

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 *Heteropneustes 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.



Fig. 10: Naturally infected *Macrogathus aculeatus* showing severe ulcer on the dorsal region of the body.

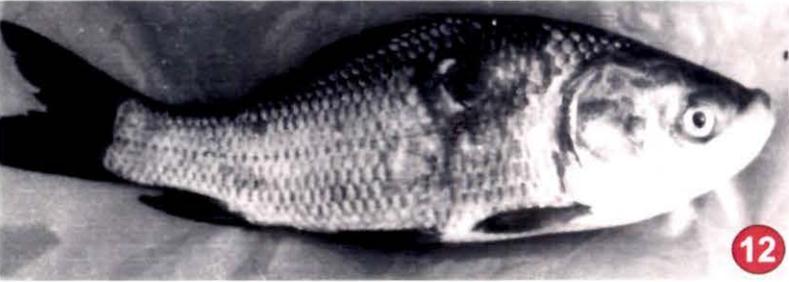
Fig. 11: Naturally infected *Labeo rohita* showing deep necrotic ulcer on the body.

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.



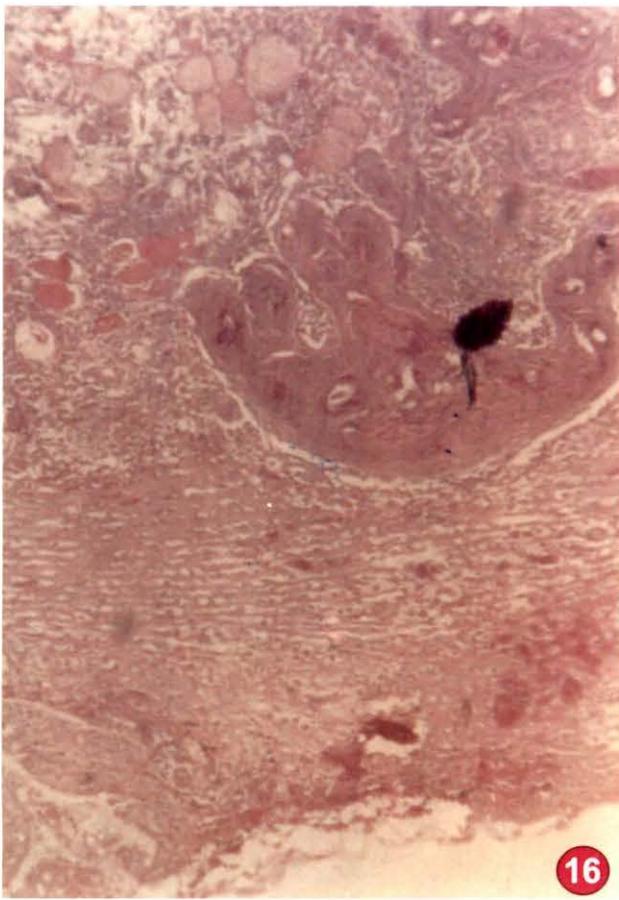
4.1. Observation of the fish affected with epizootic ulcerative syndrome

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).

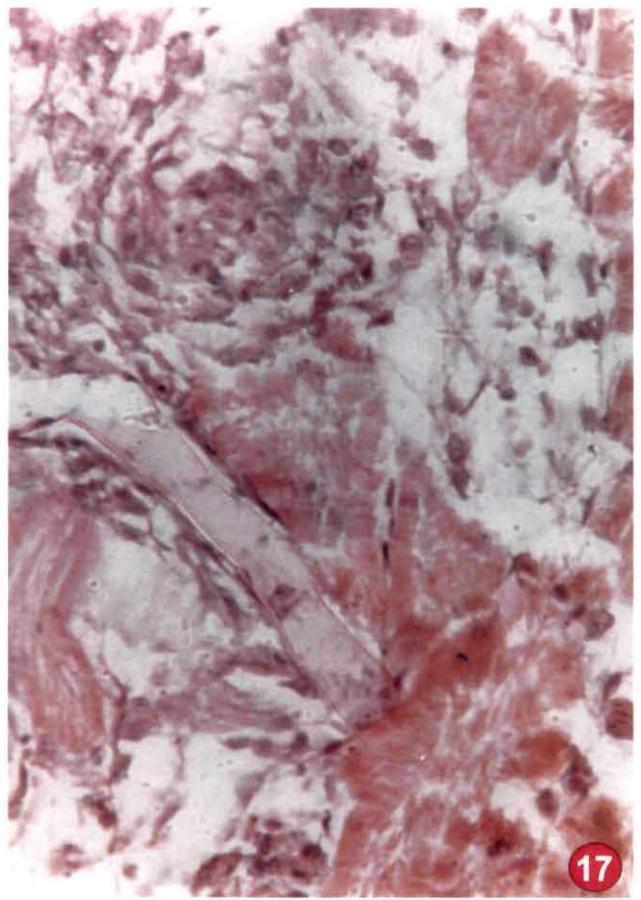
Table 2 : Collection of diseased fish

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



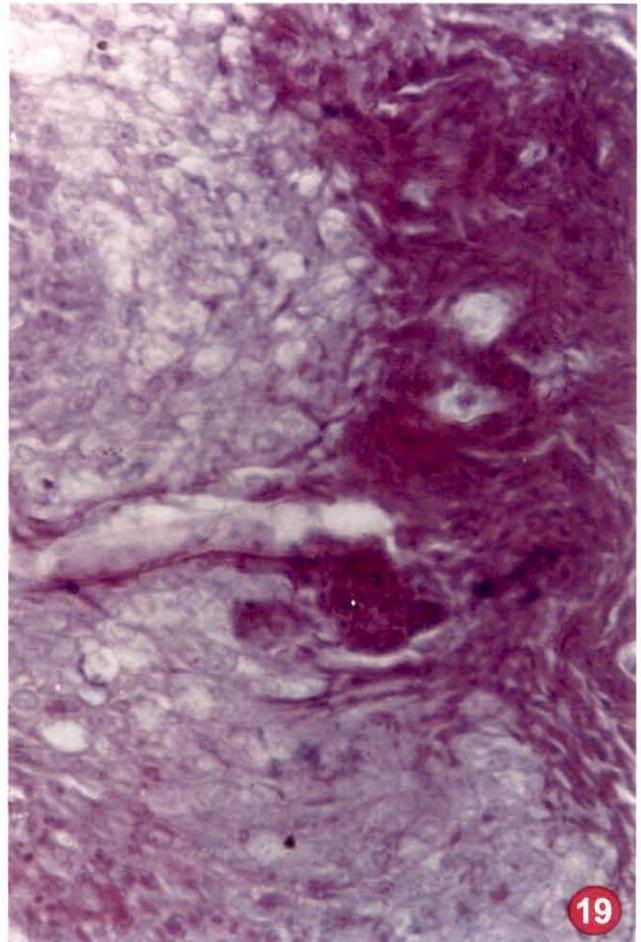
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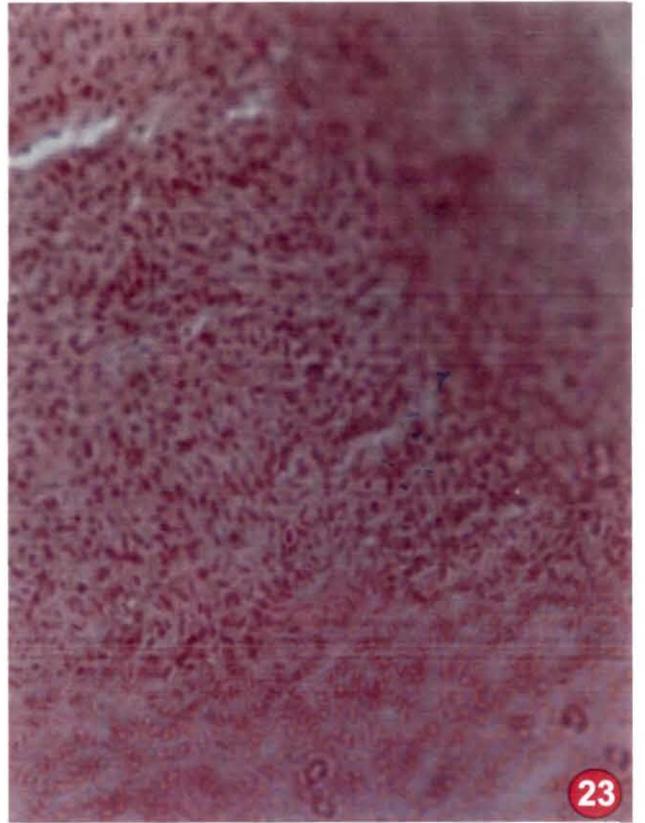
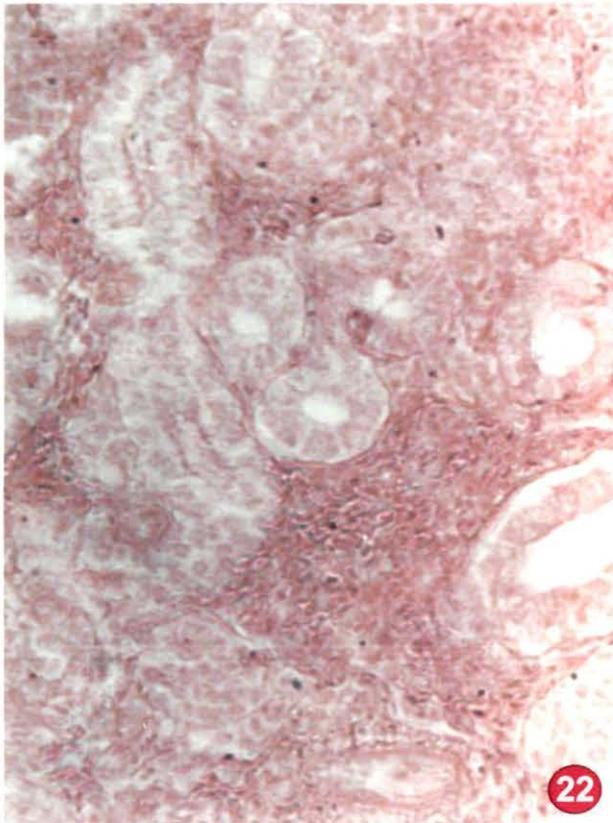
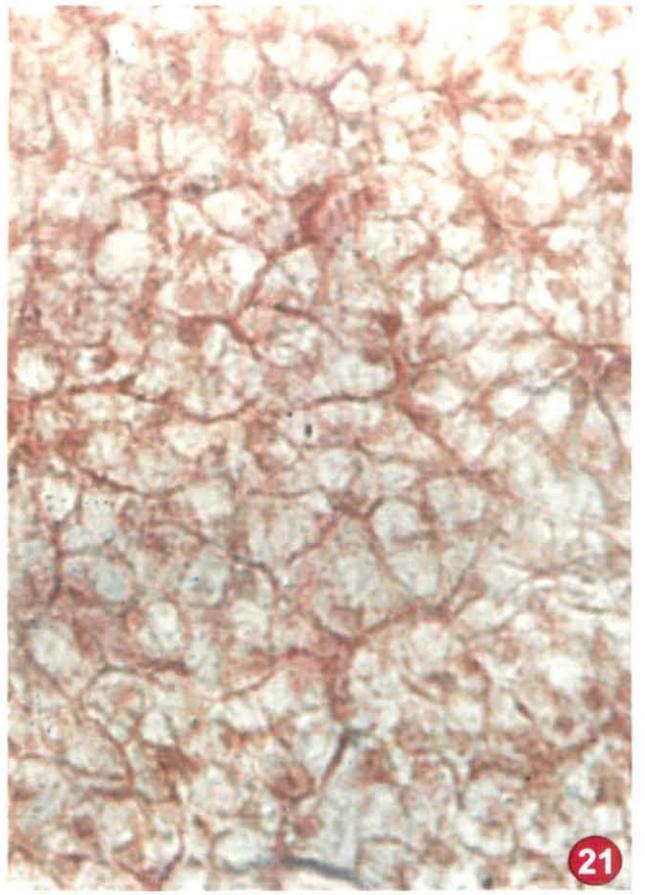
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Fig. 20: Section of the ulcer of naturally infected *C. striata* showing presence of fungal hypha with bud (GMS, x 400).

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).



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 underlying 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 haemopoietic regions and haemorrhages were observed in the section of the kidney. Tubular vacuolation were also observed (Fig. 22).

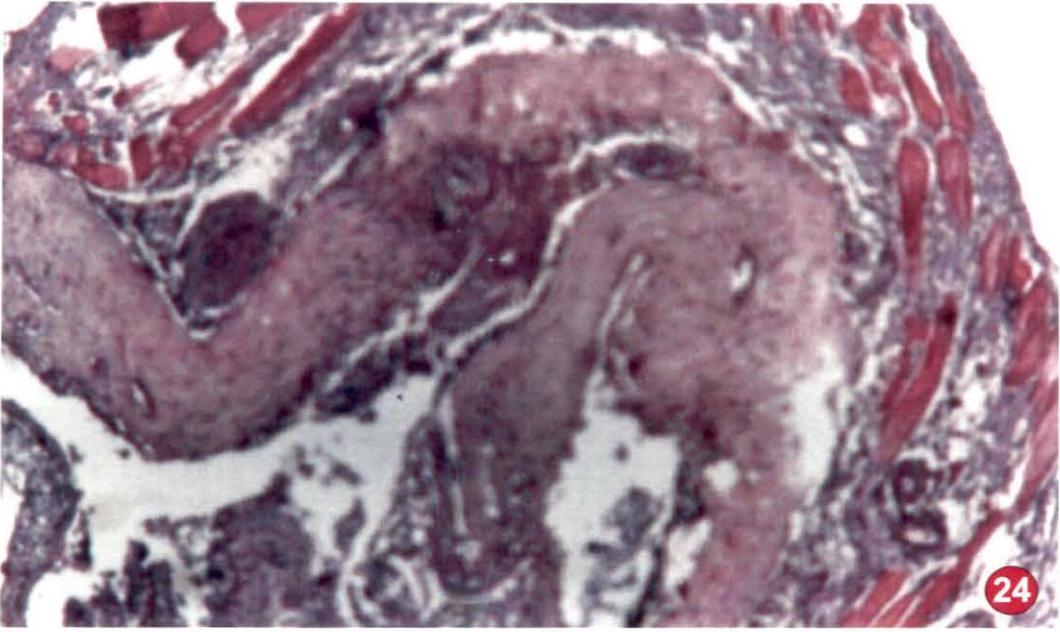
Spleen

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

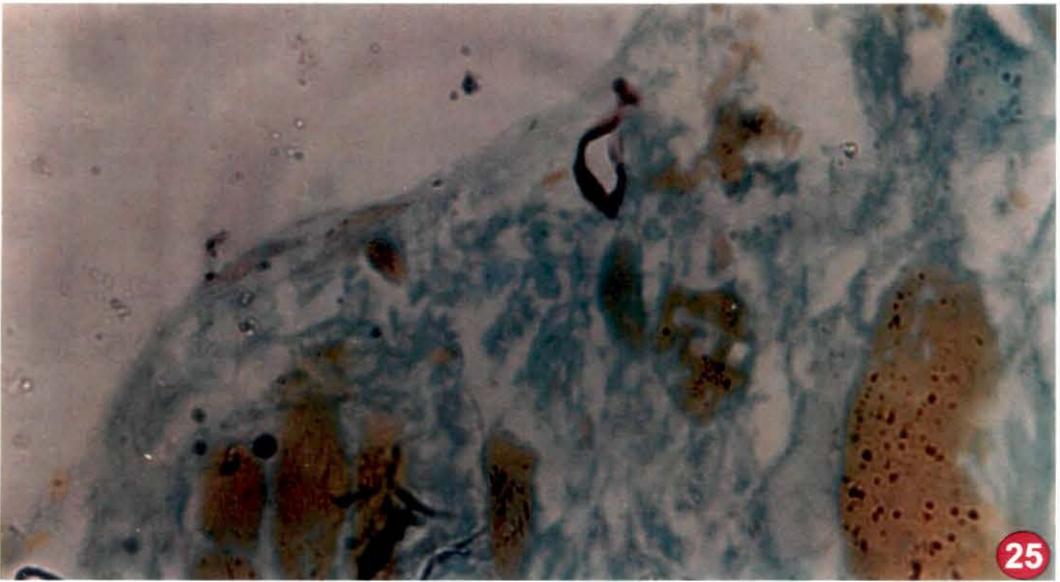
Fig. 24: Section of the ulcer of naturally infected *L. rohita* showing loss of normal architecture of epidermis and musculature (H-E, x 400).

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

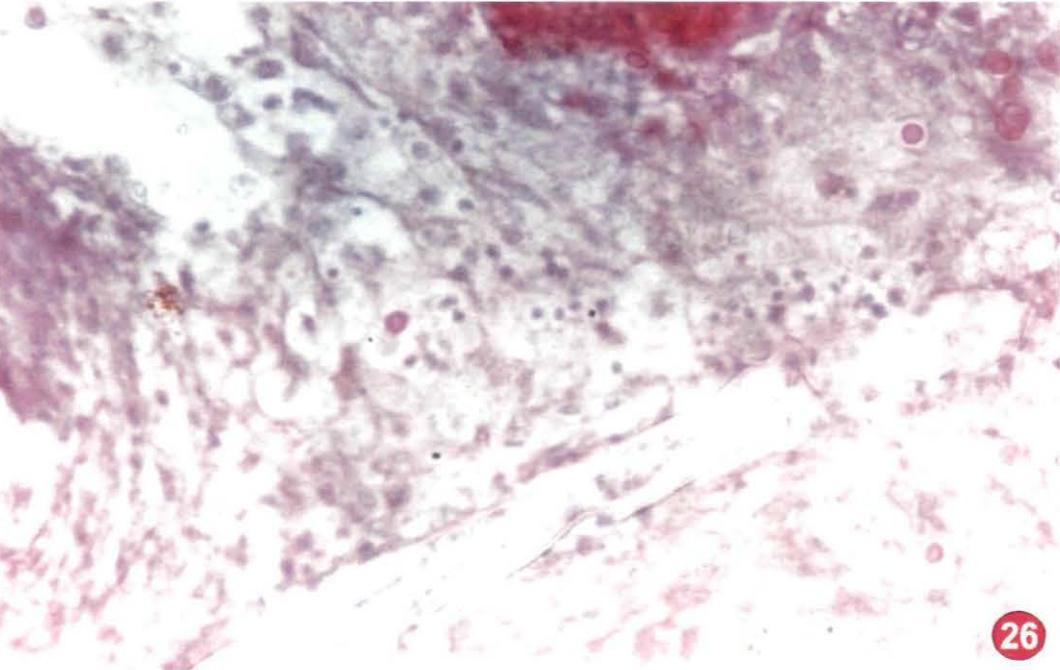
Fig. 26: Section of the ulcer of naturally infected *L. rohita* showing presence of fungal hypae (H-E, x 400).



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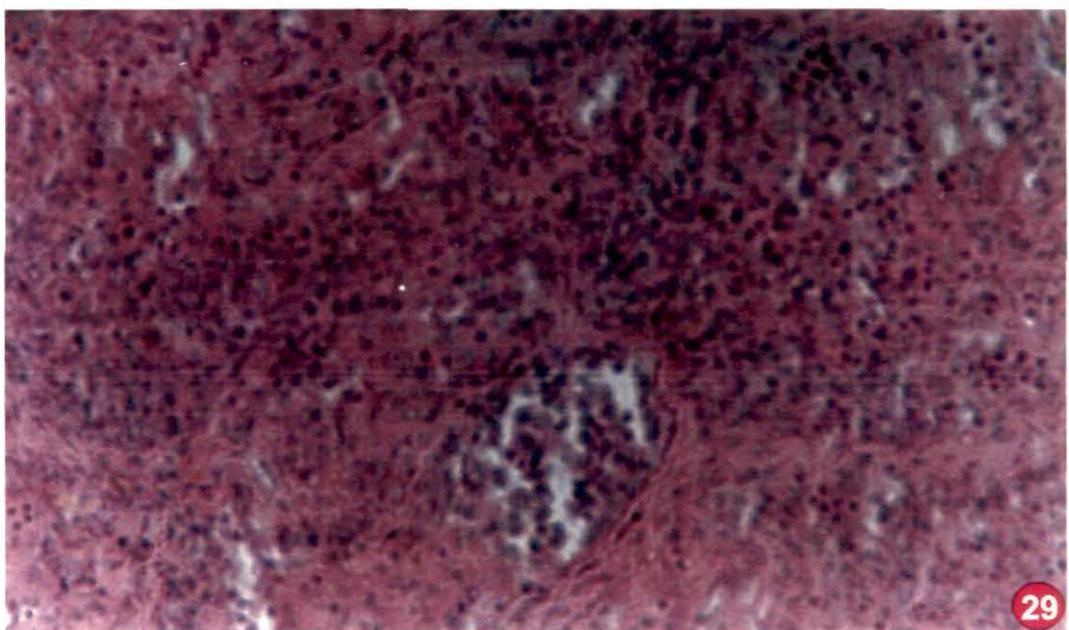
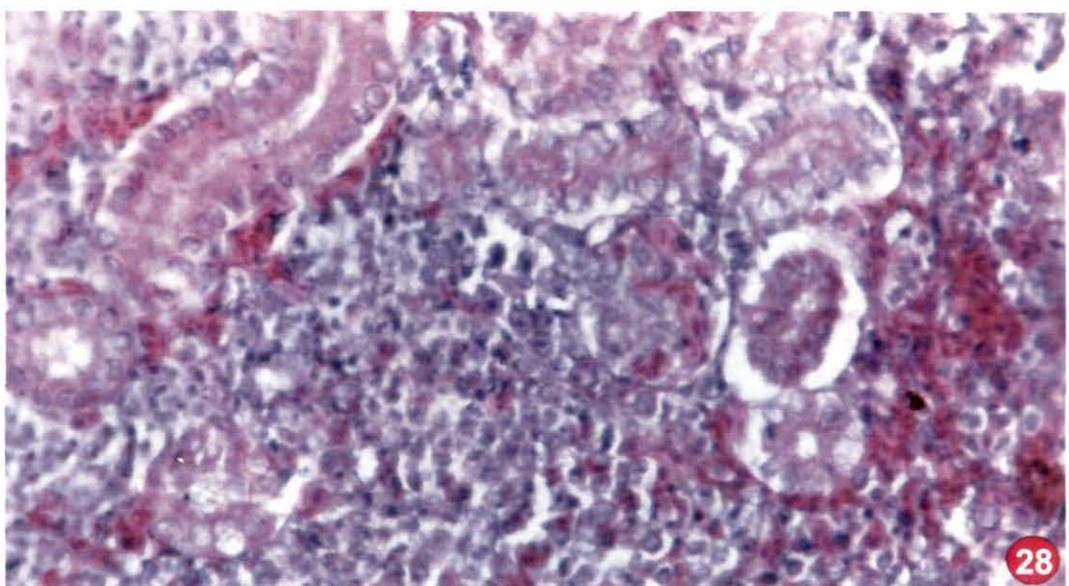
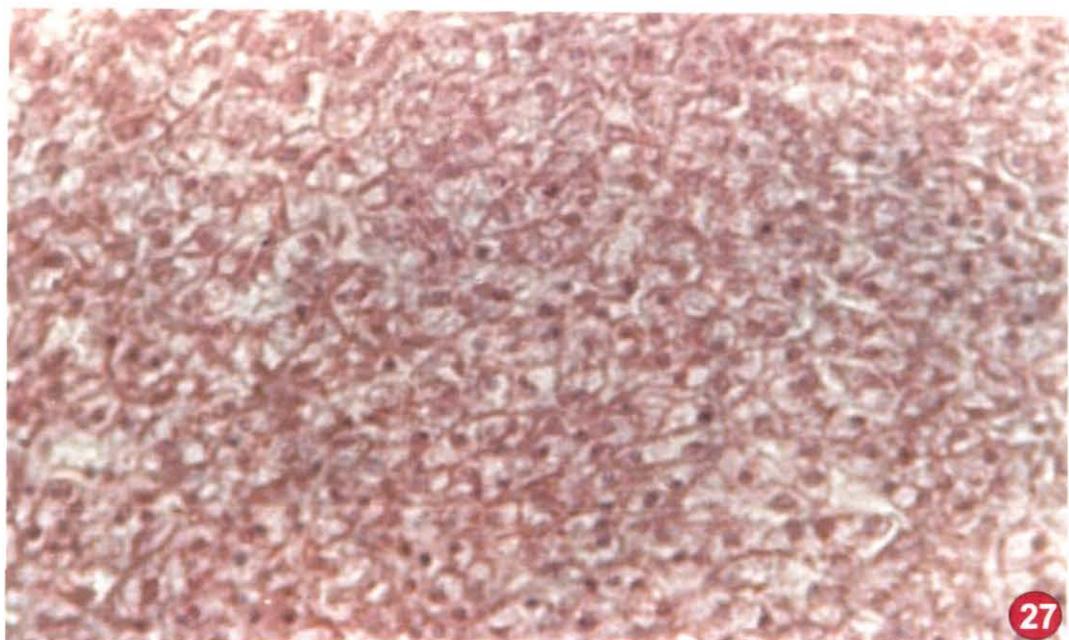


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Fig. 27: Section of the liver of naturally infected *L. rohita* showing degenerative changes (H-E, x 400).

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).



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 Haematoxyline 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 demis

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).

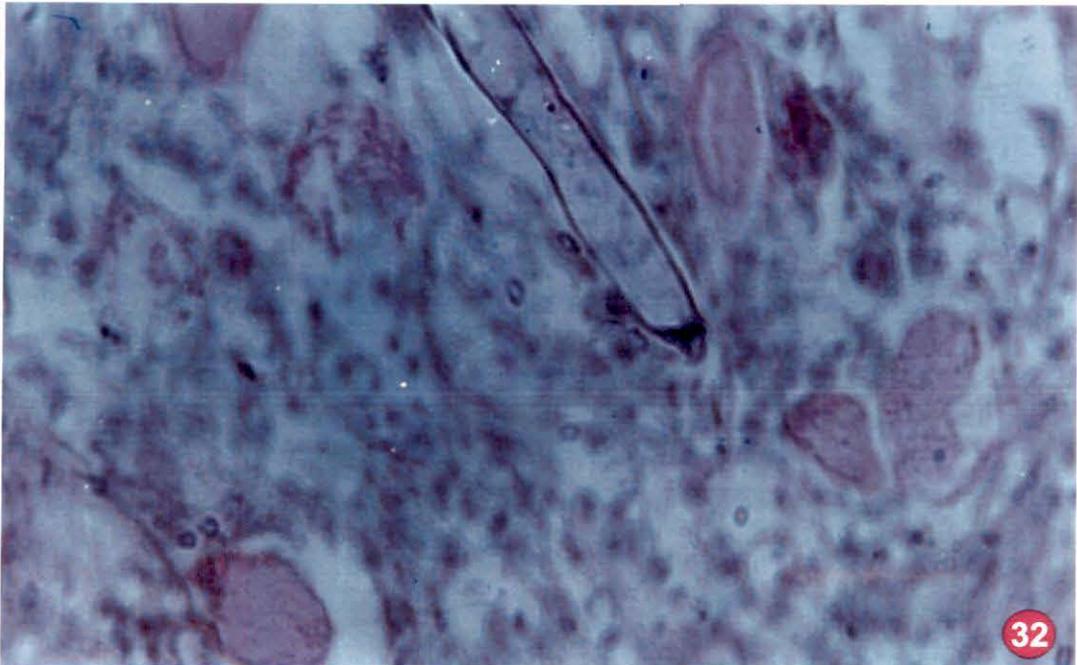
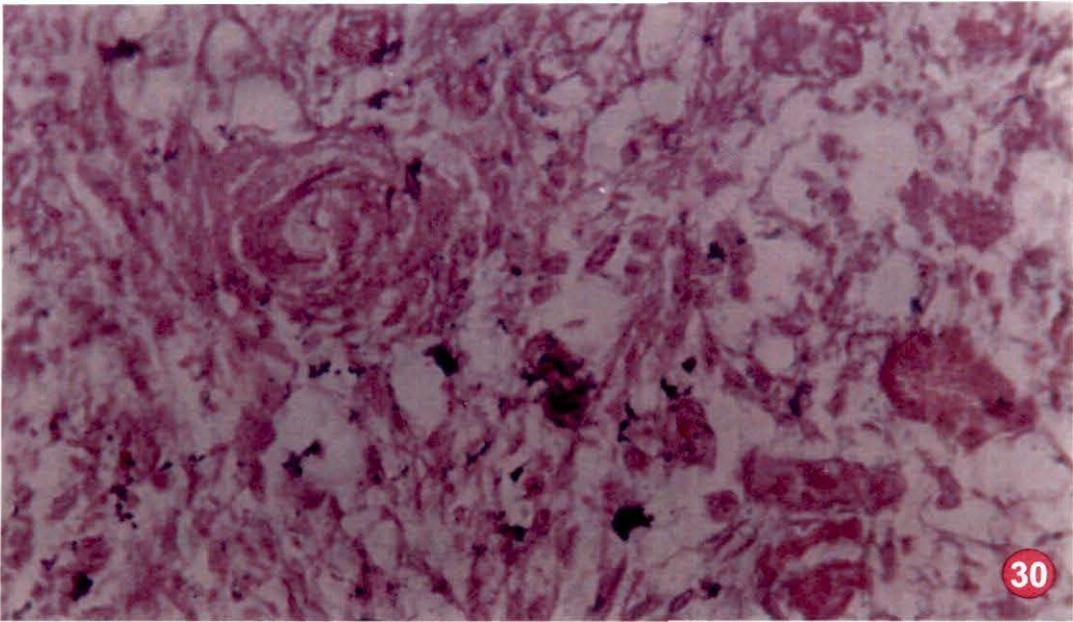
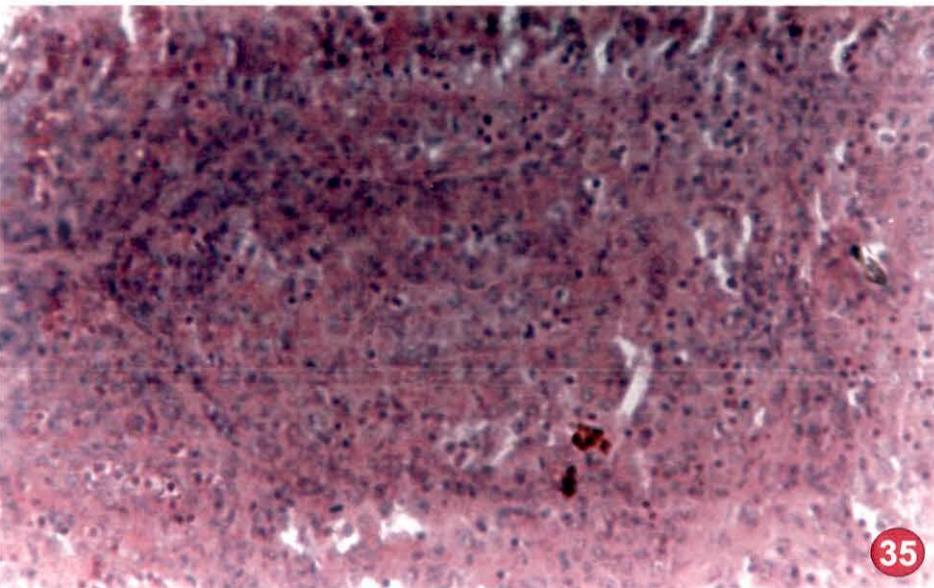
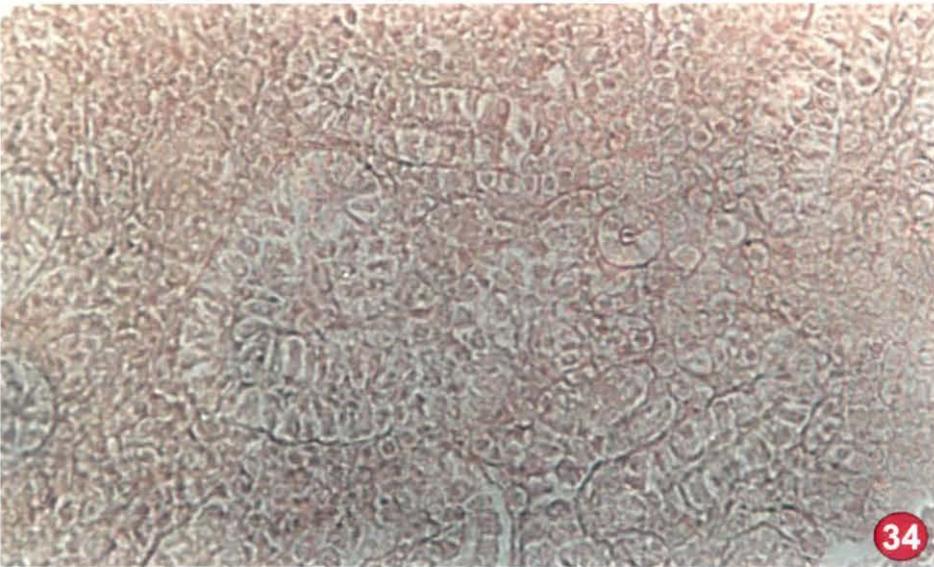
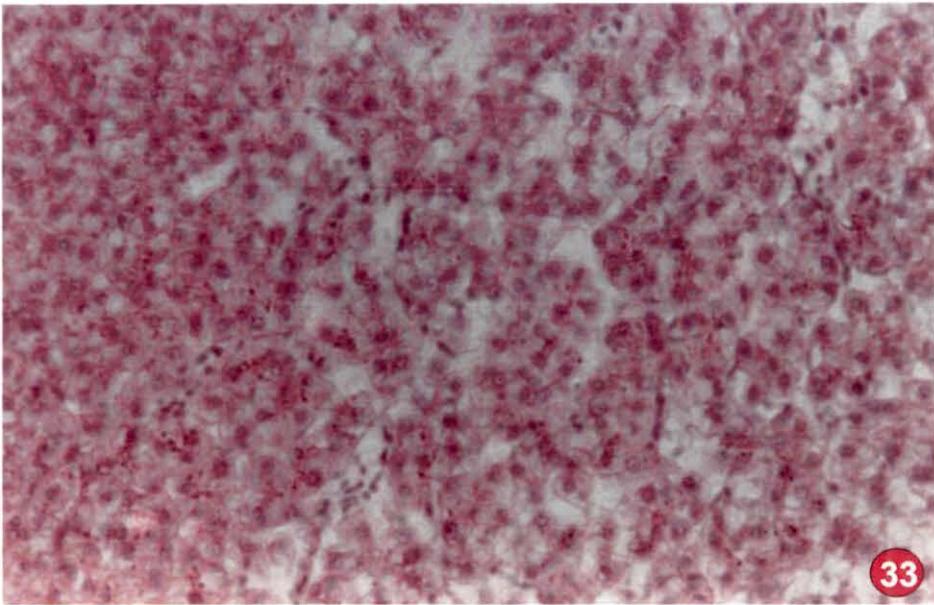


Fig. 33: Section of the liver of naturally infected *L. bata* showing vacuolation (H-E, x 400).

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 naturally infected *L. bata* showing necrotic changes (H-E x 200).



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 vacuolation 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.

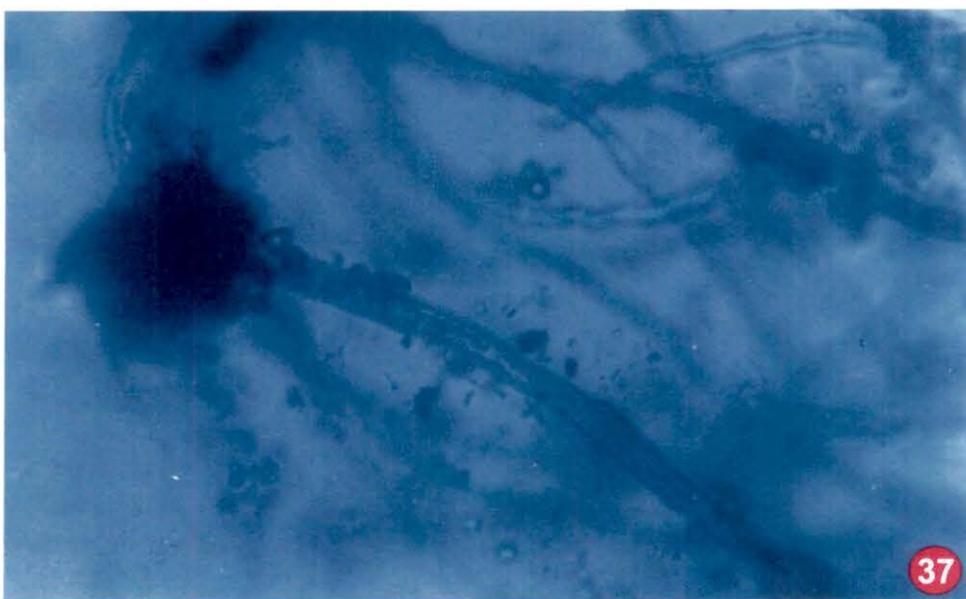
4.3. Isolation of fungus and their characterization

Newly formed hyphal tips were noticed after 6 h of incubation at 25°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 (8-15 μ) 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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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).

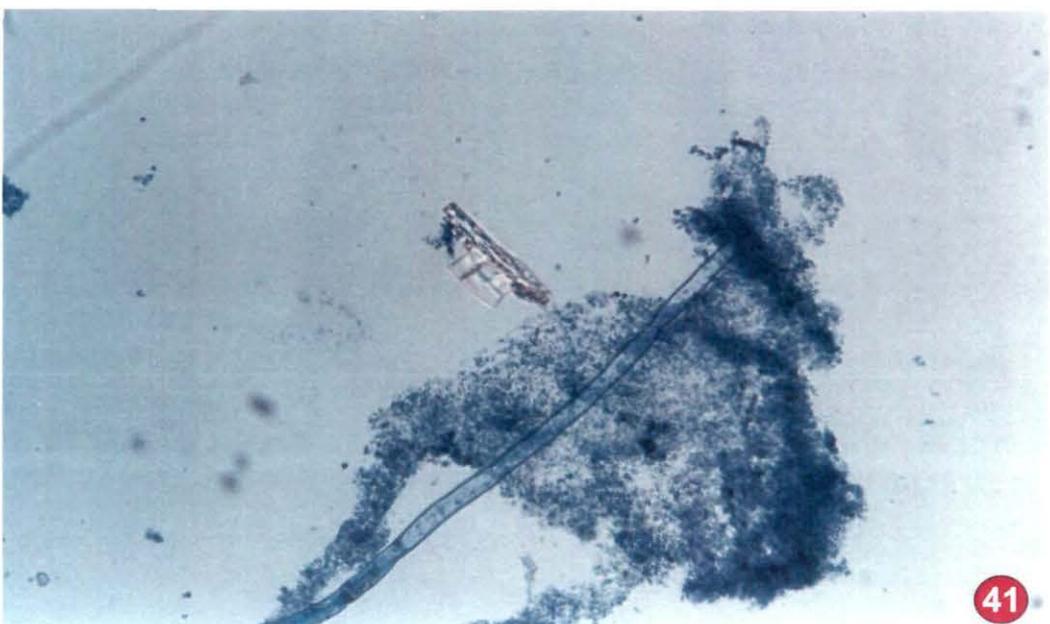
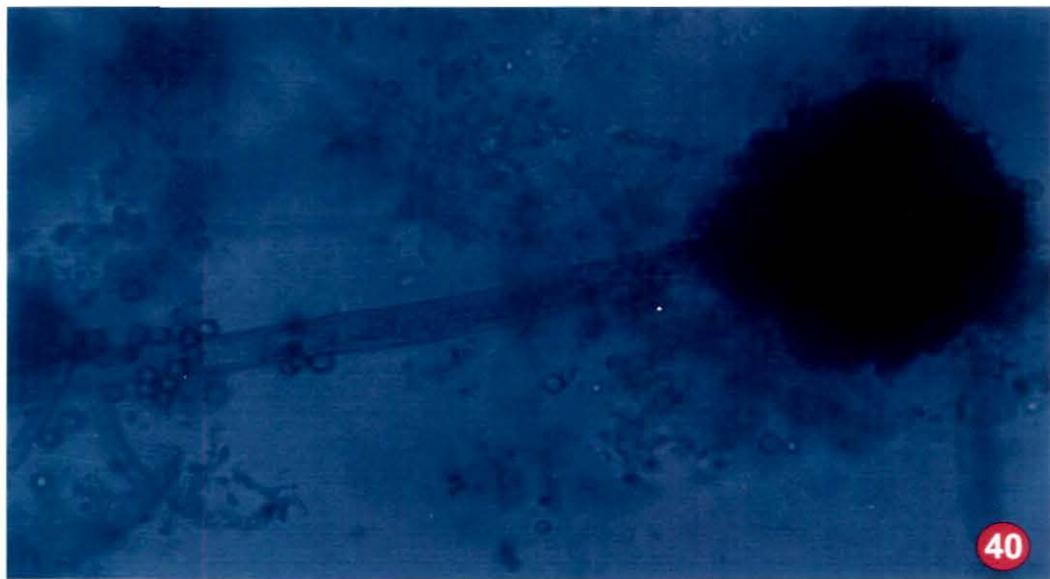


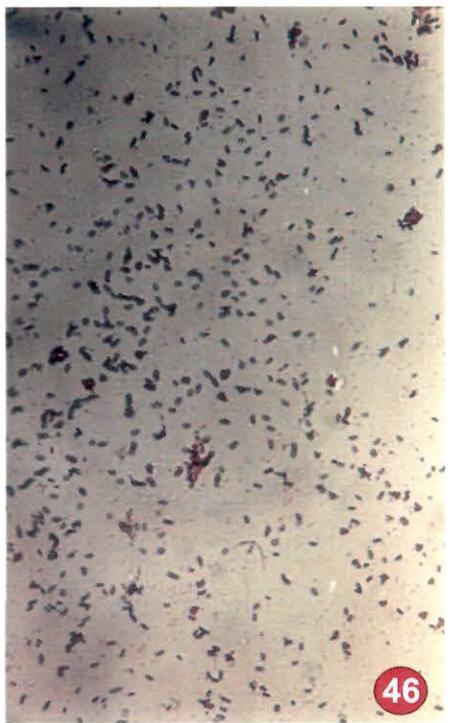
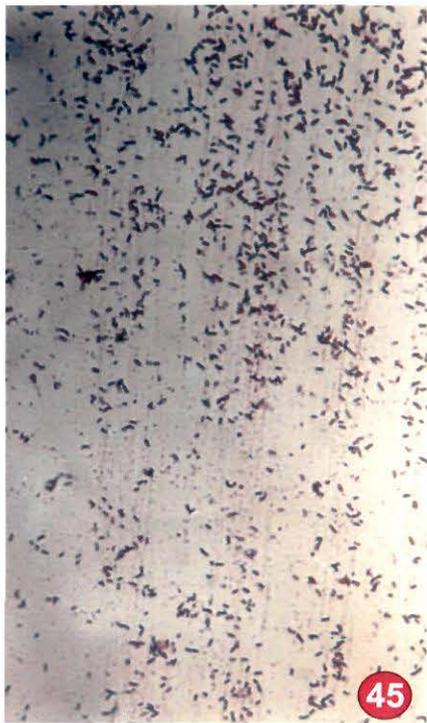
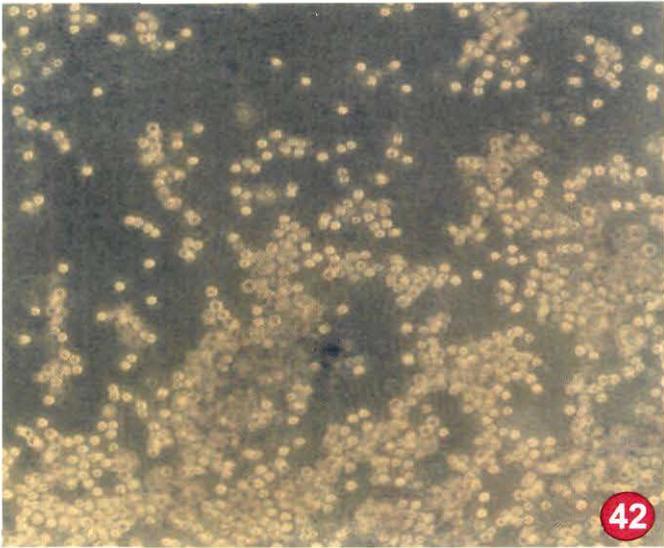
Fig. 42: Phase contrast micrograph of bacterium L_{r3} (*Micrococcus varians*).

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*).



(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 row 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}	<i>C. punctatus</i>	16.02.02	<i>Aphanomyces</i> sp.
F _{cs1}	<i>C. striata</i>	18.02.02	<i>Aphanomyces</i> sp.
F _{lr1}	<i>L. rohita</i>	09.03.02	<i>Aphanomyces</i> sp.
F _{lb1}	<i>L. bata</i>	11.03.02	<i>Aphanomyces</i> 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 ₁	Rod	2.5-3.5 x 0.6-0.8 μ m	Singles.	Nil	Circular	Turbid
L ₂	Rod	2.5 – 3.6 x 0.67- .82 μ m	Singles	Nil	Circular	Turbid
L ₃	Sphere	1.3-1.7 μ m (diameter)	Singles, pairs tetrads or in irregular cluster	Nil	Spherical	Turbid with sediment
L ₄	Rod	2.7-3.3x 0.76-0.86	Singles		Circular	Turbid with sediment
L ₅	Rod	2.8-3.6x 0.63- 0.72 μ m	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	<i>Bacterial Isolates</i>				
	Lr ₁	Lr ₂	Lr ₃	Lr ₄	Lr ₅
Gram Reaction	-	-	+	-	-
Motility	+	+	-	+	+
Growth at					
25 ^o C	m	m	m	m	m
30 ^o C	g	g	g	g	g
37 ^o C	g	g	g	g	g
42 ^o 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 ₂ S from cystein	+	+	-	+	-
Citrate utilization	+	+	+	+	+
Pigment formation	-	-	Bright yellow	-	Green in kings 'B' media

+ = positive; - = 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 of cells	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	-	-	-	-	-
Mortality	+	+	+	+	+
Growth at 25 ^o	m	m	m	m	m
30 ^o	g	g	g	g	g
37 ^o	g	g	g	g	g
42 ^o	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 ₂ S 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

+ = positive; - = 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.



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
<i>Aeromonas</i>	<i>C. striata</i>	1	C _{s5}	4
	<i>L. rohita</i>	3	L _{r1} , L _{r2} , L _{r4}	
<i>Pseudomonas</i>	<i>C. striata</i> ,	4	C _{s1} , C _{s2} , C _{s3} , C _{s4}	5
	<i>L. rohita</i>	2	L _{r5} ,	
<i>Micrococcus</i>	<i>L. rohita</i>	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

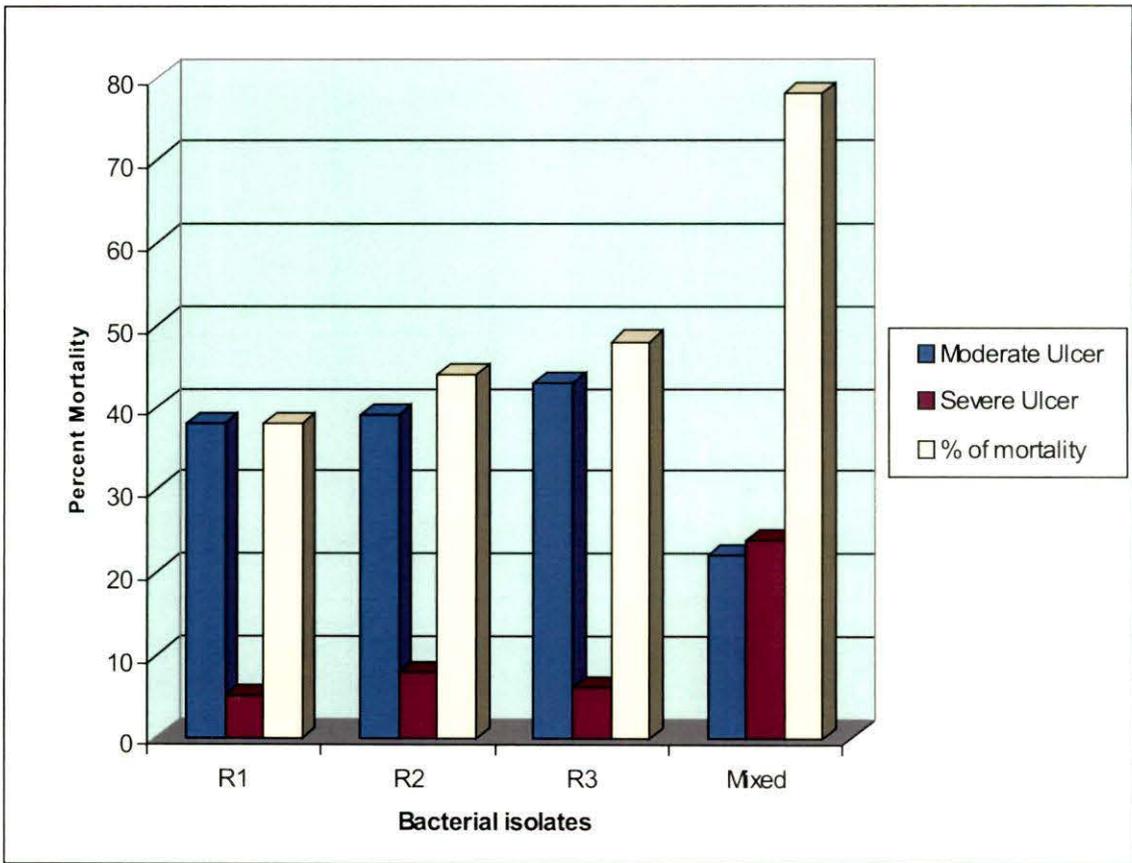


Fig. 52

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

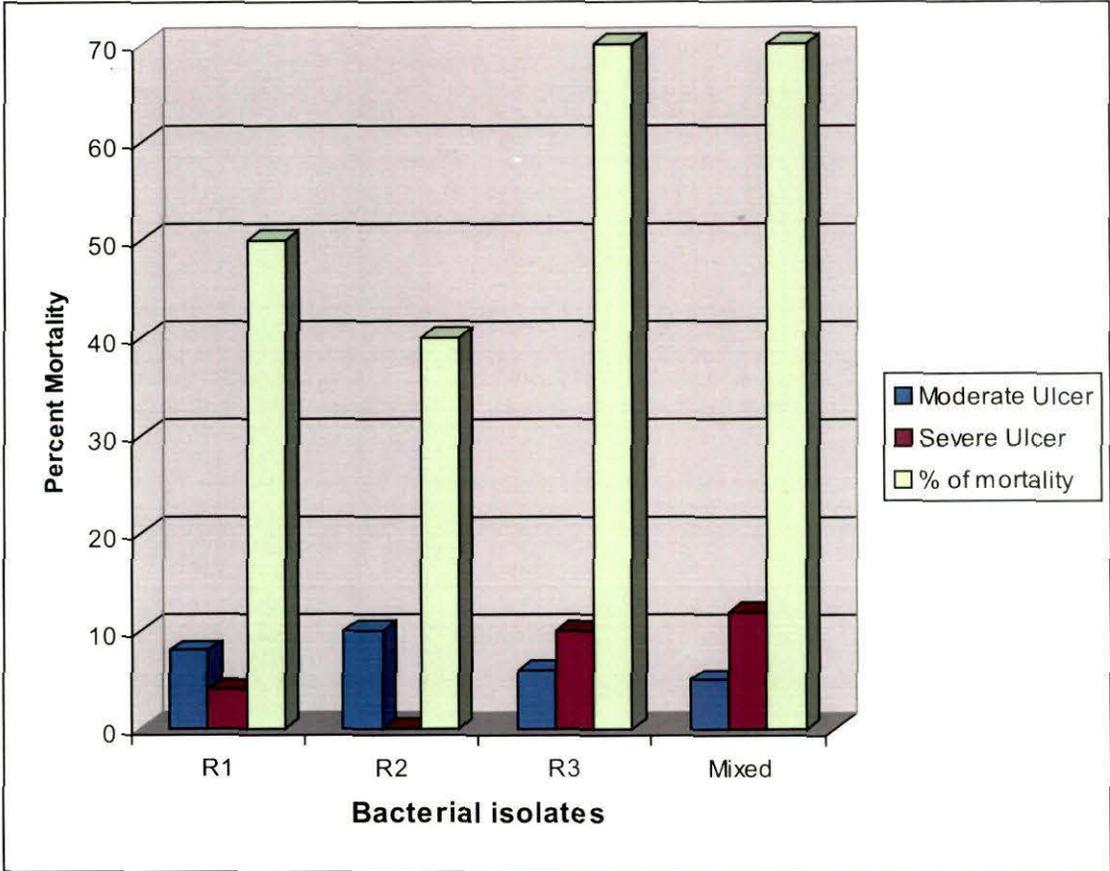


Fig. 59

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.



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*

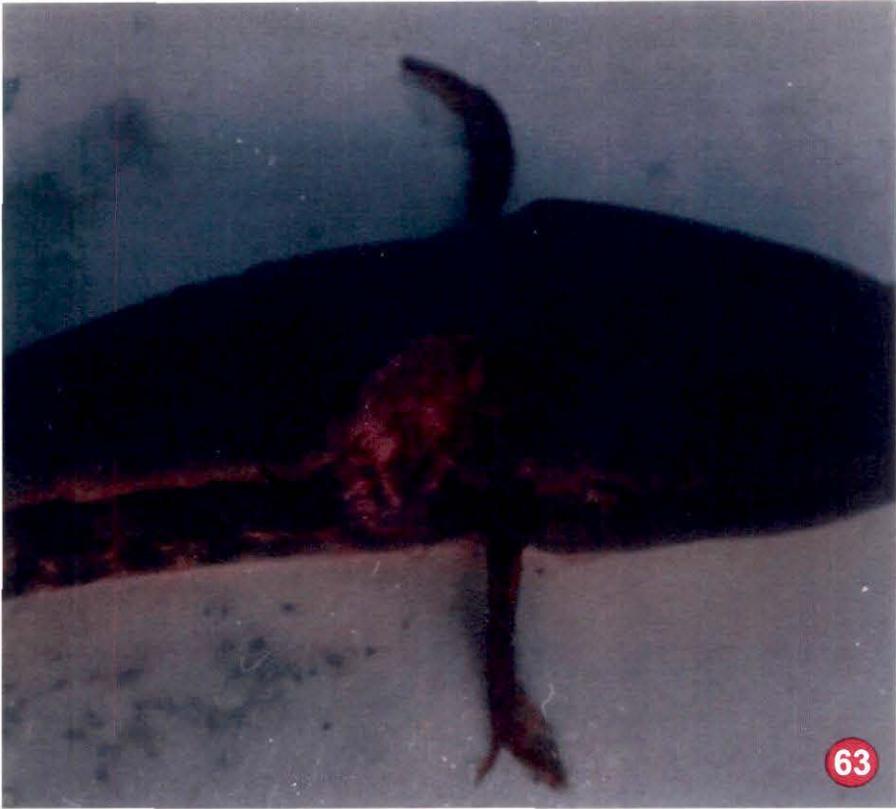
Within 48 h of inoculation some fishes showed red spot at the site of injection. The skin was intact at this stage. After 72 h red spot increased in size and ulcer developed (Figs. 60 & 61).

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

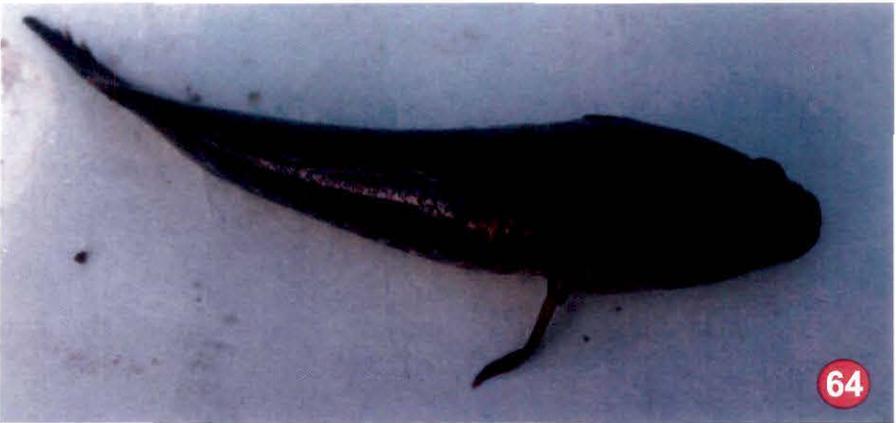
In the control set of fishes no ulcer formation and mortality were noticed within 15 days of observation (Fig. 62). The results are summarized in Table 11.

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} (*Pseudomonas* sp.).



63



64

Table 11: Percentage mortality and nature of ulcer formation in *Channa punctatus* injected intramuscularly with saline suspensions of fungal zoospore (*Aphanomyces* 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 (<i>Aphanomyces</i>) 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 behaviour 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*

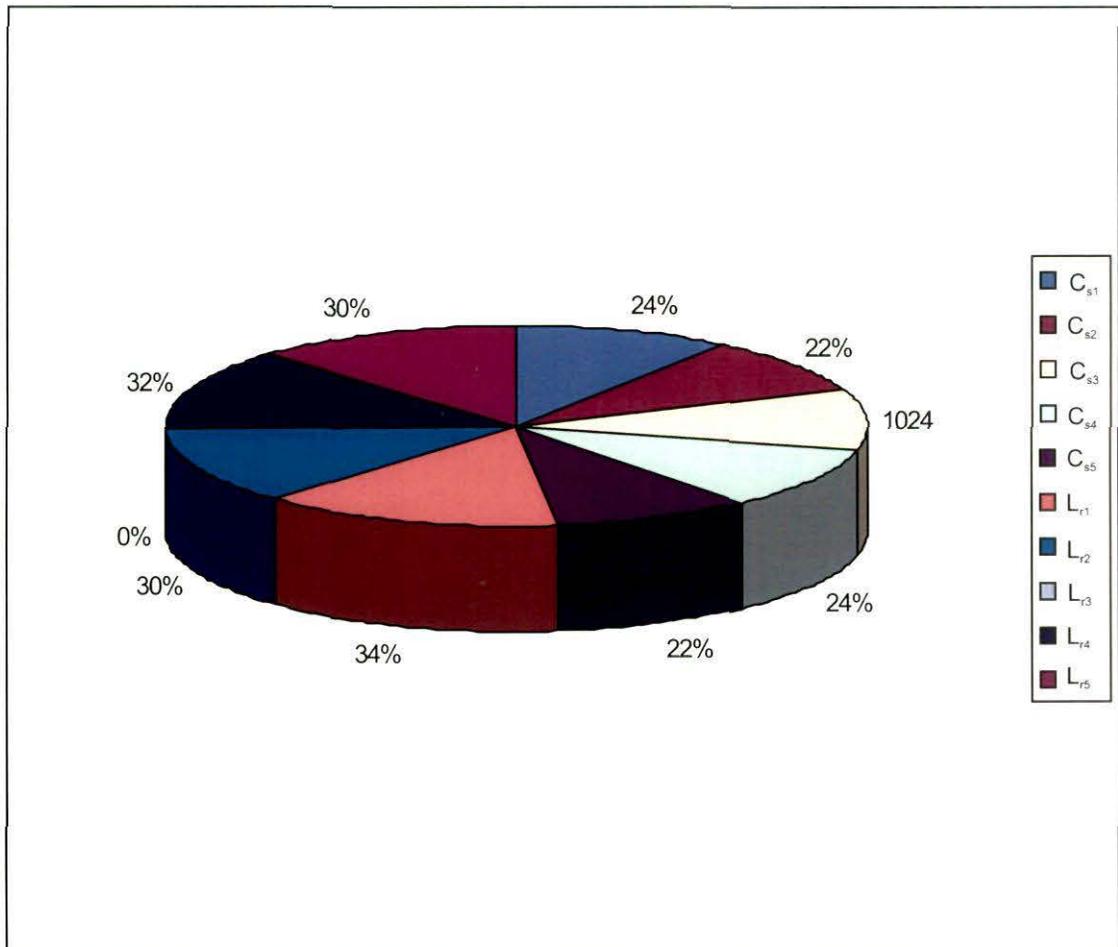


Fig. 71

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.

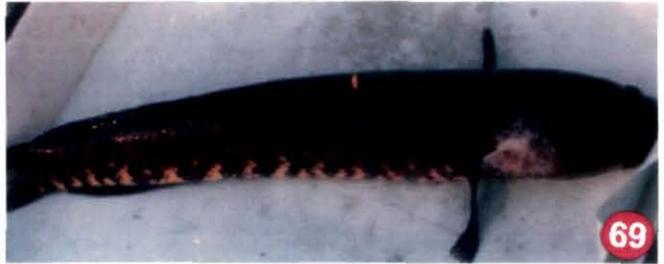
Fig. 66: *Channa punctatus* showing ulcer formation after intramuscular injection with R1+ fungal spores suspension.

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.



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 (*Aphanomyces* 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 <i>Aphanomyces</i> sp. and R1 bacteria	50	28	11	21	14	56%
Saline suspension of zoospores of <i>Aphanomyces</i> sp. and R2 bacteria	50	27	08	09	29	54%
Saline suspension of zoospores of <i>Aphanomyces</i> 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})

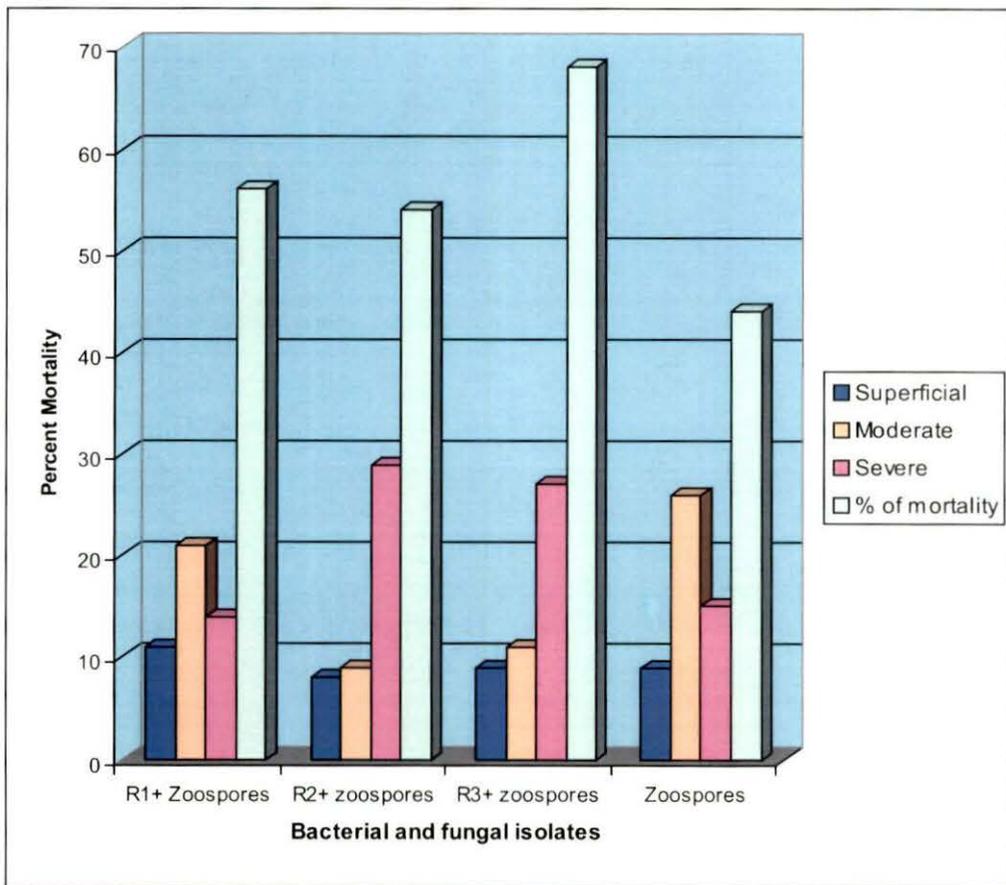


Fig. 72

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 histopathological 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 necrotic 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).

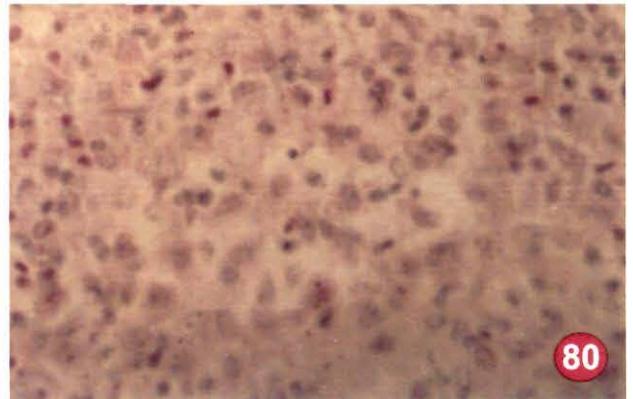
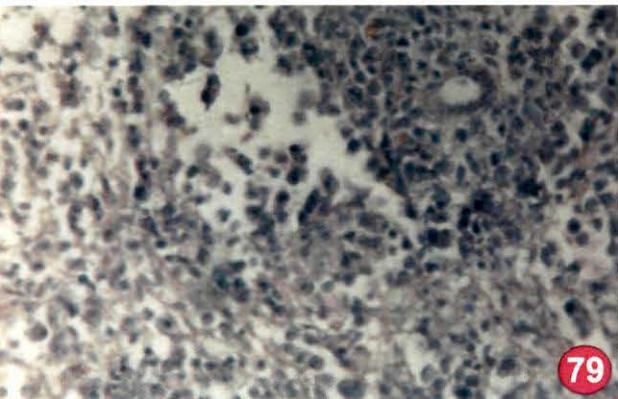
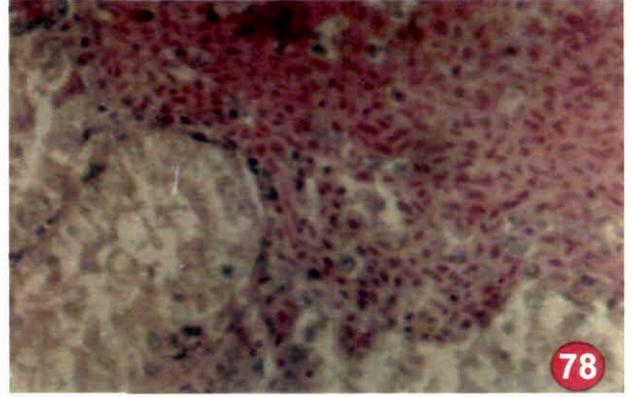
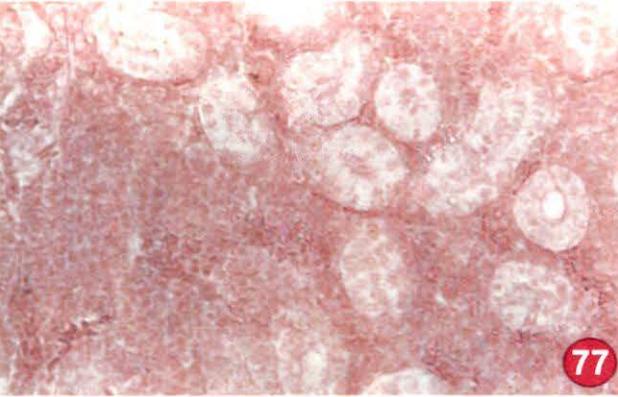
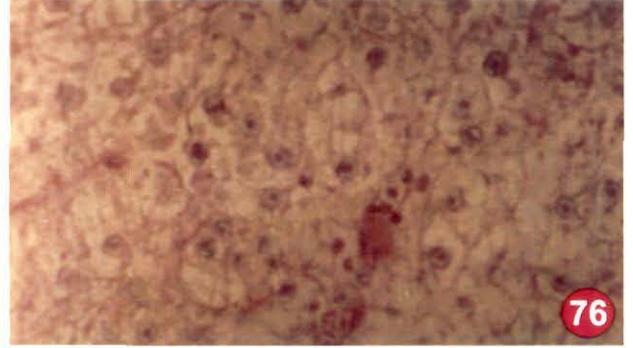
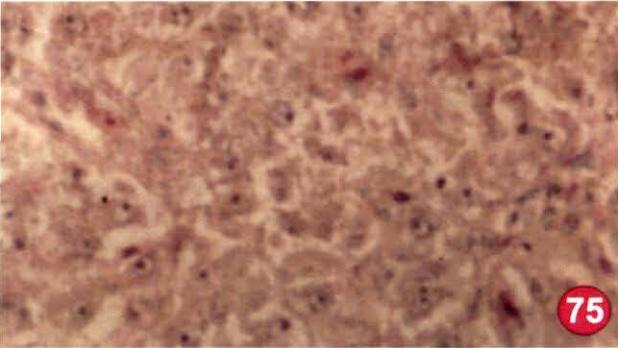
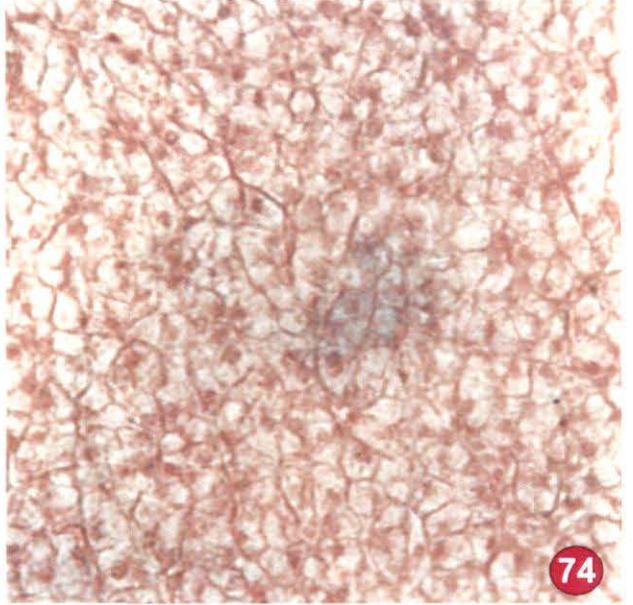
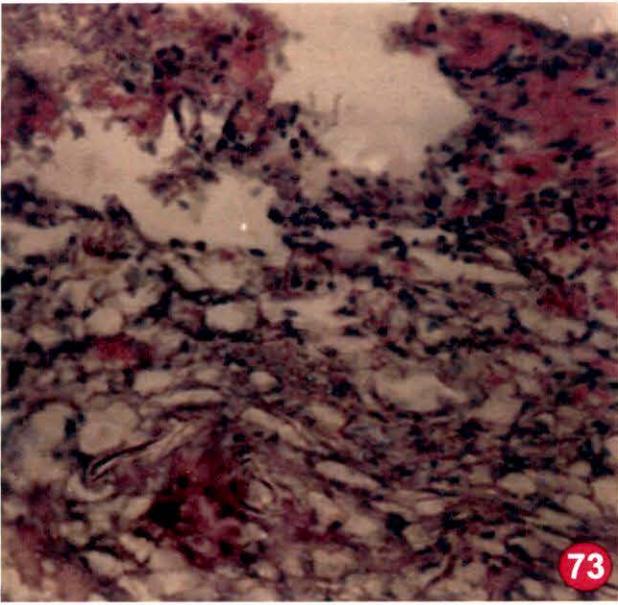


Fig. 81: Section of ulcer of *Channa punctatus* treated with zoospores suspension of *Aphanomyces* sp. showing granuloma formation. (H-E, x200).

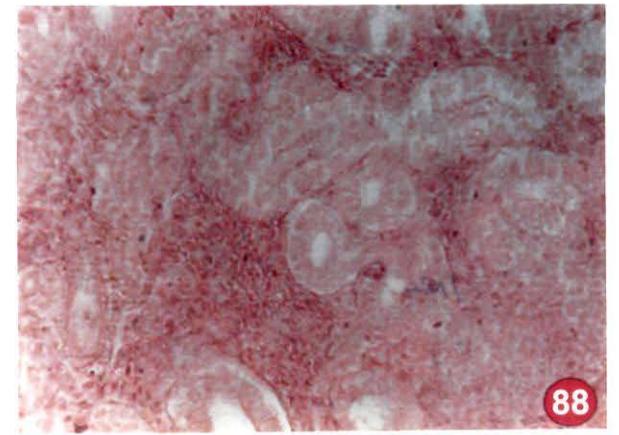
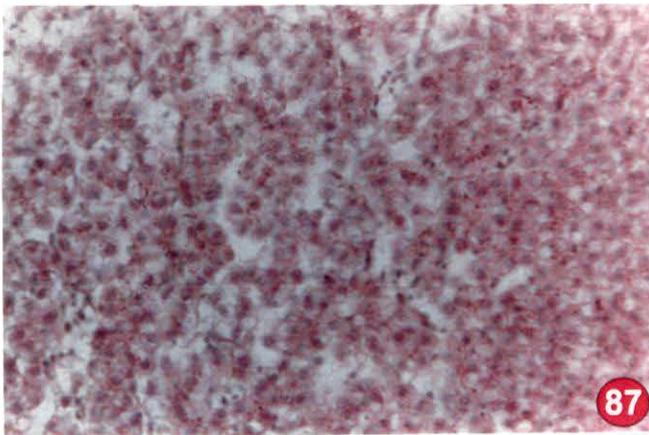
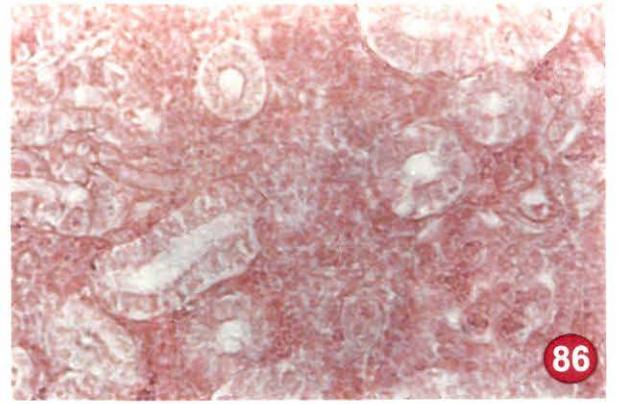
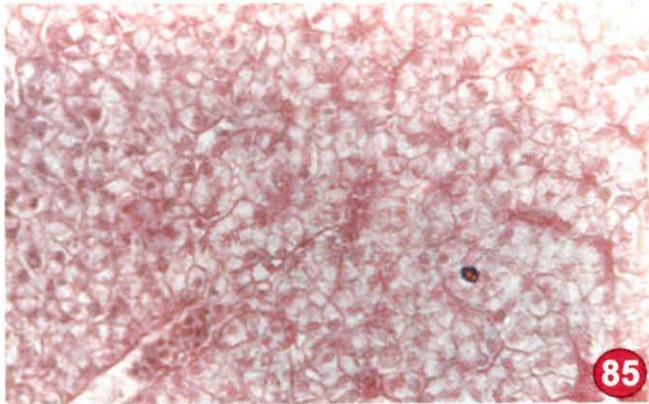
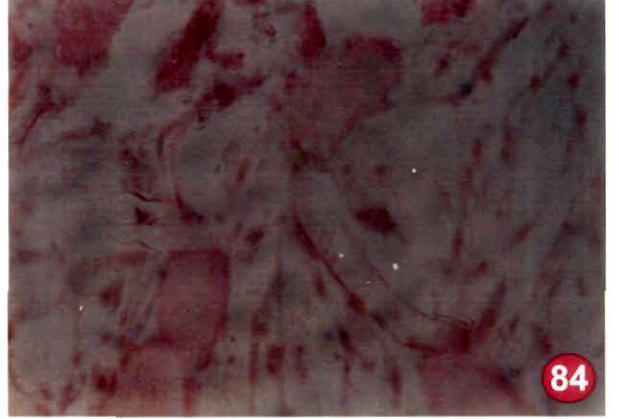
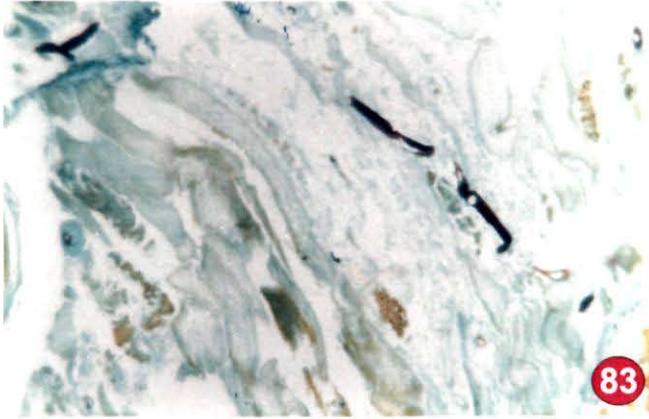
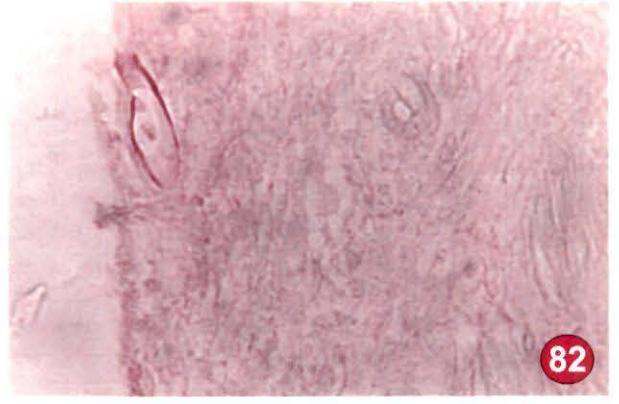
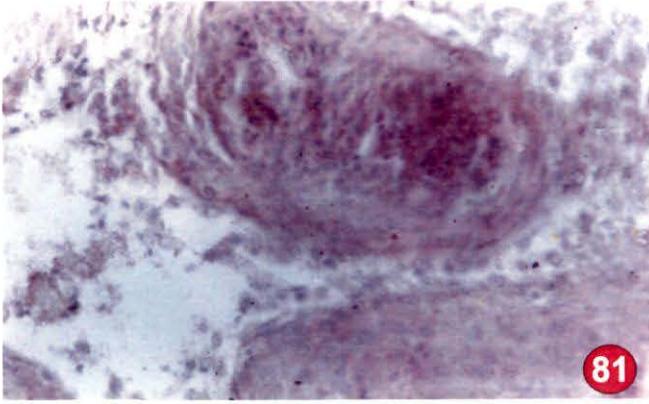
Fig. 82, 83 & 84: Section of ulcer of *Channa punctatus* treated with zoospores suspension of *Aphanomyces* sp. showing the presence fungal hyphae (H-E, x400), (GMS, x200) and (PAS, x400).

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).



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.