A N N E X U R E

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Bacterial involvement in ulcerative condition of air-breathing fish from India

J. PAL AND K. PRADHAN

Department of Zoology, Centre for Life Sciences, University of North Bengal, P.O. North Bengal University, Siliguri 734 430, West Bengal, India

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Four types of bacteria, two fluorescent pseudomonads, one aeromonad (*Aeromonas hydrophila anaerogens*) and one coccus (*Micrococcus varians*) were isolated from skin lesions of air-breathing fishes. The bacterial culture in mixed condition induced severe ulcers in healthy *Anabas testudineus*. Pure cultures of each of two pseudomonads and an aeromonad separated from the mixture induced superficial ulcer formation.

Key words: ulcers; *Anabas testudineus*; *Heteropneustes fossils*; *Clarias batrachus*; causative bacteria.

I. INTRODUCTION

Since May 1988, severe outbreaks of ulcerative fish disease have occurred in various states of north-eastern India. The disease has been reported in various types of fishes from a range of different water bodies including rivers, canals, beels (large, shallow, stagnant waters), paddy fields and ponds (Das, 1988). During the last two decades the disease has been reported from the Indo-Pacific region by several workers (Gopalakrishnan, 1963; Monohar *et al.*, 1976; Rodgers & Burke, 1977, 1981; Pal *et al.*, 1978; Haines, 1983; Pal, 1984; Tonguthai, 1985).

II. MATERIALS AND METHODS

Infected specimens of the air-breathing fishes *Anabas testudineus*, *Heteropneustes fossilis* and *Clarias batrachus* were collected from Cooch Behar, Jalpaiguri, Darjeeling and West Dinajpur districts of West Bengal, India. Disease showed as red spots on the skin of *H. fossilis* and *C. batrachius*, and grey patches on the body of *A. testudineus*; infections were at a primary stage. The minimum and maximum distances 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 × 35 × 35 cm in which the depth of water was 20 cm, at a density of 10–15 fishes per aquarium tank. Half of the septic water of the aquarium was changed every day. The water temperature varied from 28 to 30° C.

Altogether, 129 *A. testudineus* (15–35 g), 16 *H. fossilis* (15–25 g) and 11 *C. batrachus* (50–70 g) were collected. Of these, 104 (80·62%) *A. testundineus*, 12 (75·0%) *H. fossilis* and 11 (91·8%) *C. batrachus* died within 5, 6 and 4 days, respectively, in the laboratory. The remainder recovered from the infection.

One of the surviving fishes were killed and tissues from liver, kidney and spleen were fixed in Bouin's fixative and preserved in cedarwood oil. Smear preparations with the necrotic tissues of the skin lesions and blood were stained with Giemsa, Leishman and Ziehl-Neelsen fuchsin stain. A portion of the necrotic ulcer tissues was incubated in sterilized

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FIG. 1. *Clarias batrachus* showing ulcer covered with whitish membrane-like structure.

bacterial nutrient medium and in nutrient medium supplemented with 0·1% glucose at 30° C for 24 h.

Bacteria were isolated by the pour-plate method. The cultures of bacteria were maintained routinely on nutrient agar slants. Bacteria were cultured in nutrient broth when required. Intra-muscular injections of 0·1 ml of mixed culture and pure cultures of all the isolated bacteria were given to healthy *Anabas*, (20–30 g). Control fishes received only nutrient broth. Each experimental group consisted of five fishes and was replicated twice.

Morphological and physiological characteristics of four bacteria were assessed according to Cowan & Steel, (1965). All the bacteria were stained for Gram reaction and tested for (i) acid and gas production in media containing different carbohydrates, (ii) oxidation or fermentation of glucose, (iii) nitrate and nitrite reduction, (iv) production of indole, (v) hydrolysis of gelatin, (vi) catalase activity, (vii) oxidase activity, (viii) pigment production, (ix) levan formation, and (x) Voges–Proskauer test.

III. RESULTS

Within 6 days of being taken into the laboratory, 125 of the 156 infected specimens of three fish species collected from various sites died.

In the case of fishes without scales, e.g. *C. batrachus* and *H. fossilis*, the symptoms of the disease first appeared as a red spot on the skin of the body. Gradually the red spot increased in size and an ulcer developed in the affected area. Ultimately the underlying muscle layer became affected. Occasionally the ulcers remained covered by a thin whitish membrane surrounded by a reddish area (Fig. 1), and they occasionally became deep and haemorrhagic. The fins were also affected.

In scaly fishes such as *Anabas* 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. 2). The fins were also affected.

Smear preparations stained with Giemsa, Leishman and carbol fuchsin showed the presence of bacteria only; no other agent, e.g. protozoa or fungus, was detected. Four types of bacteria (rods R_1, R_2 and R_3, and coccus, C) were identified

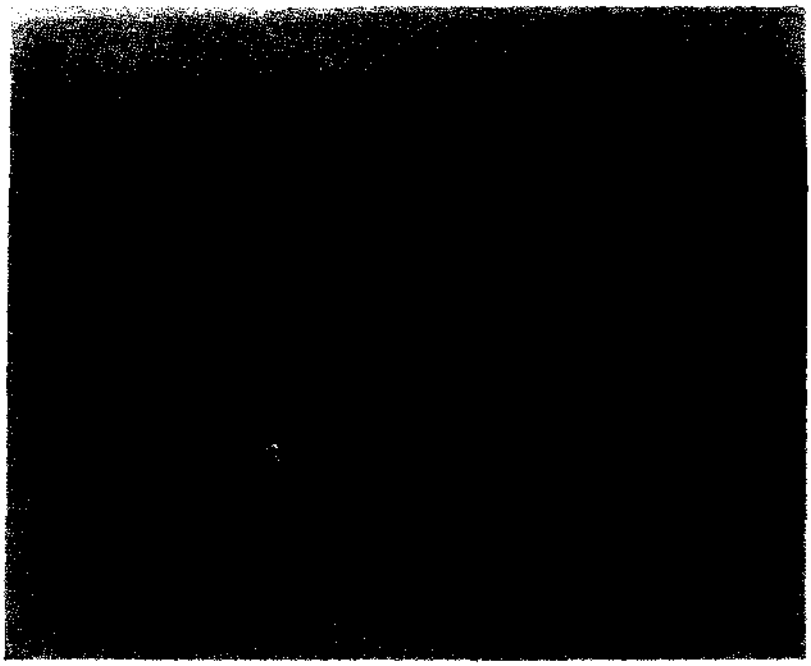

FIG. 2. *Anabas testudineus* showing haemorrhagic ulcer with affected tail fin.

from the nutrient broth supplemented with 0·1% glucose incubated with a portion of necrotic ulcer tissue. Two types of bacteria (R_3 and C) were detected from the nutrient broth incubated with necrotic ulcer tissues. All the four types of bacteria were detected from culture media after incubation with the necrotic ulcer tissues of all the three species of fishes.

The pure cultures of R_1, R_2 (pseudomonads) and R_3 (aeromonad) bacteria induced superficial ulcer formation at the point of injection in *Anabas*. R_3 bacteria induced rather more ulcer formation than did R_1 and R_2 bacteria. The mixed culture of all the bacteria induced deep ulcer formation (Fig. 3) and three fishes (30%) died. No mortality was found in other groups of experimental fishes. No ulcer formation was noticed in the control and C-treated groups of fishes.

From their morphological and physiological characteristics (Table I) it appeared that R_1 and R_2 belonged to the genus *Pseudomonas*, R_3 belonged to the genus *Aeromonas*, and C belonged to the genus *Micrococcus*.

IV. DISCUSSION

The results of the present study show that pure culture of R_1, R_2 (fluorescent pseudomonads) and R_3 (aeromonad) induced superficial ulcer formation at the point of injection whereas coccus (micrococcus) had no effect; mixed culture induced severe ulcer formation and caused 30% mortality. Gopalakrishnan (1963) reported ulcer disease caused by bacteria in Indian major carps, and Mawdesley-Thomas & Jolly (1968) reviewed ulcerative bacterial disease in trout. In furunculosis, skin lesions represent areas of necrosis and *Aeromonas salmonicida* is

FIG. 3. *Anabas testudineus* showing development of ulcer after intramuscular injection of mixed culture of four bacteria.

the causative agent. Ulcer disease is common among various species of trout and is due to the bacterium *Haemophilus piscium*. But in all cases single bacterium is involved in disease production.

Infectious dropsy of carp is associated with ascites and skin lesions. Opinions differ about the etiology of infectious dropsy. Tomasec & Fijan (1965) concluded that infectious dropsy of carp is caused primarily by a virus and that *Aeromonas punctata* or some other bacteria influences the severity of the changes and development of the disease. On the other hand, Schäperclaus (1965) regarded *A. punctata* as the etiological agent of infectious dropsy, and he thought that primary infections resulting in disease may be induced by *Pseudomonas fluorescens*. Schäperclaus (1969) indicated that three different microorganisms (*A. punctata, P. fluorescens* and a virus) may be involved in infectious dropsy of carp, which manifests itself in several forms. Pal & Pal (1986) reported induction of ulcers in *Anabas* by a mixed culture of two bacteria isolated from epithelial carcinoma in *Anabas*. Our results indicate that no one bacterium is responsible for the ulcerative condition in *Anabas*.

Experiments with *Channa punctatus* in our laboratory have shown (1) that mixed cultures induced severe ulceration in *C. punctatus* (2) that R_3 (*Aeromonas hydrophila anaerogens*) induced ulceration, but not so extensive as that caused by the mixed culture, and (3) that two pseudomonads induced superficial ulcer formation (Pradhan & Pal, 1989). Lallier *et al.* (1981) were of the opinion that, globally, *A. hydrophila* may be the bacterium associated with fish diseases, many strains of it being opportunistic and others behaving as the primary pathogen. *Pseudomonas aeruginosa, P. fluorescens* and *A. hydrophila* are normally found in water sources and soil, their opportunistic role in causing septicaemia and abscesses has been discussed by several workers (von Graevenitz, 1977; Sonnenwirth, 1980*a,b*; Lallier *et al.*, 1981).

In experiments with *Anabas*, mixed culture of bacteria caused 30% mortality in laboratory conditions, whereas the infected fishes collected from different regions

TABLE I. Morphological and physiological characteristics of the four types of bacteria (three rods R_1, R_2 and R_3, and one coccus C) isolated from ulcerated *Anabas testudineus*, *Heteropneustes fossilis* and *Clarias batrachus*

Parameter	R_1	R_2	R_3	C
Shape	Rod	Rod	Rod	Sphere
Staining	Gram negative	Gram negative	Gram negative	Gram positive
Occurrence	Single, in pairs or in chain	Single, in pairs or in chain	Single, in pairs or in chain	Single, in pairs, in regular cluster or in short chain
Motility	+	+	+	−
Agar colonies	Circular, smooth, slightly convex	Circular, smooth, slightly convex	Circular, smooth, convex	Small, smooth, convex
Broth	Turbid with pellicle and sediments	Turbid with pellicle and sediments	Turbid	At first turbid, then clear with sediments
Growth at:				
25° C	m	m	m	+ +
30° C	m	m	+ +	+ +
37° C	+ +	+ +	+ +	+ +
42° C	−	m	−	−
Gas from:				
Glycerol	−	−	−	−
Glucose	−	−	−	−
Acid from carbohydrates:				
L-arabinose	−	+	+	−
Glucose	+	+	+	+
Fructose	+	+	+	+
Sucrose	+	−	+,g	+
Lactose	−	−	+	+
O-F test	O	O	F	O
Nitrate reduction	−	+	+	+
Nitrite reduction	−	−	+	+
Indole production	−	−	+	−
Hydrolysis of gelatin	+	+	+	−
Catalase activity	+	+	+	+
Oxidase activity	+	+	+	−
Levan formation	+	−	−	−
V–P. test	−	−	−	−
Pigment	Yellowish green	Yellowish green and green	−	Yellowish

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

showed heavier mortality in the laboratory (80·62, 75·0 and 81·81% in *A. testudineus*, *H. fossilis*, and *C. batrachus*, respectively); this could be due partly to stress aggravating the infection.

The R_1 and R_2 bacteria in the present study were Gram negative, straight, non spore-forming, oxidative, catalase positive and produced yellowish green fluorescent pigment, so they belonged to the genus *Pseudomonas* (Stanier *et al.*, 1966; Doudoroff & Palleroni, 1974). The R_1 bacterium satisfied two important characteristics of *P. fluorescens*, i.e. production of fluorescent yellowish green pigment in medium B of King *et al.* (1954) and levan formation; it also satisfied two important characteristics (levan formation and denitrification inability) of biotype I of *P. fluorescens* (Doudoroff & Palleroni, 1974), but it differed from biotype I of *P. fluorescens* in that it did not break down L-arabinose. The R_2 bacterium was also a fluorescent pseudomonad, as it produced yellowish green fluorescent pigment in medium B of King *et al.* (1954), but it differed from R_1 in that it was capable of breaking down L-arabinose, could not break down sucrose into acid, reduced nitrate into nitrite, and was capable of growing at 42° C. The R_2 bacterium also produced green pigment in iron-deficient medium, nutrient broth or agar, and in old culture the green pigment became reddish. It showed some similarities with *Pseudomonas aeruginosa*, e.g. no levan formation and growth at 41° C, but it differed from *P. aeruginosa* by producing non-fluorescent green pigment. Ajellow & Hadley (1976) have reported a fluorescent pseudomonad capable of growth at 41° C but distinct from *Pseudomonas aeruginosa*. Thus, it is apparent that both R_1 and R_2 bacteria were fluorescent pseudomonads but that they differed from each other. A decision about their taxonomic status and nomenclature must await further studies.

Morphological and physiological characteristics of the R_3 bacterium satisfy the characteristics of *Aeromonas hydrophila* (Schubert, 1974). As it did not produce gas from glucose and glycerol, it was of the subspecies *anaerogens*, and, as it was Voges–Proskaur reaction negative, it was of the biotype II (Schubert, 1974). So the R_3 bacteria was *Aeromonas hydrophila anaerogens* (biotype II).

Morphological and physiological characteristics of the coccus (C) satisfied the characteristics of the species *Micrococcus variance* (Baird-Parker, 1974).

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Experimental Induction of Ulcer in the Fish *Channa punctatus* by Bacteria

K. PRADHAN AND J. PAL

Department of Zoology, Center for Life Sciences, North Bengal University, Siliguri 734430, India

Abstract

Healthy fish *Channa punctatus* were inoculated with mixed culture and pure cultures of four bacteria, two fluorescent pseudomonads, one aeromonad (*Aeromonas hydrophila anaerogens*) and one coccus (*Micrococcus variance*). The mixed culture induced severe ulcer, aeromonad induced ulcer but not to that extent as caused by the mixed culture and two pseudomonads induced superficial ulcer formation. Mortality was high in mixed culture treated group compared to aeromonad and pseudomonad treated group. No ulcer formation and mortality were recorded in the coccus treated and control group of fish.

The outbreaks of the ulcerative fish disease in epizootic form have taken place in most of the eastern Indian states in 1988. The disease has affected various types of fishes of rivers, canals, beels, paddy fields and ponds (1). Pal and Pradhan (2) isolated four types of bacteria, three rods (R_1, R_2 and R_3) and one coccus (C) from ulcer tissues of airbreathing fishes namely, *Anabas testudineus*, *Clarias batrachus* and *Heteropneustes fossilis*. R_1 and R_2 bacteria showed some similarity with *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* respectively but their exact taxonomic and nomenclatural status is yet to be decided. R_3 bacteria was *Aeromonas hydrophila anaerogens* and coccus (C) was *Micrococcus variance*. Prior to the outbreak of ulcerative fish disease in epizootic form in different states of North East India in 1988, several Indian workers have reported ulcerative skin lesions in fishes (3—6). In 1988, severe outbreaks of the ulcerative fish disease have occurred in North Bengal districts such as Cooch Behar, Jalpaiguri, West Dinajpur, Malda and plains of Darjeeling districts. The incidence of the disease has been reported from some South Bengal districts such as Murshidabad, Nadia, North and South 24 Parganas and Midnapore. In 1989, the disease have spread in all most all the South Bengal districts such as Nadia, North and South 24 Parganas, Midnapure, Howrah, Hooghly, Burdwan and Birbhum. The ulcerative fish disease has affected some areas of West Dinajpur, Jalpaiguri and Cooch Behar districts in 1989.

During the last two decades the outbreak of the ulcerative fish disease have been reported from different countries of the Indo-Pacific region. In Queensland, Australia, the disease is named "red spot disease", which has affected marine and estuarine fishes in 1972 with recurrence in the subsequent years (7). Fish disease characterized by dermal ulcer from the rivers of the South Papua, New Guinea in 1975-76 has been reported by Haines (8). In Indonesia the disease, infectious dropsy or hemorrhagic septicemia has spread to West Central and Eastern Java in 1980 (9). The outbreaks of the disease in epizootic form in 1980 and recurrence thereafter have been reported from Thailand (10). The ulcerative fish diseases have also been reported from different European countries (11—14). The present work deals with the

effects of the bacteria (two) pseudomonads, one aeromonad and one coccus) on *Channa punctatus* in mixed and in pure condition.

Methods

The culture of the four bacteria (R_1, R_2, R_3 and C) isolated from the necrotic ulcer tissues of air-breathing fish, were maintained routinely on agar slants and in nutrient broth. The bacteria were cultured in nutrient broth at 30 C when required. Intramuscular injections of 0.1 ml of bacterial culture in mixed and in pure condition were given to healthy *Channa punctatus*, weighing 25—30 g. For each set of experiment, ten fish were taken. Each experiment was done two times. The control fish received only 0.1 ml of nutrient broth. The gradual development of ulcers after injection was carefully observed. Mortality caused by the injections of bacterial cultures was also recorded.

Results and Discussion

The control group of fish and coccus treated group showed neither any development of ulcer nor any mortality (Fig. 1). Fish injected with the mixed culture of all the four bacteria showed reddish area around the point of injection on the following day of injection. The reddish area gradually increased followed by a swelling in that region. Most of the fish died at this stage. Then scales of the affected area fell down exposing an open sore. Ultimately the ulcer became deep and the muscle layers were also affected. The surviving fish showed gradual healing of the ulcer after six or seven days of injection and it took 10—15 days for complete healing of ulcer. The fish injected with the culture of R_3 (*Aeromonas hydrophila anaerogens*) developed ulcer but was not as severe as induced by the mixed culture of the four bacteria. The culture of R_1 and R_2 bacteria (pseudomonads) developed superficial ulcers at the point of injection. The percent of mortality of fish was 70, 40, 25 and 35% in mixed culture, R_3, R_2 and R_1 injected groups, respectively.

Results have shown that the pure culture of R_1 and R_2 (pseudomonads) have induced superficial ulcers at the point of injections and C (coccus) induced no ulcer formation at all. On the other hand, R_3 (aeromonad) and mixed cultures have induced ulcers but induction of ulcers by mixed culture are severe. Pal and Pradhan (2) have reported similar effects in experiment with the four bacteria on *Anabas testudineus*. All the four bacteria were isolated from three different species of air-breathing fishes such as *A. testudineus*. *H. fossilis* and *C. batrachus*. The present experiment gives an indication that these bacteria may be responsible for ulcerative fish disease in various types of air-breathing fishes and in inducing ulcer some sort of opportunistic association of these bacteria may be involved. Pal and Pal (15) have reported induction of ulcer in *Anabas* by mixed culture of two bacteria isolated from epithelial carcinoma in *Anabas*.

Lallier et al. (16) have believed that *Aero-*

Table 1. Percent of mortality and nature of ulcer formation by pure and mixed cultures of bacteria in healthy fish *Channa punctatus*. (a) Percent mortality is given in parenthesis. C, *Micrococcus variance*; R_1, Pseudomonad, which has some similarities with *Pseudomonas fluorescens*; R_2, Pseudomonad, which has some similarities with *Pseudomonas aeruginosa*; and R_3, *Aeromonas hydrophila anaerogens*.

Parameter	No. of fish injected	No. of fish died[a]	Nature of ulcer development
Control	20	Nil	No ulcer
Mixed	20	14 (70)	Severe deep ulcer
R_3	20	8 (40)	Ulcer developed but not severe
R_2	20	5 (25)	Superficial ulcer
R_1	20	7 (35)	Superficial ulcer
C	20	Nil	No ulcer

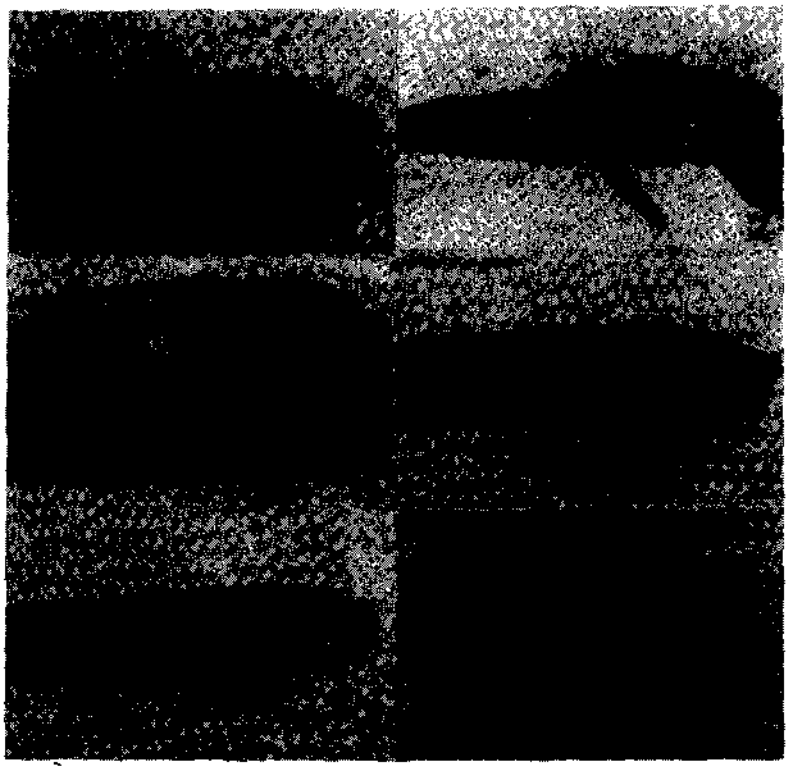

Figure 1. Experimental induction of ulcer in the fish *Channa punctatus*. (1) Fish showing no development of ulcer after intramuscular injection of bacterial nutrient broth ; (2) fish showing a swelling at the region of injection after intramuscular injection of mixed culture of four bacteria (R_1, R_2, R_3 and C) ; (3) fish showing development of deep ulcer after intramuscular injection of mixed culture of four bacteria (R_1, R_2, R_3 and C) ; (4) fish showing ulcer development after intramuscular injection of pure culture of aeromonad bacteria (R_3) ; (5) fish showing superficial ulcer development after intramuscular injection of pure culture of pseudomonad (R_1) ; and (6) fish showing superficial ulcer development after intramuscular injection of pure culture of pseudomonad (R_2).

monas hydrophila may be the bacterium globally associated with fish diseases, many strains of which are opportunistic and other clearly behave as primary pathogen. The opportunistic role of *Pseudomonas aeruginosa, P. fluorescens* and *A. hydrophila* in causing septicemia and abscess in human being had also been reported (17—19). Tomasec and Fijan (12) have reported that infectious carp dropsy associated with ascities and skin lesion is caused by a virus and *Aeromonas punctata* or some other bacteria. Though Schaperclaus (11) have regarded *A punctata* as the etiological agent of infectious carp dropsy, he also considered *Pseudomonas fluorescens* as a primary infectious agent. Manifestation of several forms of infectious carp dropsy and involvement of three microorganism such as *A. punctata, P. fluorescens* and a virus have been discussed by Schaperclaus (20).

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Addendum

We do not have the instrument to meet the level of precision asked for.

Figure 13. A part of liver of infected A.testudineus showing slight atrophy in some regions and degeneration in some other regions x 125.

Figure 14. A part of liver of infected A.testudineus showing enlarged sinusoids (dilation of sinusoids) with cord like arrangement of atrophied hepatic cells x 500.

Figure 18. A part of liver of infected H.fossilis showing degenerative changes x 500.

Figure 19. A part of liver of infected H.fossilis showing cord like arrangement of atrophied hepatic cells with dilated sinusoids. x 500.

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

Figure 22. A part of kidney of infected A. testudineus showing enlarged eosinophilic cells of a tubule. x 1250.

Figure 23. A part of kidney of infected A.testudineus showing accumulation of eosinophilic materials (may be erythrocytes) within the lumen of the tubules. x 500.

Figure 24. Necrosis of haematopoietic region of kidney of infected A.testudineus x 500.

Figure 26. A part of kidney of infected C.batrachus showing tubular breakage (may be due to necrosis of tubular epithelial cells). x 500.

Figure 27. A part of kidney of infected C.batrachus showing necrosis of haematopoetic region. x 500.

Figure 28. Showing a part of kidney of H.fossilis (control) x 500.

Figure 30. A part of kidney of infected H.fossilis showing tubular degeneration. x 500.

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

Figure 33. A part of spleen of infected A.testudineus showing vaculation may be due to disappearance of lymphoid elements and erythrocytes and dilation of sinusoids. x 500.

Figure 34. A part of spleen of infected H.fossilis showing necrosis in some regions and edematous dilation of sheathed tissue. x 500.

Figure 35 & 36. Staining has been done following the method of Mackie and McCartney's Practical Medical Microbiology 1989. (eds : J.G.Collee, J.P.Duguid, A.G.Fraser and B.P.Marmion). Churchill Livingstone.

Figure 57. A part of liver of C.batrachus showing degenerative changes in some regions and central area showing necrosis of hepatic cells after intramuscular injection of mixed bacteria. x 500.

Figure 60. A part of liver of C.bactrachus showing vaculation of hepatic cells due to necrotic changes after intramuscular injection of R_1 bacteria (fluorescent pseudomonad). x 1250.

Figure 61. A part of kidney of C.batrachus showing cloudy swelling after intra-muscular injection of mixed bacteria. x 1250.

Figure 62. A part of kidney of C. batrachus showing necrotic changes in the haematopoietic region after intramuscular injection of mixed bacteria. x 500.

Figure 63. A part of kidney of C.bactrachus showing cloudy swelling (vaculation and tubular degeneration) after intra - muscular injection of R_3 bacteria (Aeromonas caviae) x 500.

Figure 64. A part of kidney of C.batrachus showing degeneration of tubules and necrosis after intra-muscular injection of R_1 bacteria (fluorescent pseudomonad) x 500.

Figure 65. A part of kidney of C.batrachus showing degeneration of tubules and necrosis after intra-muscular injection of R_2 bacteria (fluorescent pseudomonad) x 500.

Figure 67. Vaculation due to disappearance of lymphoid elements and erythrocytes from pulp region of spleen of C.batrachus afte inoculation of mixed bacteria x 1250.

Figure 68. Showing necrosis in some regions and dilated centra vein in the spleen of C.batrachus after inoculation of mixed bacteria. x 500.