus was isolated from India (Boonyaratpalin, 1989a), Pakistan (AAHRI,ACIAR, IOA and NACA, 1997), Bangladesh, Lao PDR, Malaysia, Indonesia. Siddhi (1989) conduced virological studies on EUS affected *C. idella* , *Colisa* sp, *P. javanicus* , *H. molitrix* and *P. sophore* from Assam, *C. catla* , *C. carpio* from Tripura, *C. punctatus, M. armatus, N. nandus, P. sophore* from West Bengal and showed no evidence of virus by inoculation of tissue extracts. Kar et al, (1990) revealed the presence of viruses by electron microscope in the muscle and gills of ulcerative disease affected fishes in Assam. Kumar et al. (1991) reported that inoculums from affected *Channa* sp. *Puntius* sp. and *Mastocembelus* sp. when injected in confluent cultures of BB, FHH, EPC, cell lines showed cytopathic effect within seven days in culture. Microscopic studies showed spherical virus like particle.

Frerichs *et al.,* (1991) reported isolation of a retrovirus which was capable of inducing cytopathological effects (CPE) in a wide variety of tissue culture. A reovirus was isolated from a diseased snakehead in 1992 (Frerichs, 1995). Thus a number of rhabdo viruses, a birnavirus and a retrovirus have been isolated from EUS affected fish. The heterogenicity of viral isolates and their low recovery rate led to the conclusion that these were adventitious agents (Frerichs, 1995).

(iv) Animal parasite

Association of several metazoans (*Dactylogyrus* sp. and *Gyrodactylus* sp.) and protozoans (*Chilodonella* sp., *Trichodena* sp., *Costia* sp. *Henneguya* sp. and *Ichthyophthirus* sp.) parasites from 273 EUS affected fish during 1982-83 outbreaks in Thiland was reported by Reungprach *et al.*, (1983). A large number of protozoans (*Epistylis* sp.) were reported from several fish with tiny red spots on the skin before the second outbreak in Thiland (Tonguthai, 1985). Callinan and Keep (1989) and Pearce (1990) observed protozoan and metazoan parasites on some affected fish in Australia.

Jhingran (1990) recorded some commonly found parasites, such as *Dactylogyrus* sp. *Tripartiella* sp. and several myxozoans from EUS affected fishes in India. He also reported that as these parasites were found at a very low intensity they could not be they primary cause of ulceration. Mandal *et al.*, (1990) reported the presence of *Costia necatrix* in the the ulcer of EUS affected fish in West Bengal, India. Myxozoan parasites in the skin and kidney of Catla catla and kidney and liver of *Clarias* sp. were detected by Kumar *et al.*, (1991). Ram (1992) reported that myxozoans, Myxobolus sp. and *Thelohanellus* sp. were associated with EUS outbreak in Haryana, India.

Subasinghe (1993) conducted experiments for possible relationship between *Trichodina* sp. infection and induction of EUS in *Channa striata* by feeding with infected fish and by direct contact with water from infected environments. He observed that heavy primary infection of *Trichodina* sp. at >400 parasites per gill filament level, accelerated the appearance of EUS like lesion and ulcers when induced by feeding and cohabition with EUS infected fish. He suggested that heavy *Trichodina* sp. infection suppresses the natural defence mechanism of *C. striata* causing acceleration of the clinical sign of EUS.

Environmental factors

Disease is not a simple result of contact between host, pathogen and environment (Snieszko, 1974). Many types of stress have been implicated in epizootics of fish (Wedemeyer, 1970; Snieszko, 1974; Plumb *et al.*,1976; Wedemeyer *et al.*, 1977; Walters and Plumb, 1980). Tonguthai (1985) reported that EUS outbreaks are frequently precipitated by adverse environmental conditions.

Temperature

Rodgers and Burke (1981) reported that maximum EUS prevalence in estuarine fish populated with seasonal aggregation of fish was due to stress by low or rapidly changing water temperatures and rapid or prolonged depression of salinity. Roberts *et al.* (1986) have suggested that EUS outbreaks occur in a cyclic manner when the temperature falls after heavy rainfall. There is pronounced similarity in the geo-climatic conditions of the affected countries, which generally have rainfall followed by dry season. There is noticeable decrease in temperature, accompanied by wide fluctuation in the diurnal temperature regime (Jhingran, 1990). Phillips and Keddie (1990) reported that in Bangladesh, China, India and Lao PDR during 1988 and 1989 outbreaks which occurred in months in which the mean daily temperature was below the annual mean daily temperature. They also reported outbreaks in warmer months in Philippines and Thailand. Lumanlan-Mayo *et al.*, (1997) conducted field and laboratory experiments in Philippines and suggested that low water temperatures ($<30^0$C) plays an important role in EUS outbreak. They also showed that rainfall and decrease in alkalinity and calcium and magnesium hardness were associated with the disease outbreak in *Channa striata,* but at the onset of winter, as the temperature began to decrease, artificial maintenance of high level of alkalinity and hardness failed to prevent the outbreaks.

Immunosuppression at low temperatures was suggested as a likely mechanism for seasonality of EUS (Chinabut *et al.*, 1995). Several other

workers also indicated that low temperature was an important factor for some EUS outbreaks (Das *et al.*, 1990; Lilley *et al.*,1992; Das and Das, 1993; Mohan and Shankar, 1994; Ahamed and Hoque, 1998; Sanaullah *et al.*, 2001).

Water quality variables

The intensity of disease outbreak in various states of India was high in water of low alkalinity and hardness i.e. waters closely related to acidic low calcium soil (Jhingran, 1990; Jhingran and Das, 1990). The disease was not solely located in such waters and it was linked to periods of heavy rainfall in more alkaline environments with consequent drop in pH and alkalinity. Bondad-Reantaso *et al.*, (1992) revealed that there were variations in temperature, chloride, rainfall and hardness of water at the time of EUS outbreak in Philippines. Mohan and Shankar (1994) reported that during monsoon months of 1993 when the salinity was very low (<0.5 ppt) EUS occurred in estuaries of Dakshina and Uttara Kanada districts of Karnataka, India. Palisoc and Aralar (1995) observed that the depth, secchi disc transparency, temperature (surface and bottom), chloride and alkalinity were significantly correlated with EUS outbreaks during January 1988 to December 1989 in Laguna lake, Phillippines and outbreak in lake Nujan were associated with temperature only. Callinan *et al.*, (1995) suggested that sub-lethal exposure of susceptible fish to acidified run-off water from acid sulphate soils in Australia was a causal factor for EUS outbreak in some estuarine settlings.

Sardesai (1996) mentioned that the disease started occurring last week of July of September, 1993 in Goa as salinity in estuarine water bodies at the onset of the monsoon was reaching to zero. He also reported that during 1994 and 1995 monsoon season, the disease spread in several parts of Goa affecting water bodies in rivers, ponds etc.

Ahmed and Hoque (1998) reported that water quality parameters such as temperature alkalinity and hardness were reduced in December, January and February in comparison with other months. Clinically and histologially in

the colder months (December to February) all fish specimens were more affected by EUS in comparison with other months. Sanaullah *et al.* (2001) concluded that rapidly declining seasonal temperature and changing water quality, particularly lower chloride and alkalinity with respect to hardness might cause severe stress, depress immunity and reduce resistance to pathogen which ultimately may result to EUS.

Pathiratne and Jayasingha (2001) observed that declining dissolved oxygen concentration in water coincided with initiation of EUS outbreak in Bellancoila-Attidiya wetlands in Srilanka. Ray and Pal (2003) reported that EUS outbreak occurred when dissolved oxygen content, hardness and total alkalinity of pond water remained low.

Heavy metals

Jhingran and Das (1990) analysed the concentration of various metals, e.g. Fe, Zn, Cu, Cr. Cd, Pb and Hg in the affected areas. The values for zinc ranged from 21.0 to 26.8 mg/l in water and 9.13 to 21.6 mg/l in fish where as the respective values for copper ranged from 1.2 to 3.92 mg/l and 2.39 to 2.47 mg/l. The available information did not suggest any perceptible role of the heavy metal contamination in creating stress to the fishes leading to outbreak of the disease (Jhingran, 1990, Jhingran and Das, 1990, Das *et al.*, 1990).

Pesticides and other agrochemical

The incidence of the disease is quite high in rice field environment in all the affected countries. It has been suggested that pesticides may have a role as a predisposing factor for the outbreak of the disease. Jhingran (1990) reported presence of isomers of DDT and BHC not only in water of an affected site, Antpur, Hoogly, India, but also in the muscle of affected fishes. Choudhury *et al.* (1994) analysed pesticides residue in water, fish and plankton of some specific EUS affected water areas in India. They reported occasional higher concentrations of organochlorine and organophosphorus pesticide in water and fish samples, but did not find any correlation with the

presence of pesticide residue and disease outbreak. Palisoc and Aralar (1995) studied levels of pesticides (heptachloride, endosulphar, heptachlorepoxide) in sediment and water of Lake Laguna and Lake Naujan in Philippines but did not find any correlation between the levels of pesticides and EUS outbreak in those lakes.

Flooding

Saha *et al.,* (1992) reported that in the Sunderbans after a devastating cyclone in 1988, EUS was detected in many confined waters polluted by carcass of domestic animals and rotten leaves of plants. Barua (1994) suggested that floods in Bangladesh in 1988 resulted in the rapid spread of EUS in that country. Mohan and Shankar (1994) reported that EUS first appeared in Karnataka in the Cauvery river system during August-September, 1991, immediately after the flood.

Abdul Hameed (1996) reported that EUS outbreak in Karnataka occurred after major flood (from July to September) for four consecutive years (1991-1994).

Histopathology of EUS infected fishes

Kumar *et al.,* (1991) conducted histopathological examination of ulcer, kidney, liver and heart of *Puntius* sp. *Mastocembelus* sp. and *Channa* sp. and found that complete loss of the epidermis of the skin at the ulcer region where dermis and hypodermis showed cyst like or nodule like granulomatous formations in huge numbers. Granuloma formations were also seen in the muscle layers of the skin. Most of the granuloma formations seemed to contain a highly basophilic material inside. Kidney haematopoietic tissue showed proliferation of macrophage cells especially indicating inflammatory reaction. Almost all the renal tubles and glomeruli were found necrotic. In the liver, most of the sinusoidal spaces and blood vessels were congested and wandering lymphocytes were plenty in the liver parenchyma. However, Das *et al.* (1990) did not find any significant changes in the liver except vacuolization in certain cases.

Mohan and Shankar (1995) observed non-septate fungal hyphae in dermis and epidermis with severe necrosis. In advanced ulcers massive invasion of the skeletal musculature by the fungal hyphae was consistent with myofibrillar necrosis. Pal and Pradhan (1995) reported the presence of different bacteria from the histopathological studies of experimentally infected *Clarias batrachus*

Viswanath *et al.* (1997) found that initially (Type I lesion) there were inflammatory changes in the section of the epidermis. Sections stained with Grocott's methanamine silver staining technique (GMS) revealed the presence of fungal hyphae in some of nodular structures. The fungal presence and associated inflammatory changes were restricted to the epidermis. In early stages the dermis and the skeletal musculater were free of pathological changes. In later stage (Type II lesion) they observed mycotic granulomas in the epidermis, dermis and the skeletal musculature associated with numerous non-septate fungal hypae. In the advanced stage of the lesion (Type III lesion), the epidermis and scales were completely lost with partial loss of dermis at the site of ulcer. In most cases the underlying musculature was exposed and was almost replaced by fungal granulomas and host inflammatory tissue. There was considerable myofibrillar necrosis.

Epizootic ulcerative syndrome has been described in more that 100 species of fresh water and brakish water fishes. It was expected that there will be variations not only in the clinical features but also in the histological picture of the ulcer and internal organs between different affected species. These variations in clinical and pathological feature depend on the degree of susceptibility and size of the fish concerned. Chinabut and Roberts (1999) have categorized EUS into five clinical types and described thoroughly the underlying histopathological changes.

Haematological studies

Das and Das (1993) showed higher counts of phagocytic cells in EUS affected fishes that reflected initiation of defence phagocytosis in blood

circulation. There was a decline in counts of RBC followed by a drop in haemoglobin content indicating anaemic condition. Prasad and Qureshi (1995) reported that there were remarkable changes in the numbers of RBC, WBC, differential count of WBC and haemoglobin content.

Declining trends of TEC and Hb contents in experimentally infected fishes, *Channa punctatus* (Pradhan and Pal, 1995) and (Saha, 1998) *Heteropneustes fossilis* with two Pseudomonads (R1 and R2) and one Aeromonad, *Aeromonas caviae* (R3) were reported. Pathiratne and Rajapaksha (1995) studied the total erythrocyte and leucocyte contents, haemattocrit and haemoglobin content, mean corpuscular volume, mean corpuscular haemoglobin concentration and differential leukocyte count in healthy and EUS positive fish, *Etroplus susatengis* in Sri Lanka. Results showed that EUS positive fish were anaemic through loss of blood and destruction of erythrocyte as shown by a significant reduction in TEC, haematocrit and Hb content. In addition, the total and differential counts of severely affected EUS positive fish indicated leucocytosis coupled with marginal increased in macrophages like cell population.

Control measures

Various types of prophylactic and theraputic measures were taken to avoid the disease attack or to minimize the disease attack.

Potassium permanganate

Jain (1990) reported that if the EUS affected fishes were given bath treatment with potassium permanganate @ 5 ppm the red spots on the body of the fishes turned blackish within a week and complete healing and regeneration of scales started within two weeks. Jhingran (1990) reported that the potassium permanganate @ 0.5-2ppm in water showed a good result in curing the ulcers.

Lime

Application of lime in the pond of EUS prone area @ 200-600kg. per hectare showed very good results either in checking the outbreak of EUS or in healing of the ulcers. (Jhingran *et al.* 1990). Jain (1990) reported that better results were found when liming @ 200-600kg per hectare was followed by bath treatment of the infected fish in 5ppm potassium permanganate solution or common salt at 3% to 4% solution.

Antibiotics

The most commonly used antibiotics to treat EUS affected fishes are erythromycin, nalidixic acid, oxytetracyclin, terramycin. To get effective result the above mentioned antibiotics were recommended @ 60-100mg per kg. of feed for 7 days (Jhingran, 1990).

CIFAX

A drug formulated by CIFA for application in EUS affected captive water @ 1 liter / hectare meter of water was reported to show encouraging results in controlling EUS (Das and Das, 1993).

Materials and Methods

3.1. Collection of diseased fish

Infected fishes *Catla catla, Labio rohita, Cirrhinus mrigala, Channa punctatus, C. gachua, Clarias batrachus, Heteropneustes fossilis, Puntius* sp., *Mystus* sp. were collected from affected ponds of Ambari, Fulbari, Gajaldoba of Jalpaiguri district and Chathat, Leusipakuri of Darjeeling district. Infected fish were also collected from local markets. The fish were brought to the laboratory in live condition and were kept in glass aquaria measuring 90 x 35 x 35 cm in which the depth of water was 20 cm.Half of the static water was changed every day.

3.2. Collection and maintenance of healthy fish

Healthy *Channa punctatus* were collected from ponds of Sonapur of Darjeeling district with no record of EUS outbreak in various quantities time to time for the experimental work.

The healthy fish were kept kin glass aquaria measuring 90 x 35 x 35 cm in which depth of water was 20 cm. The fishes were fed regularly with chopped earthworms. All fishes were acclimatized under laboratory conditions for at least 15 days before using them for experimental work.

Healthy carp, *Cyprinus carpio* (30 - 50 g) were collected from local sources and acclimatized in glass aquaria measuring 90 x 35 x 35 cm in which depth of water was 20 - 25 cm at a density of 5 fishes per aquarium tank with constant blowing of air through water by air pump in laboratory. The water temperature varied from 28 to 30^{0}C. The fishes were fed Tokyu fish food.

3.3. Fungal culture

3.3.1. Source of culture

Fungi were isolated from infected fishes, during the course of the work. Culture were maintained aseptically by transferring the fungus to freshly prepared fungal media. Code names were given to each of the isolates. Fungal isolate (F_{cs1}) was used for experimental work.

3.3.2. Composition of fungal media

Various types of culture media were used during this study. The names and composition of those media are given below.

GP (Glucose-peptone) Broth (Lilley *et al.*, 1998)

Glucose	3 gm
Peptone	1 gm
$MgSo_4.7H_2O$	0.128 gm
KH_2PO_4	0.014 gm
$CaCl_2.2H_2O$	0.029 gm
$Fecl_3.6H_2O$	2.4 mg
$MnCl_2.4H_2O$	1.8 mg
$CuSO_4.5H_2O$	0.4 mg
Distilled water	1000 mL

All the ingredients were dissolved in distilled water and then autoclaved at 121^0C for 15 minutes.

GP (Glucose-peptone) Agar: 12 mg/L of agar powder was added to GP medium to prepare GP agar.

GPY (Glucose-peptone-yeast extract) Broth: 0.5 gm/L of yeast extract was added to GP medium to prepare GPY medium.

GPY (Glucose-peptone-yeast extract) Agar: 0.5 gm/L of yeast extract and 12 gm/L of agar powder were added to GP medium to prepared GPYA medium.

Penicillin-K, 100 units/mL and oxonilic acid were added at concentration of 100 mg/L to the media after autoclaving and cooling to 50^0C. (Roberts *et al.*, 1993).

Autoclaved pond water: The pond water is known to promote the fungal growth. Pond water was first filtered through Whatman filter proper. One part

of the filtered pond water was mixed with two part of distilled water and then autoclaved.

Material for fungal stain

1% Lactophenol cotton blue

Cotton blue powder	1 gm
Dissolved in Lactophenol solution	100 mL

Composition of Lactophenol

Phenol	20 mg
Glycerol	33.3 mL
Lactic acid	1.68 mL
Distilled water	20 mL

3.3.3. Methods for fungal isolation (Willoughby and Roberts, 1994; Lilley *et al.*, 1998)

For fungal isolation the lesions which have not yet completely ulcerated are most suitable. For isolation of fungus the following techniques were adopted.

1. The scales around the periphery of the lesion were removed and underlying skin was seared with a red hot spatula to sterilize the surface.
2. The anesthesized fish pinned on a dissecting tray was then taken to a laminar flow cabinet.
3. The underlying muscle of the lesion was then exposed by cutting the superficial tissues.
4. The affected underlying muscles were then excised into 4mm pieces and placed on a petridish containing the isolation medium.
5. Inoculated media were incubated at 25^0C and examined under phase contrast inverted microscope and first transfer was given after 6 h.

6. The emerging hyphal tips were repeatedly transferred to fresh plates of GP medium containing antibiotic until the cultures were free of bacterial contamination.
7. The isolates were then subcultured on GP agar and transfer was given at an intervals of 5 days.

3.3.4. Methods for sporulation of isolated fungus

For sporulation of fungus an agar plug containing actively growing mycelium was placed in a petridish containing GPY broth and was incubated for 4 days at about 20^0C. The resulting mat was washed by sequential transfer through 5 petridishes containing autoclaved pond water finally was and left overnight at 20^0C in autoclaved pond water. After about 12 hours motile secondary zoosperes were observed under microscope.

3.3.5. Microscopical examination of isolated fungus

A portion of the ulcer tissue was taken and smeared on a clean glass slide. The smear was stained with cotton blue and was observed under the microscope.

Similarly, fungal hyphae and sporangium from the pure culture maintained in and GPYA, were observed under microscope after staining with cotton blue and measured with standard ocular micrometer.

3.3.6. Effect of temperature on the growth of isolated fungus

The fungal isolates were cultured at different temperatures from 20^0C –37^0C to observe its growth in GPA and GPYA media.

3.4. Bacterial cultures

3.4.1. Source of culture

Three types of bacteria, R1, R2 (fluorescent pseudomonad) and R3 (*Aeromonas caviae*) were isolated in our laboratory from infected air breathing fish of North Bengal (Pal and Pradhan, 1990, Pradhan 1992) and

were maintained routinely. These bacteria were used for different experimental works. Bacteria were also isolated from different infected fish.

3.4.2. Composition of bacterial media

Various types of culture media were used during this study. The names and composition of these media are given below:

Nutrient Broth

Peptone	100 gm
Beef extract	10 gm
Nacl	5 gm
Distilled Water	1000 mL

The ingredients were dissolved in distilled water. The pH was adjusted to 8.0-8.4 with 10(N) NaOH and steamed for 10 minutes. It was then filtered and pH was adjusted to 7.2-7.4. with sulphuric acid. The medium was sterilized at 115^{0}C for 20 minutes.

Nutrient Agar

Nutrient agar was prepared by adding 2% agar powder to the final nutrient broth solution. The agar was melted by heating the medium before sterilization.

Glucose Peptone Broth (GPB)

Glucose	3 gm
Peptone	1 gm
$MgSo_4.7H_2O$	0.128 gm
KH_2PO_4	0.014 gm
$CaCl_2$	8 mg
$FeSO_4$	0.5 mg
$MnSO_4$	0.5 mg
$CuSO_4.5H_2O$	0.1 mg
$ZnSO_4$	0.1 mg
Distilled water	1000 mL

All the ingredients were dissolved in distilled water and after dispensing into test tubes and 100 mL conical flasks (containing 30ml of broth) was sterilized at 115^{0}C for 20min.

Nitrate Broth

KNO_3	1gm
Nutrient Broth	100 mL

Potassium Nitrate was dissolved in nutrient broth and distributed into test tubes containing inverted Durham's tubes filled with the medium and sterilized at 115^{0}C for 20min.

Gelatin Agar

Gelatin	4g gm
Distilled water	50 mL
Nutrient agar	1000 mL

The gelatin was soaked in water for 30min and added to the molten nutrient agar. It was then mixed and sterilized at 115^{0}C for 20 minutes and distributed into sterile petridishes.

Glucose-Phosphate medium

Peptone	5 gm
K_2HPO_4	5 gm
Distilled water	1000 mL
Glucose	5 gm

The first two materials were steamed with distilled water until the solids were dissolved, then filtered and the pH was adjusted to 7.4. Glucose was then added and 5ml volumes of medium was distributed into tubes. It was then sterilized at 115^{0}C for 10 minutes.

Hugh and Leifson's of medium (Hugh and leifson, 1953)

Peptone	2 gm
NaCl	5 gm
K_2HPO_4	0.3 gm
Agar	3 gm
Distilled water	1000 mL

Bromothymol blue, 0.2% aquous soln. 15mL

The solids were dissolved by steaming. The pH was adjusted to 7.1, filtered and the indicator was added. Then it was sterilized at 115^{0}C for 20 minutes.

The carbohydrate solution was sterilized separately, cooled to 45^{0}C and mixed to the OF media to give a final concentration of 1%. It was then distributed asceptically into sterile tubes.

Medium A of King (King *et al.*, 1954)

Peptone	20 gm
Glycerol	10 gm
K_2SO_4 anhyd	10 gm
$MgCl_2$ anhyd	1.4 gm
Agar	20 gm
Distilled water	1000 mL

The constituents except agar were dissolved by steaming and the pH was adjusted to 7.2. The agar was then added and dissolved. The medium was sterilized by autoclaving at 121^{0}C for 10min.

Medium B of King for Fluorescin (King *et al.*, 1954)

Proteose peptone	20 gm
Glycerol	10 gm
K_2HPO_4	1.5 gm
$MgSO_4, 7H_2O$	1.5 gm
Agar	20 gm
Distilled water	100 mL

The pH was adjusted to 7.2.

Procedure as stated for medium A was followed

Modified motility media (Hajna, 1950)

Peptone	10 gm
Beef Extract	3 gm
NaCl	5 gm
Agar	4 gm
Gelatin	80 gm
Cystein	0.2 gm
Ferrous ammonium sulphate	0.2 gm
Sodium citrate	2 gm
Distilled water	1000 mL

Gelatin was soaked in water for 30 minutes. The other ingredients were added and heated to dissolve and sterilized at 115^{0}C for 20 minutes.

Arginine Media

Peptone	1 gm
NaCl	5 gm
K_2HPO_4	0.3 gm
Phenol red, 10% aq. Soln.	1.0 mL
L(+) Arginine hydrochloride	10 gm
Agar	3 gm
Distilled water	1000 mL

The solids were dissolved in the distilled water and the pH was adjusted to 7.2. The medium was distributed into tubes to a depth of about 20mm and sterilized at 121^{0}C for 15 min.

Stains

Carbol fuchsin

Phenol	-	85 gm
Basic fuchsin		15 gm
Ethanol		250 mL
Distilled water		1250 mL

The phenol and basic fuchsin powder were mixed and heated gently to dissolve the phenol. Ethanol and distilled water were added and filtered with Whatman filter paper. To carbolfuchsin weak 1 volume of strong carbol fuchsin were mixed (10-20) time volume of distilled water.

Gram stain

Crystal violate

Burke's Iodine

Saffranin

Crystal violate stain

Crystal violet	2 gm
95% alcohol	20 mL
1% aqueous ammonium oxalate solution	80 mL

Burke's Iodine

Iodine	1 gm
Potassium iodide	2 gm
Distilled water	1000 mL

Saffronin

Nitrite regent

Solution A

0.33% sulphanilic acid was dissolved gentle in 5 N acetic acid.

Solution B

0.5% a-naphthylamine was dissolved by gentle heating in 5N acetic acid.

10% Zinc dust suspended in 1% methylcellulose solution (Steel & Fisher, 1961).

Kovac's regent

ρ-dimethylaminobenzaldehyde	5 gm
Amyle alcohol	75 mL
Concentrated HCl	25 mL

The aldehyde was dissolved in alcohol by gently warming in water bath (about 50-55^{0}C). Then it was cooled. HCl was added with care. It was stored at 4^{0}C protecting from light.

Methyl red

Methyl red	0.04 gm
Ethanol	40 mL
Distilled water	100 mL

The methyle red was dissolved in the ethanol and diluted with the distilled water.

Acetylmethyl carbinol

5% α-naphthol solution 0.6 mL 40% potassium hydroxide (KOH) aquous solution 0.2 mL.

3.4.3. Methods for isolation of bacteria

The ulcerated area of the diseased fish was dissected out asceptically from the fish and then surface sterilization was done with 0.1% mercuric chloride. The dissected tissues were placed in 100ml conical flasks containing 15 ml nutrient broth medium supplemented with glucose. The flasks were incubated at 30^{0}C for 72 hours. The tissues were then removed and the cultures were observed under microscope. Then the cultures were diluted by serial dilution (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}) and bacteria were isolated by poor-plate method. Single separated colonies on agar plates were selected and streaked in nutrient agar slants and incubated for 24 hours at 30^{0}C. Thus isolated pure cultures were obtained. Code names were given to

each of the isolates and stored at 4^0C for characterization and identification of bacteria Barrow, G.I. and R.K.A. Feltham (1993) was followed.

3.5. Morphological and biochemical tests for bacterial identification

Shape of Cells

A drop of cell suspension of the test organism was placed on a clean grease-free slide, air dried and stained with carbol fuchsin and was observed under microscope. Diameter was measured with a standard ocular micrometer.

Motility

Tubes of motility medium were stab inoculated to a depth of about 5 mm to detect the motility. The tubes were incubated at 28^0C and the turbidity pattern of the medium was observed for 5 days. The bacterial cultures in nutrient broth were also observed under a phase contrast microscope.

Gram staining

Smears of test organisms were made from 24 hour old culture on nutrient agar slant on clean grease free glass slide.

The smears were air dried heat fixed and flooded with crystal violet stain for 1 minute. Slides were washed in 95% ethanol, which was poured drop-by-drop holding the slides in slanting position against white background till no colour came out from the lower edge of the slides. After washing with tap water these smears were stained with saffranin for 1 minute and rinsed in distilled water. The slide were air dreid and observed under microscope.

Production of catalase

Organisms from 24 hours old cultures were incubated for 24 hours on a slope of nutrients agar and 1 ml of 3% H_2O_2 solution was poured down the slope. Immediate evolution of gas indicate the presence of catalase activity.

Production of Oxidase

Organisms from 24 hour old cultures grown on nutrient agar (glucose free) were taken and smeared across a filter paper moistened with freshly prepared 1% tetramethyl-p-phenylenediamine dihydrochloride with a glass rod. The appearance of a dark purple colour within 30 second indicated a positive reaction

Oxidation or Fermentation of glucose

Oxidation or fermentation of glucose was done by inoculating dupliate tubes of Hugh and Leifson's medium containing glucose as the carbohydrate, by stabbing. After inoculation, sterile molten paraffin was poured on to the top of the tubes to a depth of 10 cm to seal it from air. The other tubes was left open. The tubes were incubated at 30^0C and observed for 14 days. If the blue colour of the medium changes to yellow in the open tube only, it indicated acid production from glucose by oxidation. If both tubes changes to yellow it indicated that glucose can also be broken down fermentatively (Huge and Leifson, 1953).

Acid and gas production in media containing different carbohydrates

The medium used to detect acid production from carbohydrates was a modified Hugh and Leifson's medium without agar. The following carbohydrates were tested: D-glucose, D-fructose, D-sucrose, D-Maltose, D-sorbitol, manitol, adonitol meso-inositol, L-arabinose. The liquid medium was inoculated and incubated at 30^0C for 14 days. If the colour of the medium changed from blue to yellow, it indicated acid production. To test for gas production inverted Durham's tubes filled with the medium was inserted into the broth. The gas, when produced, was accumulated at the top of the inverted Durham's tube

Nitrate reduction

Test tubes containing nitrate broth and filled in inverted Durham's tubes were inoculated and incubated for 5 days. The presence of nitrate (after reduction of nitrate to nitrate) was tested by addition of 1 mL of nitrite reagent

A (0.8% Sulphanalic acid in 5N Acetic acid) followed by 1 mL of reagent B (0.5% a napthylamine in 5N Acetic acid). Appearance of red colour indicated presence of nitrite. Zinc dust was added to the culture tubes showing negative test for nitrite appearance of red colour indicated presence of nitrate in culture tubes that was not reduced to nitrite by the test organisms. Any gas production in the Durham's tubes was noted.

Indole production

Tryptone broth was inoculated and incubated at 30°C for 48 hours. Inddole prodction was tested by adding 0.5ml of Kovac's reagent and examined for 1min. Appearance of a red colour in the regent layer indicated indole production.

Methyl red reaction

Glucose phosphate medium was inoculated and incubated at 30°C for 5 days. Two drops of methyl red solution was then added, shaken and examined. A red colour at the surface shown the positive M R reaction.

Acetylmethylcarbinol production: the Voges-Proskauer test

After completion of the methyl red test 0.6ml of 5% α-naphthol solution in ethanol and 0.2 mL of 40% potassium hydroxide aqueous solution was added and well shaken. The tubes were then held in a sloping position to increased the area of the air liquied interface and examined after 15 minutes and 1 hour. A strong red colour shows positive V.P. test .

Gelatin Hydrolysis

Gelatin agar plates were inoculated and incubated for 3 days. The plates were then flooded with 30% trichloroacetic acid, clear zones indicated areas of glatin hydrolysis.

Gelatin hydrolysis was also tested in the modified motility medium along with testing of motility and H_2S production. The medium was stabbed inoculated with a straight wire, inoculated at 28°C and observed daily for 30 days for presence of liquefaction.

Argining hydrolysis

The arginine agar was inoculated by stabbing with a straight wire and a layer of sterile molten paraffin was poured with a pipette to a depth of about 2cm. The tubes were incubated at 30°C and observed daily for 5 days for a colour change of the medium from yellow orange to red.

H_2S production

Tubes of the modified motility medium containing cystein and ferrous ammonium sulphate wre stabbed inoculated with the bacteria. A lead acetate paper was inserted between the cotton plug and the tube and inocubated at 28°C. The tubes were examined daily for blackening of the media and the paper.

Pigment production

To test pigment production by Pseudomonads, King, Ward and Raney's media (A and B) were used. After inoculating, medium A was incubated at 30°C for 24 to 96 hours and examined daily for pigment production. Medium B was incubated at 30°C for 24 hours followed by room temperature (22-25°C) for 72 h. To test for pigment production by coccus, organisms were grown on nutrient agar plates at room temperature and were kept under diffused day light.

3.6. Inoculation of fishes with bacterial suspensions

3.6.1. *Channa punctatus*

Intramuscular injection of 0.5 mL/100 g body weight (6-8 x 10^9 cells/mL) of bacterial culture, in pure (R1, R2 and R3) and in mixed culture of three bacteria (R1, R2 and R3) were given to *Channa punctatus*. Control fishes received only nutrient broth. For each set of experiment ten fishes were taken and each experiment was done five times. The gradual development of ulcer after injection was carefully observed. Mortality caused by the injection of bacterial culture was recorded up to 10 days of injection.

3.6.2. *Cyprinus carpio*

Intramuscular injection of 1mL/100 g body weight (6-8 x 10^9 cells/mL) of bacterial culture, in pure (R1, R2 and R3) and in mixed condition of three bacteria (R1, R2 and R3) were given to *C. carpio*. Control fishes received only nutrient broth. For each set of experiment 5 fishes were taken and each experiment were done four times. The gradual development of ulcer after injection was carefully observed. Mortality caused by the injection of bacterial culture was recorded up to 10 days of injection.

3.7. Inoculation of fishes with zoospore suspension

Healthy *C. punctatus* (20 – 30 g) were collected from the ponds of Sonapur. After bringing to the laboratory, the fishes were acclimatized in the laboratory condition in the glass aquarium at 25± 1^0C for 15 days and then 10 fishes were injected spores suspension of *Aphanomyces* sp. (F_{cs1}) at the rate of 0.5 mL/100 g of body weight intramuscularly as described by Roberts *et al.* (1993). The control set received only sterile saline (0.85% NaCl) solution @ 0.5 mL / 100 g of body weight. Simultaneously, five sets of experiment, each set consisting of ten fishes, were carried out.

3.8. Pathogenicity test of bacterial isolates

Attempts were made to determine the pathgogenicity of the isolated bacteria from *C. striata* and *Labio rohita*. For this purpose healthy fishes were collected from ponds of Sonapur, of the Darjeeling district that did not have any previous report of EUS outbreak. The fishes weighing about 20-30 g were brought to the laboratory and acclimatized in the glass aquarium measuring 90 x 35 x 35 cm. Water temperature was maintained at 28 - 30^0C. Intramuscular injection was given with 0.05 ml of cell suspension containing 3 – 5 x 10^7 cell cells / ml of each isolate (C_{s1}, C_{s2}, C_{s3}, C_{s4}, C_{s5}, L_{r1}, L_{r2}, L_{r3}, L_{r4} and L_{r5}). The control set received sterile saline (0.85% NaCl) solution @ 0.5 mL/100 g of body weight of the fishes. For each set of experiment ten fishes were taken and each experiment was repeated five times under identical conditions.

3.9. Inoculation of fishes with bacterial suspension (R1, R2, R3 and mixed culture of R1, R2 and R3 for histopathological studies

Healthy fishes (*Cyprinus carpio*) each weighing about 30 – 50 g were inoculated by intramascular injection with 0.85% saline suspension of R1, R2 and R3 bacteria in pure and mixed condition. The concentration of bacterial suspensions was $1x10^7$ cfu/mL. Five fishes were injected with each bacterial suspension and mixed suspension. The control set of ten fishes received 1.00 mL sterile saline solution. The fishes were observed daily for appearance of symptoms. The moribund fishes from each aquarium were taken out and sacrificed for histological studies. Two fishes from control set were also sacrificed and the tissue from liver, kidney and spleen were processed for histological studies. Histological sections were prepared and stained with Hematoxylene-Eosin stain.

3.10. Inoculation of fishes with mixed suspension of fungal zoospore and bacteria (R1, R2 and R3) in pure condition

Healthy fishes (*Channa punctatus*) each weighing about 20 – 30 g were inoculated by intramascular injection with 0.85% saline suspension of mixed R1 and zoospore, R2 and zoospore and R3 and zoospore. Ten fishes were injected with each bacterial suspension and mixed suspension. The control set of ten fishes received 0.05 mL sterile saline solution. Each experiment was repeated five times. The fishes were observed daily for appearance of symptoms. Nature of ulcer formation and mortality was recorded upto 15 days.

3.11. Histopathology of the experimentally infected fish with isolated fungus

Healthy *C. punctatus* (20 – 30 g of body weight) were inoculated with zoospores of isolated *Aphanomyces* sp. @ 0.5 mL/ 100 g of body weight. The fishes with ulcer were sacrificed after five days of inoculation for

histopathological observation. The sections of different organs of the experientially infected fishes were prepared and stained with Haematoxylin-eosin, Grocott stain (GMS) and PAS stain.

3.12. Materials for histopathological techniques

The ulcer tissue and other internal organs of diseased and healthy fish were fixed in Bouin's fixative and preserved in cedarwood oil if necessary. For histological studies sections of 6μ thickness were done after preparing the paraffin block and were stained with haematoxylin and eosin, Grocott hexamine (methenamine) silver stain and Periodic Acid Schiff's stain.

Bouin's fixative

Saturated aqueous picric acid solution	75 mL
Formalin	25 mL]
Glacial acetic acid	5 mL

Materials for Grocott hexamine (methenamine) silver staining for fungi (Gomori, 1946; Grocott, 1966)

Solution (a) 5 percent aqueous sodium tetraborate, i.e. Borax soln.

Solution (b) 5 percent silvernitrate in distilled water-5 mL

3 percent methenamine or hexamethylentetramin in distilled water 100 mL

Silver nitrate was added to the methenamine solution and shaked until the precipitation with first forms dissolved.

This mixture was kept for 1-2 months at $4^{0}C$

Solution (c) Incubating solution

Borax solution	5 mL
Distilled water	25 mL
Methenamine silver soln. B	25 mL

The methenamine silver / water solution and the borax were heated to 56^{0}C and then mixed prior to use as the silver solution to degenerate when the borax was added.

Solution (d) Arzac's counter stain

Orange Green	0.25 gm
Light Green	1.00 gm
Phsphotungstic acid/	
Tungstophosperic acid	0.50 mg
50% alcohol	100 mL
Glacial acetic acid	1.25 mL
Sodium metabisulphate	1%
Gold chloride	0.1%
Sodium thiosulphate	3%
Chromic acid	5%

Materials for periodic acid schiff stain (PAS)

(i) **Basic fuchsin solutin**

(a) Basic fuchsin	0.1gm
(b) 95% alcohol	5.00 mL
(c) Water	95.00 mL

(ii) **Zinc (Sodium) hydrosulfite solution**

(a) Sodium hydrosulfite (Sodium dithionite)	1.0 gm
(b) Tartaric acid	0.5 gm
(c) Water	100 mL

(iii) **Light green stain**

(a) Light green	1.0 gm
(b) Glacial acetic acid	0.25 mL
(c) 80% alcohol	1000 mL

3.13. Methods of Histopathological techniques

3.13.1. Sampling and fixation

Live fish were quickly transferred to a container of benzocain solution (25 mg/L.). After two minutes, the fish was removed from the solution and pinned on a dissection tray. The external lesions were first excised from the fish body and immediately placed in Bouin's fixative. The internal organs were carefully dissected out from the body cut into small pieces of 3 to 5mm and immediately placed in the fixative. All tisues were kept in a volume of fixative at least 20 times the volume of the tissue and kept overnight.

3.13.2. Processing

The fixative was washed out of the sample by 70% alcohol. The tissue were subjected to routine processing (Schaperclaus 1986). Dehydration of the tissue was done by passing through a graded serried of alcohol solutions (70%, 90% and 100%). The tissue was then placed in xylene and infiltration was done with molten paraffin (melting point 58-60^{0}C) and allowed to cool harden. Sometimes tissues were preserved in cedarwood oil followng fixation and dehydration.

3.13.3. Sectioning

The hardened paraffin containing the tissue were trimmed into rectangular block, mounted on microtome and sections of 6μ thickness were cut. The sections were placed on grease free slides with albumin serving as an adhesive. The section were stretched on warm distilled water and the slides were dreid by keeping them overnight at room temperature.

3.13.4. Staining

Before staining the sections stretched slides were completely dewaxed by placing in xylene for 5 to 10 minutes.

3.13.5. Procedure for Haematoxylin – Eosin stain

After dewaxing the slides with stretched sections were transferred to absolute alcohol for 5 minutes and then through descending grades of alcohol e.g. 90%, 70%, 50% and 30% to water. The sections were then stained with haematoxylin and washed thoroughly with water to remove the excess stain and observed under the microscope to check the differentiation level. The nuclei were stained blue. The sections were then passed through ascending grades of alcohol and stained with alcoholic eosin. The excess eosin was washed in 90% alcohol and again observed under the microscope to check the differentiation level. If the counterstaining was satisfactory, the tissues were further dehydrated in absolute alcohol, cleared in xylene and mounted in DPX mountant.

3.13.6. Procedure for Grocott hexamine (methenamine) Silver staining for fungi

1. After dewaxing the sections were hydrated through graded alcohols to water.
2. Then the sections were oxidized in 5 percent aqueous chromium trioxide (chromic acid) for 1 hour.
3. The sections were then washed in tap water and rinsed in 1 percent sodium metabisulphite solution.
4. The sections were rinsed in distilled water and placed in preheated ($56^{o}C$) incubating solution in the dark, upto 1 hour.
5. The sections were rinsed well in distilled water and toned in 0.1 parcent gold chloride for 4 minutes.
6. Then the sections were rinsed in distilled water and fixed in 3 percnet sodium thiosulphate, for 5 minutes.
7. Then the sections were countrstained inArzac's stain for 15-30 seconds.
8. The sections were then blotted, dehydrated and mounted in D.P.X. mountant.

3.13.7. Procedure for periodic acid-schiff stain (PAS)

1. The sections were rinsed in 100% alcohol after dewaxing.
2. Then the sections were brought down gradually to distilled water and immersed in 1% periodic acid for 10 munites.
3. The sections were then rinsed in tap water for 5 to 10 minute.
4. Then the section were immersed in Basic fuchsin solution for 2 minutes and then rinsed in tap water for 30 seconds.
5. The sections were then placed in sodium hydrosulphite solution for 30 minutes.
6. The sections were then rinsed in tap water for 3 to 5 minutes and then placed in light-green stain for 2 minutes.
7. Then the sections were rinsed for a short time in tap water
8. The section were then dehydrated in 96% alcohol for 10 seconds and in 100% alcohol for 1 minutes.
9. The sections were then rinsed twice in xylol for about 1 minute each time and mounted in D.P.X. mountant.

Observations and Results

Fig. 4: Naturally infected *Channa striata* showing ulcer near the head region

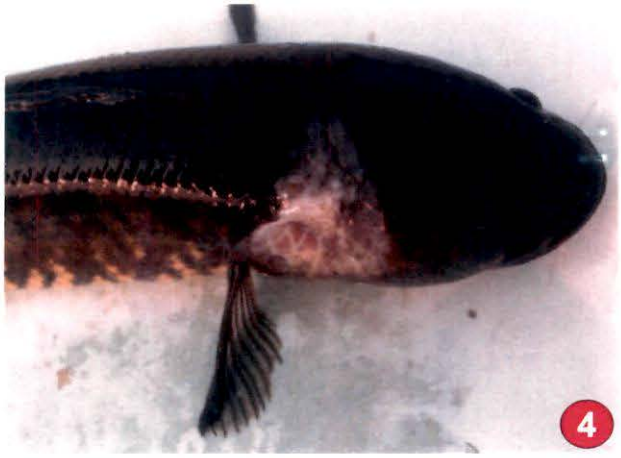
Fig. 5: Naturally infected *Channa striata* showing deep ulcer near the tail region.

Fig. 6: Naturally infected *Channa punctatus* showing primary infection on the body.

Fig. 7: Naturally infected *Hteropneustes fossilis* showing ulcer on the body.

Fig. 8: Naturally infected *Clarias batrachus* showing deep necrotic ulcer on the body.

Fig. 9: Naturally infected *Mystus* sp. showing primary ulcer on the body.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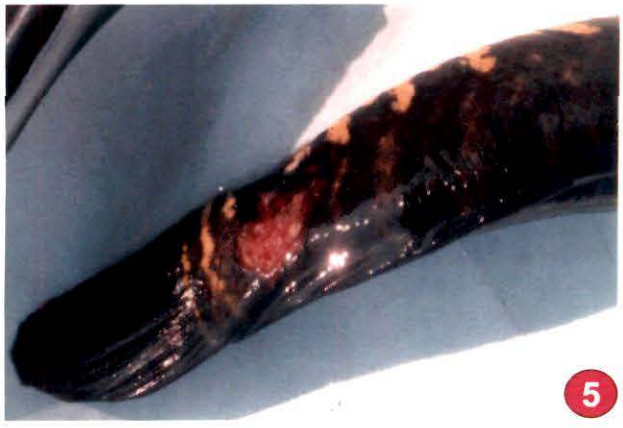
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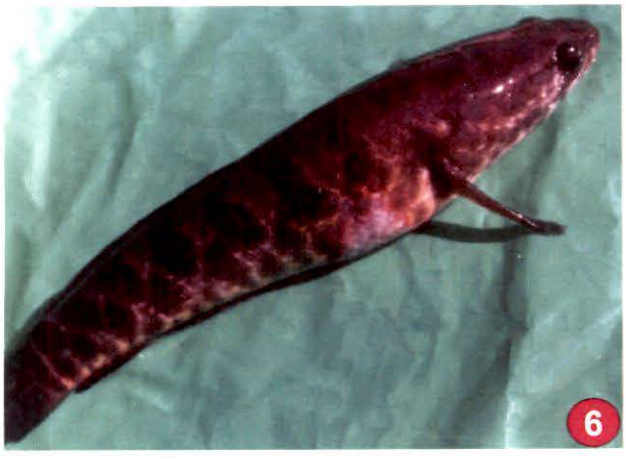
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Fig. 10: Naturally infected *Macrognathus aculeatus* showng severe ulcer on the dorsal region of the body.

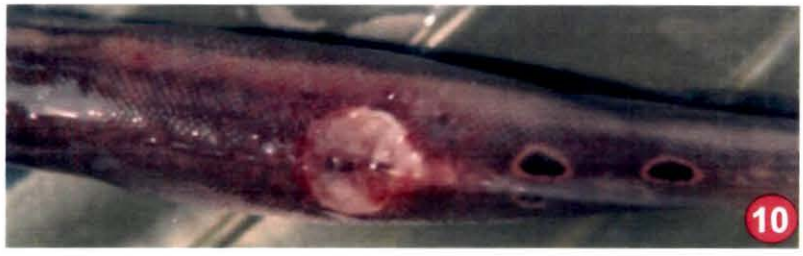
Fig. 11: Naturally infected *Labeo rohita* showing deep necrotic ulcer on the body.

Fig. 12: Naturally infected *Catla catla* showing severe ulcer on the body.

Fig. 13: Naturally infected fishes *Cirrhinus mrigala* showing severe ulcer on their bodies.

Fig. 14: Naturally infected *Labeo bata* showing multiple ulcer on the body.

Fig. 15: Naturally infected fishes *Puntius* sp. showing haemorrhagic ulcer on their bodies.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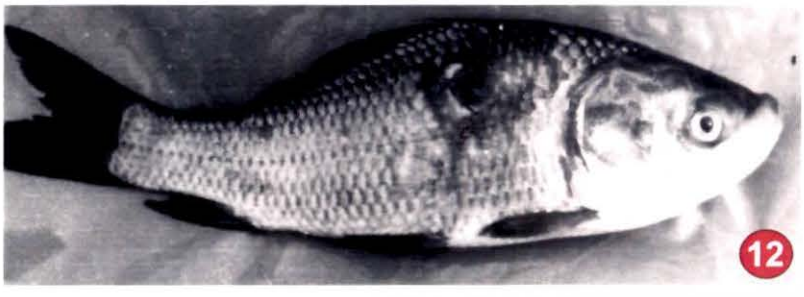
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4.1. Observation of the fish affected with epizootic ulcerative syndrome

Fifty two *C. punctatus* (20-45 g), ten *C. gachua* (15-35 g), twenty three *L. rohita* (30-75 g), thirty five *C. mrigala* (20-50 g), ten *C. catla* (50-125 g), twenty five *L. bata* (20-40 g), hundred eight *Puntius* sp. (5-10 g), fourteen *Mystus* sp. (5-15 g), three *M. aculeatus* (20-40 g), seven *C. striata* (50–70 g) and thirteen *C. batrachus* (weighing 30-80 g) were collected (Table 2) with different stages of ulcers. Some infected fish showed presence of single or multiple red spots on the body surface. Some fish showed moderate type of ulcers. In few cases ulcers were deep and necrotic and some times hemorrhagic. Occasionally a whitish rim was noticed surrounding the reddish ulcer. In some fish tail and fins were also affected. In scaly fish sloughing of scales took place when the ulcer was moderate. Altogether 300 (three hundred) EUS affected fish were collected from different Districts of North Bengal. (Fig 4-15).

Table 2 : Collection of diseased fish

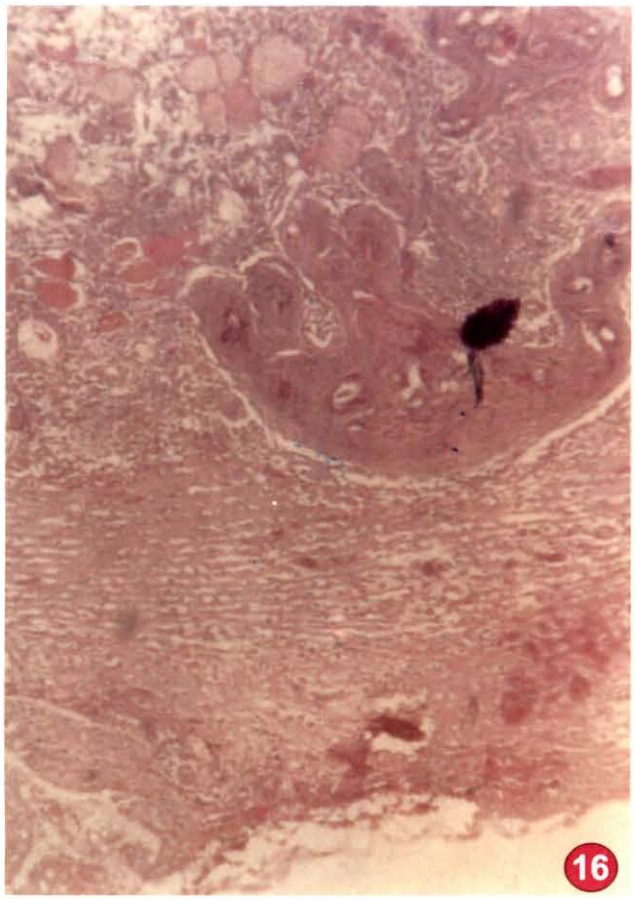
Fish species	Date of collection	No. of fish collected
Labeo rohita	18.02.2002	08
Channa punctatus	19.02.2002	04
Puntius sp.	22.02.2002	24
Catla catla	28.02.2002	03
Channa straita	03.03.2002	01
Puntius sp.	11.03.2002	28
Channa punctatus	25.12.2002	08
Channa punctatus	02.11.2003	06
Channa punctatus	28.02.2003	09
Cirrhinas mrigala	04.12.2003	04
Labeo bata	06.03.2003	13
Labeo rohita	10.01.2003	07
Puntius sp	17.11.2003	26
Catla catla	23.11.2003	04
Channa straita	23.11.2003	03
Labeo rohita	23.11.2003	08
Channa gachua	24.11.2003	05
Cirrhinas mrigala	24.11.2003	06
Channa punctatus	18.12.2003	08
Channa straita	14.01.2004	03
Mystus sp.	14.01.2004	14
Cirrhinas mrigala	28.03.2004	10
Channa punctatus	04.01.2004	09
Clarias batrachus	22.04.2004	04
Labeo bata	05.02.2004	12
Cirrhinas mrigala	06.01.2004	05
Clarias batrachus	09.02.2004	04
Clarias batrachus	19.10.2004	05
Catla catla	22.11.2004	03
Channa gachua	22.11.2004	05
Channa punctatus	30.11.2004	08
Puntius sp.	12.01.2004	30
Macrognathus aculeatus	18.12.2004	03
Cirrhinas mrigala	18.12.2004	10

Fig. 16: Section of the ulcer of naturally infected *C. striata* showing granuloma formation in the muscle layer and myonecrosis (H-E, x 200).

Fig. 17: Section of the ulcer of naturally infected *C. striata* showing the presence of fungal hyphae and degenerative changes in the musculature (H-E, x 400)

Fig. 18: Section of the ulcer of naturally infected *C. striata* showing presence of fungal hyphae (GMS, x 200).

Fig. 19: Section of the ulcer of naturally infected *C. striata* showing presence of fungal hypha and accumulation of cells around it (H-E, x 400).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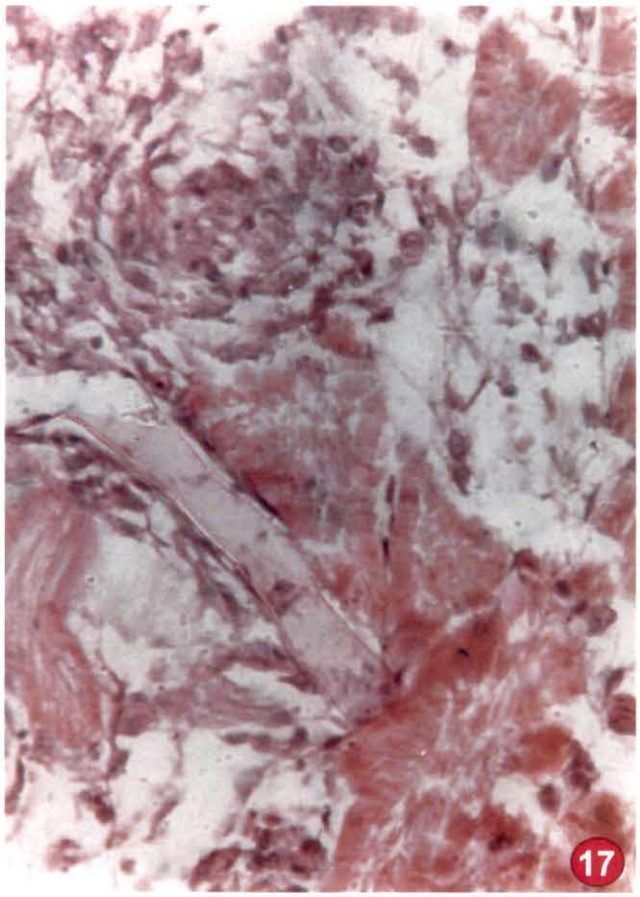
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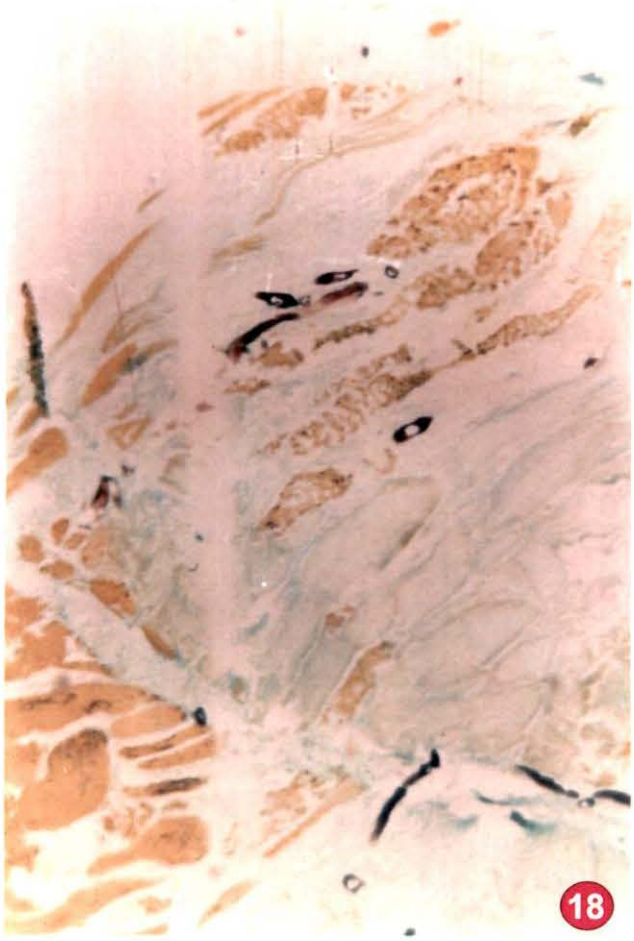
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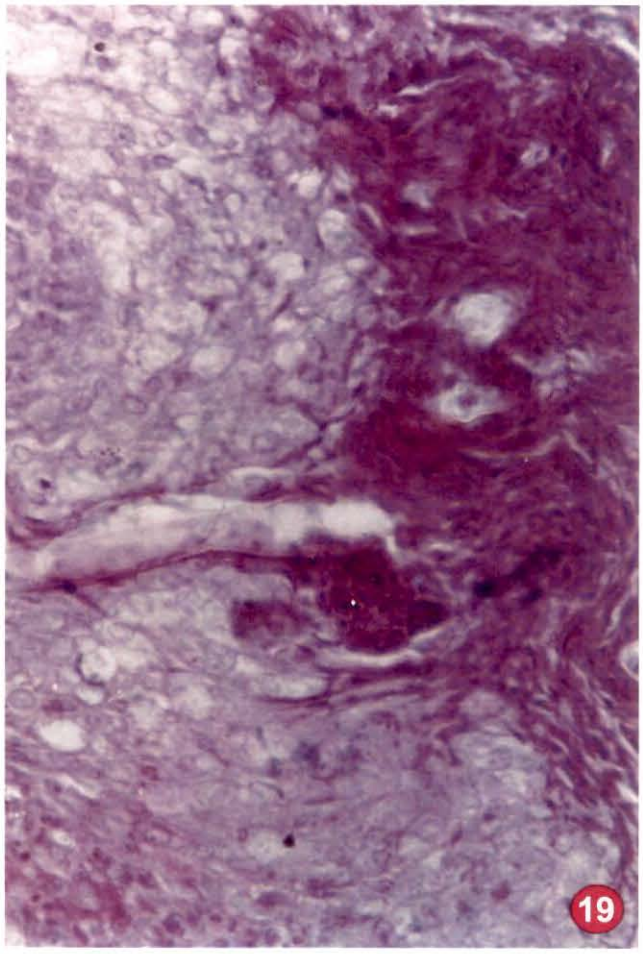
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Fig. 20: Section of the ulcer of naturally infected *C. striata* showing presence of fungal hypha with bud (GMS, x 400).

Fig. 21: Section of the liver of naturally infected *C. striata* showing infiltration of blood capillaries and vacuolation of hepatocytes (H-E,x 400).

Fig. 22: Section of the kidney of naturally infected *C. striata* showing tubular vacuolation and haemorrhages in certain haemopoietic region (H-E, x 400).

Fig. 23: Section of the spleen of naturally infected *C. striata* showing the degenerative changes and vacuolation (H-E, x 200).


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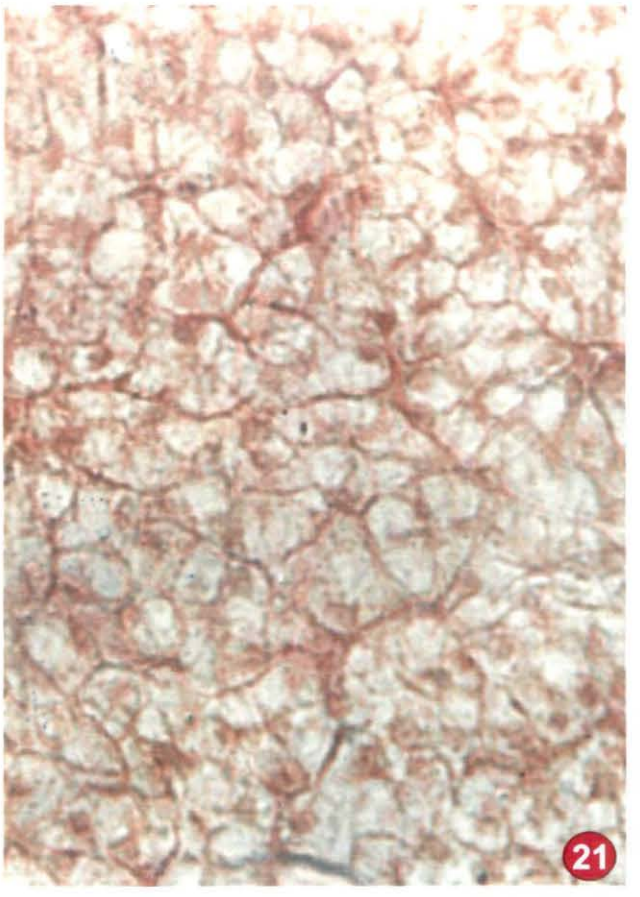
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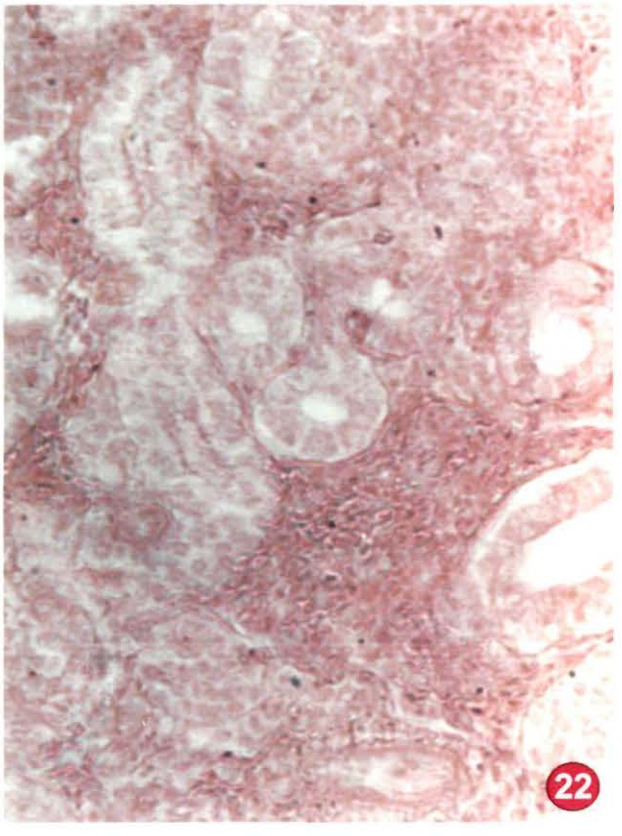
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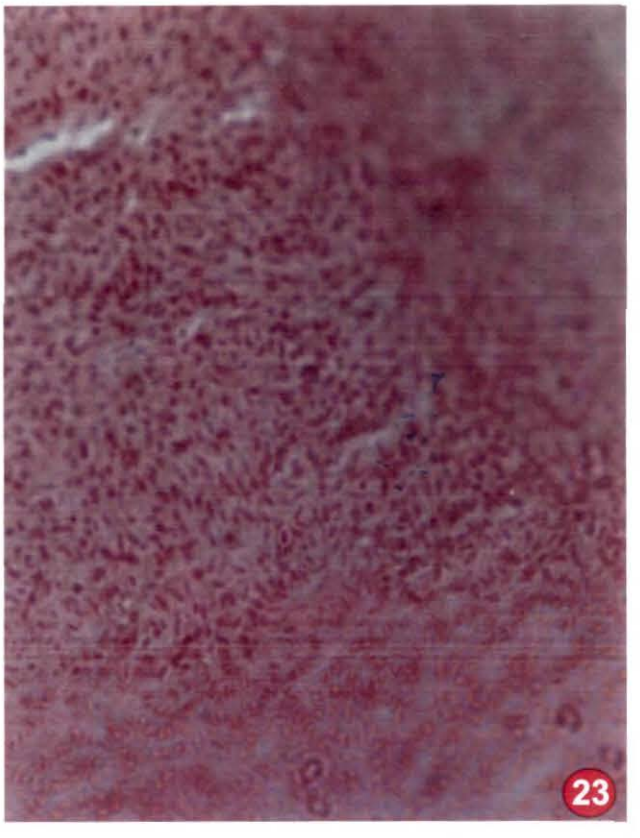
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4.2. Histopathological observations of the EUS affected fish

4.2.1. *Channa striata*

Ulcer

The epidermis of early lesions showed loss of normal architecture. In the sections of advanced lesion non septate fungal hyphae were frequently observed in the dermis and also in the underling musculature. The most important changes were granuloma formation and myonecrosis (Fig. 16). Section of the ulcer of naturally infected *C. striata* showed the presence of fungal hyphae and degenerative changes in the musculature and accumulation of cells around it (Fig. 17, 18, 19). In the GMS and H-E stained sections fungal hyphae with bud were also found (Fig. 20).

Liver

The sections of liver showed mild focal degenerative changes of hepatic cells. Haemorrhagic spots were also observed in the sections of the liver. Necrotic changes were also observed in some areas. Infiltration of blood capillaries and vacuolation of hepatocytes were also noticed (Fig. 21). No fungus was detected.

Kidney

No fungal hyphae was found in the section of kidney of *C. striata*. Necrotic changes in certain haemopoitic regions and haemorrhages were observed in the section of the kidney. Tubular vacuolation were also observed (Fig. 22).

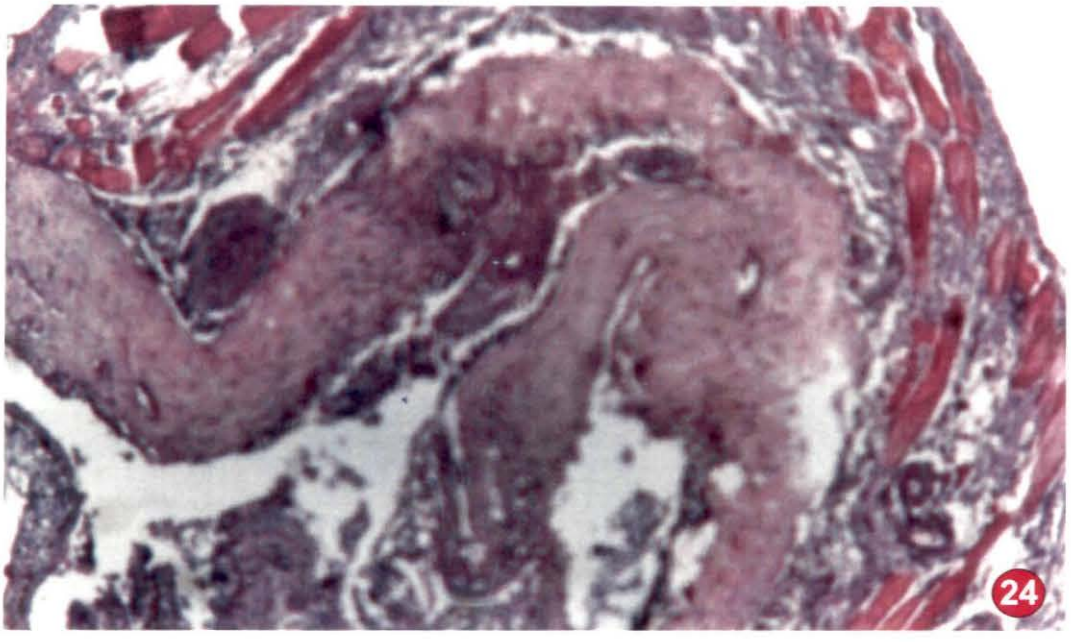
Spleen

The sections of the spleen of naturally infected *C. striata* showed haemorrhages and necrosis with vacuolation in many places (Fig. 23).

Fig. 24: Section of the ulcer of naturally infected *L. rohita* showing loss of normal architecture of epidermis and musculature (H-E, x 400).

Fig. 25: Section of the ulcer of naturally infected *L. rohita* showing presence of fungal hyphae (GMS, x 200).

Fig. 26: Section of the ulcer of naturally infected *L. rohita* showing presence of fungal hypae (H-E, x 400).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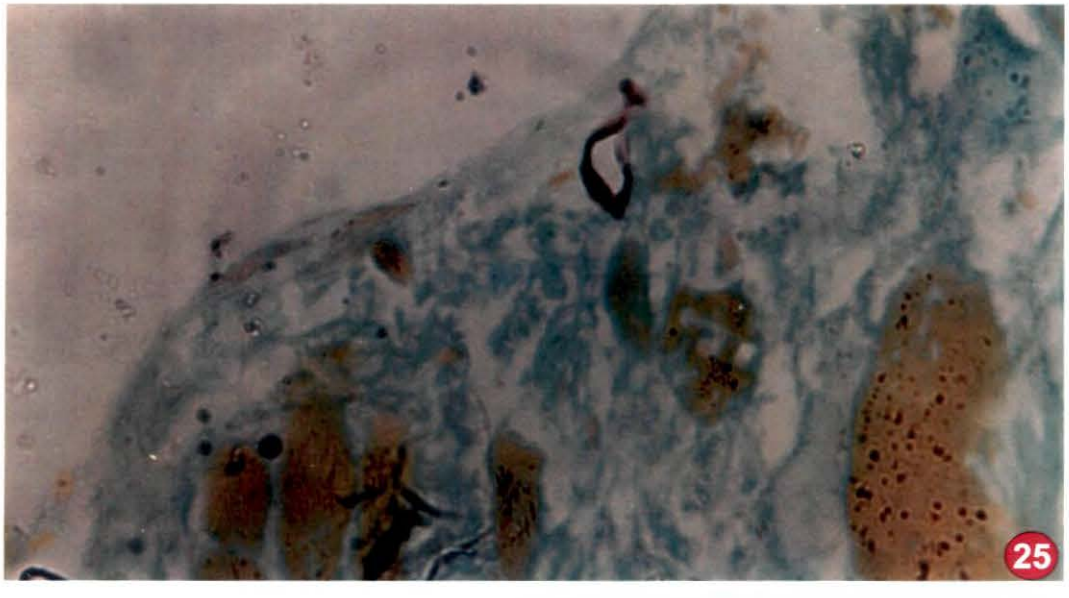
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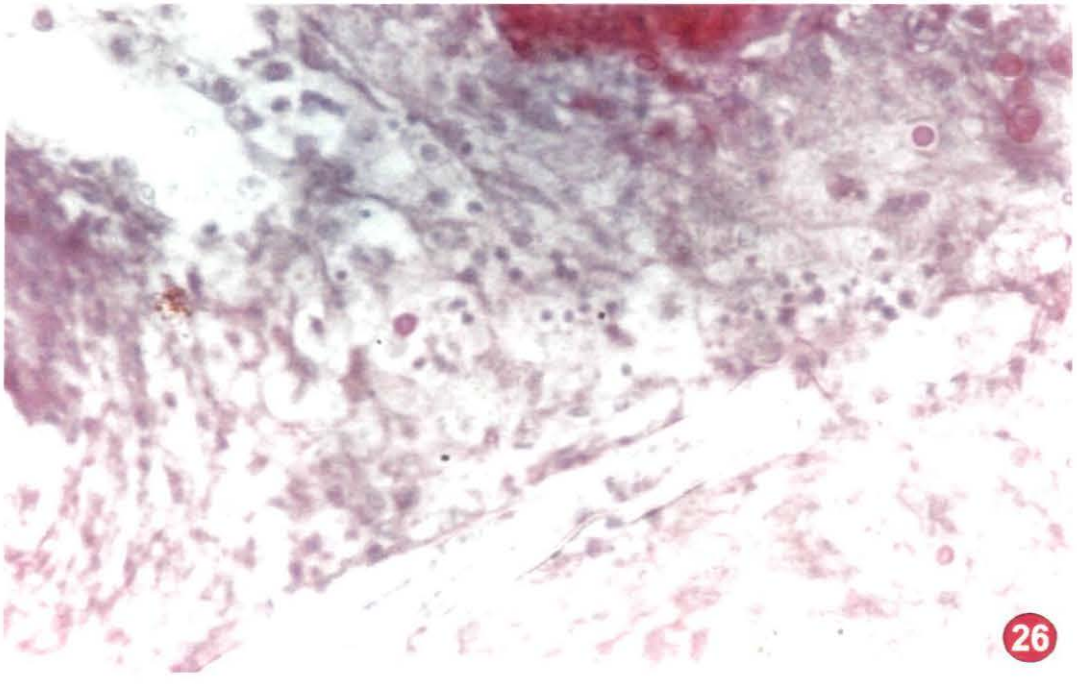
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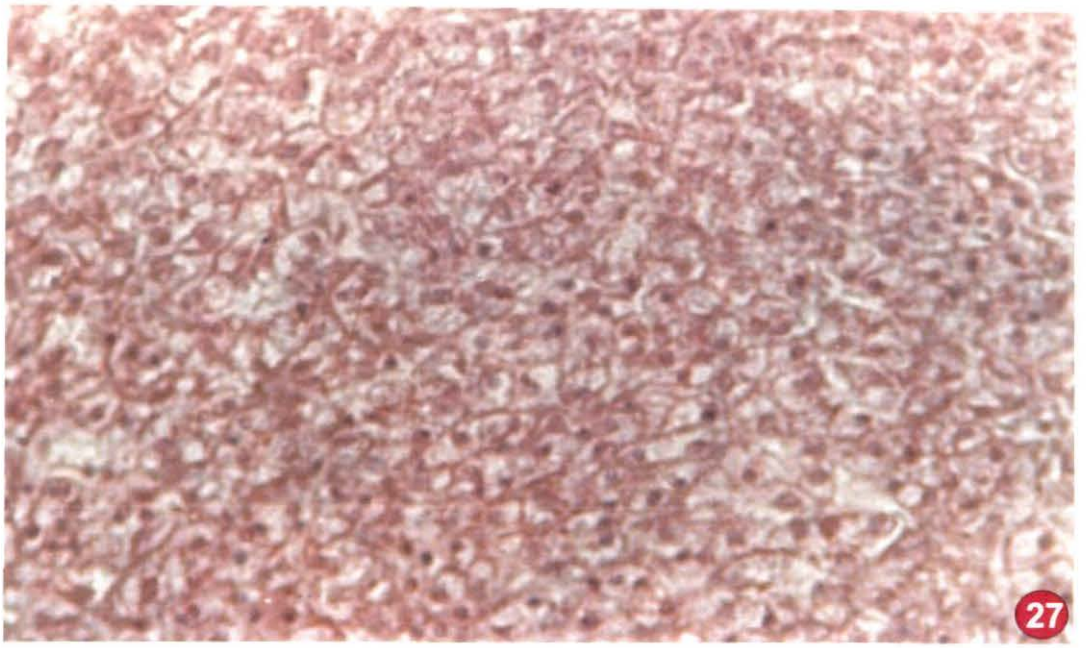
Fig. 27: Section of the liver of naturally infected *L. rohita* showing degenerative changes (H-E, x 400).

Fig. 28: Section of the kidney of naturally infected *L. rohita* showing tubular degeneration and vacuolation (H-E, x 400).

Fig. 29: Section of the spleen of naturally infected *L. rohita* showing degenerative changes in some areas (H-E, x 200).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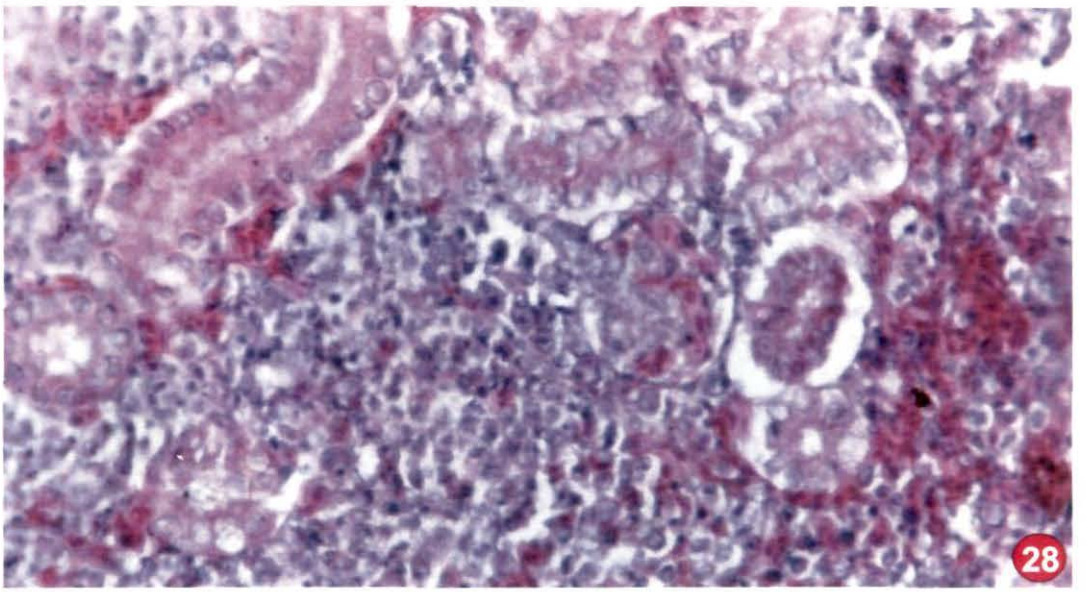
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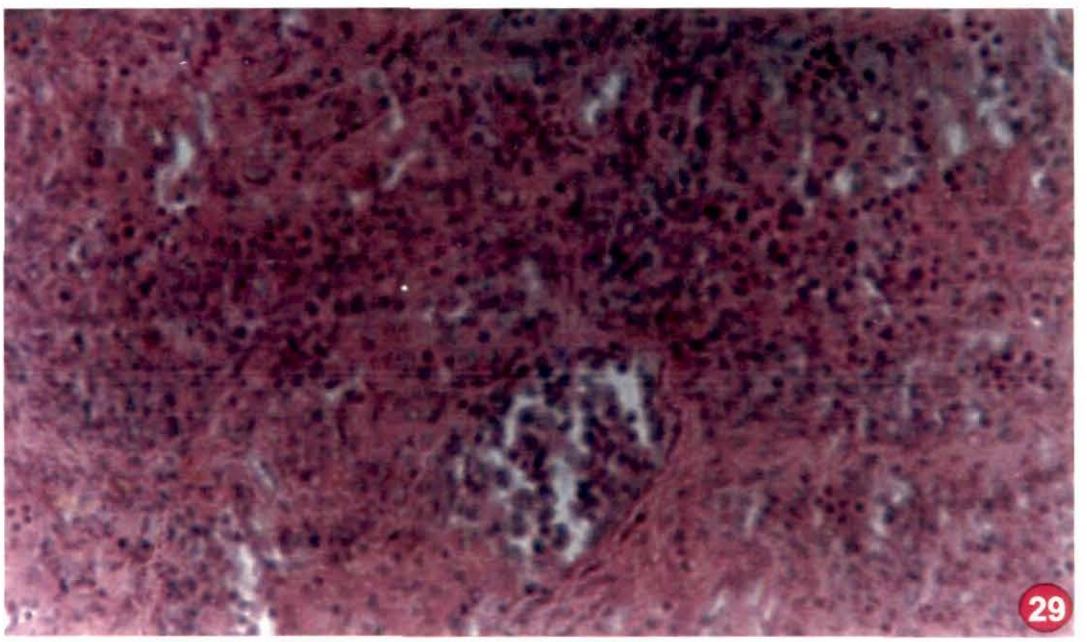
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4.2.2. *Labeo rohita*

Ulcer

The sections of early stages of ulcer showed that normal architecture of the epidermis was changed. Sections of the advanced lesions showed complete loss of epidermis and the underlying musculature were replaced by granulomatous and inflammatory tissues (Fig. 24). Myonecrosis was also observed in some areas. Fungal hyphae, stained black with GMS, were often found (Fig. 25). The sections stained with H-E showed presence of fungus (Fig. 26).

Liver

The section of liver *L. rohita* showed no evidence of fungus. The Haematoxylene and eosin stained sections showed degenerative changes (Fig. 27) and infiltration of blood capillarie. Necrotic changes were also observed in some areas of the liver. Chord like arrangement with enlarged sinusoids and highly vacuolated hepatic cells were also observed.

Kidney

No evidence of fungus was observed in sections of kidney. Tubular degeneration and vacuolation of tubular cells were noticed in the sections of kidney (Fig. 28). Necrotic changes and haemorrhages were also observed in some areas of the kidney .

Spleen

Sections of the spleen showed degenerative changes in some areas (Fig. 29)

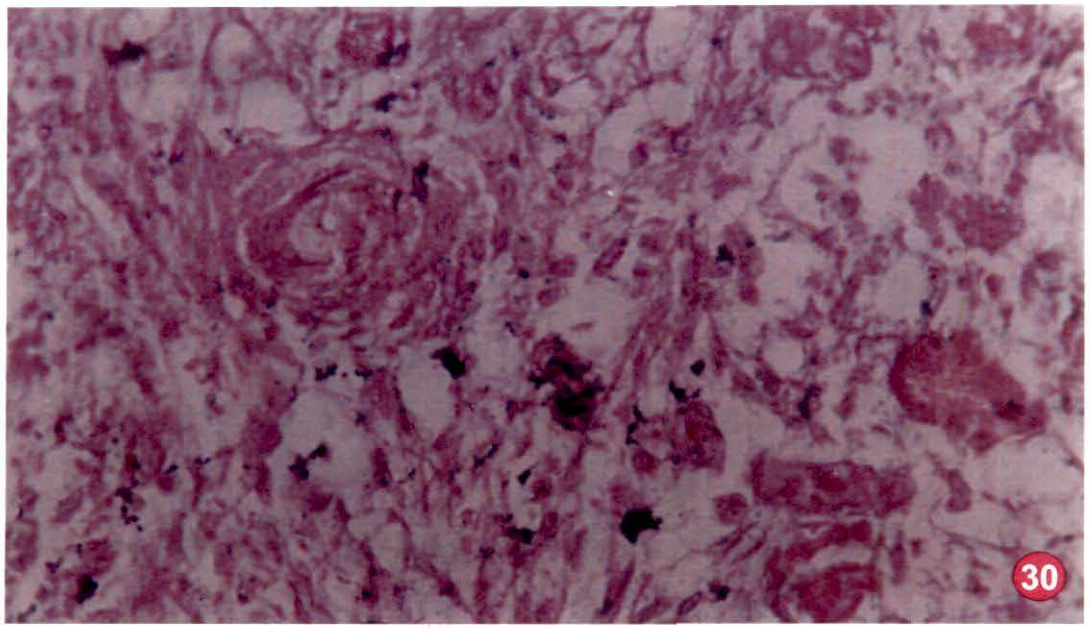
4.2.3. *Labeo bata*

Ulcer

The section of deep ulcerated area showed the complete loss of epidermis. The dermal layer lost its normal architecture and was replaced by granulomas. Several non septate fungal hyphae were observed in the dermis

Fig. 30: Section of the ulcer of naturally infected *L. bata* showing granulomatous changes.(H-E,x200).

Fig.31 & 32: Section of the ulcer of naturally infected *L. bata* showing presence of fungal hyphae in the muscle, (GMS, x 400) and (H-E, x 200).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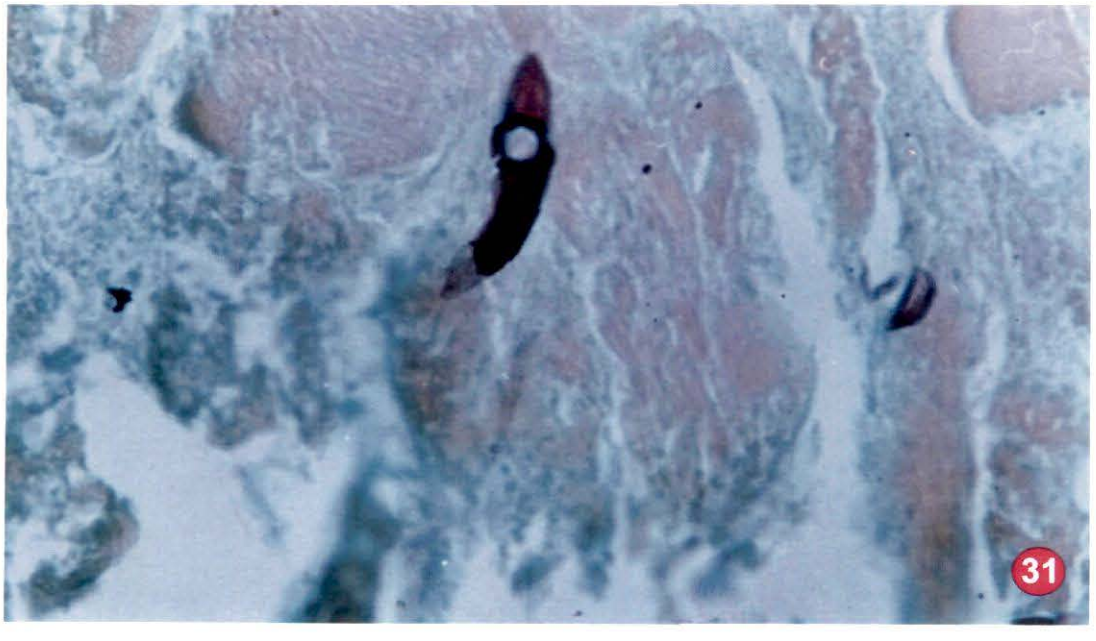
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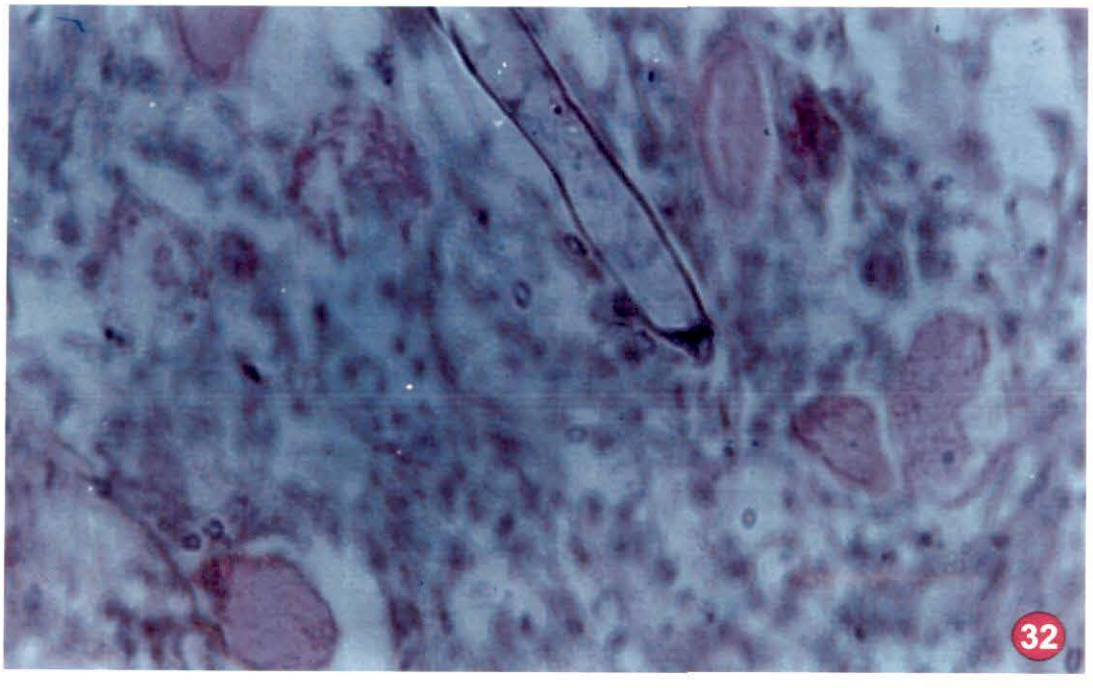
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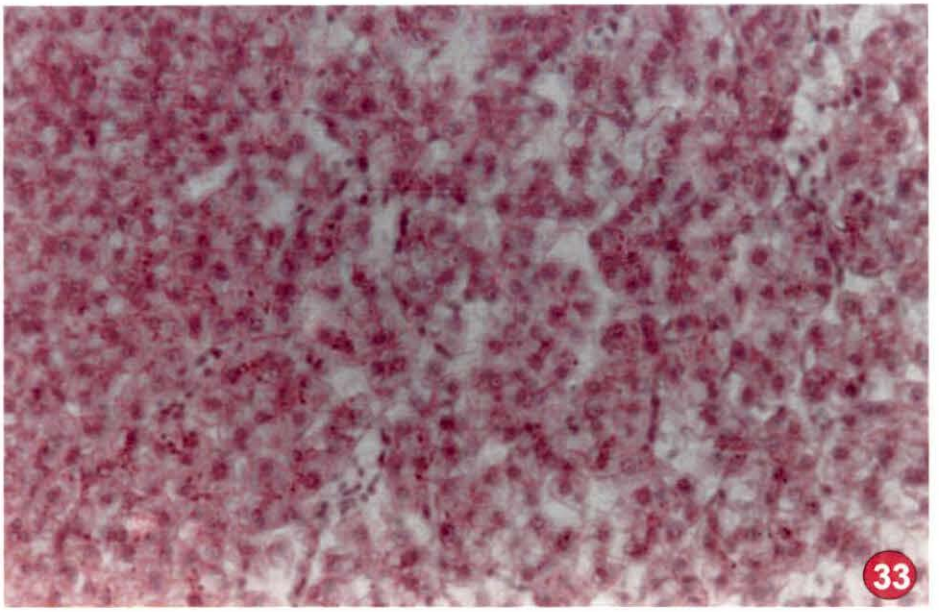
Fig. 33: Section of the liver of naturally infected *L. bata* showing vaculation (H-E, x 400).

Fig. 34: Section of the kidney of naturally infected *L. bata* showing tubular breakage and vacuolation (H-E, x 200).

Fig. 35: Section of the spleen of nnaturally infected *L. bata* showing necrotic changes (H-E x 200).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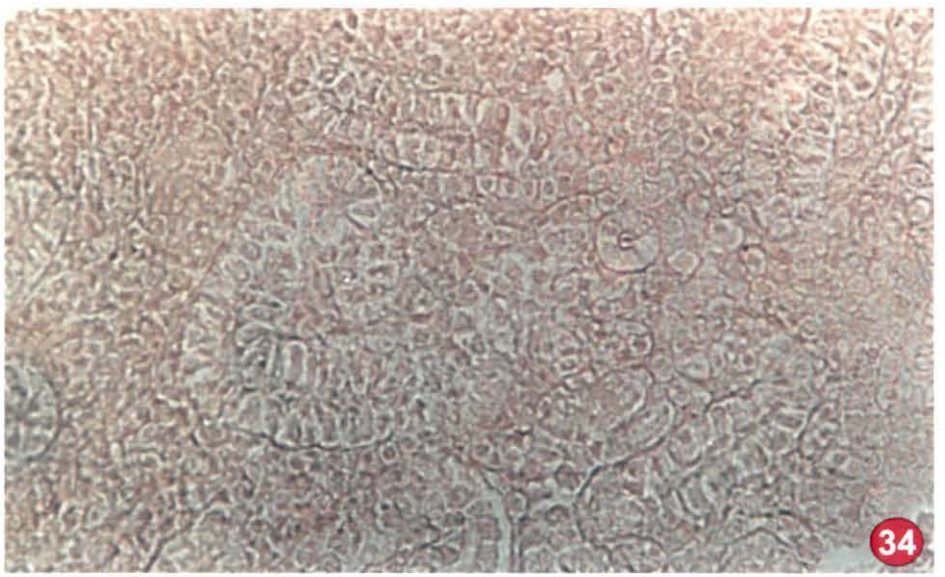
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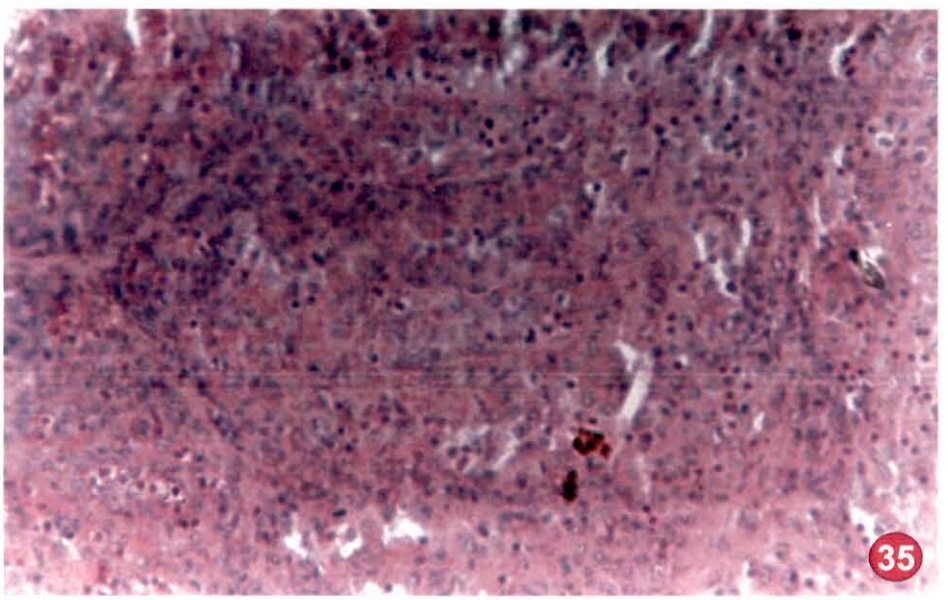
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Granuloma formation and myonecrosis were prominent in the center of the ulcer (Figs. 30, 31 & 32).

Liver

The section of liver of the naturally infected *Labeo bata* showed vacuolation (Fig. 33) and in some regions the hepatocytes were arranged in a chord like arrangement with enlarged sinusoid. Infiltration of blood capillaries were also observed in some region.

Kidney

Haemorrhages were observed in some areas of the sections of the kidney of naturally infected *L. bata*. No evidence of fungal hyphae was observed. Tubular breakage, tubular necrosis and vacuolarion of tubular cells were observed in the sections of the kidney (Fig. 34).

Spleen

In the section of spleen vacuolation were observed. In some regions haemorrhage and necrosis were also observed (Fig. 35). No fungus was detected.

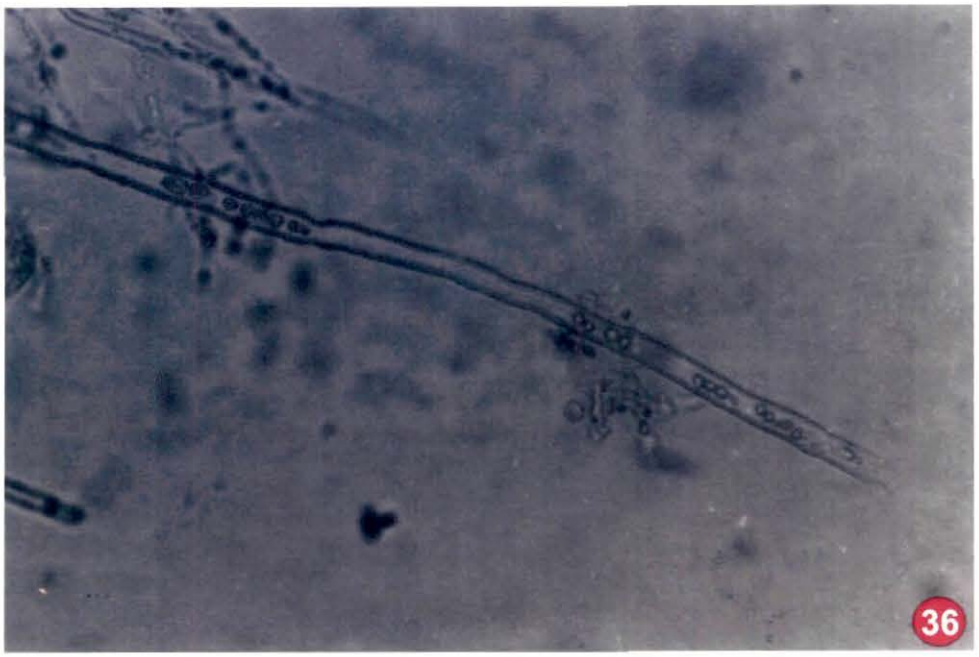
4.3. Isolation of fungus and their characterization

Newly formed hyphal tips were noticed after 6 h of incubation at 25^{0}C under inverted phase contrast microscope (CKII, Olympus). The growth of the hyphal tips were monitored routinely and next transfer was given after 24 h . The culture was made free from all contamination by repeated transfer. Finally all contamination free culture were transferred to GPA and GPYA for routine maintenance. Microscopical examination of the cotton blue stained smear of ulcer tissue revealed presence of branched, aseptate fungus mycelium in all cases. No zoosporangium was detected in the smear preparation. The mycelium of the fungal isolate grown on GPA and GPYA were also branched and aseptate but narrow in diameter (8-15μ) than those observed in ulcer tissue. The fungus grown on GPA and GPYA showed presence of terminal zoosporangia containing a single row of zoospores

Fig. 36: Zoosporangium of *Aphanomyces* sp. isolated from naturally infected *L. rohita* with a single row of primary zoospores (x200). (Isolate F_{cp1}).

Fig. 37: *Aphanomyces* sp. isolated from naturally infected *C. punctatus* showing ball of discharged zoospores at the tip of sporangium (x200) (Isolate. F_{cp1})

Fig. 38: *Aphanomyces* sp. isolated from naturally infected *C. striata* showing ball of discharged zoospores at the tip of zoosporangium under phase contrast microscope (x200). (Isolate F_{cs1}).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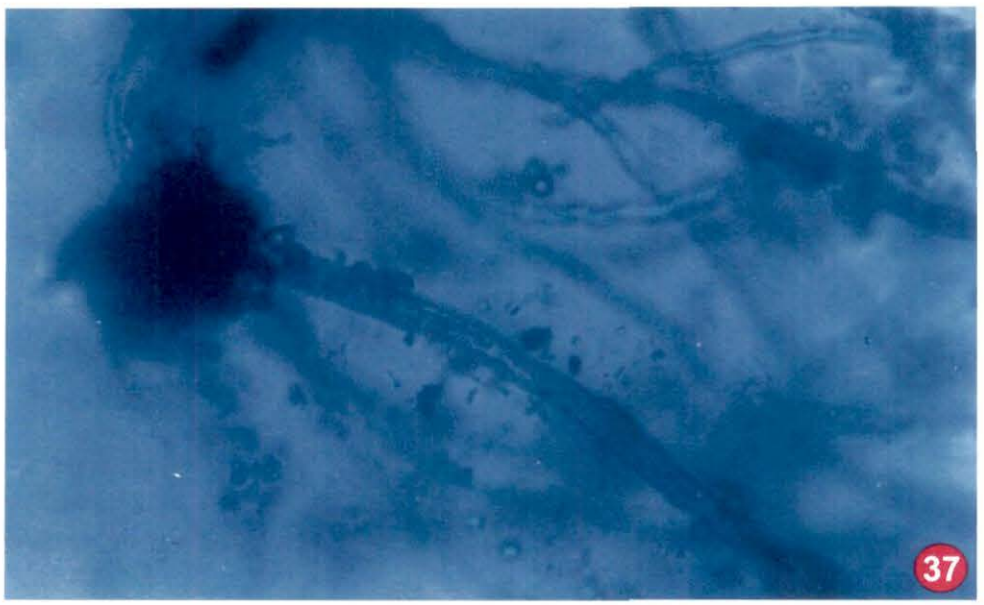
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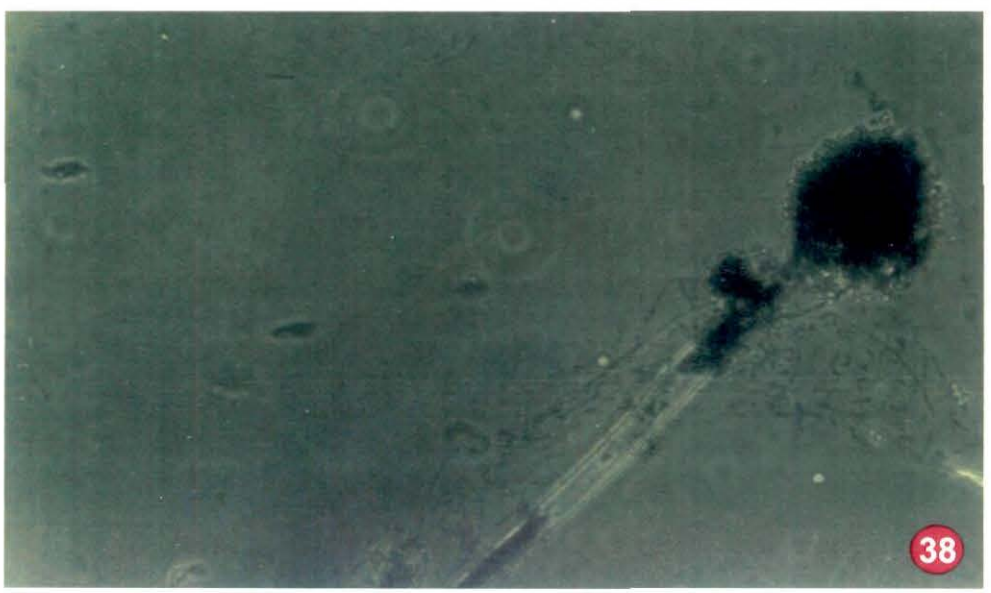
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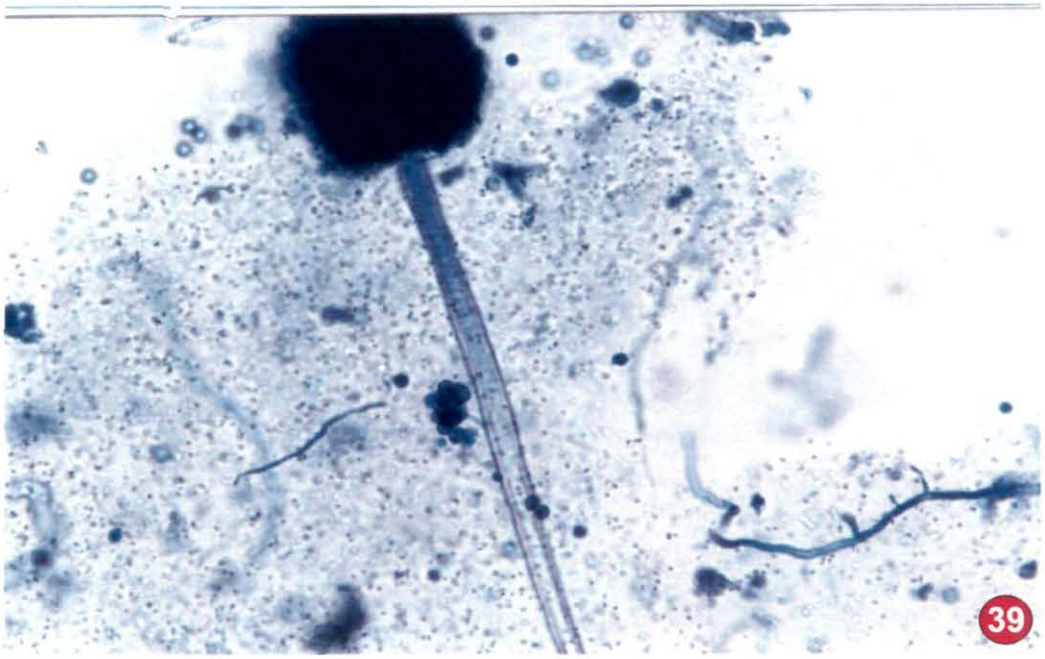
Fig. 39: *Aphanomyces* sp. isolated from naturally infected *L. rohita* showing ball of discharged zoospores at the tip of zoosporangium (x200). (Isolate F_{lr1}).

Fig. 40: *Aphanomyces* sp. isolated from naturally infected *L. bata* showing ball of discharged zoospores at the tip of zoosporangium (x400). (Isolate F_{lb1})

Fig. 41: Aseptate fungal hypha isolated from naturally infected *C. striata* (x200).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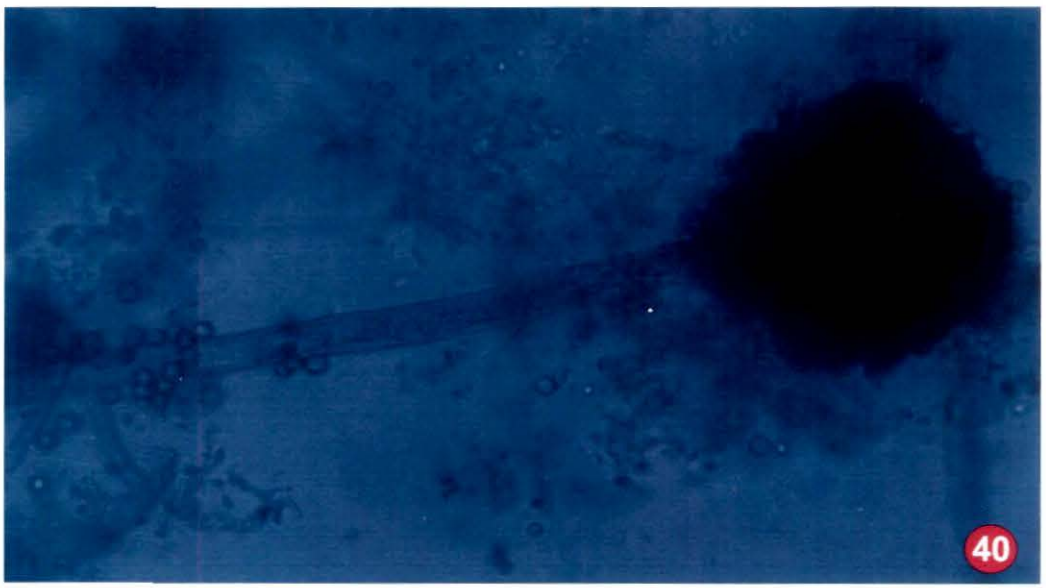
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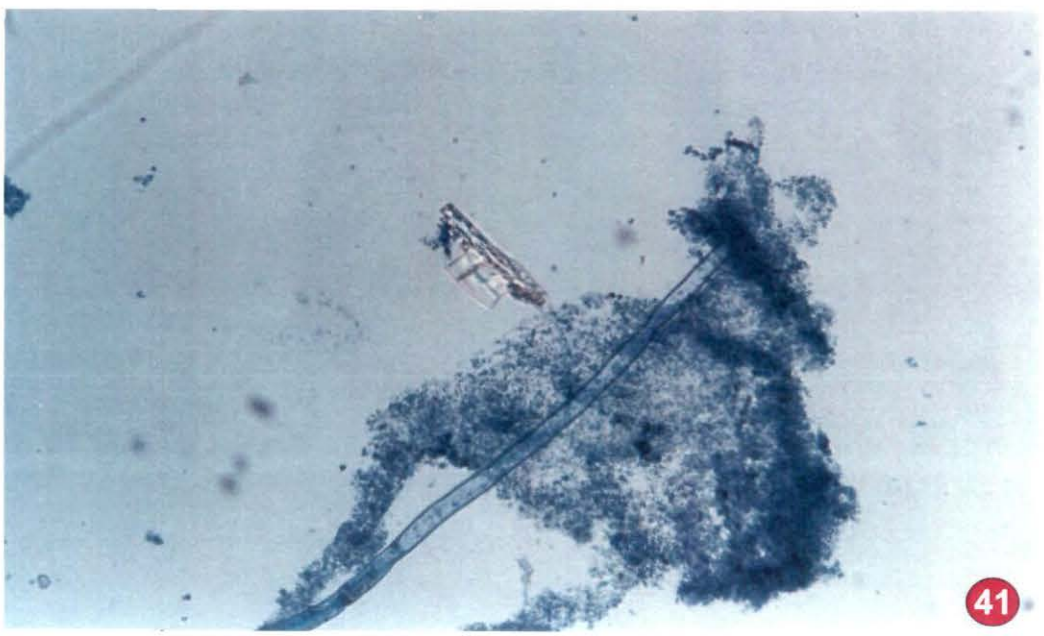
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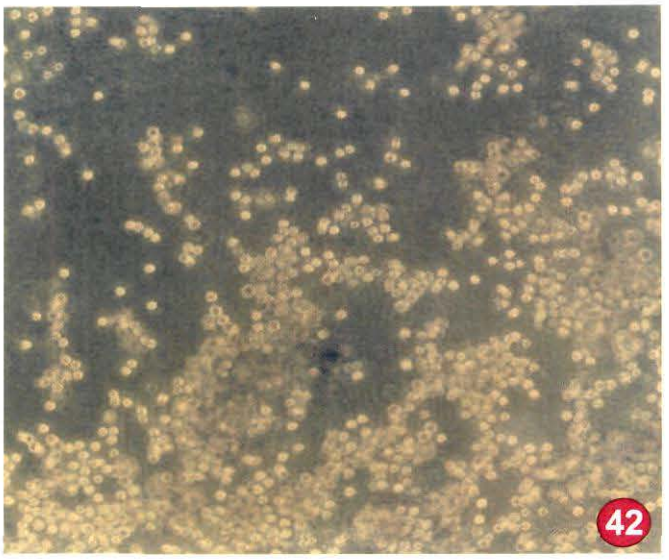
Fig. 42: Phase contrast micrograph of bacterium L_{r3} (*Micrococcus varians*).

Fig. 43: Photo micrograph of bacterium L_{r1} (*Aeromonas hydrophila*)

Fig. 44: Photo micrograph of bacterium C_{S2} (*Pseudomonas* sp.).

Fig. 45: Photo micrograph of bacterium C_{S4} (*Pseudomonas Fluorescence*).

Fig. 46: Photo micrograph of bacterium C_{s5} (*Aeromonas sobria*).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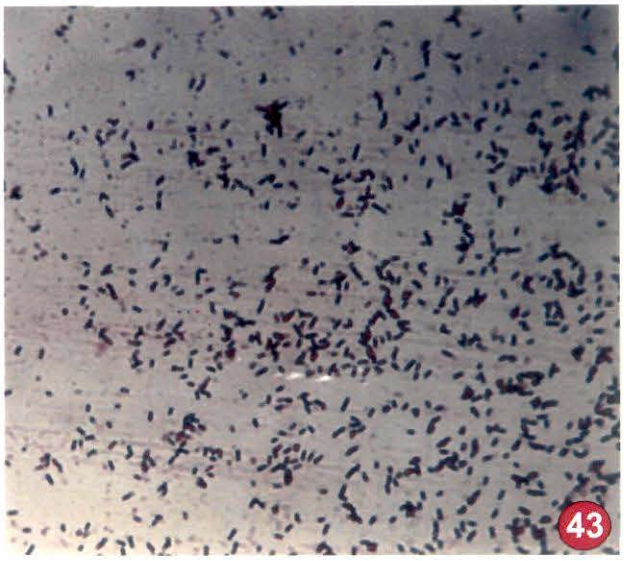
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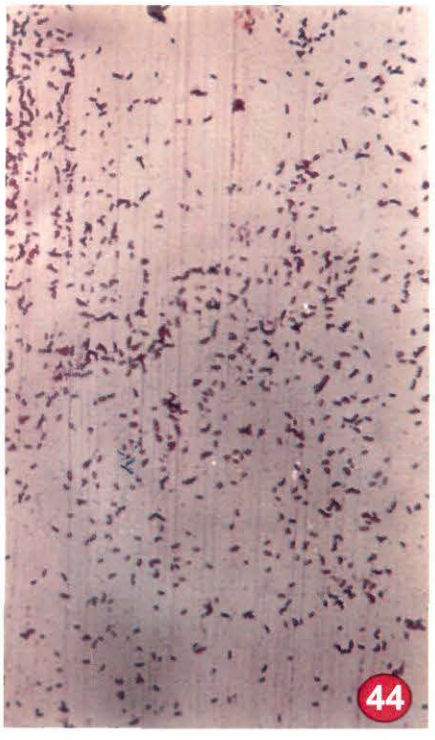
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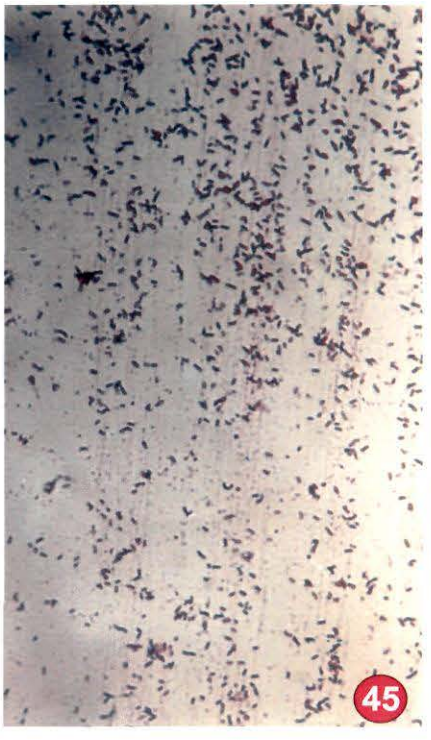
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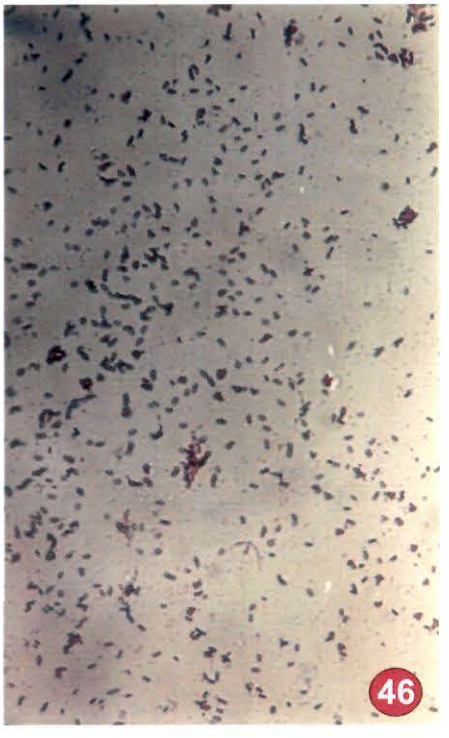
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(Fig. 36) some preparations showed ball of discharged zoospores at the tip of the zoosporangium (Fig. 37).

The fungi was identified by its asexual characteristics and particular characteristics of zoosporangia which was not wider than the hyphae (Fig.38) A single raw of primary zoospores was found with in the zoosporangia (Fig. 39). The fungal isolates grow slowly in culture media from 25°C to 30°C, but failed to grow at 37°C. *Aphanomyces* sp. was isolated from ulcer tissues of *C. punctata, C. striata, L. rohita* and *L. bata.* (Fig. 40 & 41, Table 3).

Table 3 : Details of Fungal isolates; isolated from Naturally infected fishes

Fungal Isolates	Source Fish	Date	Type of fungal isolate
F_{cp1}	*C. punchatus*	16.02.02	*Aphanomyces* sp.
F_{cs1}	*C.striata*	18.02.02	*Aphanomyces* sp.
F_{lr1}	*L. rohita*	09.03.02	*Aphanomyces* sp.
F_{lb1}	*L. bata*	11.03.02	*Aphanomyces* sp.

4.4. Isolation and characterization of bacteria

Five types of bacteria were isolated from ulcers of *L. rohita* and their morphological and biochemical characteristics were given in Table 4 and 5. respectively. Five types of bacteria were isolated from ulcers of *C. striata* (Table 6 and 7)

Among the isolates of *L. rohita* one belonged to *Micrococcus* (Fig. 42). three belonged to the genus *Aeromonas,* (Fig. 43) one belonged to the genus *Pseudomonas* and Among the isolates of *C. striata* four belonged to the genus *Pseudomonas* (Fig. 44 & 45) one belonged to the genus *Aeromonas* (Fig. 46 and Table 8).

Characterization of the bacteria isolated from EUS affected fish were done according to Barrow and Feltham (1993) on the basis of morphological and biochemical characteristics which are described in details under materials and methods.

Table 4: Morphological characteristics of bacteria isolated from ulcers of *Lebio rohita*.

Bacterial isolates	Shape	Size	Occurrence	Spore	Agar colonies	Culture in nutrient broth
L_{r1}	Rod	2.5-3.5 x 0.6-0.8μm	Singles.	Nil	Circular	Turbid
L_{r2}	Rod	2.5 – 3.6 x 0.67- .82 μm	Singles	Nil	Circular	Turbid
L_{r3}	Sphere	1.3-1.7 μm (diameter)	Singles, pairs tetrads or in irregular cluster	Nil	Spherical	Turbid with sediment
L_{r4}	Rod	2.7-3.3x 0.76-0.86	Singles		Circular	Turbid with sediment
L_{r5}	Rod	2.8-3.6x 0.63- 0.72μm	Single, pairs or in chain also	Nil	Circular	Turbid

Table 5: Bio-chemical characteristics of the Bacteria isolated from the ulcer of *Labeo rohita*.

Name of the tests	*Bacterial Isolates*				
	Lr_1	Lr_2	Lr_3	Lr_4	Lr_5
Gram Reaction	–	–	+	–	–
Motility	+	+	–	+	+
Growth at					
25°C	m	m	m	m	m
30°C	g	g	g	g	g
37°C	g	g	g	g	g
42°C	m	m	m	m	m
Indol production	+	+	–	+	–
M-R	w	+	–	+	–
V-P	–	+	–	+	–
Nitrate test	+	+	–	+	–
Gas from Glucose	+	+	–	+	–
Oxidase	+	+	+	+	+
Calalase	+	+	+	+	+
Gelatin Hydrolysis	+	+	+	+	+
O-F test	F	F	O	F	O
Acidfrom	+	+	+	+	+
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Sorbitol	–	–	+	–	–
L-Arabinose	–	–	+	–	–
m-Inositol	–	–	+	–	–
Maintol	–	–	+	–	–
Adonitol	–	–	–	–	–
Levan from sucrose	–	–	–	–	–
Arginine Hydrolysis	–	+	–	+	–
H_2S from cystein	+	+	–	+	–
Citrate utilization	+	+	+	+	+
Pigment formation	–	–	Bright yellow	–	*Green in* kings 'B' media

+ = postive; – = negative; g = good growth; m = moderate growth; o = oxidative; F = fermentative; w = weak.

Table 6 : Morphological characteristics of bacteria isolated from ulcers of *Chana striata*

Name of the bacteria	Shape & size ofcells	Shape of spores cells	Occurrence	Size	Agar colonies	Culture in nutrient broth
C_{s1}	Rod	Nil	Singles Pairs, chains	2.6-3.1x0.71-0.81μm	Circular and smooth	Turbid with Pellicle
C_{s2}	Rod	Nil	Singles, pairs, chains	2.1-2.6x0.74-0.79 μm	Circular and flat	Turbid with Pellicle
C_{s3}	Rod	Nil	Singles, pairs ichain	2.1-3.1x0.69-79 μm	Circular smooth flat	Turbid with Pellicle
C_{s4}	Rod	Nil	Singles, pairs chains	2.5-3.1x 0.71-0.8 μm	Circular smooth	Turbid with Pellicle
C_{s5}	Rod	Nil	Mostly single	2.5-3.4x 0.72-0.84 μm	Convex and circular	Turbid

Table 7: Bio-chemical characteristics of Bacteria isolated from ulcer of *Chana striata*

Name of Tests	Cs_1	Cs_2	Cs_3	Cs_4	Cs_5
Gram Reaction	–	–	–	–	–
Mortility	+	+	+	+	+
Growth at 25^0	m	m	m	m	m
30^0	g	g	g	g	g
37^0	g	g	g	g	g
42^0	n	m	n	n	n
Indole production	–	–	–	–	+
Nitrate test	–	+	–	+	+
Gas from Glucose	–	–	–	–	+
Oxidase test	+	+	+	+	+
Catalase	+	+	+	+	+
Gelatin hydroxysis	+	+	+	+	+
OF test	o	o	o	o	f
Acid form	+	+	+	+	+
Glucose	+	+	+	+	+
Fructose	+	+	+	+	+
L. - arabenose	+	+	+	+	+
Sorbitol	+	+	+	–	–
Sucrose	+	+	+	+	+
m-inositol	–	+	+	–	–
Manitol	+	+	+	+	+
Adonitol	–	–	–	–	–
Levan from sucrose	+	–	+	–	–
Argenine Hydrolysis	+	+	+	+	+
H_2S from cystein	–	–	–	–	+
Citrate utilization	+	+	+	+	+
Pigment formation	Greenish yellow in King's'B' media	Reddish Brown on Kings 'B'	Greenish yellow in Kings 'B'	Greenish yellow in Kings 'B' Media	No. Pigment ation on found in king 'B' media

+ = postive; – = negative; g = good growth; m = moderate growth; n = no growth; o = oxidative; F = fermentative; w = weak.

Fig. 47: *Channa punctatus* showing no ulcer formation after intramuscular injection with sterile saline suspension.

Fig. 48: *Channa punctatus* showing superficial ulcer formation after 48 h of intramuscular injection with R1 (fluorescent Pseudomonad).

Fig. 49: *Channa punctatus* showing moderate ulcer formation after 48 h of intramuscular injection with R2 (fluorescent Pseudomonad).

Fig. 50: *Channa punctatus* showing severe ulcer formation after 48 h of intramuscular injection with R3 (*Aeromonas caviae*).

Fig. 51: *Channa punctatus* showing severe ulcer formation after 48 h of intramuscular injection with R1, R2 and R3 in mixed condition.


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Table 8 : Bacteria isolated from surface ulcers of naturally infected fishes

Type of isolated bacteria	Source fish	No. of strains isolated	Isolate number	Total No. of strains of each type of bacteria
Aeromonas	*C. striata*	1	C_{s5}	4
	L. rohita	3	L_{r1}, L_{r2}, L_{r4}	
Pseudomonas	*C. striata,*	4	C_{s1}, C_{s2}, C_{s3}, C_{s4}	5
	L. rohita	2	L_{r5},	
Micrococcus	*L. rohita*	1	L_{r3}	1

4.5. Observation of external pathological symptoms of *Channa punctatus* treated with R1, R2, R3 in pure and mixed conditon

In the control set of fish, no ulcer formation (Fig. 47) and mortality were noticed within the 15 days of observation. The results are summarized in Table 9.

Within 24 hours of inculcation 70% of the fishes injected with the pure bacterial suspension of the three bacteria R1, R2 and R3 manifested external signs of the disease. The area around the injection site became reddish and gradually it swelled. Afterwards around the reddish area a zone of discoloration of the skin was noticed. The skin was almost intact except at the center of injection. This type of lesion was termed as superficial ulcer (Fig. 48). None of the fishes died at this stage.

Gradually the ulcers increased in size (10mm). The fish lost the scales and erosion of the skin was noticed. The fish became sluggish with irregular opercular movement. It was termed as moderate ulcer (Fig. 49). Some fishes died at this stage.

In some fish after 72 hours of inoculation the ulcers became deep and necrotic. The underlying muscle layer was affected subsequently. This type of ulcer was termed as severe ulcer (Fig.50).The fish mainly remained motionless either at the floor of the aquarium or floated near the surface.

Comparative mortality of species *Channa Punctatus* after inoculation with pure and mixed bacterial suspension of R1, R2 and R3

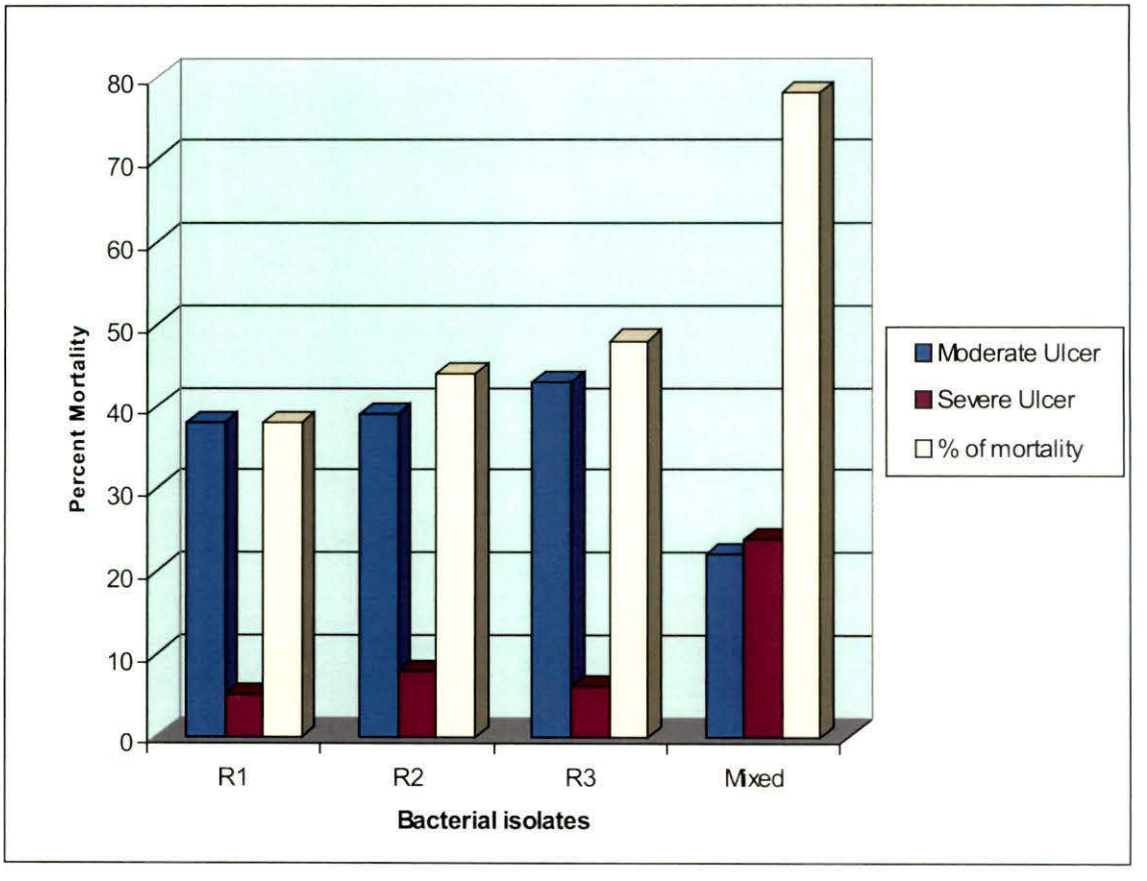

Fig. 52

Most fish at this stage died. Healing of the surviving fish was noticed after 10-12 days of injection and it took about 15 days for complete healing.

In fishes treated with mixed bacterial suspension R1, R2 and R3, about 90% of the fishes showed reddish swellings at the injection site within 24 to 48 hours of inoculation. After 48 hours, the skin was eroded and the underlying muscle layer was exposed. The scales sloughed off at this stage. The ulcer became severe within 72 hrs of inoculation (Fig.51). The fish began to die with severe ulcers at the injection site. Healing of the surviving fishes was observed after 10-12 days of inoculation and it took about 15 days for complete healing.

Table 9: Percentage mortality and nature of ulcer formation in *Channa punctatus* injected intramuscularly with saline suspensions of R1, R2 and R3 in pure and mixed condition.

	No. of fishes	No. of fishes dead	Nature of ulcer		Percentage of mortality
			Moderate	Severe	
Control*	50	0	0	0	0
R1	50	19	38	5	38%
R2	50	22	39	8	44%
R3	50	24	43	6	48%
Mixed	50	39	22	24	78%

* Control set of fishes were intramuscularly injected with sterile saline suspension. Fig.52.

Fig. 53: *Cyprinus carpio* showing no ulcer formation after intramuscular injection with sterile saline solution.

Fig. 54: *Cyprinus carpio* showing swelling after intramuscular injection of pure culture of R3.

Fig. 55: *Cyprinus carpio* showing severe ulcer formation with exposed vertebral column after intramuscular injection of pure culture of R3.

Fig. 56: *Cyprinus carpio* showing severe ulcer formation after intramuscular injection of mixed culture of three bacteria (R1, R2 and R3).

Fig. 57: *Cyprinus carpio* showing superficial ulcer formation after intramuscular injection of R1.

Fig. 58: *Cyprinus carpio* showing moderate ulcer formation after intramuscular injection of R2.


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4.6. Observation of external pathological symptoms *Cyprinus carpio* treated with R1, R2, R3 in pure and mixed condition

Nature of ulcer formation and percent of mortality in different groups of fishes treated with pure and mixed culture of bacteria were shown in (Table 10) Swelling was noticed within 24hrs, at the site of injection and its was reddish in appearance. Gradually the swelling increased in size with a rim of reddish area. The scales of the swelled area sloughed. The affected area became whitish and ultimately ulcers developed. The control fishes treated with sterile saline suspension developed no swelling and ulcer (Fig. 53). In the fishes treated with the pure culture of aeromonad (R3) and mixed culture of three bacteria (R1, R2 and R3) severe ulcer developed after 48 to 72 hrs. of injection (Figs. 54, 55 & 56). Underlying muscle layers were also severely affected. Most of the fishes died at this stage. In one fish treated with culture of aeromonad (R3) the ulcer was so severe that the vertebral column was exposed (Fig. 55). Healing of ulcers in the surviving fishes was noticed after 5-6 days of injection and took 10 to 15 days for complete healing. In fishes treated with pure culture of R1 and R2 superficial and moderate type of ulcer development respectively were observed (Figs. 57 & 58). In some R1 and R2 treated fishes swelling were regressed after 4-5 days of injection without developing into ulcers. The percent of mortality in mixed, R3, R2 and R1 treated groups of fishes were 70.0, 70.0, 40.0 and 50.0 percent respectively.

Comparative mortality of species *Cyprinus carpio* after inoculation with pure and mixed bacterial suspension of R1, R2 and R3

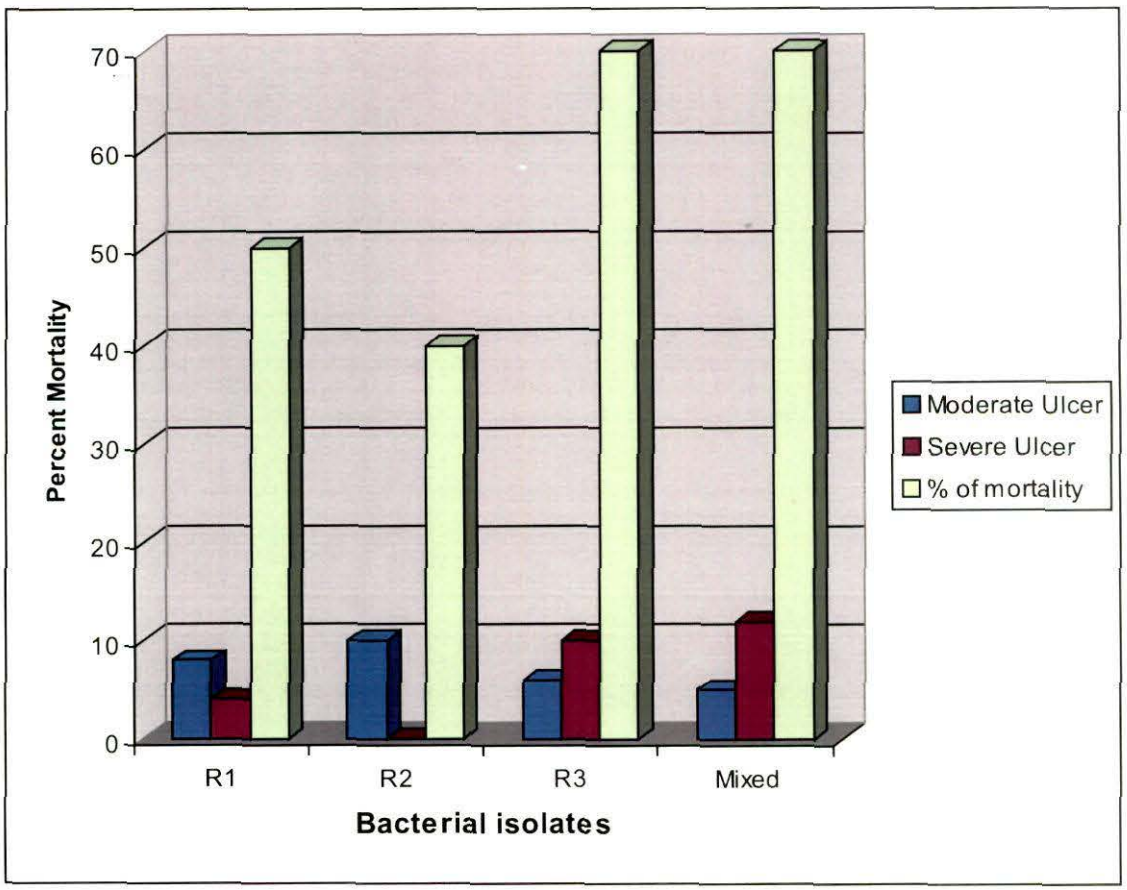

Fig. 59

Fig. 60: *Channa punctatus* showing severe ulcer formation after 72 h of intramuscular injection with fungal spore suspension.

Fig. 61: *Channa punctatus* showing formation of severe ulcer at the site of injection after 96 h of intramuscular injection with fungal spores suspension.

Fig. 62: In control set *Channa punctatus* showing no ulcer formation after intramuscular injection with sterile saline suspension.


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Table 10: Percentage mortality and nature of ulcer formation in *Cyprinus carpio* injected intramuscularly with saline suspensions of R1, R2 and R3 in pure and mixed condition. Fig. 59.

	No. of fishes	No. of fishes dead	Nature of ulcer		Percentage of mortality
			Moderate	Severe	
Control*	20	0	0	0	0
R1	20	10	8	4	50%
R2	20	08	10	Nil	40%
R3	20	14	06	10	70%
Mixed	20	14	05	12	70%

4.7. Pathogenecity test of isolated fungus; *Aphanomyces* sp. in *C. punctatus*

Within 48 h of inoculation some fishes showed red spot at the site of injection. The skin was intact at this stage. After 72 h red spot increased in size and ulcer developed (Figs. 60 & 61).

44% mortality was found in fishes treated with zoospore suspension of the isolate F_{cs1}.

In the control set of fishes no ulcer formation and mortality were noticed within 15 days of observation (Fig. 62). The results are summarized in Table 11.

Fig. 63: *Channa punctatus* showing severe ulcer formation after 72 h of intramuscular injection with isolate L_{r1} (*Aeromonas* sp.).

Fig. 64: *Channa punctatus* showing superficial ulcer formation after 72 h of intramuscular injection with isolate C_{s1} (*Pheudomonas* sp.).

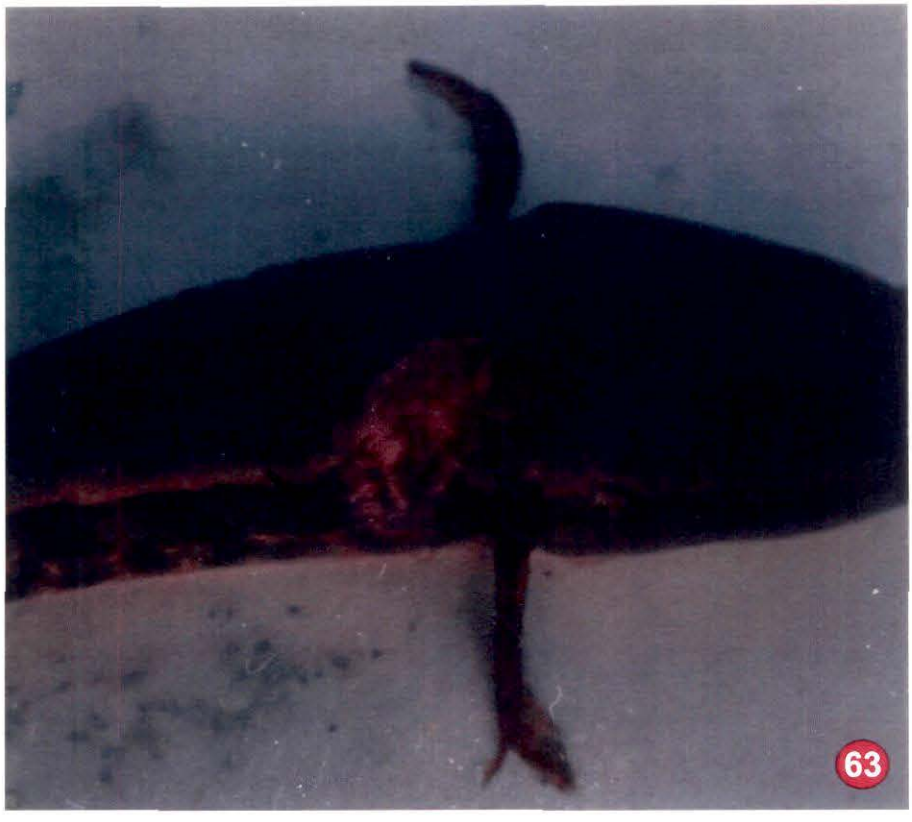
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Table 11: Percentage mortality and nature of ulcer formation in *Channa punctatus* injected intramuscularly with saline suspensions of fungal zoospore (*Afphanomyces* sp.)

	No. of fishes	No. of fishes dead	Nature of ulcer			Percentage of mortality
			Superficial	Moderate	Severe	
Control*	50	0	0		0	0
Saline suspension of fungal (*Aphanomyces*) zoospores	50	22	9	26	15	44%

4.8. Pathogenicity test of isolated bacteria on *C. punctatus*

Five *Pseudomonas* strains and four *Aeromonas* strains out of ten isolates of bacteria from EUS of *L. rohita* and *C. striata* induced ulcer in healthy fishes. Healthy fish affected within 48 h of inoculation. In control set the fish received only saline suspension. No diseases sign was noticed and no mortality was found.

All fish in which ulcers developed, however, did not die. In some fish with moderate ulcers, healing was observed. Initially, the area around the injection site turned reddish. Gradually, it swelled and around the small red spot a zone of discoloration of the skin was noticed. No notable change in the swimming behviour was observed. The skin was almost intact at this stage. After 72 h, the red spots grew in size to a moderate ulcer. Ulcers developed up to the severe stage by the inoculation of L_{r1}, L_{r2} and L_{r4} (Fig. 63) bacterial isolates and up to moderate stage by C_{s1}, C_{s2}, C_{s3}, C_{s4} and L_{r5} isolates (Fig. 64). The experimental fish began to die after 72 h of inoculation with severe ulcer at the injection site. Percentage of mortality in *C. punctatus* induced by bacterial isolates are shown in (Table 12).

Percent mortality in *Channa punctatus* induced by bacteria isolated from *Channa striata* and *Labeo rohita*

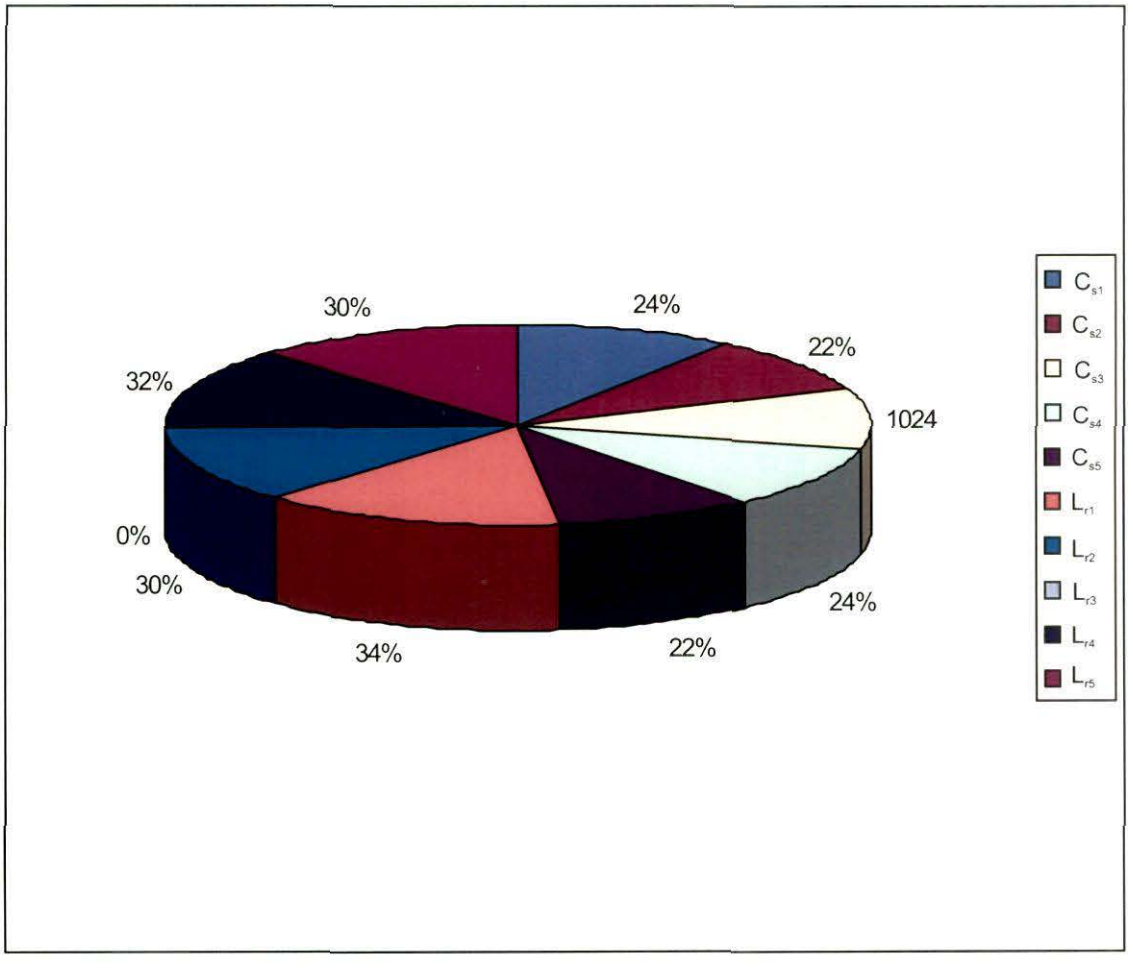

Fig. 71

Table 12: Percent mortality in *C. punctatus* induced by the bacteria isolated from *L. rohita* and *C. striata* Fig-71.

Bacterial isolates	No. of inoculated fish	No of dead fish (After 15 days of inoculation)	Percent of Mortality
Control	50	0	0%
C_{s1}	50	12	24%
C_{s2}	50	11	22%
C_{s3}	50	12	24%
C_{s4}	50	12	24%
C_{s5}	50	11	22%
L_{r1}	50	17	34%
L_{r2}	50	15	30%
L_{r3}	50	0	0%
L_{r4}	50	16	32%
L_{r5}	50	15	30%

4.9. Observation of external pathological symptoms of *C. punctatus* treated with mixed saline suspensions of zoospore of *(Aphanomyces* sp.) and bacteria R1, R2 and R3 in pure condition

In the control set of fish no ulcer formation (Fig.65) and mortality were noticed within 15 days of observation. The results is summarized in Table 13.

Within 24-48 h of inoculation with fungal spore suspension mixed with R1, R2 and R3 bacteria singly most of the experimental fishes manifested external sign of the disease. The area around the injection become reddish and ultimately turned into ulcer (Figs. 66, 67 & 68).

Fig. 65: In control set *Channa punctatus* showing no ulcer formation after intramuscular injection with sterile saline suspension.

Fig. 66: *Channa punctatus* showing ulcer formation after intramuscular injection with R1+ fungal spores suspension.

Fig. 67: *Channa punctatus* showing ulcer formation after intramuscular injection with R2+ fungal spores suspension.

Fig. 68: *Channa punctatus* showing the ulcer formation after intramuscular injection with R3+ fungal spores suspension.

Fig. 69: *Channa punctatus* showing deep ulcer formation after intermuscular injection with fungal spores suspension + bacterial isolates.

Fig. 70: *Channa punctatus* showing severe ulcer infected naturally.


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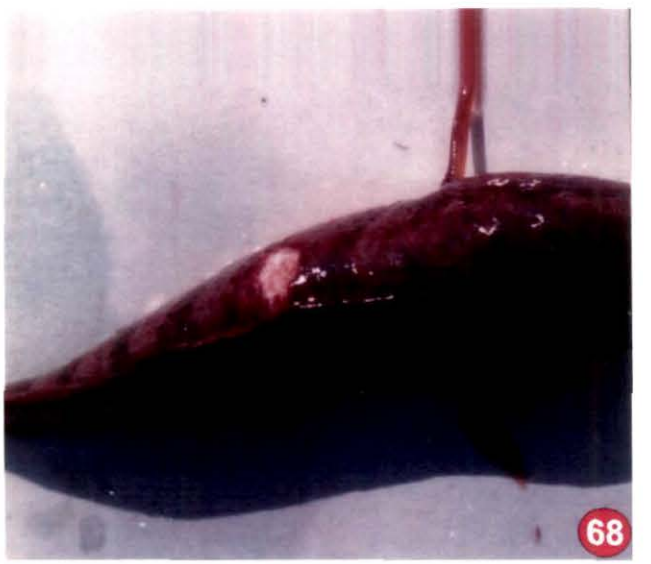
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The ulcers increased in size (15mm) by 72-96 h. . The fish became sluggish with irregular opercular movement. Some fishes died at this stage. R1 plus zoospore suspension of F_{cs1} induced 56% mortality, R2 plus zoospore suspension induced 54% and R3 zoospore suspension induced 68% mortality in experimental *C. punctatus* (Table 13).

In some fishes the ulcer became deep and necrotic (Fig.69) which were comparable of naturally infected severe ulcers (Fig. 70). In this stage fishes become motionless. Most of the fishes died at this stage with open sores on their body surface. Healing of ulcer of the fishes was noticed after 12-15 days of injection and took about 25 days for complete healing.

Table 13: Percentage mortality and nature of ulcer formation in *Channa punctatus* injected intramuscularly with saline suspensions of fungal spore (*Afphanomyces* sp.) and bacteria R1, R2 and R3. Fig.72.

	No. of fishes	No. of fishes dead	Nature of ulcer			Percentage of mortality
			Superficial	Moderate	Severe	
Control*	50	0	0	0	0	0
Saline suspension of zoospores of *Aphanomyces* sp. and R1 bacteria	50	28	11	21	14	56%
Saline suspension of zoospores of *Aphanomyces* sp. and R2 bacteria	50	27	08	09	29	54%
Saline suspension of zoospores of *Aphanomyces* sp. and R3 bacteria	50	34	09	11	27	68%

Percent mortality and nature of ulcer formation in *Channa punctatus* induced by fungal zoospore and bacteria R1, R2 and R3 mixed separately with fungal zoospore (F_{cs1})

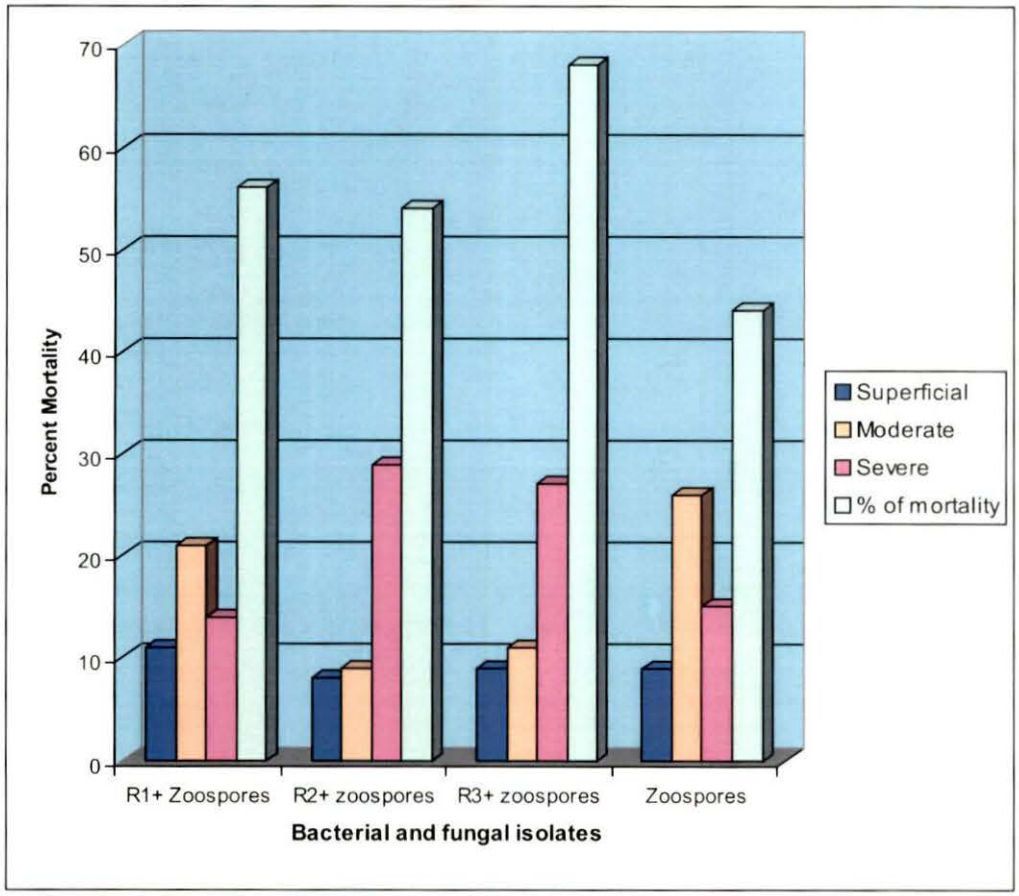


Fig. 72

4.10. Histopathological observations of experimentally infected *Cyprinus carpio* treated with R1, R2 and R3 bacteria in pure and mixed condition

Ulcer

The stained sections from all experimentally infected fishes showed various degrees of histopathoglical changes after inoculation with saline suspensions of mixed and pure R1, R2 and R3 bacteria.

Loss of epidermis was noticed in histological section of ulcers treated with mixed bacterial suspension. The dermis became highly fibrous with infiltration of blood capillaries. Necrotic changes were also found in the affected area of the dermis. A nectrotic response with degeneration of the muscle fibers was observed (Fig. 73). In fish injected with suspensions of R1, R2, and R3, degenerating changes were observed in the muscle layer. The dermis became fibrous. Giemsa stained sections showed presence of rod shaped bacteria in the muscle layer. No pathological changes were found in section of the skin of fishes treated with saline solution only.

Liver

Histological sections of the liver of the fishes treated with sterile solution showed no change (Fig. 74). Section of the liver of the fishes treated with a mixed bacterial suspension showed vacuolation (Fig.75), necrosis and infiltration of blood capillaries. Vacuolated hepatic cells and chord like arrangements with enlarged sinusoid were also observed in some regions. The liver of fishes injected with R1, R2 and R3 showed vacuolation, necrotic changes and infiltration of blood capillaries (Fig. 76). Bacteria were seen in the sections of the liver of all affected fishes.

Kidney

In the kidney of fish treated with sterile saline solution showed no change (Fig. 77). In the kidney of fish treated with mixed culture showed tubular degeneration and vacuolation of tubular cells. In certain regions

Fig. 73: Section of the ulcer showing loss of epidermis, necrotic changes in the dermal layer and underlying musculature of experimentally infected fish *Cyprinus carpio* (H-E, x400).

Fig. 74: Showing section of Liver of *Cyprinus carpio* injected with sterile saline solution (Control) (H-E, x 400).

Fig. 75: Section of liver showing vacuolation of experimentally infected fish *Cyprinus carpio* after intermuscular injection of mixed culture of three bacteria R1, R2 and R3 (H-E, x400).

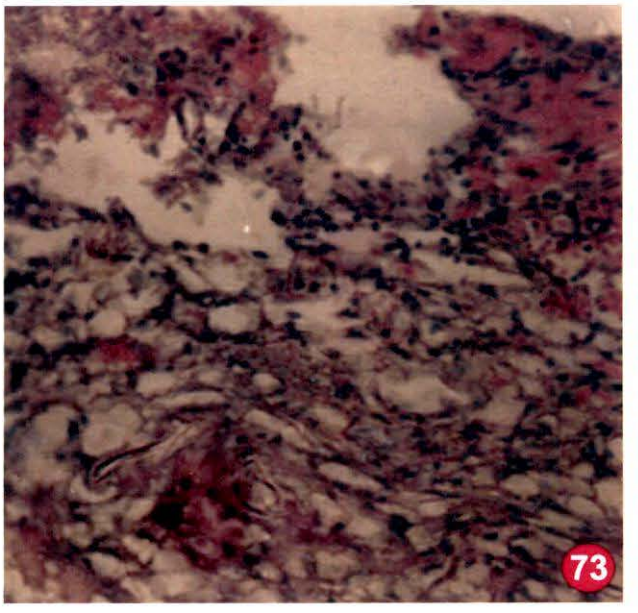
Fig. 76: Section of liver of experimentally infected *Cyprinus carpio* showing vacuolation and infiltration of capillaries after intramuscular injection of pure culture of R3, *Aeromonas caviae* (H-E, x400).

Fig. 77: Showing section of Kidney of *Cyprinus carpio* injected with sterile saline solution (Control) (H-E, x 400).

Fig. 78: Section of kidney of experimentally infected *Cyprinus carpio* showing haemorrhages and vacuolations after intramuscular injection of pure culture of R3 (H-E, 400).

Fig. 79: Section of spleen of experimentally infected *Cyprinus carpio* showing vacuolation after intramuscular injection of mixed culture of three bacteria R1, R2 & R3 (H-E, x400).

Fig. 80: Section of spleen of experimentally infected *Cyprinus carpio* showing vacuolations after intramuscular injection of pure culture of R3 (H-E, x400).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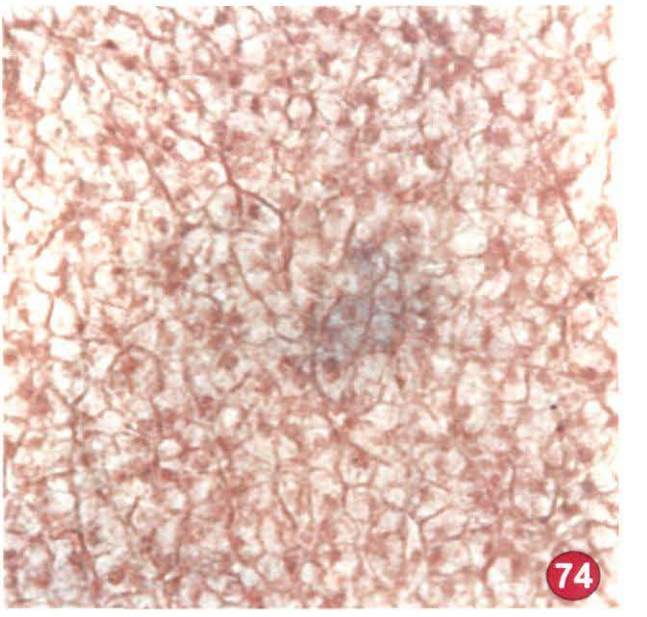
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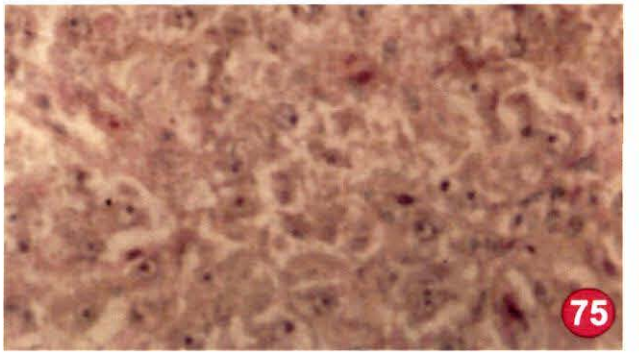
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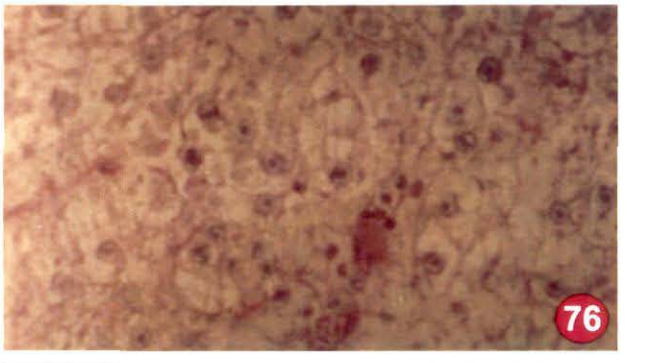
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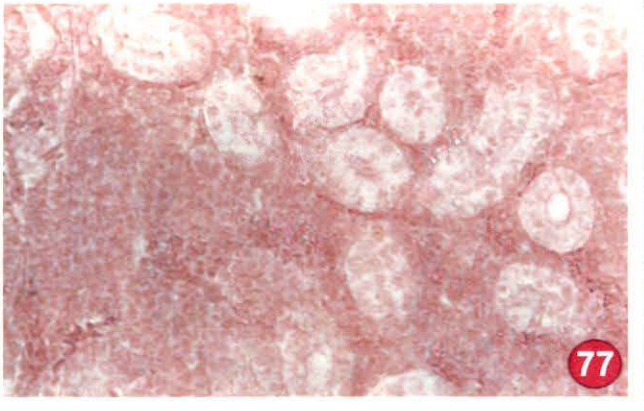
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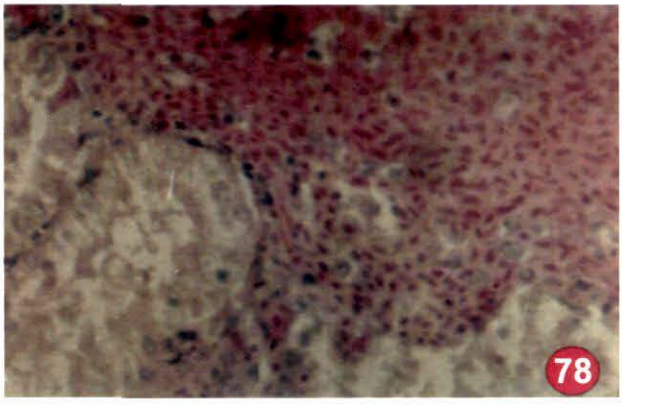
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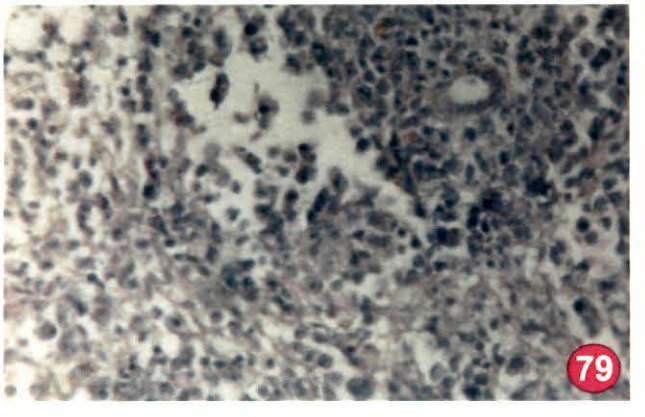
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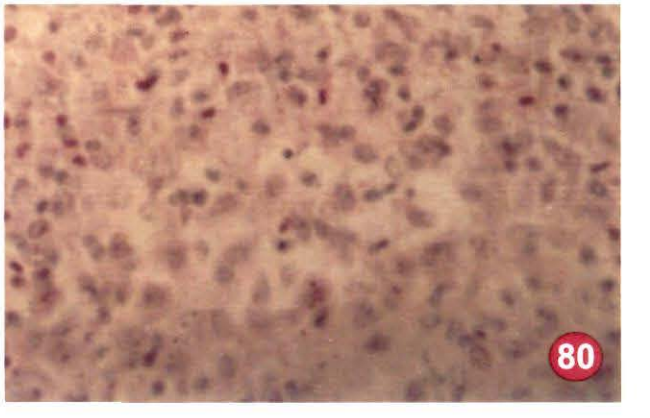
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Fig. 81: Section of ulcer of *Channa punctatus* treated with zoospores suspension of *Aphanomyces* sp. showing granuloma formation. (H-E, x200).

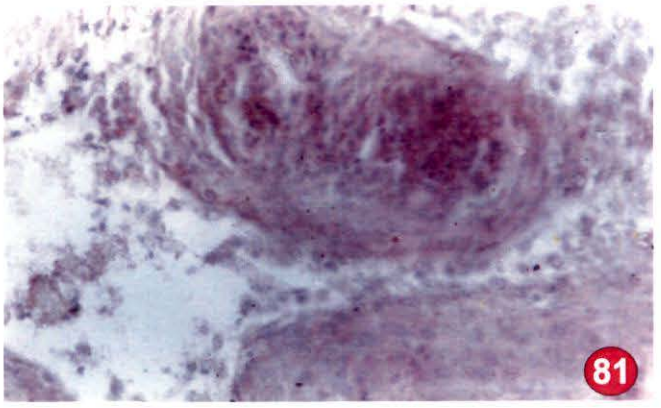
Fig. 82, 83 & 84: Section of ulcer of *Channa punctatus* treated with zoospores suspension of *Aphanomyces* sp. showing the presence fungal hyphae (H-E, x400), (GMS, x200) and (PAS, x400).

Fig. 85: Showing section of Liver of *Channa punctatus* injected with sterile suspension (Control) (H-E, x 200).

Fig. 86: Showing section of Kidney of *Channa punctatus* injected with sterile suspension (Control) (H-E, x 200).

Fig. 87: Section of Liver of *Channa punctatus* injected with fungal spores suspension showing vacuolation (H-E, x 200).

Fig. 88: Section of Kidney of *Channa punctatus* injected with fungal spores suspension showing vacuolation (H-E, x 200).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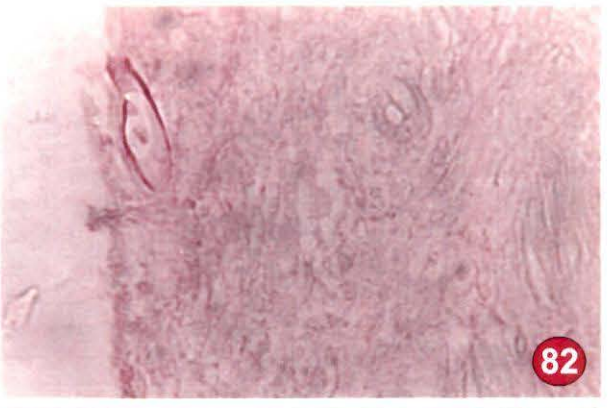
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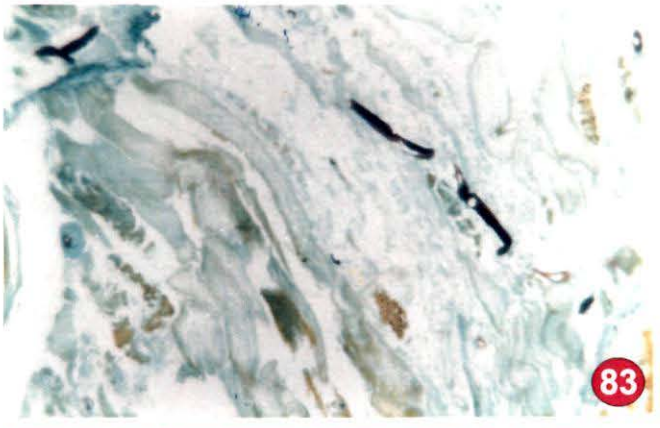
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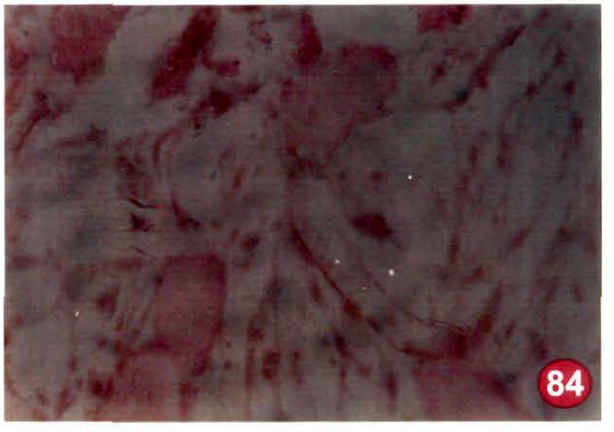
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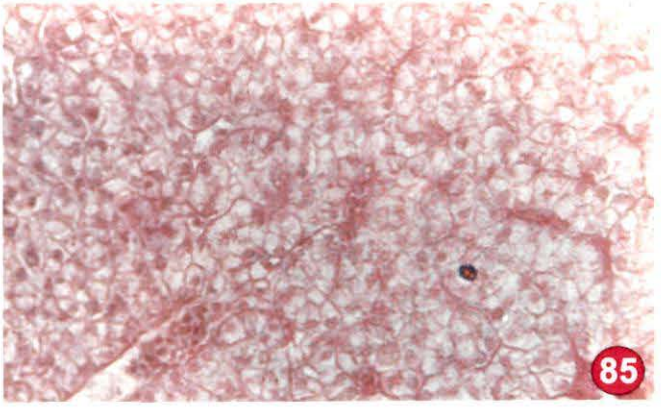
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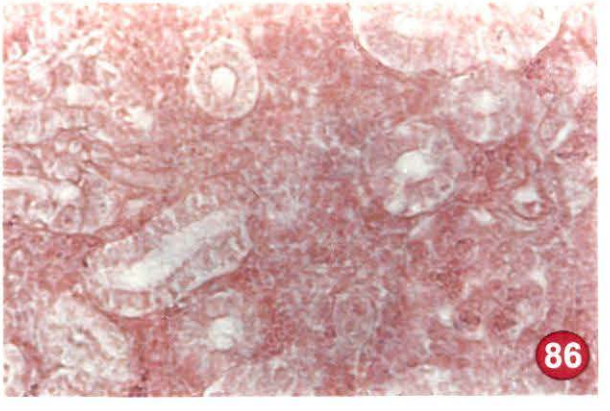
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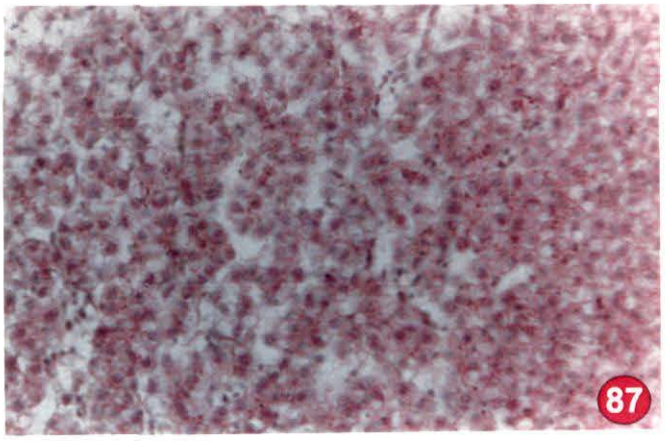
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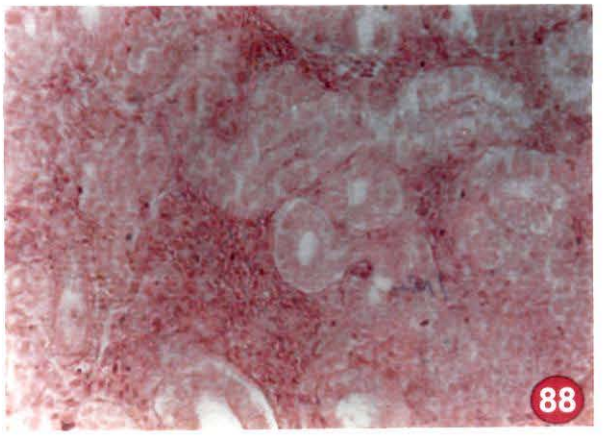
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hemorrhages were also seen. Vacuolation, tubular degeneration and necrosis in some regions were also observed in fishes treated with R1, R2 and R3. Haemosiderin laden macrophages were seen in the sections of the kidney of all experimentally infected fishes Haemorrhages and vacuolations were observed in the kidney of R3 treated fishes (Fig.78).

Spleen

In the spleen of fish treated with mixed culture showed vacuolation and degenerative changes (Fig. 79). Section of spleen of experimentally infected fishes with pure culture of R3 showed vacuolation in some region (Fig. 80).

4.11.Histopathological observations of the fish experimentally infected *Channa punctatus* treated with zoospores of *Aphanomyces* sp.

Ulcer

Histological sections of the severe ulcer showed loss of epidermis and dermis. Myonecrosis and granuloma formation were found (Fig. 81). Numerous delicate fungal hyphae were detected within the section of ulcer tissues stained with H-E, PAS and GM. (Figs. 82, 83 & 84).

Section of liver and kidney of control set showed no changes (Figs. 85 & 86). Section of liver and kidney of fishes treated with fungal spore suspension showed vacuolation (Figs. 87 & 88).

No other detectable pathological changes were found in the sections of the liver and kidney of experimentally infected fishes treated with zoospores of *Aphanomyces* sp. No evidence of the presence of fungus was also observed in the sections of liver and kidney.

Discussion

The epizootic ulcerative syndrome (EUS) first broke out in India in 1988. It is the most dreadful disease affecting various types of fresh and brackish water fishes in India. Though the degree of the occurrence of EUS is now decreased in different parts of India in North Bengal the disease has been occurring every year during a definite period of the year i.e. generally from October to March. A lot of research work is being conducted to identify the exact aetiology of the disease. In the present study an attempt has been made to find out the role of pathogenic bacteria and fungus isolated from the ulcers of EUS affected fish of North Bengal.

Two virulent strains of *Pseudomonas* sp. (R1 and R2) and one virulent strain of *Aeromonas caviae* were isolated from ulcerative air breathing fishes from North Bengal in 1988 and reported earlier to be pathogenic to *Anabas testudineus* (Pal and Pradhan, 1990). Some workers reported presence of fungus within the ulcers of EUS affected fishes. But little experimental work has been carried out on the role of fungus, *Aphanomyces* in causing ulcer in fishes in India.

During the entire study period 300 EUS affected fish of different species were collected (Sec. 4.1). The ulcers were categorized into three major types i.e. superficial, moderate and severe depending on the nature of ulcer. The histopathological observation of ulcer tissue of different fishes showed loss of epidermis granulomatoas changes and necrosis of the muscle. Sections of the liver of infected fishes showed presence of vacuolation of the hepatocytes and infiltration of blood capillaries. Sections of kidney showed tubular vacuolation and necrosis in some region. Sections of spleen showed degenerative changes and necrosis.

Callinan *et. al.* (1989) described EUS of mullets in Australia and grouped into four types: erythromatous dermatitis, intermediate types, necrotizing dermatitis and dermal ulcer. Viswanath *et. al.* (1997) categories EUS lesions into three types e.g. Type-I (early lesion), Type II (moderately advanced lesion) and Type III (advanced lesion). Chinabut and Roberts

(1990) classified EUS into five types depending on clinical patterns: Type I, Type II, Type III, Type IV and Type V.

In our study three species of naturally infected fishes were observed histopathologically (Secs. 4.2.1, 4.2.2. & 4.2.3.). The ulcer tissue of all fishes showed the presence of nonseptate invasive fungal hypae which were stained black with Grocott's methenamine silver stain (GMS) and pink with PAS stain. The sections of severe ulcer showed the complete loss of epidermis and granuloma formation with necrotic changes in the dermis and underlying musculature. In some sections budding of fungal hypha was also found.

Kumar *et. al.* (1991) reported the complete loss of epidermis of the skin at the ulcer area where dermis and hypodermis showed characteristic cyst like or nodule like granulomatous formation. The muscle layers also showed granuloma formation. The sinusoidal spaces and blood vassals were congested in the liver tissue and plenty of lymphocytes were present in the liver parenchyma. The haematopoietic tissue showed proliferation of macrophage cells which indicated the inflammatory response. Qureshi *et al.* (1995) found that the epidermis of the infected *Clarias batrachus* were destroyed completely and wherever it existed it showed spongiosis. The dermis lost its original compactness.

Aseptate fungus was isolated from EUS affected *Channa striata*, *C. punctata, Labeo rohita* and *L.* bata (Sec. 4.3). All the isolates (F_{cp1}, F_{cs1}, F_{lr1} and F_{lb1}) formed sporangia at the hyphal tip not wider than hyphae in GPY medium and ball of spores was noticed at the tip of the sporangium. They did not grow at 37^{0}C. All the isolates showed the similar characteristics and they resembled *Aphanomyces* sp.

Srivastava (1979) reported *Aphanomyces* infection in *C. batracus* in Uttar Pradesh, India, in 1974, long before the outbreak of EUS in this country.

Fraser *et al.* (1992) reported the *Aphanomyces* sp. association in RSD outbreak in Australia in 1989. Roberts *et al.* (1993) reported presence of a

morphologically typical fungus in fish affected with epizootic ulcerative syndrome taken from Asia. Paclibare *et. al,* (1994) reported fungal isolation from EUS affected fishes.Callinan *et. al.* (1995a, b) reported the isolation of *Aphanomyces* sp. from EUS in Philippines and RSD affected fish in Australia.Mohan and Shankar (1995) reported the presence of fungal hyphae with severe necrosis of the dermis and epidermis. They also reported that fungal hyphae were surrounded by one to several layers of macrophages and invested by fibroblasts. Cruz-Lacierda and Shariff (1996) reported the association of highly invasive broad (upto 24.6 μm in dioameter) branched aseptate fungal hyphae with EUS.

Lilley and Roberts (1997) reported about the isolation of *Aphanomyces* sp. from EUS infected fish of Bangladesh Philippines and Indonesia. Viswanath *et. al.* (1998) reported spread of the fungus to all direction from the center of the dermal tissue. The ulcer in the advanced stage showed mycotic granulomatous responses and massive necrotic changes associated with fungal invasion in the integument and skeletal musculature. They also reported invading fungal hyphae in the abdominal viscera and fungal granuloma in the kidney, liver and digestive tract. But in our observation we did not find the invasion of fungus in the liver, kidney and spleen.

Among the bacteria that were isolated in this study (Sec. 4.4) from the external lesion of EUS positive fishes of North Bengal the morphological features and biochemical profile of C_{s1}, C_{s2}, C_{s3}, C_{s4}, and L_{r5}, revealed that these bacteria were gram negative rods, motile, catalase positive, oxidase positive, non spore forming, attacked glucose oxidatively. All produced a yellow-green pigment except C_{s2} which diffused into the medium when grown in medium B of King. (Table 3-7). Thus they belonged to the genus *Pseudomonas* (Stanier *et al.,* 1966; Palleroni, 1984). C_{s2} satisfied most of the characteristics of *Pseudomonas fluorescens* but it produced a raddish brown pigment instead of the usual yellow-green pigment and was capable of growing at 42^{0}C. So it was not regarded as *P. fluorescens.* Some strains of *Pseudomonas aeruginosa* produces a dark red (Palleroni, 1984) or brown

(Barrow and Feltham, 1993) pigment and this bacteria is also capable of growing at 42°C. However, C_{s2} differed from *Pseudomonas aeruginosa* as it was capable of producing acid from sucrose, sorbitol and m-inositol. Ajellow and Hoadley (1976) have reported a fluorescent *Pseudomonad* capable of growing at 40°C but distinct from *P. aeruginosa*. Pal and Pal (1986a) reported isolation of a fluorescent *Pseudomonad* from epithelial carcinoma of *Anabas testudineus* which had similarities with *P. fluorescens* but was capable of growing at 40°C. The bicochemical tests that were done did not give any clue as to which species C_{s2} may belong. Thus a further detailed study is awaited in order to decide its taxonomic status and nomenclature.

Pigment production, inability to grow at 40°C and acid production from sucrose indicate that C_{s1}, C_{s3}, C_{s4} and L_{r5}, resembled *P. fluorescens*. Denitrifiction ability, inability to produce levan from sucrose and acid from sorbitol and m-inositol suggested that C_{s1} belonged to biovar III. However, this strain differed from biovar III in that it was able to produce acid from sucrose.

C_{s3} and C_{s4} could not reduce nitrate and was unable to produce acid from adonitol. The overall biochemical characters were similar to *P. fluorescens* biovar V. All reactions of C_{s3} and C_{s4} were similar except that C_{s3} could not produce acid from m-inositol.

L_{r5} was also capable of growing at 40°C it was unable to produce acid from sorbitol, m-inositol and adonitol. Thus it resembled *P. aeruginosa* (Palleroni, 1984) but it did not produce pyocyanin in King's A medium. The biochemical characteristics of L_{r5} resembled R2 (Pal and Pradhan, 1990) but it was found to be non pathogenic.

Isolates C_{s5}, L_{r1} L_{r2}, and L_{r4} were gram negative straight rods, motile, catalase positive, oxidase positive, indole positive, reduced nitrate to nitrite and attacked glucose by fermentation. Thus they belong to the genus *Aeromonas* (Popoff, 1984). They were not considered to belong to the genus *Vibrio* because all the isolates produced gas from glucose.

Among the *Aeromonas* strains isolated, C_{s5}, L_{r1} L_{r2}, and L_{r4} phenotypically resembled *Aeromonas hydrophila,* since it produced gas from glucose, H_2S from cystein, gave a positive VP test and produced acid form L-arabinose (Popoff, 1984). L_{r1} showed a negative VP reaction and also did not produce acid from L-arabinose. However, it was capable of producing gas from glucose and H_2S from cystein. Hence L_{r1} phenotypically resembled *A. sobria.*

The morphological features and biochemical characteristics of the isolate L_{r3} showed that the bacteria was coccoid, gram positive, non motile, catalase positive, oxidase positive and attacked glucose by oxidation (Kocur, 1986a). Thus it belonged to the genus *Micrococcus.* It differed from the genus *Staphylococcus* in the unlike *Staphylococcus* which ferments glucose anaerobically, the isolate L_{r3} attacked glucose oxidatively. L_{r3} was not considred to belong to the genus *Planococcus,* which is another member of the family Micrococcaceae because unlike *Planococcus* which was motile with one or two flagella, L_{r3} was non motile (Kocur, 1986c). Since L_{r3} produces a yellow pigment and was able to grow on Simmons citrate agar, it was similar to *Micrococcus varians.*

Intramuscular injections of R1, R2 (*Pseudomonads* spp.) and R3 (*Aeromonus* cavae) were given to healthy Channa *punctatus* and *Cyprinus carpio* in pure and in mixed condition (section 4.5 & 4.6). Mortality data (Table 9 & 10) showed that the mixed bacterial suspension was more virulant than the pure bacterial suspensions. Severe ulcers were induced at the injection site in fishes treated with a mixed suspension while moderate ulcers were induced in fishes injected with pure bacterial suspensions of R1, R2 and R3.

Lio-Po *et al,* 1992; Leano *et al,* 1995 found that experimentally *Aeromonads* and *Pseudomonads* caused ulcer in healthy snakehead (*C. straita*) and walking fish (*C. batrachus*) when injected intramuscularly.

Besides *Aeromonas* sp. and *Pseudomonas* sp., various other types of bacteria wre also found to be associated with epizootic ulcerative syndrome. Jhingran and Das (1990).

Ali and Tamuli (1991) isolated three types of bacteria from ulcers of four species of affected fishes and reinfection studies showed that *Aeromonas* sp. induced only mild infections, *Vibrio* sp. induced similar disease symptoms and *Micrococcus* sp. failed to induce any disease symptom.

Mukherjee *et al,* (1995) reported associateion of Chemoautotrophic nocardioform (CAN) bacteria with EUS .

Pseudomonads and *Aeromonads* were reported to be highly pathogenic while Micrococcans and Cytophagans were less pathogenic (Qureshi *et al* 1995b).

Experimentally, the zoospores of the fungal isolate (F_{cs1}) induced ulcer in helthy *C. punctatus* and produced typical granulomas in the dermis and underlying musculature (Sec. 4.7 and 4.11). It also induced histopathological changes in the liver, kidney and spleen of the experimental fishes. Presence of the fungus was detected in the sections of the ulcer of experimental fishes but no fungus was detected in the liver, kidney and spleen.

Roberts *et al.* (1993) reported that a survey of fishes affected with epizootic ulcerative syndrome taken from outbreaks in countries throughout South and Southeast Asia showed constantly the presence of a morphologically typical fungus within the lesion. When mycelium from pathogenic isolates of *Aphanomyces* sp. was placed below the dermis of healthy fishes, it caused an inflammatory response and proceeded to migrate down into the tissues of fishes inducing severe myonecrosis with chronic epithelial reaction. Chinabut *et al.,* (1995) inoculated snakeheads, *C. striata* (Bloch), with a spore suspension of *Aphanomyces* sp., isolated from EUS affected fish in Southeast Asia. Fish were held at three different temperatures : 19,26 and 31^{0}C. In the early stages of the disease degenerative changes

were observed in all samples, but inflammatory infiltrate was much more marked in fish kept at 26^{0}C and 31^{0}C while fish kept at 19^{0}C developed a severe invasive myonecrosis with limited macrophage response. From 14 to 28 days, post-infection healing become well established at 26^{0}C and 31^{0}C and surviving fish kept at these temperatures recovered completely by 28 days. The lesion was still progressing at 21 days post-injection in fishes kept at 19^{0}C and all such fish succumbed by this time. The mortalities in the fishes kept at 19^{0}C were considerably higher than in the group of fishes kept at 26 and 31^{0}C. The findings explain the mortalities from EUS occurring when water temperatures are low.

Lilley and Roverts (1997) reported that a distinct species of *Aphanomyces* was responsible for much of the characteristic pathology of epizootic ulcerative syndrome. Zoospores of 58 fungal isolates were injected intermuscularly in snakehead fishes, *C. striata* (Bloch). These fungi comprised *Aphanomyces* strains isolated from EUS affected fishes; saprophytic *Aphanomyces, Achlya* and *Saprolegnia* spp. from contaminated waters; and further saprolegniaceous fungi involved in other diseases of aquatic animals. Only the *Aphanomyces* strains isolated from fishes affected by EUS. Australian red spot disease (already considered synonymous with EUS) or mycotic granulomatosis described from Japan were able to grow invariably through the fishes muscle and produce the distinctive EUS lesions.

In the present study the mortality of experimental fishes treated with zoospore suspension was 44% at 25$\pm$1^{0}C. Chinabut *et al.* (1995) showed that at 19^{0}C the mortality of the fishes treated with zoospores was 40% and at 26^{0}C the mortality was 12%. In the present work it was also found that mortality of the experimental fishes increased (Table 13) when the fishes were treated with zoospore suspension along with a bacterial suspension (R1, R2 and R3 in pure condition) (Sec. 4.9). Similarly higher percentage of mortality was recorded in experimental fishes (Table 9 and 10) treated with mixed bacterial suspension (R1, R2 and R3 in mixed condition).

Altogether, five *Pseudomonas* sp., four *Aeromonas* and one *Micrococcus* sp. were isolated from the infected *L. rohita* and *C. straita* (4.8). Out of these ten bacteria five *Pseudomonas* sp. and four *Aeromonas* sp. were pathogenic and produced ulcer when injected intramuscularly (0.5 mL/100g body weight).

Severeal other workers (Boonyaratpalin, 1989; Subhashinghe *et al,* 1990; Chattopadhyay *et al.* 1990; McGaray *et al.* 1991; Torres *et al* 1993; Cartwright *et al* 1994) also reported that association of mainly *Aeromonas* and occasionally *Pseudonomonas* with epizootic ulcerative syndromes. Karunasagr *et al* (1989) and McGaray *et al* (1991) had recovered *Aeromonas hydrophila* and *Aeromonas sobria* more often than other bacteria. Pal and Pradhan (1990) on the other hand found *Aeromonas caviae* and two other fluorescent Pseudomonads were involved in EUS. *Aeromonas* sp. and *Pseudomonas* sp. are highly opportunistic pathogens which invade the fish once the skin barrier is breached. This however does not eliminate the fact that these bacteria are not primary pathogens.

In the present study, histological observations reveal severe hitopathoglogical changes of the liver,. kidney and spleen and at the injection site of *C. carpio* experimentally infected by the two pathogenic *Pseudomonads* (R1 and R2) and the *Aeromonad* (R3) (Sec.4.10). A nectrotic response in the dermis and degeneration of the muscle fibres was observed but no granuloma formation was detected in the dermis and underlying musculature (Figs. 73, 75, 76, 78, 79 and 80). The liver showed vacuolatin, necrosis and infiltration of blood capillaries in some regions. The kidney showed hemorrhages and necrotic changes in certain hematopoietic regions along with tubular necrosis, tubular degeneration. Bacteria were found to be present in the tissue sections of the liver, kidney and ulcer region of inoculated fishes. Presence of bacteria were also detected in the histological sections of liver, kidney and ulcer tissues of the naturally infected fishes. The changes resembled the observations by previous workers who investigated histological changes in fishes experimentally induced by pathogenic strains of

Aeromonad and Pseudomonad (Kumar *et al,* 1991; Pradhan, 1992; Pal and Pradhan, 1995; Prasad *et al,* 1995; Qureshi *et al,* 1995b).

EUS is defined as a seasonal epizootic condition of freshwater and estuarine warm water fish of complex infectious etiology characterized by the presence of invasive *Aphanomyces* and necrotizing ulcerative lesions typically leading to a granulomatous response (Roberts *et al.,* 1994). However,studies have shown that a rhabdovirus, the bacteria (*Aeromonads*) and the fungus *Aphanomyces* have been consistently associated with EUS affected snakehead *O. striata.* (Freichs *et al.,* 1986; Lio-Po *et al.,* 1992; Robert *et al.,* 1993; Callinan *et al.*, 1995). So far, none of these pathogens have been conclusively proven to be the primary agent of EUS (Freichs *et al.,* 1993; Chinabut *et al.,* 1995; Lio-Po *et al.,* 1996; Lilley and Roberts, 1997; Lio-Po *et al.,* 1998).

So from the above discussion and from our study it can be concluded that an aseptate fungus *Aphanomyces* sp. which is capable of growing by budding weaken the tissue of affected fishes exerts the pathological changes in the infected areas. Bacteria are also playing important role in the manifestation of the disease which is revealed from the severe histopathological changes in the liver, kidney, spleen and at the injection site of experimental fishes. Fungal zoospore suspension induced typical granulama formation in the dermis and muscle layer in experimental fishes very similar to the naturally infected fishes. Higher percentage of mortality was recorded in experimental fishes treated with mixed culture of bacteria and in fishes treated with zoospore suspension along with a bacteria. The mortality rate of initial outbreak of the disease was higher. It was quite possible in the initial outbreaks involvement of more than one etiological factor. The present work does not rule out the involvement of virus in the disease. So it can be concluded that both bacteria and fungus play important roles in the manifestation of EUS.

Summary

The present study dealt with "Evaluation of the role of fungi and bacteria in causing epizootic ulcerative syndrome in fishes", consisting of (1) Observation on the epizootic ulcerative syndrome (EUS) affected fishes, (2) Histopathological observations of some EUS affected fishes, (3) Isolation of fungus, *Aphanomyces* sp. from some EUS affected fishes, (4) Isolation of bacteria from naturally infected fishes, (5) Studies on the pathogenicity of R1, R2 (fluorescent pseudomonads) and R3 (*Aeromonas caviae*), isolated from EUS affected fishes on *Channa punctatus* and *Cyprinus carpio*, (6) Studies on the pathogencity of fungus (F_{cs1}) isolated from EUS positive *Channa striata,* (7) Studies on the pathogenicity of the bacteria isolated from EUS positive fishes, (8) Studies on the pathogenicity of zoospore of fungus (F_{cs1}) and bacteria (R1, R2 and R3), (9) Histopathology of experimentally infected fishes, *Channa punctatus* and *Cyprinus carpio* after intramuscular injection with R1, R2 and R3 singly and in mixed condition, (10) Histopathology of experimentally infected fishes, *Channa punctatus* after intramuscular injection of zoospore suspension of fungus, *Aphanomyces* sp. isolated from *C. striata*. (F_{cs1}).

In May 1988, EUS first occurred in India. From the beginning it affected a wide variety of fish species in both wild and cultured water. The disease spreaded all over India barring a few states by the year 1993. Some areas of North Bengal witnessed the disease recurringly every year.

Altogether 300 infected fish such as, *Channa punctatus, C. striata, C. gachua, Mystus* sp. *Clarias batrachus, Labeo rohita, Labeo bata, Catla catla, Cirrhinus mrigala, Puntius* sp. and *Macrognathus aculeatus* where collected. The ulcers were categorized into superficial, moderate and severe.

Histopathological studies were conducted on naturally EUS affected fishes, *Labeo rohita, Labeo bata* and *Channa* striata. The Grocott methenamine siliver stain and Periodic acid-schiff stained section of ulcer showed the presence of fungal hyphae. H-E stained sections showed that there was loss of epidermis. Necrosis of muscle, granulomatous changes and

blood capillary infiltration in the dermal and subdermal layers at the site of ulcers were the prominent changes. Liver showed vacuolation of hepatocytes, necrosis and infiltration of blood capillaries. In the kidney, vacuolation, tubular degeneration and haemorrhage were the major changes. Spleen also showed degenerative changes.

Fungus was isolated from the ulcers of naturally infected *C. striata, C. punctatus, L. rohita* and *L. bata*. The isolated aseptate fungus was identified as *Aphanomyces* sp. by its characteristic zoosporangia which was not wider than the hyphae A ball of spore at the tip of sporangium was observed. A single row of primary zoospores was found within the zoosporangia. In culture media the fungus became slender. The isolated fungus did not grow at 37^0C. All the four isolates (F_{cs1}, F_{cp1}, F_{lr1} andF_{lb1}) showed similar characteristics.

Bacteria were isolated from the ulcers of naturally infected *Labeo rohita* and *C. striata*. Among the isolates of *L. rohita* one belonged to the genus *Pseudomonas,* three belonged to the genus *Aeromonas,* one belonged to the genus *Micrococcus*. Among the isolates of *C. striata* one belonged to the genus *Aeromonas*, and four belonged to the genus *Pseudomonas*.

Pathogenicity of R1, R2 and R3 were tested on *Channa punctatus* and *Cyprinus carpio*. The experimental fishes were injected intramuscularly pure and mixed culture of three bacteria (R1, R2 and R3) @0.05 mL/100 g body weight (6-8 x 10^9 cells / mL) and @ 1 mL/100 g body weight (6-8 x 10^9 cells / mL) respectively. The result showed that all the three bacteria were virulent. The virulence of R3 higher than R2. The virulence of R1 was slightly low which caused superficial ulcer at the site of injection with slight redness on the surrounding. Results also showed that the mixed bacterial suspension was more pathogenic than the pure bacterial suspensions. Severe ulcers were induced at the injection site in fishes treated with a mixed and R3 suspension while superficial and moderate ulcers were induced in fishes injected with pure bacterial suspensions of R1 and R2 respectively. Both the fish species were equally susceptible to R1, R2 and R3 when injected intramuscularly either in pure or in mixed form.

The pathgenicity studies with the zoospores of isolated fungus (F_{cs1}) *Aphanomyces* sp. showed that the isolated fungus was pathogenic and it induced ulcer at the site of injection and caused 44% mortality in experientally infected fishes, *Channa punctatus*.

Pathogenicity studies with the bacterial isolates showed that nine strains of bacteria belonging to the genus *Aeronomonas* and *Pseudomonas* were virulent. *Micrococcus varians* was non pathogenic. Virulent L_{r1}, L_{r2}, L_{r4}, and C_{s5} belonging to *Aeromonas* and C_{s1}, C_{s2}, C_{s3} C_{s4} and L_{r5} belonging to *Pseudomonas* were found to be pathogenic.

Pathogenicity studies with mixed suspension of fungal zoospore (F_{cs1}) and bacteria (R1, R2 and R3) in pure condition showed that zoospore and R3 suspension is more virulent than that of the suspension of zoospore and R1. Zoospore and R2 suspension is less virulent than zoospore and R1 suspension. The pathogenicity studies showed mixed suspension of fungal zoospore (F_{cs1}) and R3 is most virulent.

Histopathological studies were conducted on *Cyprinus carpio* after intramuscular injection given to healthy fishes with pure and mixed bacterial suspension of R1, R2 and R3. Results showed that, there was loss of epidermis, muscle necrosis, degenerative changes and blood capillary infiltration in the dermal and subdermal layers at the site of injection. Liver showed vacuolation of hepatocyes, necrosis and infiltration of blood capillaries. In the kidney, vacuolation, tubular degeneration and haemorrhage were major changes. Haemosiderin laden macrophages were detected in the kidney of all infected fishes. The degree of pathological changes were comparatively less in the section from fishes injected with pure bacterial suspension than the sections from fishes injected with mixed bacteria.

Histopathological studies were conducted on *C. punctatus* after intramuscular injection with the zoospores of isolated *Aphanomyces* sp. The loss of epidermis and dermis of the skin of ulcerative area were noticed but the dermis showed severe changes wherever it was present. Severe

myonecrosis and typical granuloma formation were found. Aseptate fungal hypae were found in the dermis and underlying musculature. In the liver vacuolation and haemorrhages were observed. Necrotic and degenerative changes were observed in the kidney and spleen. No evidence of fungal hyphae were observed in the liver, kidney and spleen.

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