1. ULCERATIVE DISEASE IN AIR-BREATHING FISHES AND ISOLATION OF BACTERIA FROM ULCER TISSUES

Materials and Methods

Infected air-breathing fishes such as *Anabas testudineus*, *Clarias batrachus* and *Heteropneustes fossilis* were collected from affected areas of Cooch Behar, Jalpaiguri, Darjeeling and West Dinajpur districts of West Bengal. The minimum and maximum distance of collecting sites from the laboratory were 15 and 200 km respectively. The fishes were brought to the laboratory in an earthenware container and there kept in glass aquaria measuring 90 x 35 x 35 cm in which the depth of water was 20 cm at a density of 10-15 fishes per aquarium tank. Half of the static water of the aquarium was changed everyday. The water temperature varied from 28 to 30°C.

In the month of November, 1988 altogether, 129 *A.testudineus* (15-35 gms), 16 *H. fossilis* (15-25 gms) and 11 *C. batrachus* (35-70 gms) were collected. Of these 104 (80.62%) of *A.testudineus*, 12 (75.0%)

H. fossilis and 9 (81.8%) C. batrachus died within 5, 6 and 4 days respectively, in the laboratory. The remainder recovered from the infection.

Some of the surviving fishes were killed and tissues from liver, kidney and spleen were fixed in Bouin's fixative and preserved in cedarwood oil. For histological studies section of 6 µm thickness were done and stained with haematoxylin and eosin (H-E), and carbol fuchsin (Ziehl's).

Smear preparations with the sliced liver, kidney and spleen were done by impression method and were stained with Giemsa and carbol fuchsin (Ziehl's). Blood smear preparations were done and stained with Giemsa and Leishman. Smear preparations were made with the ulcer tissues of the skin and were stained with Giemsa and carbol fuchsin (Ziehl's). A portion of the ulcer tissue was incubated in sterilized bacterial nutrient broth medium (Beef extract 10g, Peptone 10g, NaCl 5g in 1000 ml dist.water) and nutrient broth medium supplemented with 0.1% glucose, after surface sterilization in 0.1% mercuric chloride, at 30°C for 24 hrs. Bacteria were isolated by the pour-plate method. The culture of bacteria were maintained routinely on nutrient agar slants (nutrient broth with 2% agar). Bacteria were cultured in nutrient broth when required.

79 A. testudineus, 147 C. batrachus and 88 Channa punctatus were also collected from local market (Shivmandir, Bagdogra and Siliguri) during the months of September to December 1989. Of these 55 (69.62%) A. testudineus, 93 (63.26%) C. batrachus and 46 (52.27%) C. punctatus died within 2 to 05 days of collection.

Morphological and physiological characteristics of the isolated bacteria were assessed according to the "Manual for the identification of medical bacteria" (Cowan and Steel, 1965). The morphological characteristics examined included the shape and size of the cells. The colour, texture and shape of the colonies on agar media were also noted. Presence of spores and capsules were noted after appropriate staining (Cowan and Steel, 1965).

The bacteria were grown for 24 hrs. in nutrient broth at different temperature e.g. 25°, 30°, 37° and 42°C and optical density at 630 nm was taken in a spectrophotometer to determine the growth at different temperature.

All the isolated bacteria were stained for Gram reaction and tested for (i) catalase activity, (ii) oxidase activity, (iii) Oxidation or fermentation of glucose (O-F test), (iv) acid and gas production in media containing different carbohydrates, (v) utilization of amino acids, (vi) nitrate reduction,

(vii) Nitrite reduction, (viii) production of indole, (ix) arginine dihydrolase (x) hydrolysis of gelatin, (xi) levan formation, (xii) Voges-Proskauer test and (xiii) pigment production.

Gram staining

The method of Bartholomew (1962) was followed. A suspension of 24 hrs-old bacterial culture on slant was prepared in distilled water. A drop of that suspension was taken on a grease free slide and a smear was made. It was then heat fixed, followed by crystal violet stain for 1 min, and washed for 5 sec with water. The smear was flooded with Burke's iodine solution, allowed to react for 1 min, and washed again for 5 sec with water. Holding the slide against a white surface, 95% ethanol was poured drop wise from the top edge of the slide until no more colour comes out from the lower edge of the slide. After washing, the smear was stained with saffranin for 1 min and washed again with water. The slide was air dried and observed under oil immersion.

Catalase activity

To test for catalase activity organisms were grown on slants of nutrient agar and 1 ml of 3% H_2O_2 was poured down the slope. Evolution of gas bubble indicated catalase activity.

Oxidase activity

To test for oxidase activity 2-3 drops of the oxidase reagent (freshly prepared 1% tetramethyl-p-phenylene diamine) was placed on a piece of filter paper in a petridish and the culture of the test organism was smeared across the impregnated paper with a platinum loop. A positive reaction was indicated by the appearance of a dark purple colour on the paper within 10 sec.

Oxidation or Fermentation of Glucose (O-F test)

Oxidation or fermentation of glucose was done by inoculating duplicate tubes of O-F medium of Hugh and Leifson (1953) by stabbing. The medium contained

peptone 2 gm, NaCl 5 gm, M K_2HPO_4 0.3 gm, agar 3 gm, distilled water 1000 ml, pH was adjusted to 7.2 and appropriate amount of carbohydrate (D-glucose) to give a final concentration of 1%. Bromothymol blue, 15 ml (2% aqueous solution) was added as indicator. After inoculation of duplicate tubes sterile melted paraffin was poured on to the top of one tube to a depth of 10 mm. If the carbohydrate is broken by oxidation, only the open tube will turn yellow and in fermentation reaction both the open and closed tubes will show yellow colour.

Acid and gas production in media containing different carbohydrates

For utilization of carbohydrates the basal medium consisted of beef extract 3 gm, peptone 10 gm and distilled water 1000 ml, and pH was adjusted to 7.2. The medium was supplemented with 0.5% (final concentration) of the following carbohydrates, L-arabinose, D-glucose, D-fructose, sucrose, D-Lactose, adonitol, D-sorbitol, mannitol and meso-inositol and glycerol. To test for acid and gas production sufficient amount of indicator, bromothymol blue, was incorporated in the medium and inverted Durham's tube filled with the medium was

introduced into the broth. Acid when produced changed the colour from blue to yellow, gas, if any produced will be accumulated at the top of the Durham's tube.

Utilization of amino acids

To test for utilization of amino acids, as a sole source of carbon, the basal medium (Pridham and Gottlieb's medium) was supplimented with amino acids (β-alanine, L-arginine, L-aspartic acid and L-valin) to a final concentration of 0.2%.

Nitrate reduction

For nitrate reduction test the organisms were grown on nutrient broth containing 0.1% KNO_3 and incubated for 5 days. The presence of nitrite (after reduction of nitrate) was tested by addition of nitrite reagent, sulphanilic acid (0.8% in 5N acetic acid) and α-napthylamine (0.5% in 5 N acetic acid). Appearance of red colour indicated presence of nitrite. Zinc dust were added to the culture tubes shown negative test for

nitrite; appearance of red colour indicated presence of nitrate in culture tubes which was not reduced to nitrite by the test organism.

Nitrite reduction

For nitrite reduction test nutrient broth was supplimented with 0.001% $NaNO_2$ and incubated for 7-14 days. The presence of nitrite was tested with nitrite reagent (0.8g of sulphanilic acid in 100 ml of 5N acetic acid and 0.5 g of α-napthylamine in 100 ml of 5N acetic acid). Absence of red colour indicated nitrite reduction.

Indole production

To test for indole production, organisms were grown on tryptone broth (Tryptone 1%, beef extract 0.3 %) and was examined with Kovac's reagent (p-dimethylamino-benzyldehyde 5 g, butyl alcohol 75 ml and conc. HCl 25 ml).

Arginine dihydrolase

To test for arganine dihydrolase Thornley's medium were used. The medium contained peptone 1 gm, NaCl 5 gm, K_2HPO_4 0.3 gm, phenol red 0.01 gm, arginine 10 gm, distilled water 1000 ml and agar 3 gm, pH was adjusted to 7.2. Test medium were inoculated by stabbing. Immediately after inoculation a layer (10 mm) of sterile melted paraffin was added over the slab. The tubes were incubated at 30°C for 3 days. Positive reaction was shown by colour change from yellow to red.

Hydrolysis of gelatin

To test for hydrolysis of gelatin, organisms were grown on plates and on slants of gelatin agar (Nutrient agar 1000 ml, gelatin 4 gm and distilled water 50 ml) and incubated for 3 days. The plates and slants were flooded with acid-mercuric chloride solution (mercuric chloride 1 gm, water 80 ml and conc. HCl 16 ml) Appearance of clear zones indicated gelatin hydrolysis.

Levan formation

To test for levan production, bacteria were grown on plates and on slants of nutrient agar containing 3 4% sucrose.

Voges - Proskauer test

To test for Voges-Proskauer reaction organisms were cultured 2 days at 30°C in glucose - phosphate medium containing 5 gm peptone, 5 gm K_2HPO_4 and 5 gm glucose in 1000 ml. distilled water and pH was adjusted to 7.2. This reaction was tested by addition of 0.6 ml of α-napthol solution and 0.2 ml of 40% KOH aqueous solution. Appearance of strong red colour indicated positive test.

Pigment production

To test for pigment production, King, Ward and Raney's media (A and B) were used. The medium A (for pyocyanin) contained peptone 20 gm, glycerol 10 gm, K_2SO_4 (anhydrous) 10 gm, $MgCl_2$ (anhydrous) 1.4 gm, distilled water 1000 ml and agar 20 gm, the medium B (for fluorescin) contained proteose peptone 20 gm,

glycerol 10 gm, K_2HPO_4 1.5 gm, $MgSO_4 \cdot 7H_2O$ 1.5 gm, distilled water 1000 ml and agar 20 gm. After inoculating medium A was incubated at 30°C for 24-96 hrs. and medium B was incubated at 30°C for 24 hrs. followed by room temperature (22-25°C) for 72 hrs. To test for pigment production by the coccus, organisms were grown on nutrient agar plates at room temperature and were kept in diffuse day light.

Observations and results

Within 6 days of being taken into the laboratory, 125 of the 156 infected specimen of three fish species, i.e. Anabas testudineus, Clarias batrachus and Heteropneustes fossilis collected from various sites died. Similarly, 194 of the 314 infected fish such as C. batrachus, A. testudineus and Channa punctatus died within 5 days of collection from local market. Infected fish showed presence of lesion in fins, and on different regions of the body. In the case of fishes without scales, e.g. C. batrachus and H. fossilis the symptoms of the disease first appeared as red spot on the skin of the body. Gradually the red spot increased in size and an ulcer developed in the infected region,

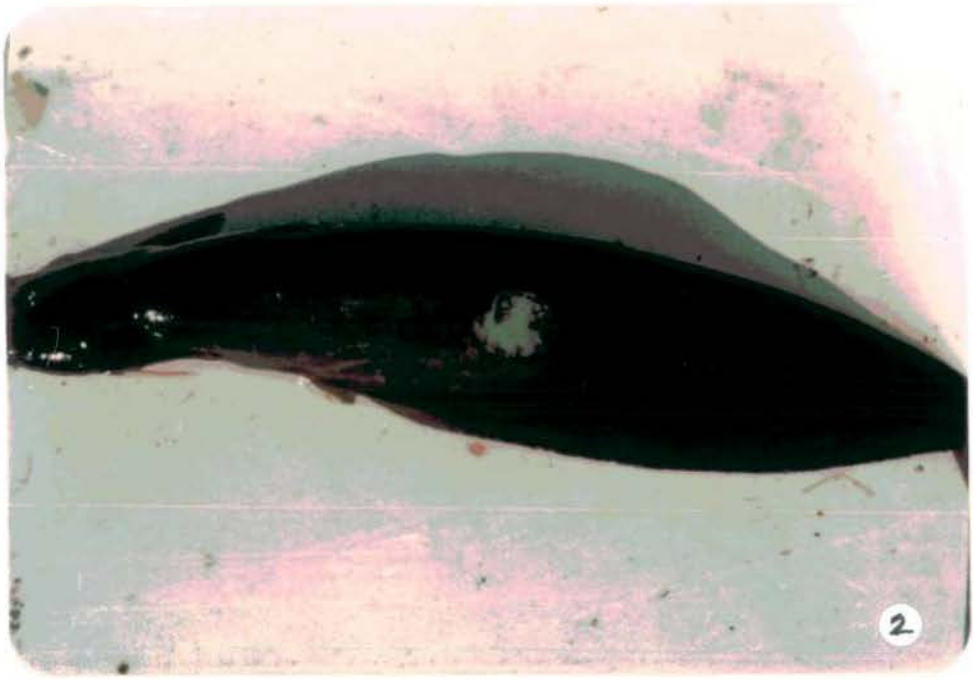
ultimately the underlying muscle layer became affected. Occasionally the ulcer remained covered by a thin whitish membrane surrounded by a reddish area (Fig. 2), Occasionally the ulcers became deep and haemorrhagic (Fig. 3). The Tails were also affected and in severe cases the lesion erodedthe total peduncle portion (Fig. 4). In some cases haemorrhages were seen on the ventral side of the body surface (Fig. 5). In scaly fishes such as Anabas testudineus the mucous layer covering the scales was first affected. Red spots appeared in some region of the body. The normal colour of the affected region of the body became changed to grey, scales were sloughed and the ulcer became deep and necrotic (Fig. 6). The fins were also affected.

In laboratory, it was found that some infected fishes A. testudineus, C. batrachus, H. fossilis and C. punctatus remained most of the time sitting on the floor of the aquarium. They performed quick or irregular opercular movement. Some infected C. batrachus remained motionless making 45-90° angle of their body to the surface of water with their head directed upward (Fig. 7).

Plate I

Fig. 2. *Heteropneustes fossilis* showing ulcer covered with whitish membrane-like structure.

Fig. 3. *Clarias batrachus* showing deep ulcer.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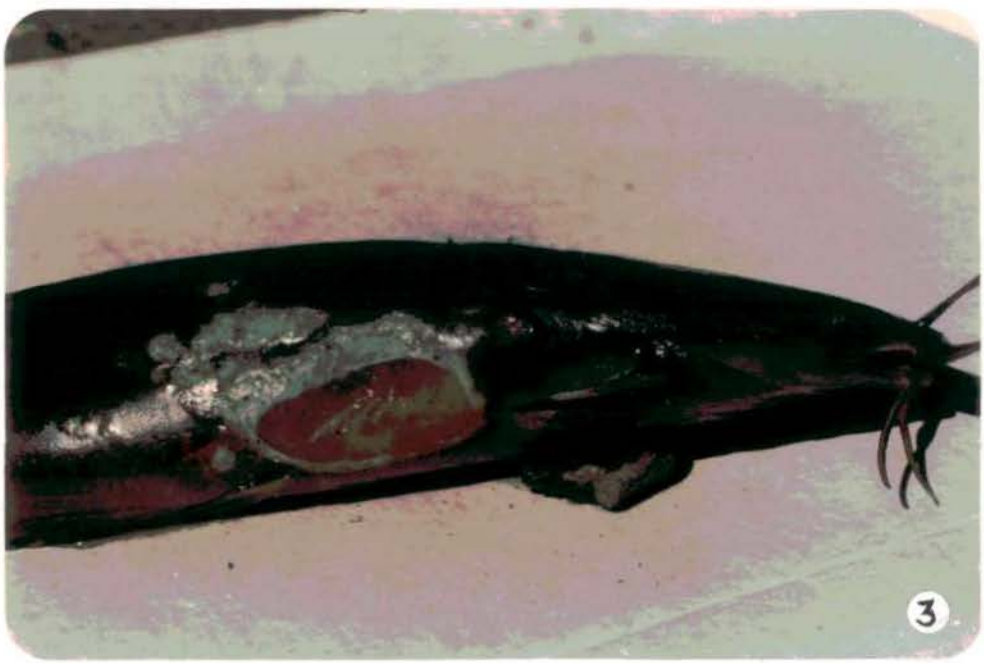
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Plate II

Fig. 4. Clarias batrachus showing loss of tail region.

Fig. 5. Clarias batrachus showing haemorrhage on the ventral side of the body.


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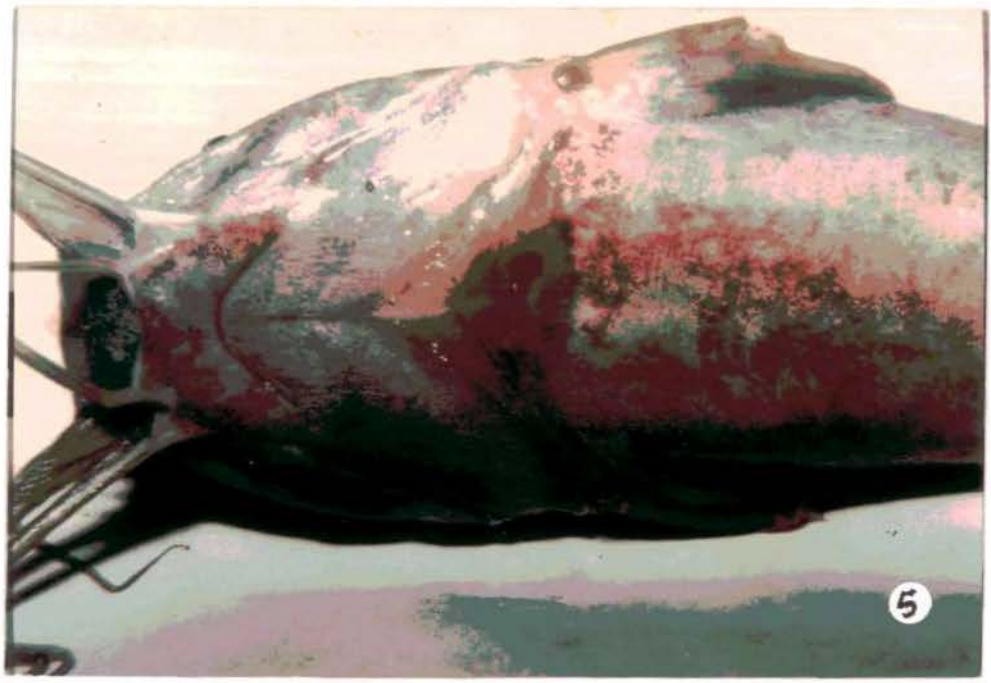
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Plate III

Fig. 6. Anabas testudineus showing haemorrhagic ulcer with affected tail fins.

Fig. 7. Clarias batrachus at moribund stage in an aquarium in the laboratory.


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Smear preparation of ulcer tissues and impression of liver, kidney and spleen stained with Giemsa and Carbol-fuchsin showed the presence of bacteria only (Fig. 8). No other agent such as protozoa or fungus were detected. Smear preparations of blood from infected fishes showed vaculation in the nucleus and cytoplasm of erythrocytes (Fig. 9). Perinuclear hallow was found in some blood cells (Fig. 10). Nuclear shadows of erythrocytes (degenerating erythrocytes) were also observed. No such changes were found in the smear preparations of blood from healthy fishes. Presence of bacteria were also detected in the smear preparations of blood from infected fishes only (Fig. 11).

In all three species of infected fishes various histopathological changes in the liver, kidney and spleen were detected. Histological observations of the livers of A. testudineus, C. batrachus and H. fossilis showed that in some regions the normal architecture of the livers were lost when compared to the sections of the livers of control fishes (Figs. 12, 15 and 17). In A. testudineus section of liver showed various degrees of degeneration (Fig. 13), vaculation of hepatocytes. In some regions parenchymal cells were arranged in cords with enlarged sinusoids (Fig. 14). In C. batrachus cord like arrangement of parenchymal cells were observed in some regions of the liver. Vaculations of

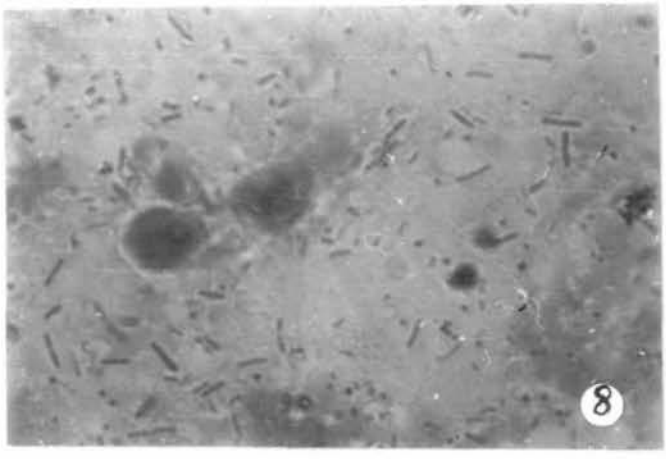
Plate IV

Fig. 8. Smear preparation with the ulcer tissues of infected Clarias batrachus showing bacteria, rods and coccus.

Fig. 9. Smear of blood from infected Clarias batrachus showing vacoules in the nucleus of a blood cell.

Fig. 10. Smear of blood of infected Clarias batrachus showing perinuclear hallow in a blood cell.

Fig. 11. Smear of blood of infected Clarias batrachus showing bacteria (arrow).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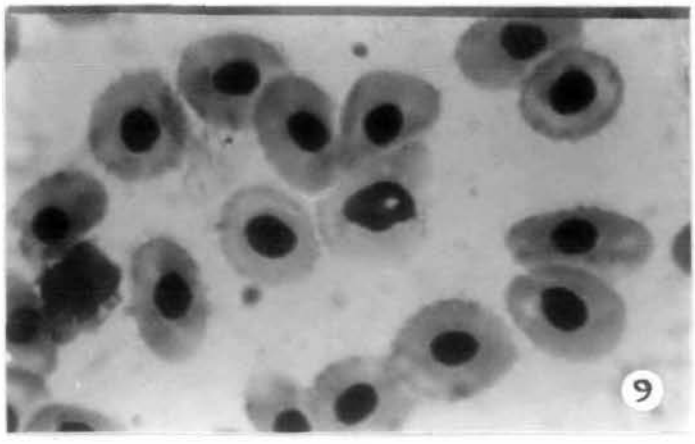
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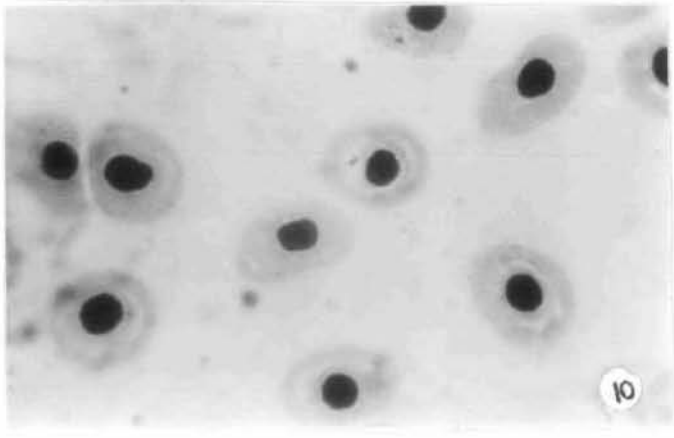
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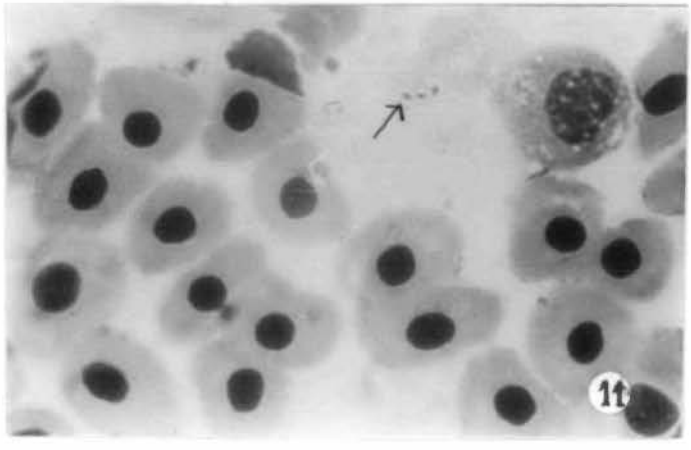
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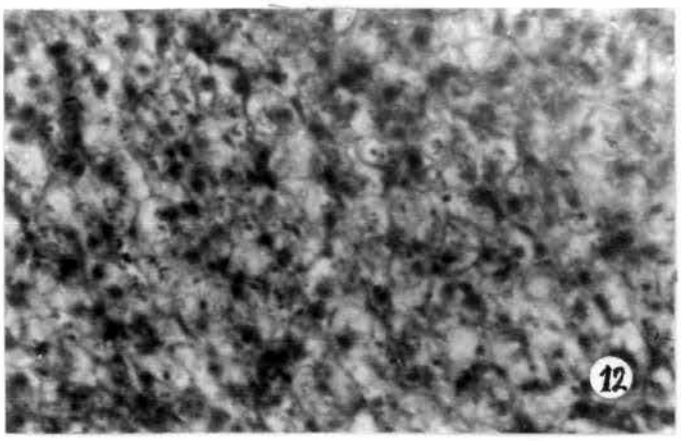
Plate V

Fig. 12. Showing a part of liver of A. testudineus (Control). x 500.

Fig. 13. A part of liver of infected A. testudineus showing degenerations. X 125.

Fig. 14. A part of liver of infected A. testudineus showing enlarged sinusoids with cord like arrangement of liver cells. X 500.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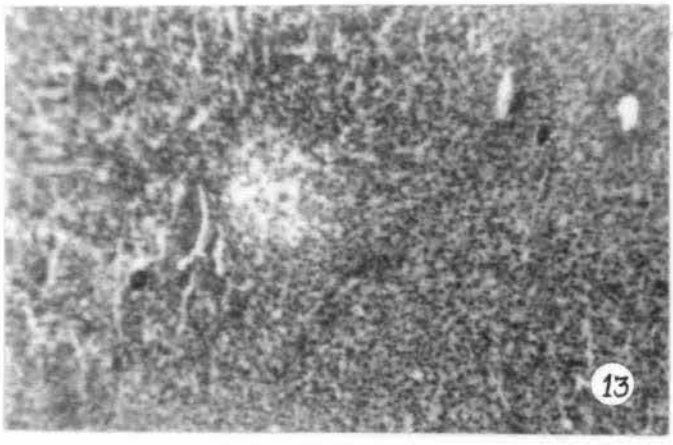
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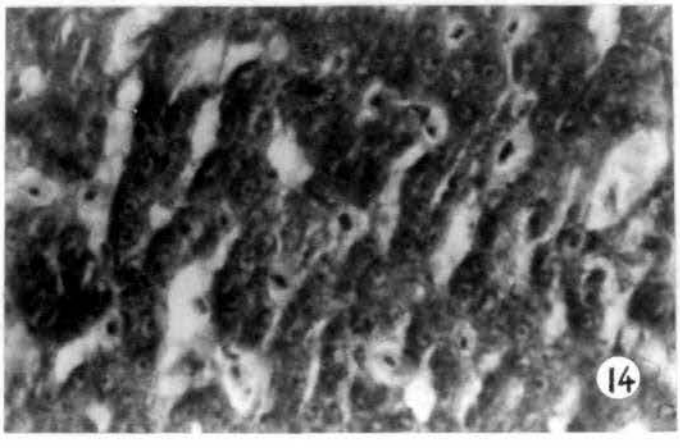
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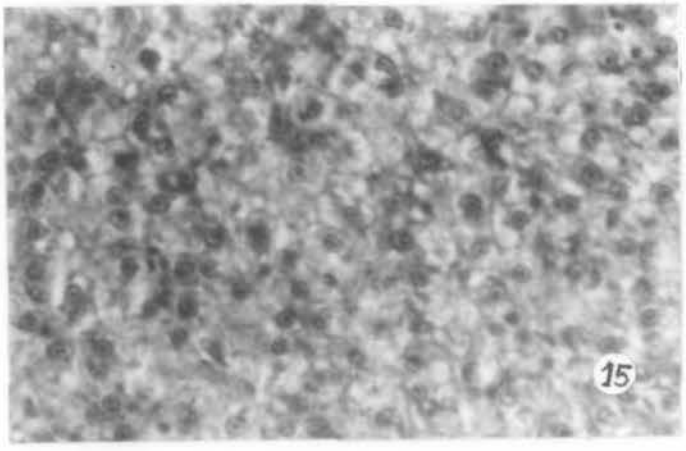
Plate VI

Fig. 15. Showing a part of liver of C. batrachus (Control). X 500.

Fig. 16. A part of liver of infected C. batrachus showing vaculation of hepatic cells. X 1250.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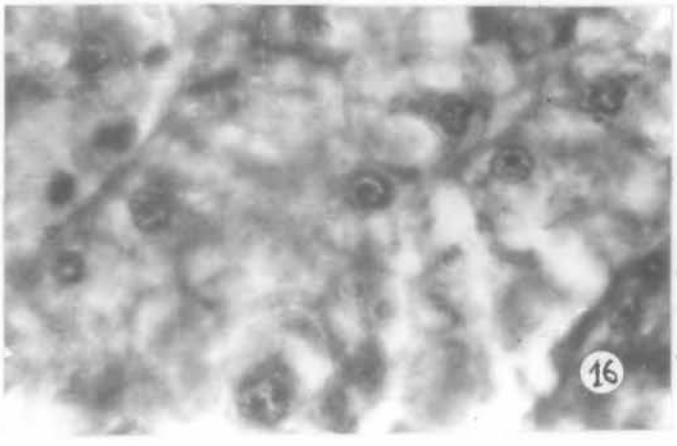
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the hepatocytes were also detected in some regions of the liver (Fig. 16). The sections of the livers of H. fossilis showed degenerative changes as well as cord like arrangement of hepatic cells (Figs. 18 and 19).

Microscopic observation of naturally infected kidney of A. testudineus revealed severe changes in renal tubules. Tubular breakage, tubular degeneration and vaculation of tubular cells were the most frequent changes (Fig. 21) in comparison to that of the normal kidney (Fig. 20). In some tubules enlarged eosinophilic tubular cells were detected (Fig. 22). Accumulation of eosinophilic materials were seen within the lumen of tubules (Fig. 23). Besides the major tubular changes, haemorrhages were detected in some regions of the kidney. Necrosis of the haematopoietic region of the kidney were also observed (Fig. 24). Vaculation, degeneration and necrosis of the tubular cells were also observed in the kidney of infected C. batrachus and H. fossilis (Figs. 25-30).

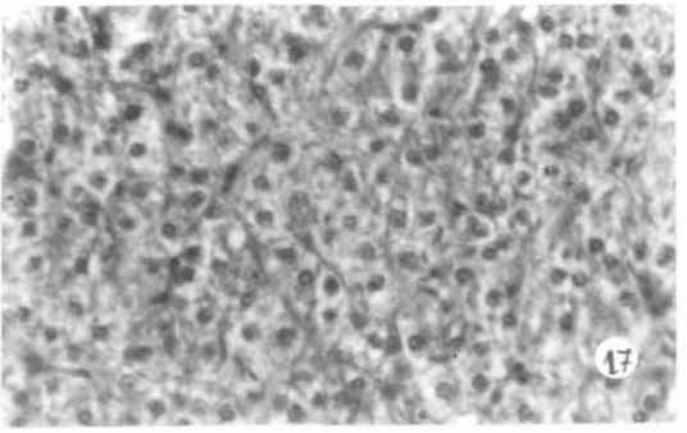
Sections of the spleens of the three species of fish showed vaculation and necrosis in some regions of the spleens (Figs. 31-34). Cord like arrangement of splenocytes were also observed in some regions of the spleens of C. batrachus and H. fossilis.

Plate VII

Fig. 17. Showing a part of liver of H. fossilis (Control). X 500.

Fig. 18. A part of liver of infected H. fossilis showing degenerative changes. X 500

Fig. 19. A part of liver of infected H. fossilis showing cord like structure. X 500.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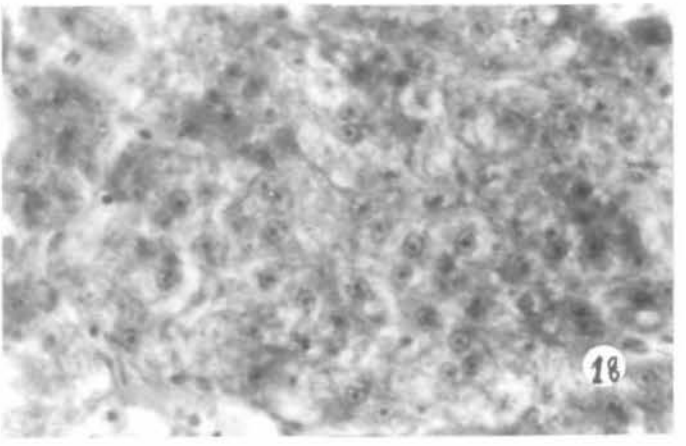
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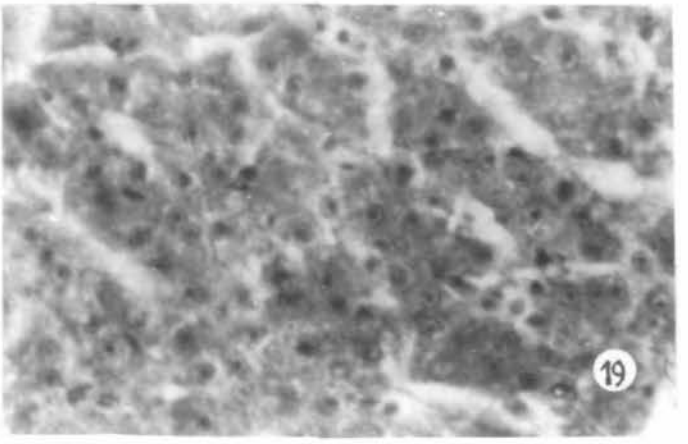
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Plate VIII

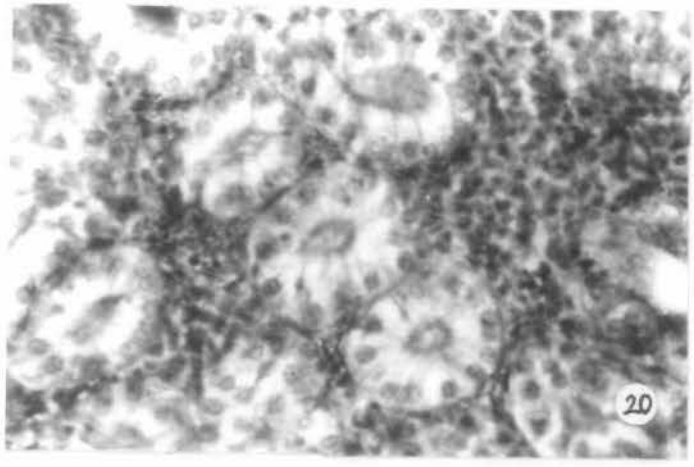
Fig. 20. Showing a part of kidney of *A. testudineus* (Control) . X 500.

Fig. 21. A part of kidney of infected *A. testudineus* showing a tubular vaculation x 500.

Fig. 22. A part of kidney of infected *A. testudineus* showing enlarged eosinophilic cells of a tubule. X 1250.

Fig. 23. A part of kidney of infected *A. testudineus* showing accumulation of eosinophilic materials within the lumen of tubules X 500.

Fig. 24. Necrosis at haematopoietic region of kidney of infected *A. testudineus* . X 500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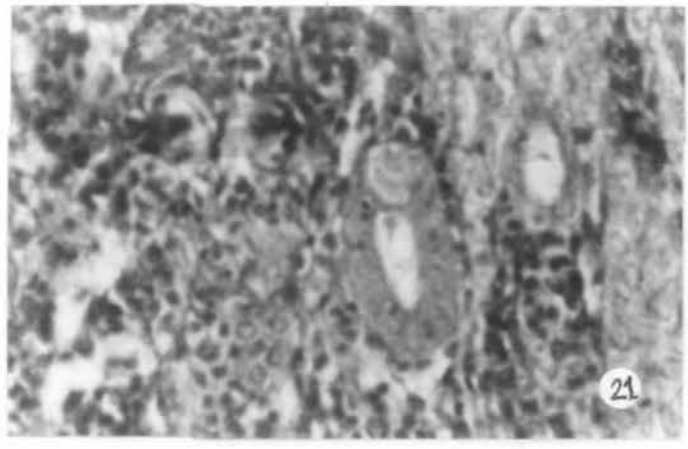
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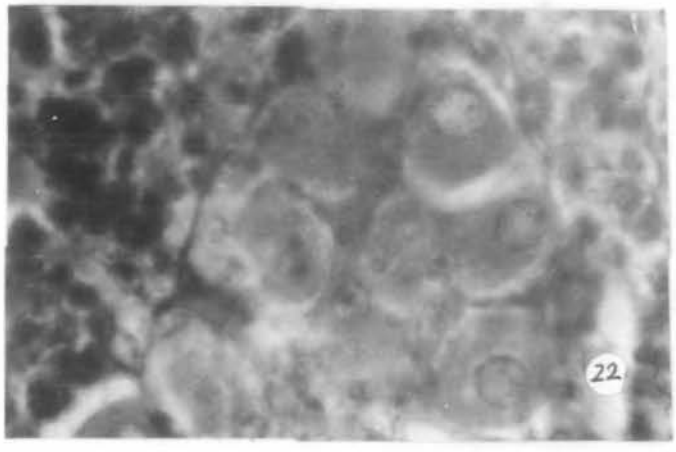
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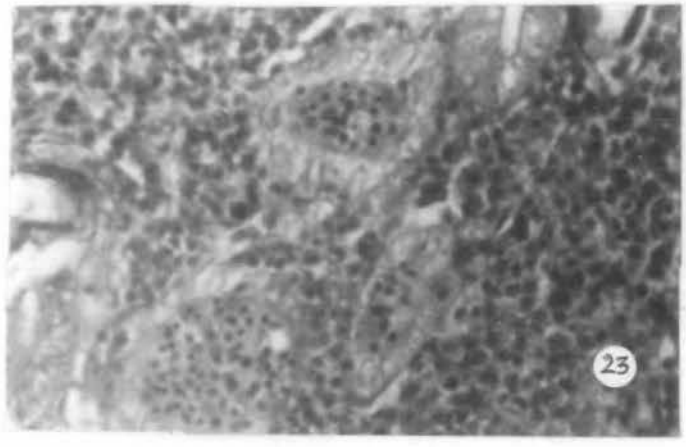
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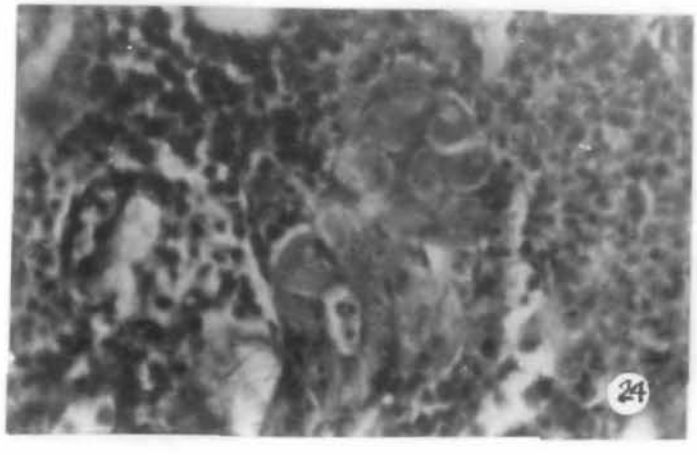
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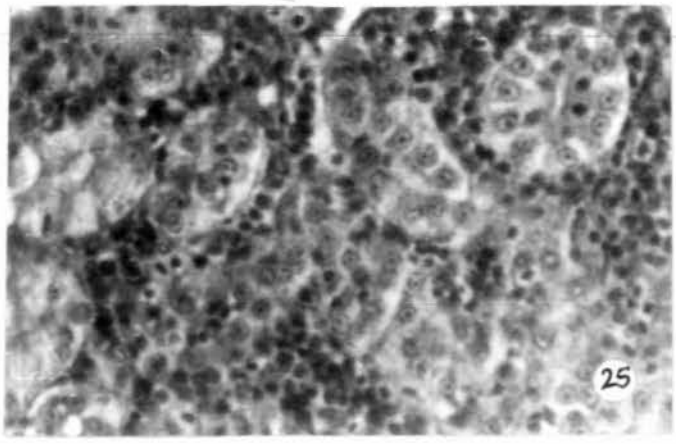
Plate IX

Fig. 25. Showing a part of kidney of C. batrachus (Control) X 500.

Fig. 26. A part of kidney of infected C. batrachus showing tubular breakage. X 500

Fig. 27. A part of kidney of infected C. batrachus showing necrosis at haematopoietic region X 500.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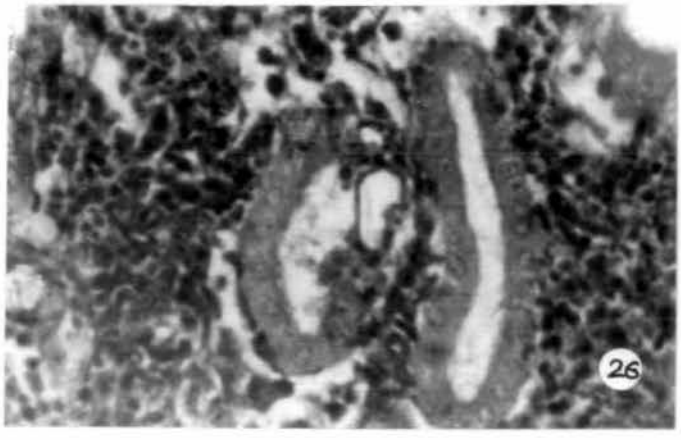
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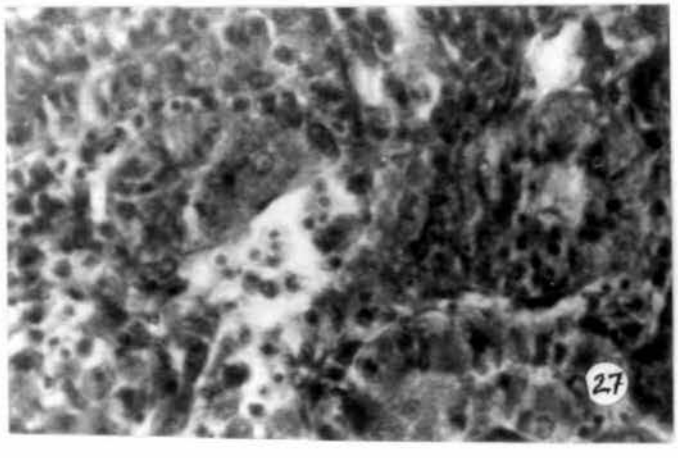
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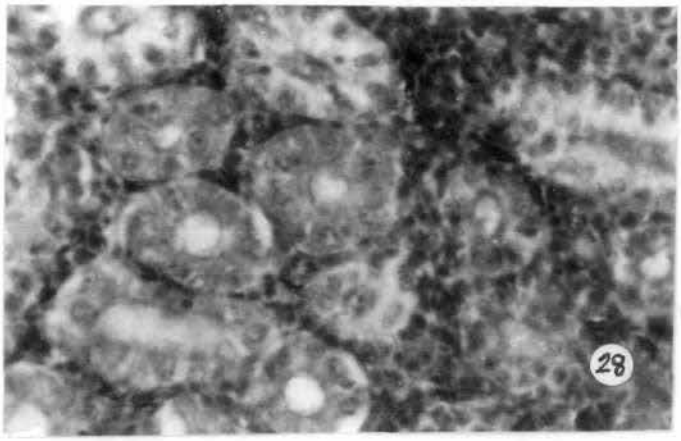
Plate X

Fig. 28. Showing a part of kidney of H. fossilis (Control) X 500.

Fig. 29. A part of kidney of infected H.fossilis showing tubular vaculation, and necrosis at haematopoietic region X 500.

Fig. 30. A part of kidney of infected H.fossilis showing tubular degeneration. X 500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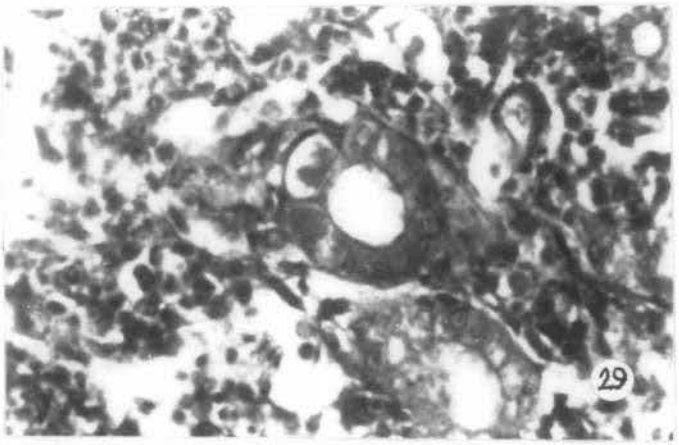
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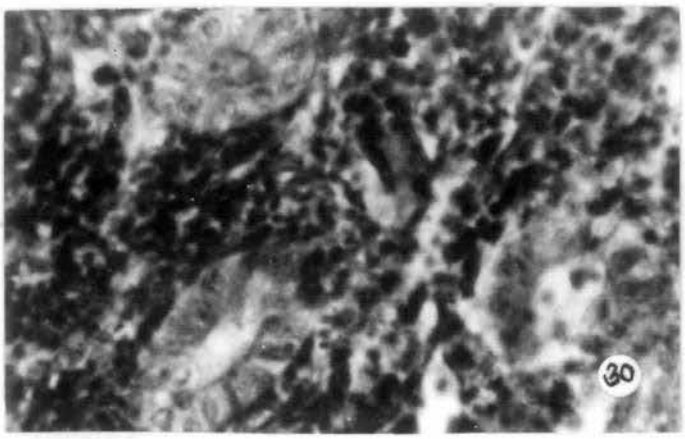
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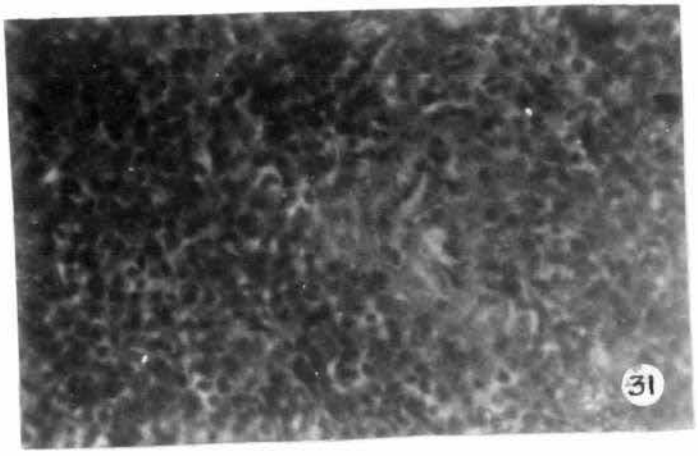
Plate XI

Fig. 31. Showing a part of spleen of C. batrachus (Control) X 500.

Fig. 32. A part of spleen of infected C. batrachus showing necrosis at white pulp region X 500.

Fig. 33. A part of spleen of infected A. testudineus showing vaculation X 500.

Fig. 34. A part of spleen of infected H. fossilis showing necrosis at white pulp region X 500.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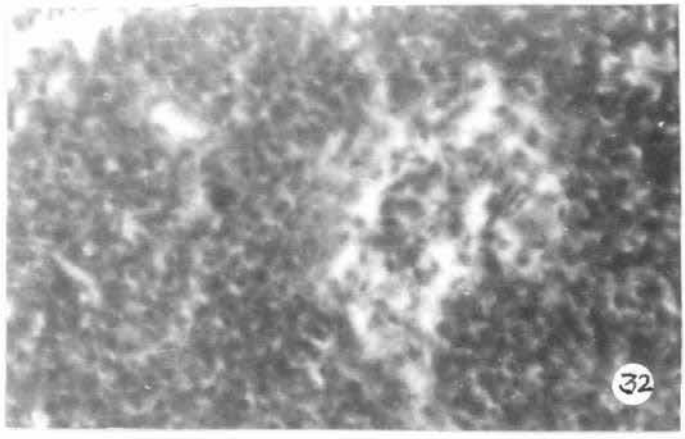
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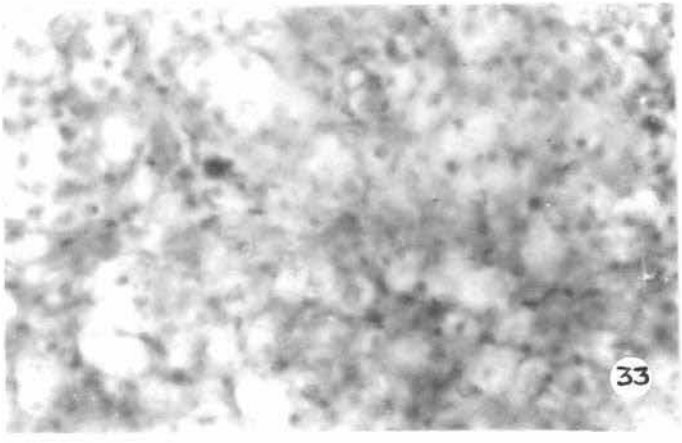
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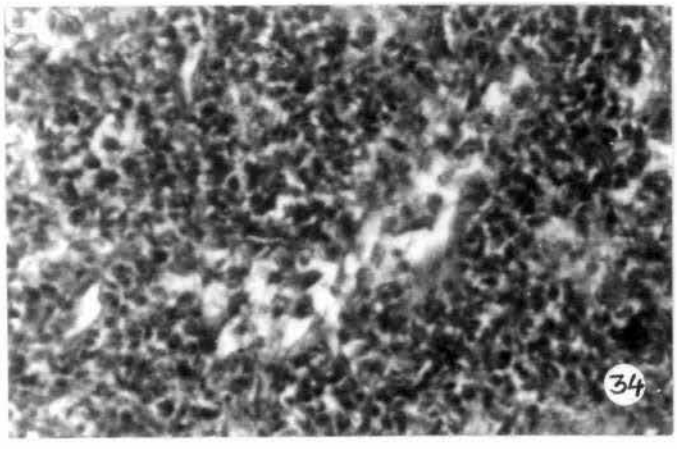

34

Histological sections of liver, kidney and spleen stained with carbol-fuchsin (Ziehl's) showed presence of bacteria rods and coccus (Figs. 35 and 36). But no such bacteria were seen in the sections of liver, kidney and spleen of healthy fishes.

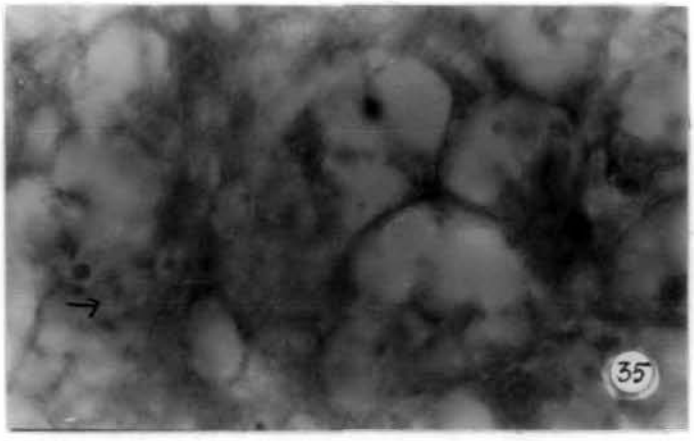
Four types of bacteria (rods, R_1, R_2, R_3 and sphere C) were detected from the nutrient broth supplemented with 0.1% glucose incubated with a portion of ulcer tissue. Two types of bacteria (R_3 and C) were also detected from nutrient broth incubated with ulcer tissues. All the four types of bacteria were detected from culture media after incubation with the ulcer tissues of the three species of air-breathing fish such as C. batrachus, A. testudineus and H. fossilis.

From their morphological and physiological characteristics (Table 2) it appeared that three bacteria R_1, R_2 and R_3 were rod shaped and straight measuring about 0.68 - 0.75 by 2.2 - 2.7 μm, 0.68 - 0.72 by 1.8 - 2.2 μm and 0.65 - 0.68 by 1.8 - 2.7 μm, respectively. (Figs. 37-39). They were found singly, in pairs, and some times in short chains. All three rod shaped bacteria showed motility in hanging drop suspension. No spores were found. The cells were gram negative, Colonies on agar plates were circular, smooth and slightly convex.

Plate XII

Fig. 35. Section of liver of infected C. batrachus showing bacteria. (arrow) X 1250 (carbol-fuchsin stain).

Fig. 36. Section of kidney of infected C.batrachus showing bacteria (arrow) X 1250 (carbol-fuchsin stain).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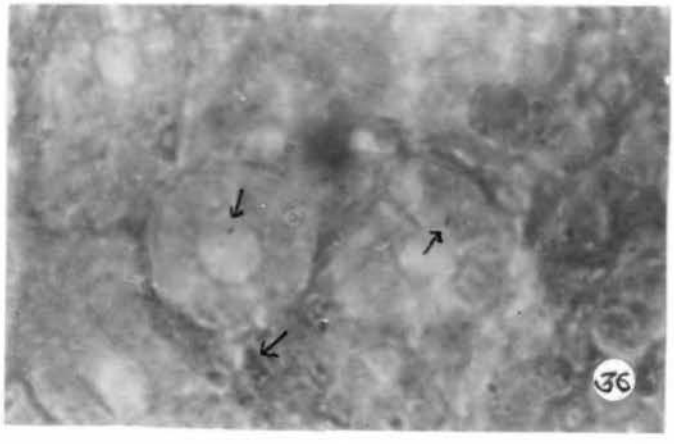

36

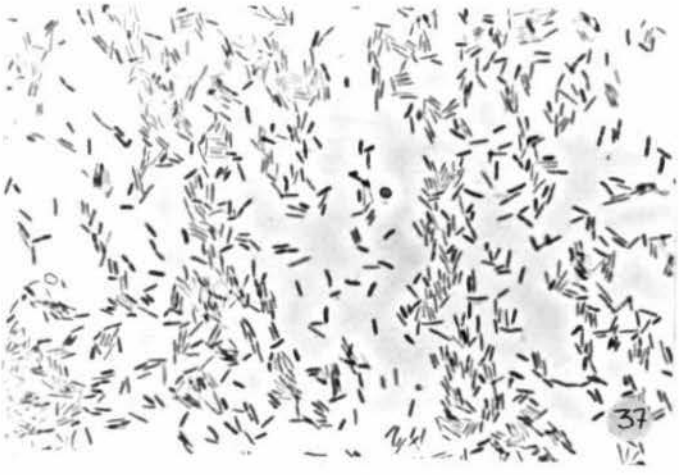
Plate XIII

Fig. 37. Showing rod shaped bacteria, R_1 (Pseudomonad) phase contrast photomicrograph. X 350.

Fig. 38. Showing rod shaped bacteria, R_2 (Pseudomonad) phase contrast photomicrograph. X 350.

Fig. 39. Showing rod shaped bacteria, R_3 (Aeromonas caviae) phase contrast photomicrograph. X 800

Fig. 40. Showing coccus C.(Micrococcus varians) phase contrast photomicrograph. X 800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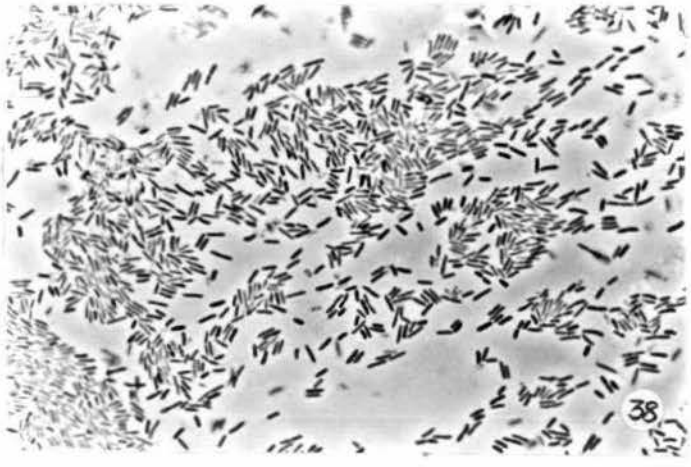
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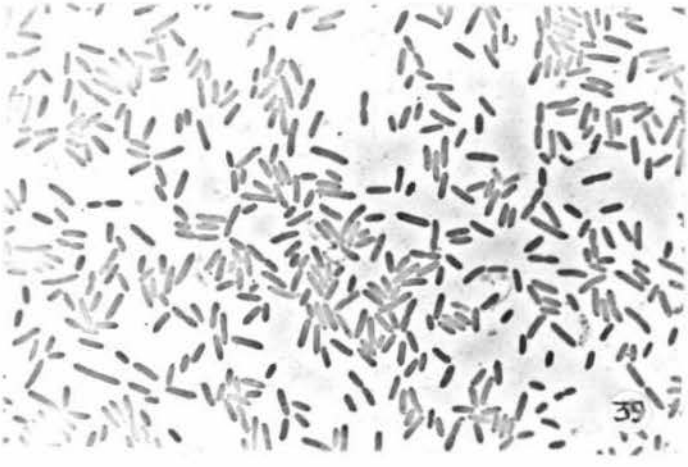
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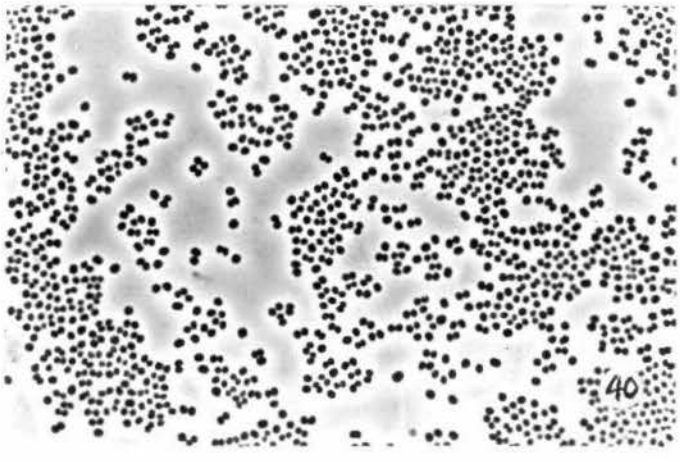
40

Table 2: Morphological and physiological characteristics of the four bacteria (three rods R_1, R_2 and R_3 and one coccus C) isolated from ulcers of Anabas testudineus, Heteropneustes fossilis and Clarias batrachus.

Parameter	R_1	R_2	R_3	C
Shape	Rod	Rod	Rod	Sphere
Occurence	Single, in pairs or in chain	Single, in pairs or in chain	Single, in pairs or in chain	Single, in pairs in tetrad, in irregular cluster or in short chain.
Size	2.2 - 2.7 x 0.68 - 0.75 µm.	1.8 - 2.2 x 0.68 - 0.72 µm	1.8 - 2.7 x 0.65 - 0.68 µm	0.9 - 1.2 µm
Spore	-	-	-	-
Gram stain	-	-	-	+
Agar colonies	Circular, smooth slightly convex.	Circular, smooth slightly convex.	Circular, smooth, convex.	Small, smooth, Convex.
Catalase activity	+	+	+	+
Oxidase activity	+	+	+	-
Motility	+	+	+	-
Broth	Turbid with pellicle and sediments	Turbid with pellicle and sedi-ments.	Turbid	At first turbid then clear with sediments.

Contd.....

Parameter	R_1	R_2	R_3	C
Growth at				
25°C	m	m	m	++
30°C	m	m	++	++
37°C	++	++	++	++
42°C	-	m	-	-
O-F test	O	O	F	O
Acid from carbohydrates				
L-arabinose	-	+	+	-
D-glucose	+	+	+	+
D-Fructose	+	+	+	+
Sucrose	+	-	+,g	+
D-Lactose	-	-	+	+
Adonitol	-	-	-	-
D-sorbitol	-	-	-	+
meso-Inositol	+	-	-	-
Mannitol	+	+	+	+
Utilization of amino acids				
β-Alanine	+	-	-	-
L-arginine	+	+	+	-
L-Aspartic acid	-	-	-	-
L-valin	+	+	-	-

Contd.....

Parameter	R_1	R_2	R_3	C
Gas from Glycerol	-	-	-	-
Glucose	-	-	-	-
Nitrate reduction	-	+	+	+
Nitrite reduction	-	-	+	+
Indole production	-	-	+	-
Arginine dihydrolase	+	+	+	-
Hydrolysis of gelatin	+	+	+	-
Levan formation	+	-	-	-
V-P test	-	-	-	-
Pigment	Yellowish green (in medium 'B' of King et al.,)	Yellowish green (in medium 'B' of King et al.,) and green pigment.	-	Yellowish

+, positive, ++, good; -negative, ; m,- moderate, O, oxidative; F , fermentative; g, gas formation.

Nutrient broth culture of two rod shaped bacteria (R_1 and R_2) were turbid with pellicle and sediments and another rod shaped bacteria (R_3) showed turbidity only in nutrient broth. All three rod shaped bacteria showed good growth at 37°C, and no growth was observed at 42°C except R_2 which showed moderate growth. At 30°C R_1 and R_2 showed moderate growth where as R_3 showed good growth, at 25°C all showed moderate growth.

The other bacterium was spherical (0.9 - 1.2 µm in diameter) found singly, in pairs, in tetrad (Fig.40), in irregular cluster in fresh cultures and in short chain in old cultures. The cells were gram positive and no spore were found. Colonies on agar plates were small, circular, smooth and convex. Cultures in nutrient broth first appear turbid then became clear with sediments. It showed good growth at 25°C, 30°C and 37°C but there was no growth at 42°C.

R_1 bacterium produced acid from D-glucose, D-fructose, sucrose, meso-inositol and mannitol and no acid was produced from L-arabinose, D-lactose, adonitol and D-sorbitol. R_2 bacterium produced acid from L-arabinose, D-glucose, D-fructose and mannitol but no acid was formed from sucrose, D-lactose, adonitol, D-sorbitol and meso-inositol; R_3 bacterium produced acid from L-arabinose, D-glucose, D-fructose, sucrose, D-lactose and

mannitol but no acid was formed from adonitol, D-sorbitol, and meso-inositol. R_3 bacteria produced gas from sucrose and no gas production was detected from glucose and glycerol.

C bacterium produced acid from D-glucose, D-fructose, sucrose, D-lactose, D-sorbitol and mannitol and no acid was produced from L-arabinose adonitol and meso-inositol.

O.F.test clearly indicated that two rod shaped bacteria (R_1 and R_2) and coccus (C) utilized glucose by oxidation as only the unsealed (with out paraffin) tube turns yellow, another rod shaped bacterium (R_3) utilized glucose by fermentation as both the tubes turned yellow.

No red colour developed in the culture tubes containing nitrate broth inoculated with rod shaped bacterium R_1 after addition of nitrite reagents which indicated absence of nitrite in the culture tubes. But after addition of zinc dust appearance of red colour indicated presence of nitrate in the culture tubes. So it clearly indicated that the R_1 bacteria was unable to reduce nitrate to nitrite. However, other two rod shaped bacteria (R_2, R_3) and Coccus (C) developed red colour after addition of nitrite reagents in the culture tubes

containing nitrate broth inoculated with R_2, R_3 and C bacteria respectively which indicated that these three bacteria were capable of reducing nitrate to nitrite.

The culture tubes containing nitrite broth inoculated with R_1 and R_2 bacteria, developed red colour after addition of nitrite reagent, which indicated presence of nitrite and was not reduced by these two rod shaped bacteria. On the other hand,no red colour were found after addition of nitrite reagents in the culture tubes containing nitrite broth inoculated with the R_3 and C bacteria. It indicated absence of nitrite in the culture tubes which was reduced by the two bacteria (R_3 & C).

Three bacteria (R_1, R_2 and C) could not produce indole, whereas culture medium inoculated with R_3 bacteria produced red colour after addition of Kovac's reagent indicating indole production.

All three rod shaped bacteria hydrolysed gelatin as clear zones surrounding the colonies of the bacteria were developed both on culture plates and on slants after addition of acid mercuric chloride solution. On the other hand, coccus, C bacteria could not hydrolyse gelatin.

All four bacteria, three rods (R_1, R_2 and R_3) and one a coccus (C) showed positive test for catalase activity as bubble of gas appeared after pouring down hydrogen peroxide (H_2O_2).

Positive oxidase activity was shown by all three rods as a dark purple colour appeared within 10 sec, on the paper impregnated with oxides reagent. The coccus showed negative result for oxidase activity.

Only colonies of R_1 bacteria on agar plates and on slants, containing 4% sucrose, became slimy due to levan formation. Colonies of R_2, R_3 and C bacteria did not produce levan.

Three rods R_1, R_2, R_3 and coccus C bacteria showed negative result in Voges-Proskauer reaction.

R_1 utilized β-alanine, L-arginine and L-valin but L-aspartic acid was not utilized as a sole carbon source. R_2 utilized L-arginine and L-valin but β-alanine and L-aspartic acid were not utilized as a sole carbon source. R_3 utilized L-arginine but β-alanine, L-aspartic acid and L-valin were not utilized. C did not utilize any supplied amino acids.

Yellowish green pigments were produced by the two rod shaped bacteria R_1 and R_2 in medium B of King et al., (1954); and the pigments showed its fluorescent nature.

under UV light. No pigments were produced on medium A of King *et al.*, (1954), R_3 and C did not produce any pigment in either medium of King *et al.*, (1954). However yellowish pigment was produced by the colonies of C bacteria on nutrient agar kept at room temperature in diffused day light. Occasionally, R_2 produced green pigment and in old cultures the green pigment turned into reddish.

Therefore, from their morphological and physiological characteristics it appeared that R_1 and R_2 bacteria belonged to the genus *Pseudomonas*, R_3 bacterium belonged to the genus *Aeromonas* and the coccus (C) belonged to the genus *Micrococcus*.