Experiments and Results

4.1. Studies on the involvement of the three bacteria two fluorescent Pseudomonads (R1 and R2) and one Aeromonad (R3) in the ulcerative disease of fishes

In 1988, soon after the initial outbreak of epizootic ulcerative in North Bengal two fluorescent Pseudomonads (R1 and R2) and one Aeromonad (R3) were isolated from the skin ulcers of affected air breathing fishes (Pal and Pradhan, 1990). Since these bacteria were able to induce ulcers when injected to healthy fishes, it was considered worthwhile to study the clinical symptoms, histopathology and haematology of fishes that were experimentally infected by these pathogenic bacteria along with a study on the pathogenicity and virulent characteristics of these bacteria.

4.1.1. Studies on the pathogenicity of the bacteria R1, R2 and R3

Though initial findings suggested that R1, R2 and R3 were able to induce ulcers when injected in high doses to healthy fish, there was no report regarding the LD_{50} values of these bacteria in any fish (Pradhan, 1992). Therefore, an attempt was made to determine the LD_{50} values of these bacteria and a comparative study was done on the susceptiblity of three species of air breathing fishes to these three pathogenic bacteria.

4.1.1.1. Determination of LD_{50} value

Cell suspensions of the three bacteria R1, R2 and R3 were injected to healthy fishes (*Heteropneustes fossilis*) in serially diluted doses which ranged from $5x10^7$ to $5x10^3$ cfu in 0.05 ml of the inoculum. The details of the methodology followed is given in materials and methods. The mortality rates of the fishes increased with increasing concentrations of all the three bacterial suspensions. R1, R2 and R3 could be isolated from the external lesion and liver and kidney of the dead fishes. At the highest concentration ($5x10^7$ cfu), 80% of the fishes inoculated with R1and R2 and 90% of the fishes inoculated with R3 died. At the lowest concentration ($5x10^3$ cfu), only 20% of the fishes injected with R1 and 30% of the fishes injected with R2 and R3 died. The LD_{50} values of

R1, R2 and R3 was calculated to be $9.97x10^5$ cfu, 4.45×10^5 cfu and 6.29×10^5 cfu respectively (Table 2). It was observed that R1 was slightly less pathogenic than R2 and R3.

Table - 2 : Cumulative mortalities of *Heteropneustes fossilis* after 15 days of inoculation with serial 10 fold dilutions of R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*).

	Number of dead fishes / number of fishes inoculated		
Dose[a] (c.f.u.)	R1	R2	R3
5×10^7	7/10	8/10	8/10
5×10^6	5/10	6/10	6/10
5×10^5	4/10	5/10	4/10
5×10^4	3/10	3/10	3/10
5×10^3	2/10	2/10	2/10
LD_{50}[b]	9.97×10^5	4.45×10^5	6.29×10^5

[a] c.f.u. / 0.05 ml of inoculum

[b] Calculated number of bacteria required to kill 50% of injected fish. (Calculated from relationship between probits of percentage mortalities and the logs of the dilution series of bacterial suspension).

4.1.1.2. Susceptibility of three species of air breathing fishes to R1, R2 and R3

Susceptibilities of different species of fish to the same bacterial pathogen are known to differ considerably (Al-Harbi, 1996; Sakai *et al*, 1991). Since air

breathing fishes are commercially important freshwater fish species in West Bengal, an attempt was made to study the comparative pathogenicity of R1, R2 and R3 in *Channa punctatus*, *Anabas testudineus* and *Heteropneustes fossilis*. A comparative study on the progressive development of the clinical symptoms of the disease in fishes with scales (*A. testudineus*) and those without scales (*H. fossilis*) was also attempted.

Accordingly, saline suspension of the three fish pathogenic bacteria R1, R2 and R3 were injected intramuscularlly (0.5 ml / 100 gm body weight) to healthy *C. punctatus*, *A. testudineus* and *H. fossilis* in pure and mixed conditions. The dose was adjusted to $5x10^5$ cfu. Each bacteria was injected to 10 fishes of each species, the control set was injected with sterile saline (0.85% NaCl). The experiment was repeated 4 times under identical conditions. Thus 40 fishes of each species was injected by each bacterial suspension. All fishes were kept under observation for 15 days.

4.1.1.2.1. Observation of external pathological symptoms

All species developed similar type of ulcers and dead fishes with ulcers on their body were noted in all aquariums. In the control set of fishes, no change was noticed within the 15 days of observation (Figs. 9a, 10a and 11). All fishes that developed ulcers, however, did not die. In some fishes with moderate ulcers, healing was observed. The results are summarised in table 3,4 and 5.

Fishes treated with mixed bacterial suspension

Within 24 hours of inoculation 95% of the total number of fishes injected with the mixed bacterial suspension of the three bacteria R1, R2 and R3 manifested external signs of the disease. The external clinical symptoms did not vary much among the three fish species. Initially, the area around the injection site turned reddish. Gradually it swelled and around the small red spot (3 to 4 mm diameter) a zone of discolouration of the skin was noticed. No notable change in the swimming behaviour was observed. The skin was almost intact at this stage and this type of lesion was termed as superficial ulcer. In fish with scales, the scales were almost intact and only the mucous layer was affected (Fig. 12). None of the fishes died at this stage.

These superficial ulcers were observed between 20 to 30 hours after inoculation after which the ulcers increased in size (8 mm to 14 mm). In *H. fossilis* the surface layer of the skin was eroded. In *A. testudineus* and *C. punctatus* the scales fell off and erosion of the skin was noticed (Figs. 9e and 13). The fish became sluggish with irregular opercular movement. This stage of ulcer was termed as moderate ulcer. Some fishes died at this stage.

After 48 to 72 hours, in some of the surviving fishes, the ulcers became deep and necrotic, the underlying muscle layer was eroded and the skeletal musculature was exposed. The fish mainly remained motionless either at the floor of the aquarium or floated near the surface making 45 to 90^0 angle of their body to the surface of the water. This type of ulcer was termed as severe ulcer (Fig. 10e). All fishes at this stage ultimately died with deep open sores on their body surface. Healing of the surviving fishes with moderate ulcers was noticed after 7 or 8 days of injection and it took about 12 days for complete healing.

Fishes treated with pure bacterial suspension

In fishes treated with pure bacterial suspension of R1, R2 and R3, about 75% of the fishes showed reddish swellings at the injection site within 24 to 48 hours after inoculation. Gradually after 48 hours, the skin was eroded and the underlying muscle layer was exposed. In scaly fishes, the scales sloughed at the site of ulcer and the skin was also eroded (Figs. 9b, 9c and 9d). The ulcer produced was moderate and measured about 8 to 14 mm in diameter. The fishes began to die after 72 hours with moderate ulcers at the injection site (Figs. 10b, 10c and 10d). Healing of the surviving fishes was observed after 7 to 8 days of inoculation and it took about 12 to 14 days for complete healing.

4.1.1.2.2. Comparative mortality rate of the three fish species

In case of *A. testudineus*, the mortality rate was 65.0% when inoculated with a mixed bacterial suspension of R1, R2 and R3. With pure bacterial suspensions, R1 induced 32.5% mortality, R2 induced 40.0% mortality and R3 induced 42.5% mortality (Table 3).

Plate IV

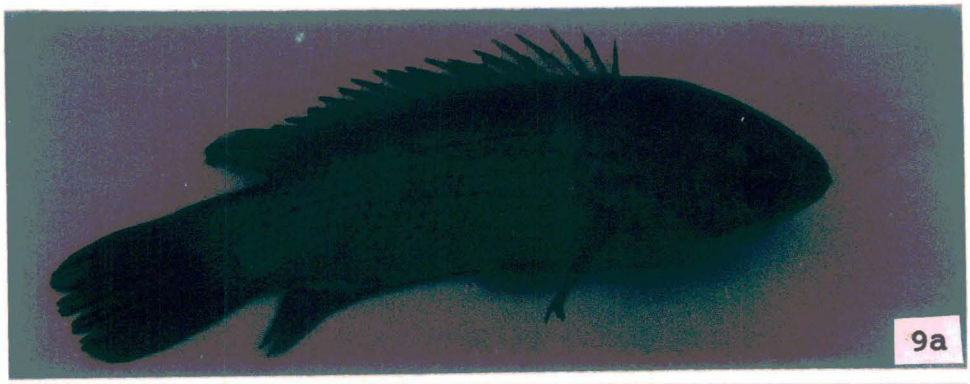
Fig. 9a. *Anabas testudineus* showing no ulcer formation after intramuscular injection with sterile saline suspension.

Fig.9b. *Anabas testudineus* showing ulcer formation 48 hours after intramuscular injection with R1 (fluorescent Pseudomonad).

Fig.9c. *Anabas testudineus* showing ulcer formation 48 hours after intramuscular injection with R2 (fluorescent Pseudomonad).

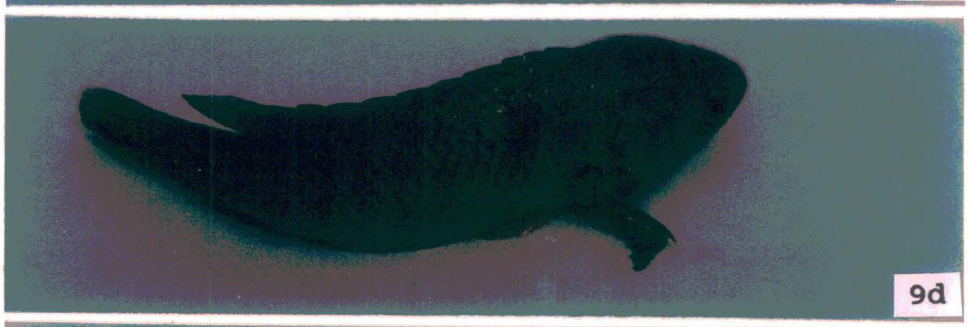
Fig.9d. *Anabas testudineus* showing ulcer formation 48 hours after intramuscular injection with R3 (*Aeromonas caviae*).

Fig.9e. *Anabas testudineus* showing ulcer formation 48 hours after intramuscular injection with a mixed suspension of R1, R2 and R3.


9a


9b


9c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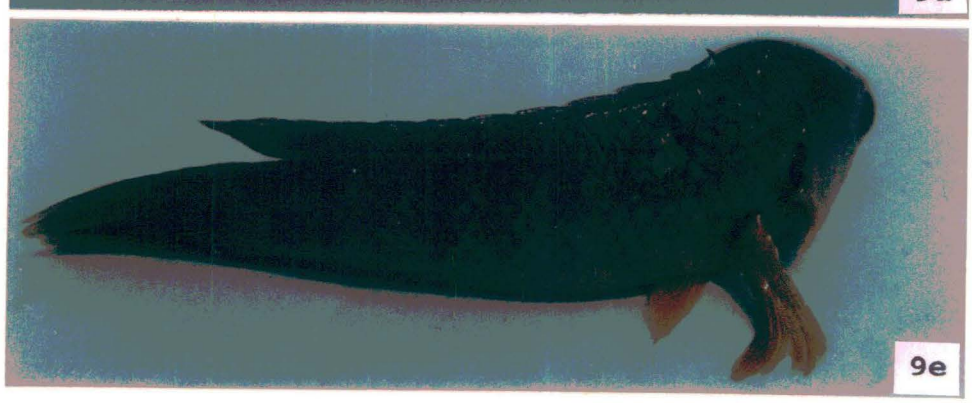

9e

Plate V

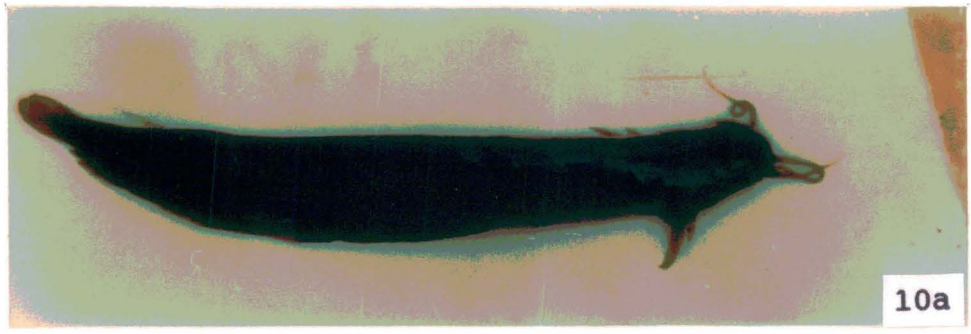
Fig.10a. *Heteropneustes fossilis* showing no ulcer formation after intramuscular injection of sterile saline suspension.

Fig.10b. *Heteropneustes fossilis* showing ulcer formation 72 hours after intramuscular injection with R1 (fluorescent Pseudomonad).

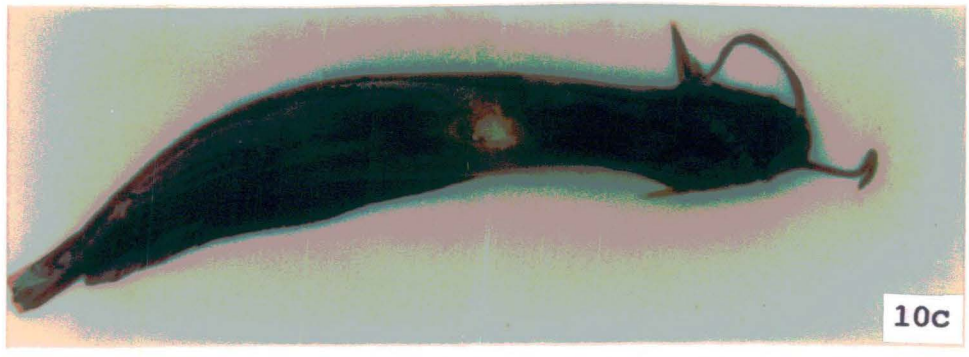
Fig.10c. *Heteropneustes fossilis* showing ulcer formation 72 hours after intramuscular injection with R2 (fluorescent Pseudomonad).

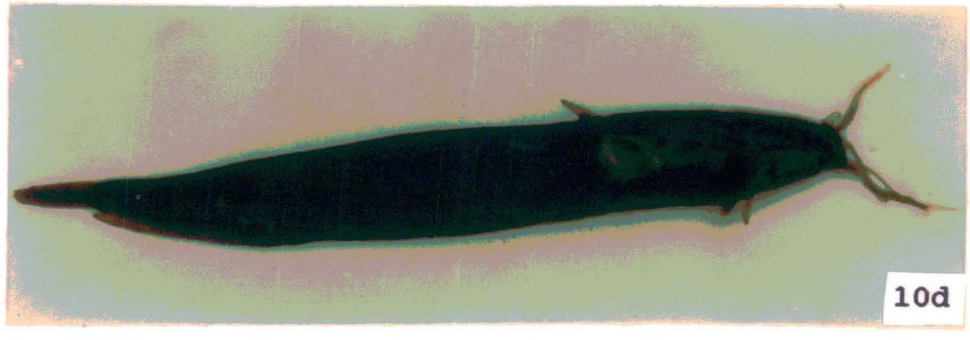
Fig.10d. *Heteropneustes fossilis* showing ulcer formation 72 hours after intramuscular injection with R3 (*Aeromonas caviae*).

Fig.10e. *Heteropneustes fossilis* showing ulcer formation 72 hours after intramuscular injection with a mixed suspension of R1, R2 and R3.


10a


10b


10c


10d

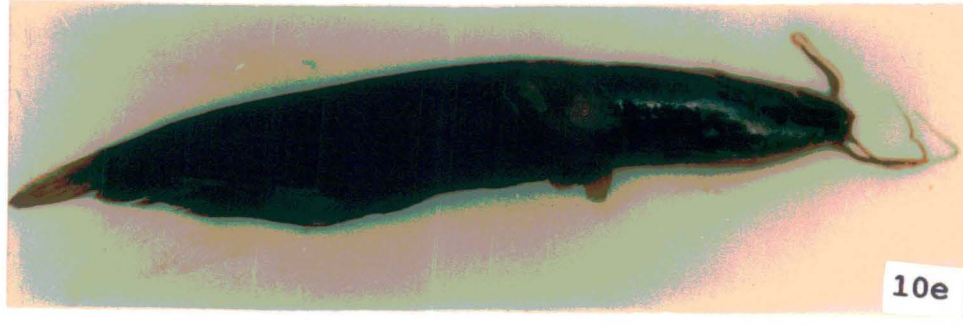

10e

Plate VI

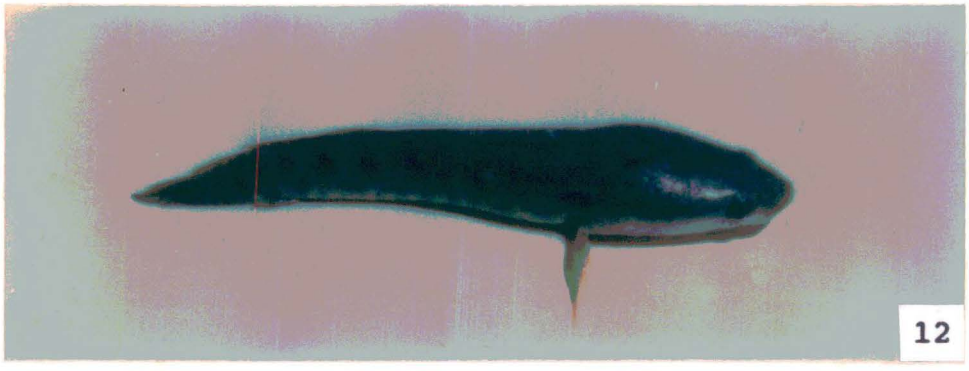
Fig. 11-13. *Channa punctatus* showing gradual development of ulcer following intramuscular injection of mixed bacterial suspension of R1, R2 (fluorescent pseudomonads) and R3 (*Aeromonas caviae*) :

Fig. 11. Showing *C. punctatus* immediately after injection.

Fig. 12. Development of ulcer after 24 hours.

Fig. 13. Development of ulcer after 48 hours.


11


12


13

In *C. punctatus*, the percentage mortalities of fishes infected with R1, R2 and R3 were 40.0%, 42.5% and 47.5% respectively. The mixed bacterial suspension induced 77.5% mortality (Table 4).

In *H. fossilis*, 72.5% of fishes injected with mixed bacterial suspension died. The percentage mortalities of fishes injected by R1, R2 and R3 were 37.5%, 45.0% and 42.5% respectively (Table 5).

Cumulative mortalities of fishes with respect to number of days after inoculation is shown graphically in Figs. 14-17. The figures show that in all cases there is a steep rise in the number of mortalities 24 hours after injection with the pathogenic bacteria. The maximum number of mortalities occur between 48 to 96 hours after injection. After 96 hours, the mortality rate again falls sharply and no fishes were found to be dead 7 days after inoculation.

In all three fish species (*C. punctatus*, *A. testudineus* and *H. fossilis),* the mortality rate of fishes injected with R1 was slightly less than R2 and R3. The percentage mortality however, did not vary much among the three fish species (Fig. 18).

Table : 3. Percentage mortality and Nature of ulcer formation in *Anabas testudineus* injected intramuscularly with saline suspensions of R1, R2 and R3 in pure and mixed condition.

	Number of fishes inoculated	*Number of fishes dead*	*Nature of ulcer*	*Percentage mortality*
Control[a]	40	0	-	-
R1	40	13	moderate	32.5%
R2	40	16	moderate	40.0%
R3	40	17	moderate	42.5%
Mixed	40	26	severe	65.0%

[a] Control set of fishes were intramuscularly injected with sterile saline suspension

Table : 4. 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.

	Number of fishes inoculated	*Number of fishes dead*	*Nature of ulcer*	*Percentage mortality*
Control[a]	40	0	-	-
R1	40	16	moderate	40.0%
R2	40	17	moderate	42.5%
R3	40	19	moderate	47.5%
Mixed	40	31	severe	77.5%

[a] Control set of fishes were intramuscularly injected with sterile saline suspension

Table : 5. Percentage mortality and Nature of ulcer formation in *Heteropneutes fossilis* injected intramuscularly with saline suspensions of R1, R2 and R3 in pure and mixed condition.

	Number of fishes inoculated	*Number of fishes dead*	*Nature of ulcer*	*Percentage mortality*
Control[a]	40	0	-	-
R1	40	15	moderate	37.5%
R2	40	18	moderate	45%
R3	40	17	moderate	42.5%
Mixed	40	29	severe	72.5%

[a] Control set of fishes were intramuscularly injected with sterile saline suspension

Cumulative mortalities of the three air breathing fishes after injection with R1

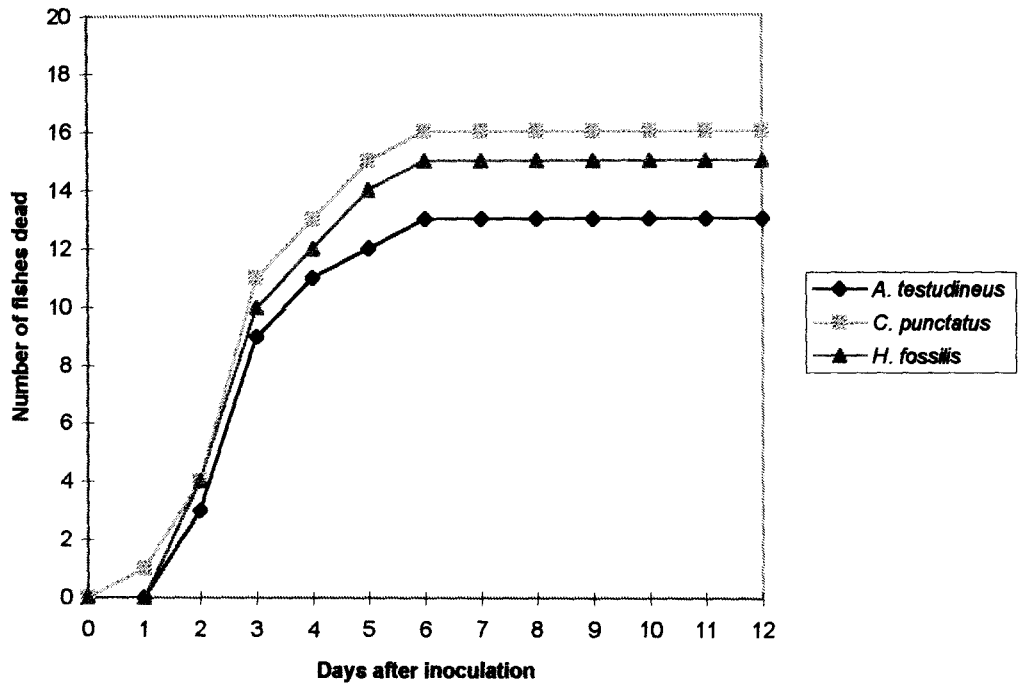


Fig. 14

Cumulative mortality of the three air breathing fishes after injection with R2

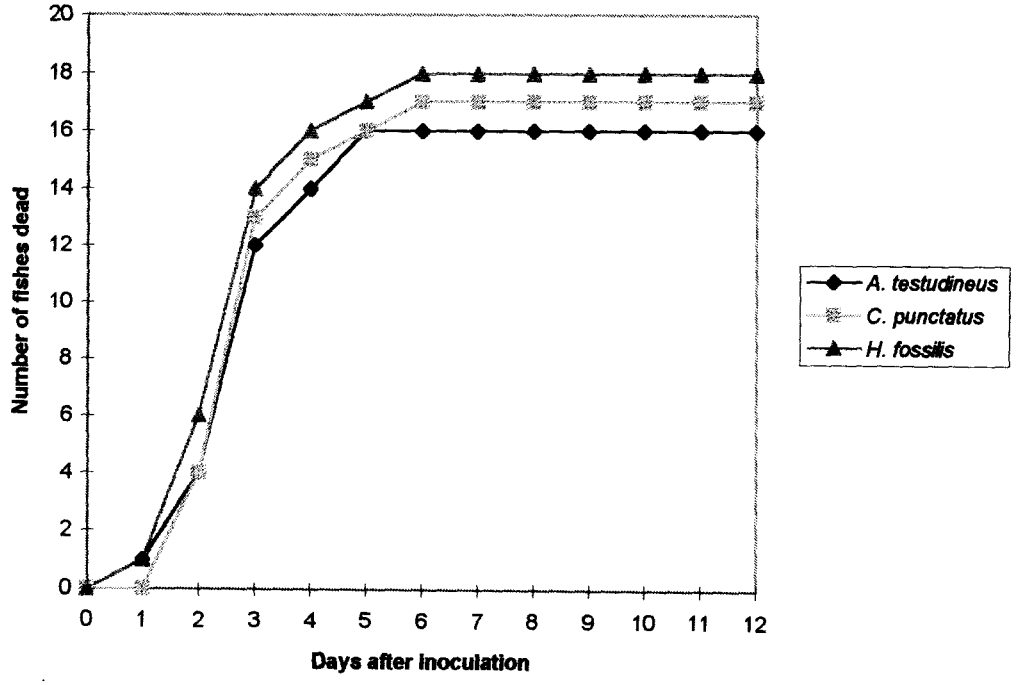


Fig. 15

Cumulative moratalities of the three air breathing fishes after injection with R3

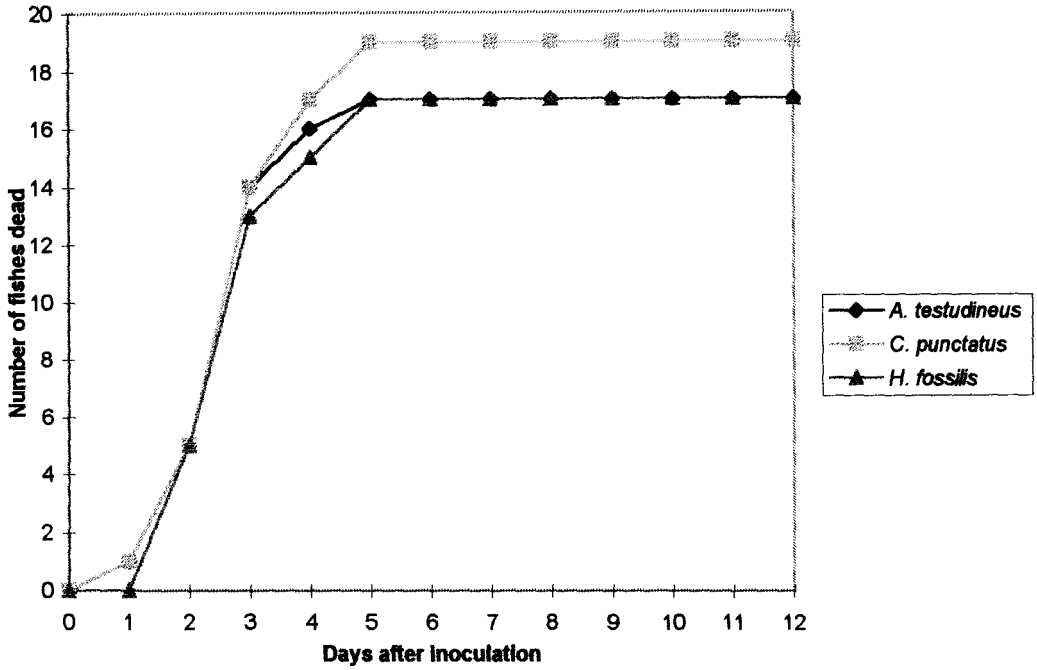


Fig. 16

Cumulative mortalities of the three air breathing fishes after inoculation with a mixed culture of R1, R2 and R3

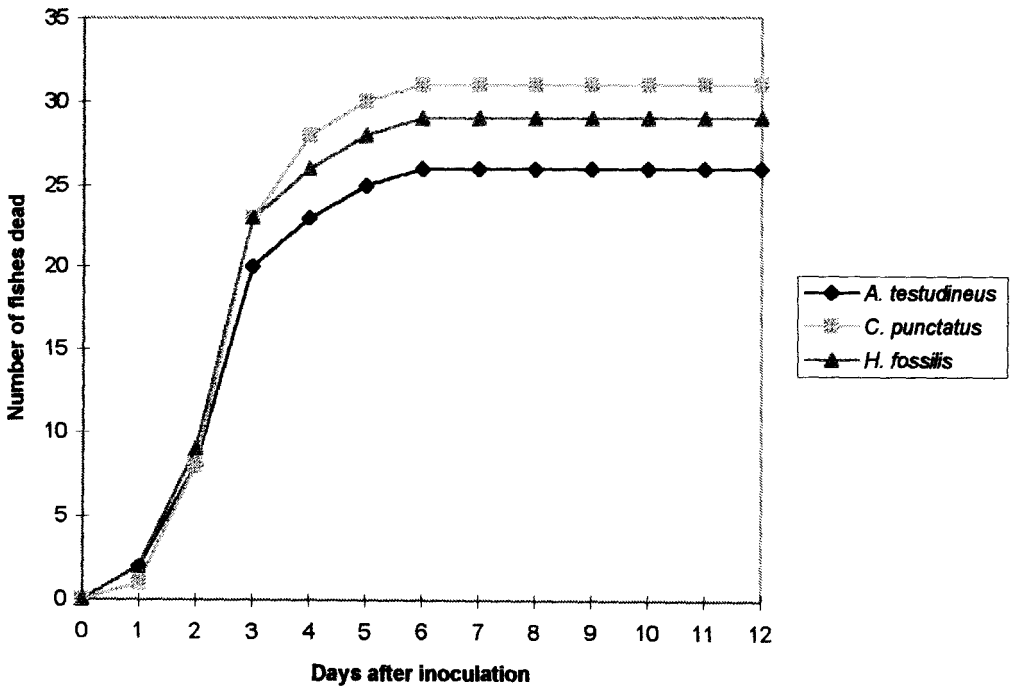
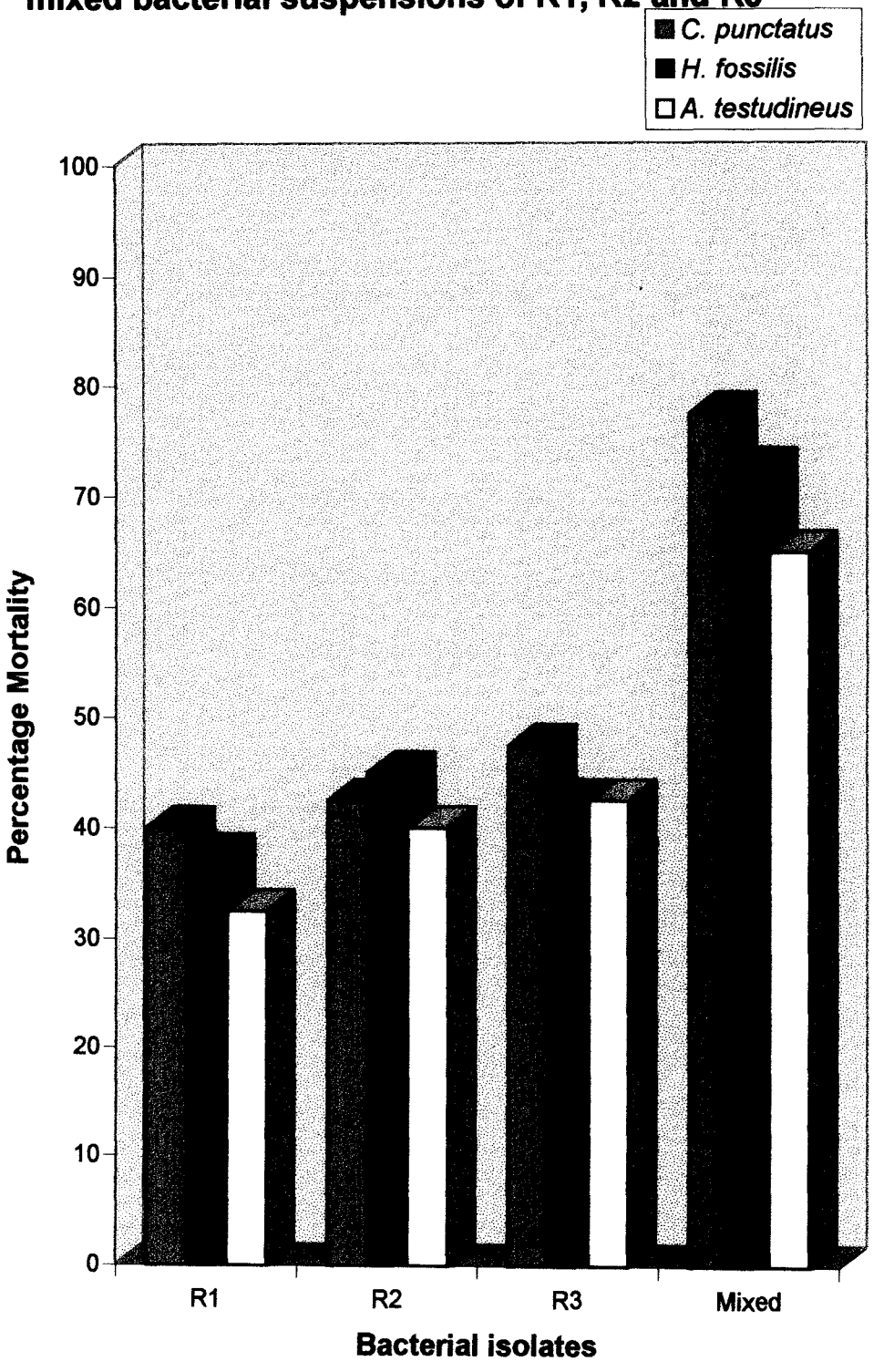


Fig. 17

Comparative mortality of three species of air breathing fishes after inoculation with pure and mixed bacterial suspensions of R1, R2 and R3



4.1.2. Histopathology of experimentally infected fish *Heteropneustes fossilis*

Healthy fishes (*Heteropneustes fossilis*) each weighing about 10 to 15 gms were inoculated by intramuscular injection with 0.85% saline suspension of R1, R2 and R3 bacteria in pure and mixed condition. The concentration of bacterial suspensions was $1x10^7$ cfu / ml. Twenty fishes were injected with each bacterial suspension and the mixed suspension. The control set of twenty fishes received 0.05 ml sterile saline solution. Inoculation techniques are described in materials and methods. The fishes were observed daily for appearance of symptoms. Initial symptoms appeared as small reddish swellings at the site of injection within 24 hours of inoculation. The lesion gradually grew in size and in some fishes, the surface layer of the skin was eroded. The first mortality was recorded after 48 hours. After 72 hours, two fishes each inoculated with R1 and R3 and 3 fishes inoculated with R2 were found dead. At least some fishes in each aquarium having ulcers at the site of injection were found to be moribund. At this stage, the moribund fishes from each aquarium were taken out and sacrificed daily for histological studies till the 7th day. In the control set, no disease symptom appeared in any fish and no mortality was recorded. Two fishes from this set were also sacrificed and their tissues were processed for comparison with the diseased fishes.

Histological sections were prepared and stained with Giemsa and Hematoxylene - Eosin stain. The sections from all diseased fishes showed various degrees of histopathological changes after inoculation with saline suspensions of mixed and pure R1, R2 and R3 bacteria. Giemsa stained sections showed presence of bacteria. No changes were noticed in the sections of liver and kidney of the control set of fishes (Figs. 20a and 21a).

Ulcer

Histological observations of ulcers of fishes treated with a mixed bacterial suspension of R1, R2 and R3 showed complete loss of epidermis and scale. There was inflammatory infiltration and areas of haemmorrhage at the centre of the lesion. The dermis became highly fibrous with infiltration of blood

capillaries. Inflammatory and necrotic changes were also found in the affected area of the dermis. A nectrotic granulomatous response with cloudy degeneration of the muscle fibres was observed. In some areas, the underlying musculature became necrotic with wide separation of individual bundles. In fishes injected with sterile saline suspensions of R1, R2 and R3, changes with cloudy degeneration were observed in the muscle layer. The dermis became fibrous and showed inflammatory changes (Figs. 19a-19d). Giemsa stained sections revealed presence of rod shaped bacteria in the muscle layer beneath the skin in all the affected fishes.

Liver

Observation of histological sections of the liver of the fishes treated with a mixed bacterial suspension revealed vacuolation, necrosis and infiltration of blood capillaries. Focal necrosis were also observed (Fig. 20e). Highly vacuolated hepatic cells and chord like arrangements with enlarged sinusoids were also observed in some regions. The liver of fishes injected with R2 and R3 showed vacuolation, necrotic changes and infiltration of blood capillaries (Figs. 20c and 20d). Vacuolations in the hepatocytes were observed in fishes treated with R1, but the degree of pathological changes were comparatively less (Fig. 20b). Bacteria were seen in the liver of all affected fishes.

Kidney

In the kidney of fishes treated with a mixed culture, tubular degeneration and vacuolation of tubular cells were the prominent changes. Necrotic changes in certain hematopoetic regions and hemorrhages were also seen. Vacuolation, tubular degeneration and necrosis in some regions were also observed in fishes treated with R2 and R3 (Fig 21b). R1 treated kidney showed vacuolation of tubular cells and necrosis in some regions. Haemosiderin laden macrophages were seen in the kidney sections of all infected fishes (Fig. 21c). Presence of bacteria was also observed in the Giemsa stained sections of all affected fishes.

Plate VII

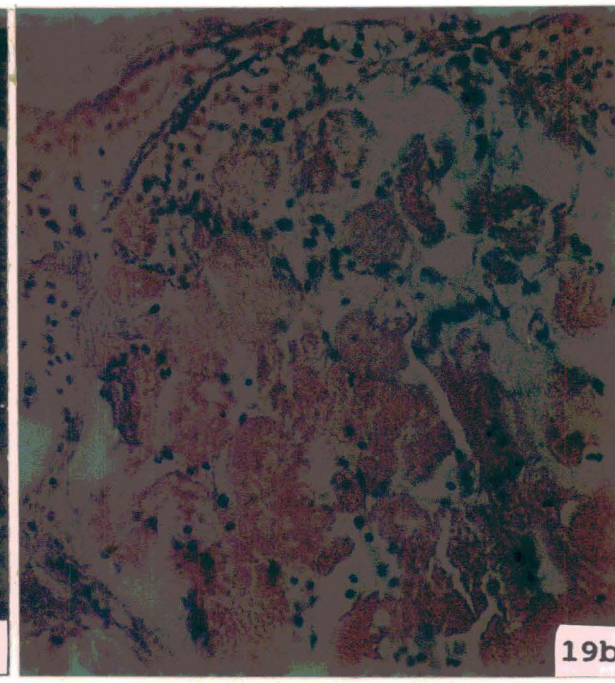
Fig. 19 a. Section of ulcer showing loss of epidermis and early granulomatous changes in the dermal and subdermal layers in *Heteropneustes fossilis* after intramuscular injection with R2 (fluoresent Pseudomonad). (x400) (H-E).

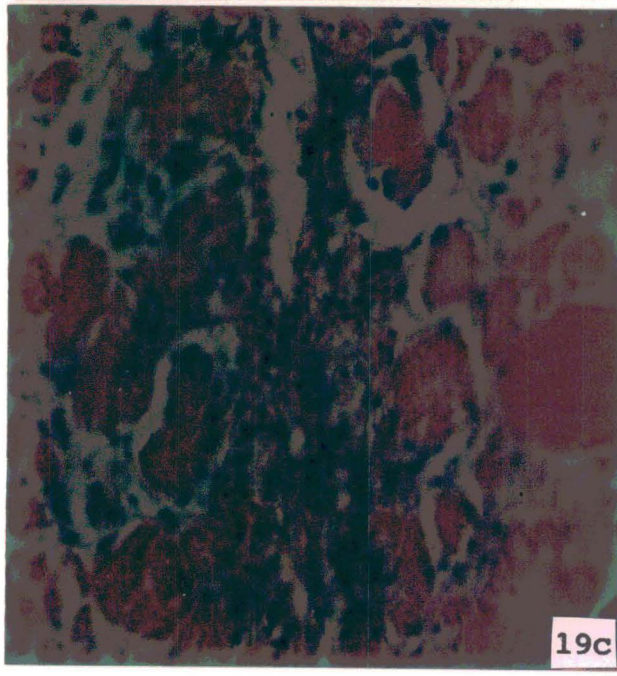
Fig.19b. Section of ulcer showing infiltration of blood cappillaries and degenerative changes in *H. Fossilis* after intramuscular injection with R2 (fluoresent Pseudomonad). (X400) (H-E).

Fig. 19 c. Showing Muscle necrosis and granulomatous response in *H. fossilis* after intramuscular injection with R3 (*Aeromonas caviae*). (x400) (H-E).

Fig. 19 d. Granulomatous response in the muscle of *H. fossilis* injected with a mixed bacterial suspension of R1, R2 and R3. (x 400) (H-E).


19a


19b


19c

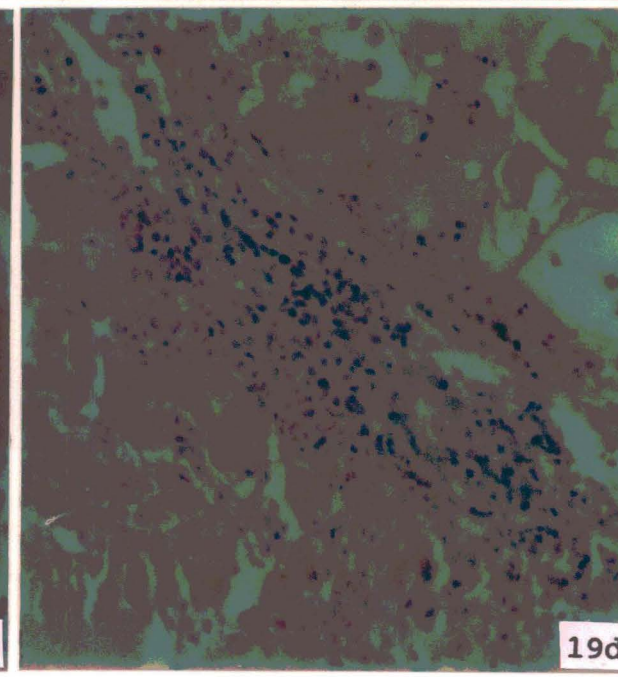

19d

Plate VIII

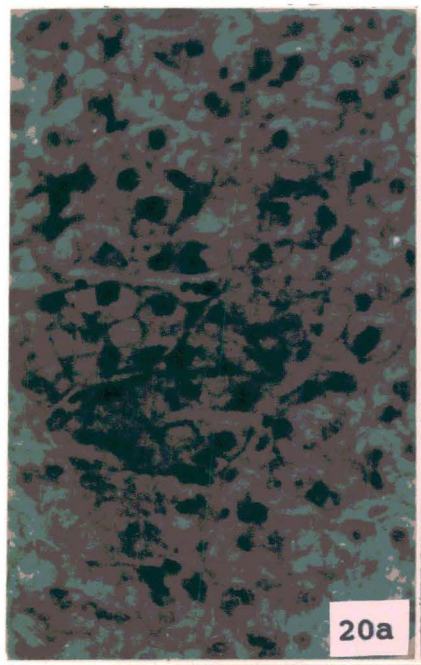
Fig. 20. a. Showing a section of liver of *Heteropneustes fossilis* injected with sterile saline suspension (Control) (x400). (H-E)

Fig. 20 b. Section of liver of *H. fossilis* showing infiltration of blood cappillaries 72 hours after intramuscular injection with R1 (fluoresent Pseudomonad) (x400). (H-E)

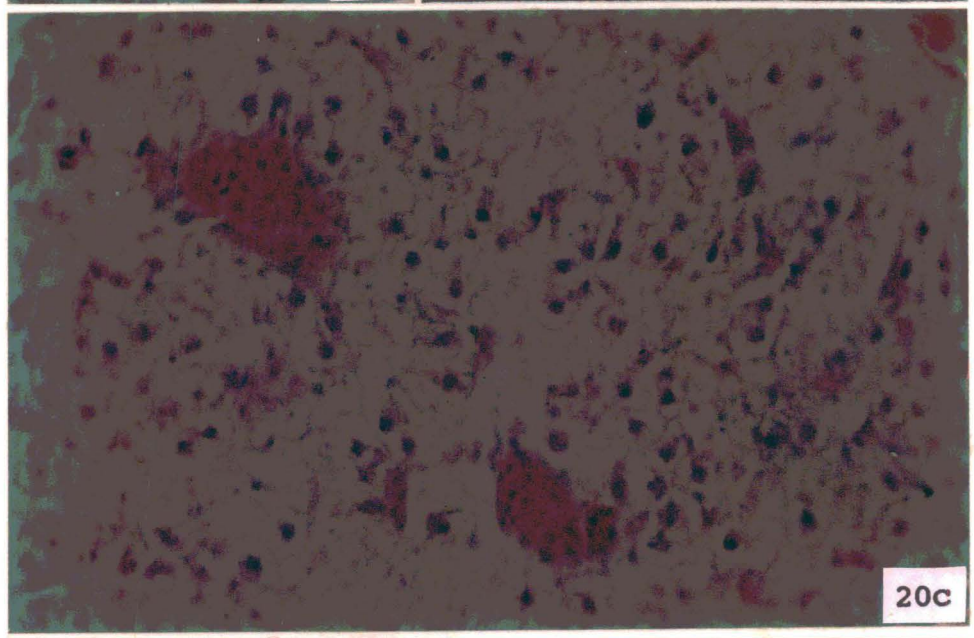
Fig. 20 c. Section of liver of *H. fossilis* showing infiltration of blood cappillaries and vacuolation of hepatocytes 72 hours after intramuscular injection with R2 (fluoresent Pseudomonad) (x400) (H-E)

Fig. 20 d. Section of liver of *H. fossilis* showing vacuolation of hepatocytes 72 hours after intramuscular injection with R3 (*Aeromonas caviae*) (x400). (H-E)

Fig. 20 e. Section of liver showing necrotic changes 72 hours after injection with a mixed suspension of R1, R2 and R3. (x400). (H-E)


20a


20b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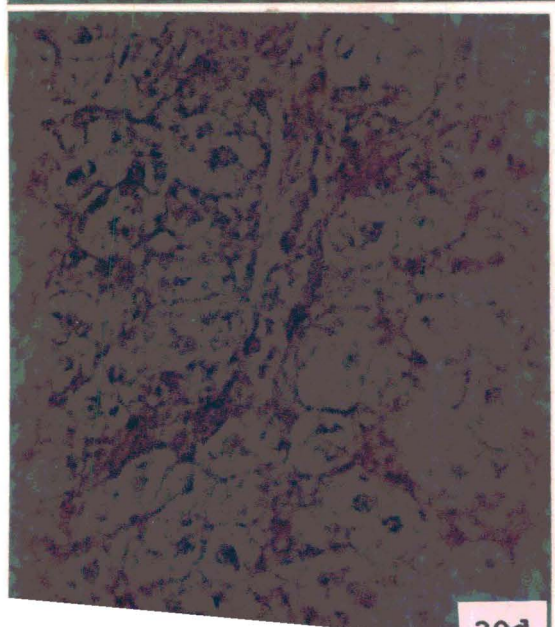
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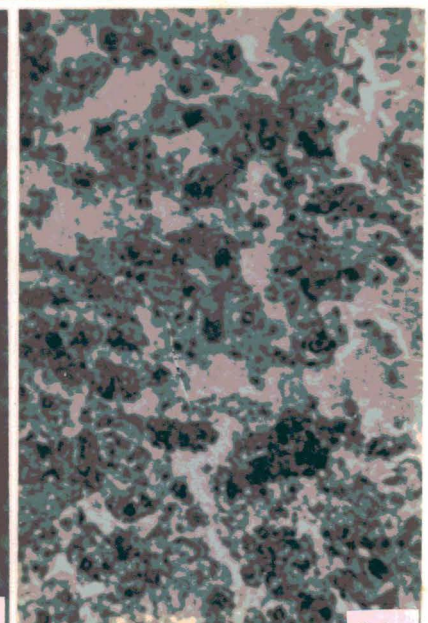
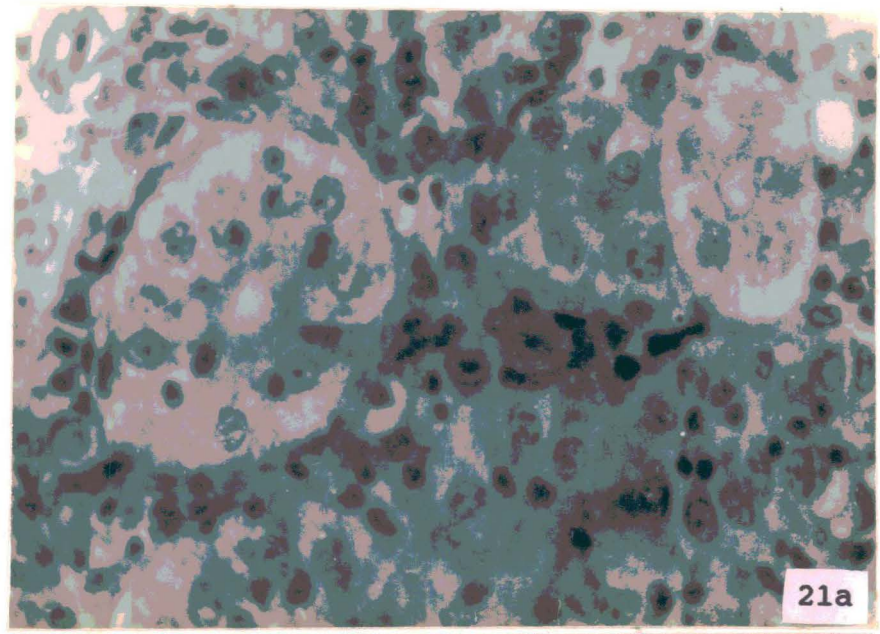


Plate IX

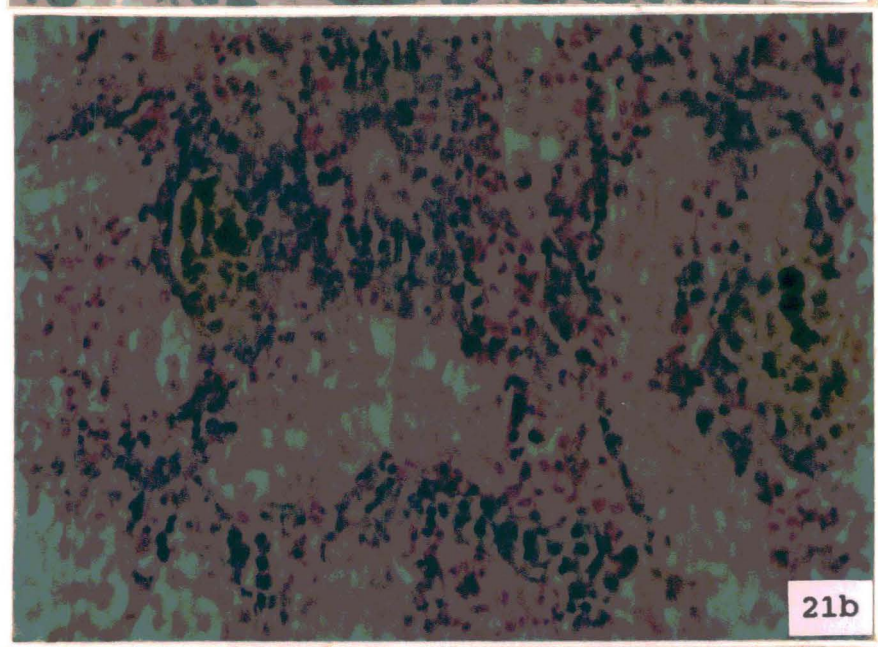
Fig. 21. a. Showing a section of kidney of *Heteropneustes fossilis* injected with sterile saline suspension (Control) (x500). (H-E)

Fig. 21 b. Section of kidney of *H. fossilis* showing haemosiderin laden macrophages, tubular vacuolation and blood cappillary infiltration 72 hours after intramuscular injection with R2 (fluoresent Pseudomonad) (x400). (H-E)

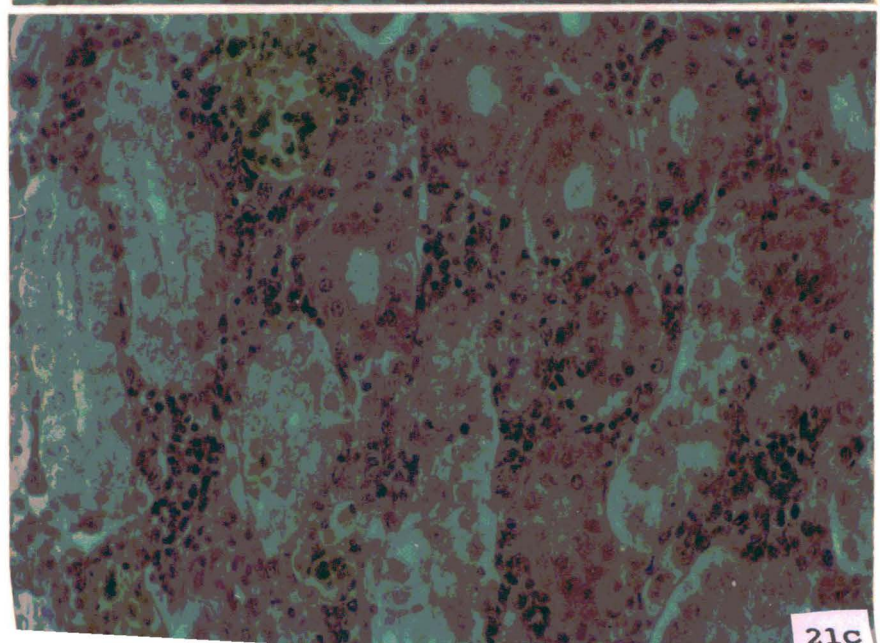
Fig. 21 c. Section of kidney of *H. fossilis* showing haemosiderin laden macrophages and necrotic changes in the tubules 72 hours after intramuscular injection with a mixed suspension of R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*). (x400). (H-E)



21a



21b



21c

4.1.3. Effect of the three pathogenic bacteria on morphology of erythrocytes, Total Erythrocyte Count and Haemoglobin content of *H. fossilis*

The decline in erythrocyte count followed by drop in haemoglobin content and hematocrit values indicated anaemic condition in the EUS affected fishes (Das and Das, 1993; Pathiratne and Rajapakshe, 1995). In view of these findings, it was necessary to ascertain whether these blood parameters varies in experimentally infected fish as it does in naturally infected fish.

Healthy fishes (*H. fossilis*), weighing 10 to 15 gms were inoculated by intraperitoneal injection with the three bacterial saline suspension of R1, R2 and R3 in pure and mixed form. Each bacterial suspension were injected to 40 fishes. The concentration of the bacterial suspensions was $1x10^7$ cfu / ml. The control set of 40 fishes received sterile saline solution. Blood was collected immediately after inoculation from five fishes of each set and TEC and Hb content of five fishes from individual sets of bacterial infection was determined after 24, 48, 72, 96, 120 and 144 hours. Blood smears were prepared from fishes of each set and after staining, the slides were observed under the microscope. The methodologies followed are described under materials and methods. The results obtained are given in table 6 and 7 and graphically represented in Figs. 22 and 23.

Changes in the morphology of the erythrocytes

Erythrocyte morphology in all sets of fishes (infected and control) was observed after 72 hours of infection. The shape of the erythrocytes of the control set of fishes was oval. Round shaped erythrocytes with vacuolation in the cytoplasm were observed in all the infected fishes. Rod shaped bacteria were also observed in the blood smears from experimentally infected fishes only. Disintegration of erythrocytes were noted in all the infected fishes, however, the degree of disintegration was very high in the fishes treated with mixed bacterial suspension. Here, majority of the cells were found to be either totally disintegrated or partially disintegrated and nuclear shadows were seen in large numbers. Vacuolation of both the cytoplasm and the nucleus was

observed in intact cells. In some portions the shape erythrocytes were totally distorted.In the fishes treated with pure bacterial suspension, some disintegrated erythrocytes could be seen. All the RBCs were either round or their shapes were distorted due to swellings.

Changes in Total Erythrocyte Count and Haemoglobin Content

Fishes experimentally infected with mixed bacterial suspension of R1, R2 and R3 showed a significant decrease in TEC and Hb Content after 48 hours($P<0.01$) and 72 hours ($P<0.01$) of inoculation respectively. Erythrocyte count in fishes treated with a mixed bacterial suspension was $3.15 \times 10^6 \pm 0.092$ / mm^3 at 0 hr. After 48 hrs of inoculation it reduced to $2.52 \times 10^6 \pm 0.036$ /mm^3 ($P< 0.01$), and after 72 hrs it reduced to $2.05 \times 10^6 \pm 0.091$ / mm^3 ($P<0.01$). After 144 hrs TEC was found to be $1.43 \times 10^6 \pm 0.067$. On the other hand, the Hb content after 48 hours, was reduced to 14.24 ± 0.14 gm /100 ml from 16.25 ± 0.12 gms / 100 ml at 0 hr ($P<0.01$). After 72 hrs, the Hb content reduced to 13.65 ± 0.13 gms / 100 ml and finally to 12.28 ± 0.09 gm / 100 ml after 144 hrs of inoculation.

Decrease in TEC and Hb Content was also noted in experimentally infected fishes with pure bacterial suspensions of R1, R2 and R3. Significant decrease ($P< 0.05$) in TEC were observed after 48 hours of inoculation by all the three bacteria. Similarly significant decrease ($P<0.01$) in Hb content of fishes treated with R3, R2 and R1 was noted at 48, 72 and 144 hrs. after inoculation respectively. TEC in fishes treated with sterile saline solution were $3.13 \times 10^6 \pm 0.074$ / mm^3 after 0 hr and $3.09 \times 10^6 \pm 0.06$ / mm^3 after 144 hrs of injection. Similarly Hb Content of the control set of fishes were 16.40 ± 0.08 gms / 100 ml and 15.92 ± 0.10 gms / 100 ml at 0 hr and after 144 hrs of injection respectively.

Table 6 : Total erythrocyte count (TEC) ($x10^6$ / mm^3)[a] of control and experimentally infected fishes (*Heteropneustes fossilis*) with mixed and pure saline suspensions of R1, R2 and R3 bacteria.

	Hours after inoculation						
	0	24	48	72	96	120	144
Control[b]	3.13 ± 0.074	3.06 ± 0.068	3.12 ± 0.061	3.16 ± 0.07	3.09 ± 0.058	3.14 ± 0.071	3.09 ± 0.06
R1	3.16 ± 0.075	2.91 ± 0.04	2.74 ± 0.055	2.48 ± 0.061	2.31 ± 0.069	2.21 ± 0.059	2.14 ± 0.049
R2	3.09 ± 0.069	2.86 ± 0.057	2.68 ± 0.055	2.37 ±0.094	2.22 ± 0.071	2.11 ± 0.073	1.97 ± 0.081
R3	3.08 ±0.089	2.97 ± 0.043	2.67 ± 0.08	2.42 ± 0.063	2.15 ± 0.074	2.05 ± 0.045	1.93 ± 0.071
M[c]	3.15 ± 0.092	2.94 ± 0.047	2.52 ± 0.036	2.05 ± 0.091	1.72 ± 0.093	1.64± 0.088	1.43 ± 0.067

[a] Data represent the mean ± SD values of five replications of each treatment.

[b] Control set received intraperitoneal injection of 0.5 ml/100gm body weight of sterile saline (0.85% NaCl) solution.

[c] M = Mixed

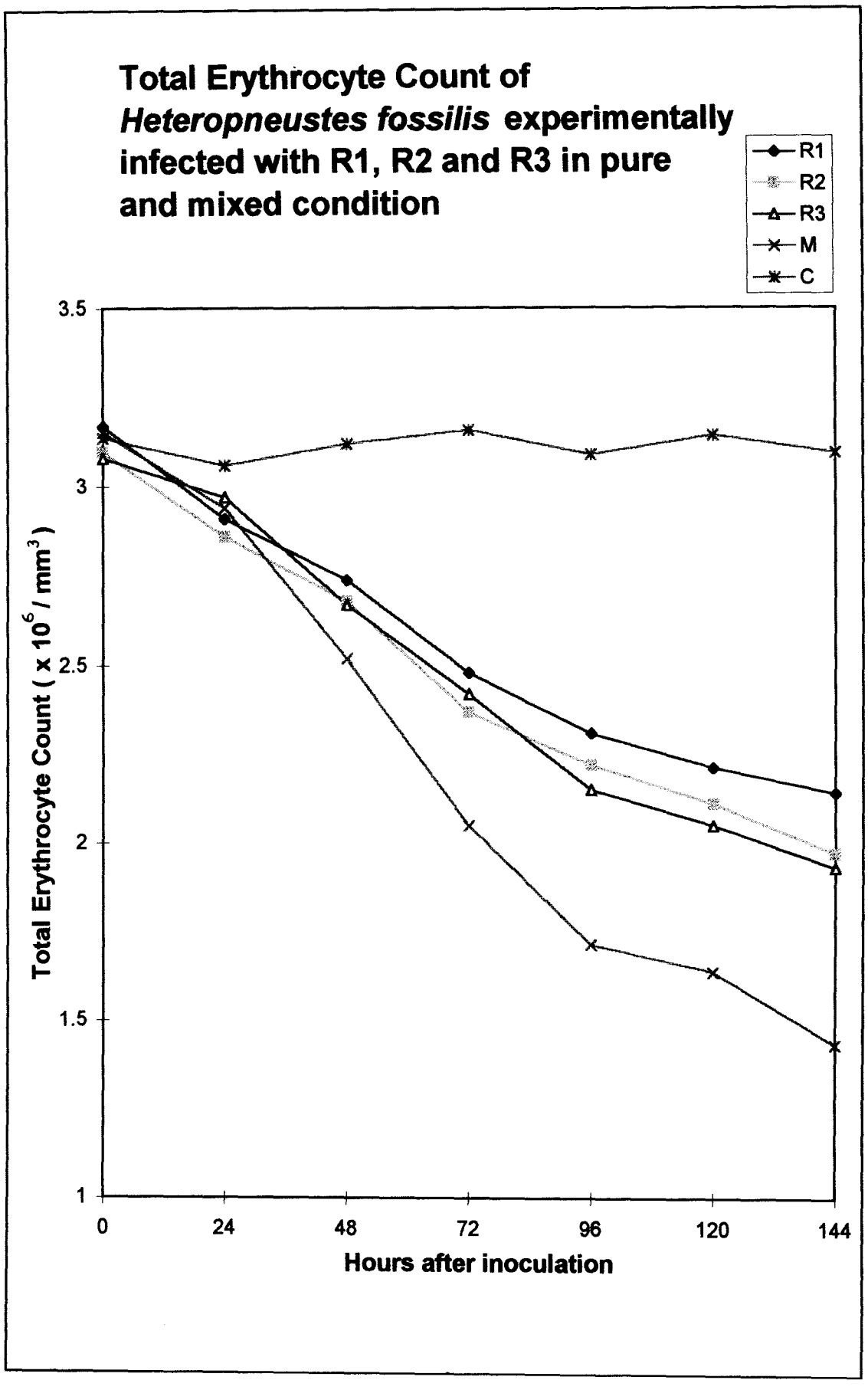
Table 7 : Haemoglobin content (grams / 100 ml)[a] of fishes (*Heteropneutes fossilis*), experimentally infected with pure and mixed saline suspension of R1, R2 and R3 bacteria.

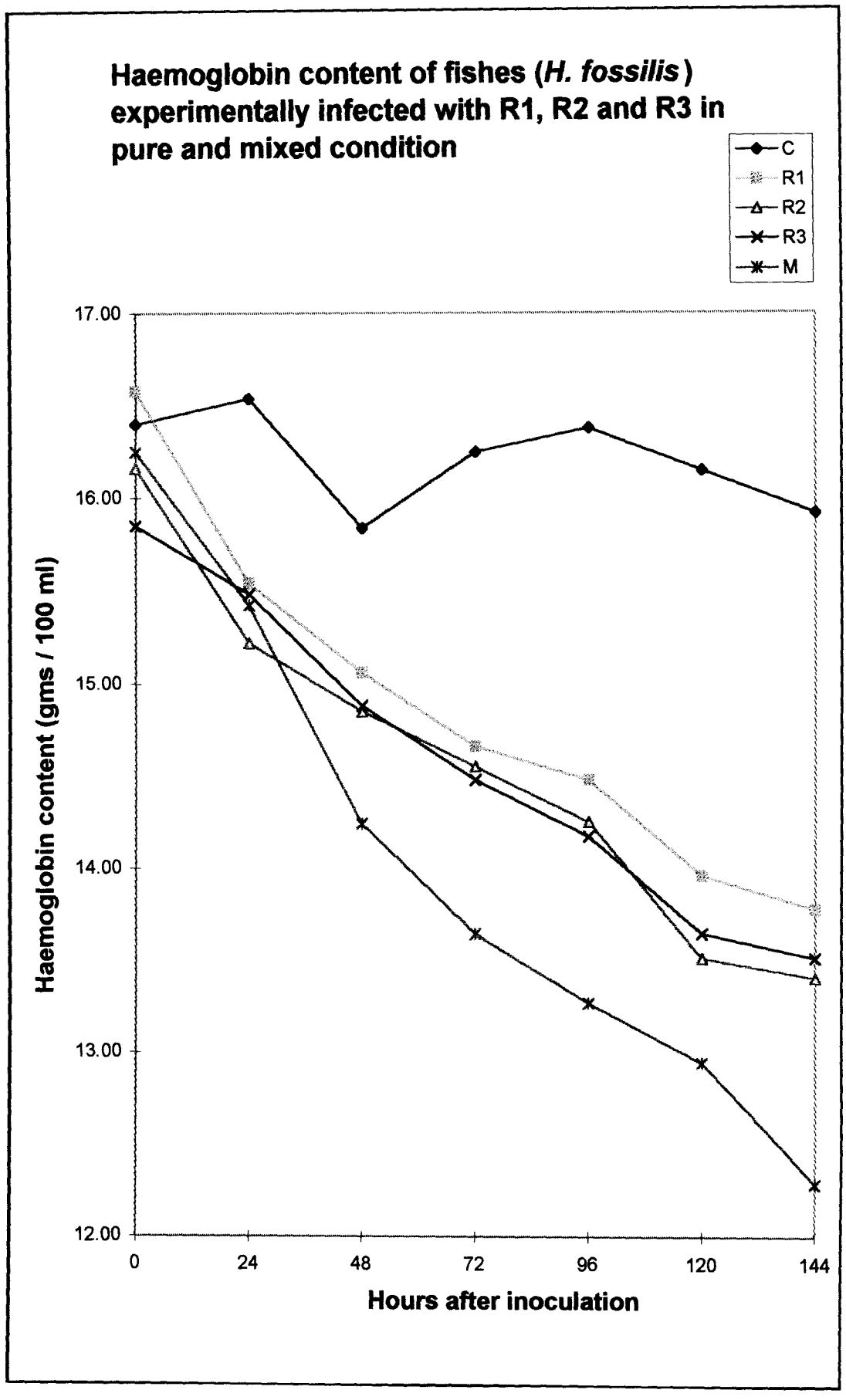
	Hours after inoculation						
	0	24	48	72	96	120	144
Control[b]	16.40 ± 0.08	16.54 ± 0.11	15.84 ± 0.09	16.25 ± 0.06	16.38± 0.09	16.15 ± 0.14	15.92 ± 0.10
R1	16.58 ± 0.11	15.54 ± 0.13	15.06 ± 0.08	14.66 ± 0.12	14.48 ± 0.15	13.96 ± 0.07	13.78 ± 0.11
R2	16.16 ± 0.07	15.22 ± 0.05	14.85 ± 0.07	14.55 ± 0.09	14.25 ± 0.11	13.52 ± 0.08	13.41 ± 0.04
R3	15.85 ± 0.08	15.48 ± 0.09	14.88 ± 0.11	14.48 ± 0.06	14.17 ± 0.10	13.65 ± 0.12	13.52 ± 0.09
M[c]	16.25 ± 0.12	15.42 ± 0.16	14.24 ± 0.14	13.65 ± 0.13	13.27 ± 0.16	12.94 ± 0.04	12.28 ± 0.09

[a] Mean of five independent experiments ± SD.

[b] Control set received intraperitoneal injection of 0.5 ml/100gm body weight of sterile saline (0.85% NaCl) solution.

[c] M = Mixed


Total Erythrocyte Count of
Heteropneustes fossilis experimentally
infected with R1, R2 and R3 in pure
and mixed condition
R1
R2
R3
M
C
3.5
3
2.5
2
1.5
1
Total Erythrocyte Count (x 10^6 / mm^3)
0
24
48
72
96
120
144
Hours after inoculation


Haemoglobin content of fishes (H. fossilis) experimentally infected with R1, R2 and R3 in pure and mixed condition
C
R1
R2
R3
M
17.00
16.00
15.00
14.00
13.00
12.00
Haemoglobin content (gms / 100 ml)
0
24
48
72
96
120
144
Hours after inoculation

4.1.4. Studies on some virulence associated characters of R1, R2 and R3

Bacteria bring about pathogenic effect especially due to the action of various extracellular lytic enzymes (Schäperclaus, 1986). which may be detected in the growth medium of the bacteria when cultured in suitable media. Various authors have reported different virulence associated characters of *Aeromonas* sp. (Mittal *et al*, 1980; Wakabayashi *et al*, 1981; Ljungh and Wadstrom, 1982; Santos *et al*, 1996) and *Pseudomonas* sp. (Morihara, 1963; Liu, 1957; Volf and Havelka, 1965; Schäperclaus, 1965; Wakabayashi and Egusa, 1972). Since, these bacteria show a wide variation in their pathogenicity to fish (Schäperclaus, 1986; Karunasagar *et al*, 1995), it is etiologically important to study their biochemical characteristics in relation to their virulence. Thus it was considered worthwhile to test the presence of various extracellular products in the culture media of the three pathogenic bacteria, two Pseudomonads (R1 and R2) and the Aeromonad (R3, *Aeromonas caviae*) associated with EUS. The results obtained (Table 8) show that all the bacteria exhibited virulence properties.

Table - 8 : Presence of extracellular virulence factors[a] in the three pathogenic bacteria R1, R2 and R3

Virulence factor	Bacterial isolates		
	R1	R2	R3
gelatenase	+	+	+
caseinase	+	+	+
Lecithinase	+	+	+
Lipase	+	+	+
Haemolysin	+	+	+

[a] Methods for detecting the extracellular virulence factors are described under materials and methods.

Gelatenase production tests showed that all the three bacteria were able to produce gelatenase. All were able to produce caseinase in skimmed milk agar. All the three bacteria produced haemolysins as was indicated by clear zones around bacterial growth in tryptic soya agar containing 2% fish blood. All the bacteria were capable of producing lecithinase as indicated by an opaque zone formed around the colonies in lecithovitellin agar media. Lipolytic activity was also exhibited by all the bacteria in Tween 80 agar plates.

4.1.5. Detection and partial purification of extracellular virulence factors of R1, R2 and R3

Earlier results showed that extracellular lytic enzymes were secreted by the bacteria into the culture medium (Table 8). Hence the bacteria free culture medium after the growth of bacteria which contained the extracellular products (ECP) was collected. The procedure is described under materials and methods. Assay of the extracellular products showed that R1, R2 and R3 produced 2.1 PU/ml, 2.75 PU/ml and 3.05 PU/ml of protease activity. Their respective specific activities were 1.842 PU/mg, 2.148 PU/mg and 1.871 PU/mg.

The amounts of haemolysin produced were 32.74 HU/ml, 35.63 HU/ml and 41.44 HU/ml by R1, R2 and R3 respectively which gave respective specific activities of 26.8 HU/mg, 27.83 HU/mg and 26.91 HU/mg (Table 9).

4.1.5.1. Induction of ulcer by cell free culture filtrates

The culture filtrate was used as an inoculum for intramuscular injection to healthy *A. testudineus* weghing 20 to 25 gms approximately (dose : 0.5 ml / 100 gm body weight). 0.1 ml of the culture filtrates of each bacteria were injected to 10 fishes, the control set received boiled culture filtrates of the bacteria. The fishes were kept under observation for 15 days. 0.1 ml of the inoculum contained 0.21 units of proteolytic activity and 3.056 units of haemolytic activity in case of R1, 0.275 units of proteolytic activity and 3.563 units of haemolytic activity in case of R2 and 0.305 units of proteolytic activity and 4.144 units of haemolytic activity in case of R3. The amount of protein in 0.1 ml of the

inoculum was 114 μg, 128 μg and 163 μg in case of R1, R2 and R3 respectively (Table 9).

Table 9 : Protease and haemolysin production and LD_{50} values of the three pathogenic bacteria R1, R2 (fluorescent pseudomonads) and R3 (*Aeromonas caviae*) present in the culture filtrate[a]

Bacteria	Enzyme activity (units / ml)		Protein (mg / ml)	LD_{50} (c.f.u.)
	Protease (PU/ml)[b]	Haemolysin (HU/ml)[c]		
R1	2.1	30.56	1.14	9.97×10^5
R2	2.75	35.63	1.28	4.45×10^5
R3	3.05	41.44	1.63	6.29×10^5

[a] : Culture filtrate was obtained by filtering 48 hr bacterial culture in BHI broth through G5 sintered filter

[b] : P.U. = Proteolytic units = Absorbance at 440 nm x dilution factor

[c] : H.U. = Haemolytic units = Absorbance produced by 0.8 ml of sample volume in the standard assay as described under materials and methods

Eighty percent of the fishes injected with culture filtrates of R2 and R3 and 65% of the fishes injected with culture filtrates of R1 manifested external signs of the disease within 24 hours of inoculation (Fig. 24). The control set of fishes showed no signs of the disease. The development of ulcers in the infected fishes was similar to that induced by bacterial suspension. The area around the injection site turned reddish, which gradually swelled and increased in size. After 48 hrs, the scales at the site of ulcer fell off and the surface layer of the skin was eroded. Thus moderate ulcers developed at the site of injection.

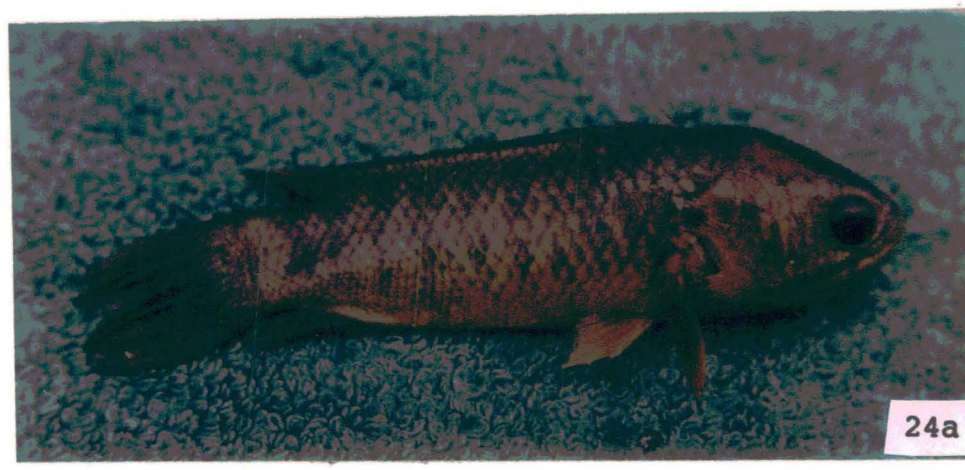
Plate X

Fig. 24. *Anabas testudineus* showing haemorrhage at the site of injection 24 hours after intramuscular injection with culture filtrates of :

a. R1 (fluorescent Pseudomonad),

b. R2 (fluorescent Pseudomonad) and

c. R3 (*Aeromonas caviae*).



24a

24b

24c

Mortality rate was 40% in fishes treated with R3, 40% in fishes treated with R2 and 30% in fishes treated with R1 (Table 10).

Table 10 : Percentage mortality and nature of ulcer formation in *Anabas testudineus* injected with cell free culture filtrates of R1, R2 and R3.

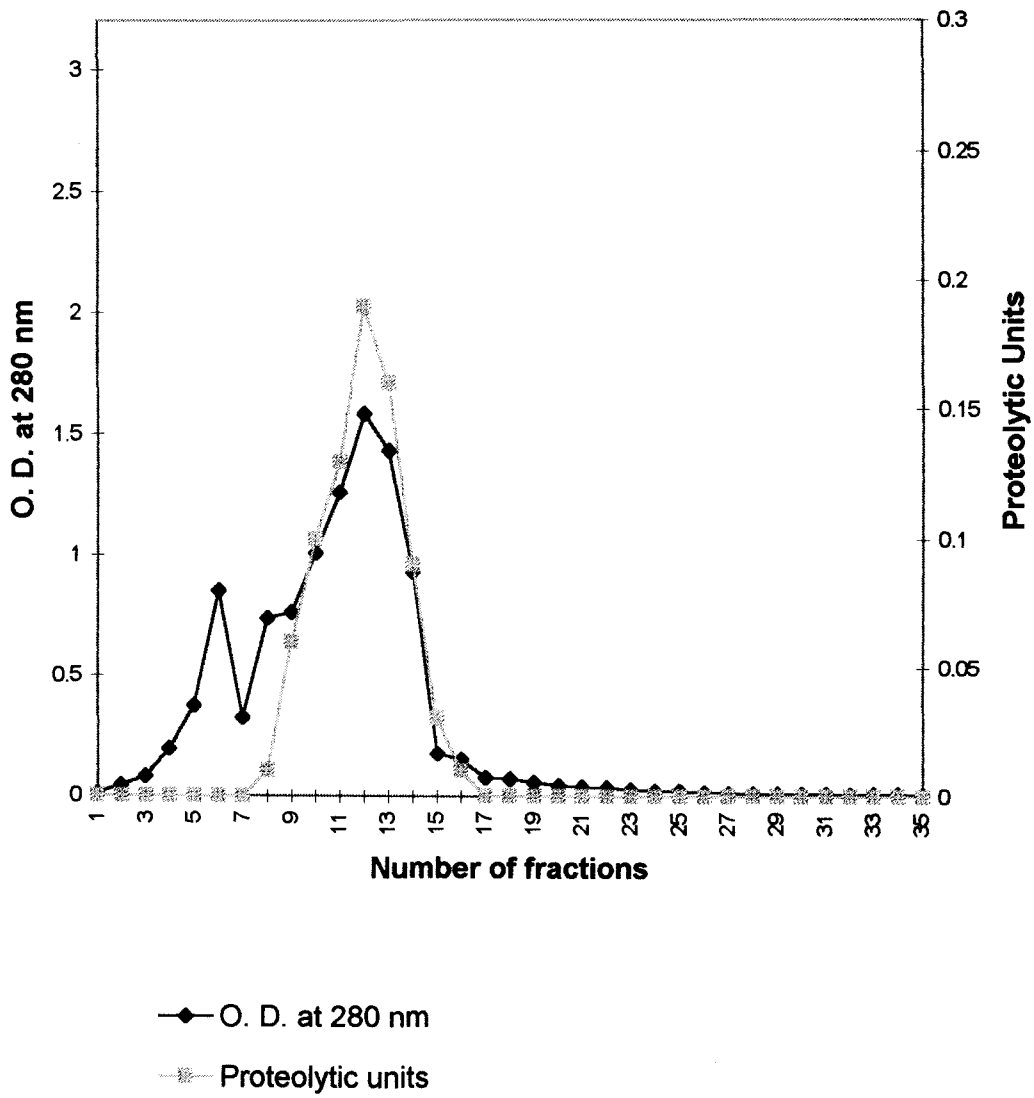
	Number of fishes inoculated	Number of fishes dead	Nature of ulcer	Percentage mortality
Control[a]	20	0	-	-
R1	20	6	moderate	30.0%
R2	20	8	moderate	40.0%
R3	20	8	moderate	40.0%

[a] Control set of fishes received boiled culture filtrates of the bacteria.

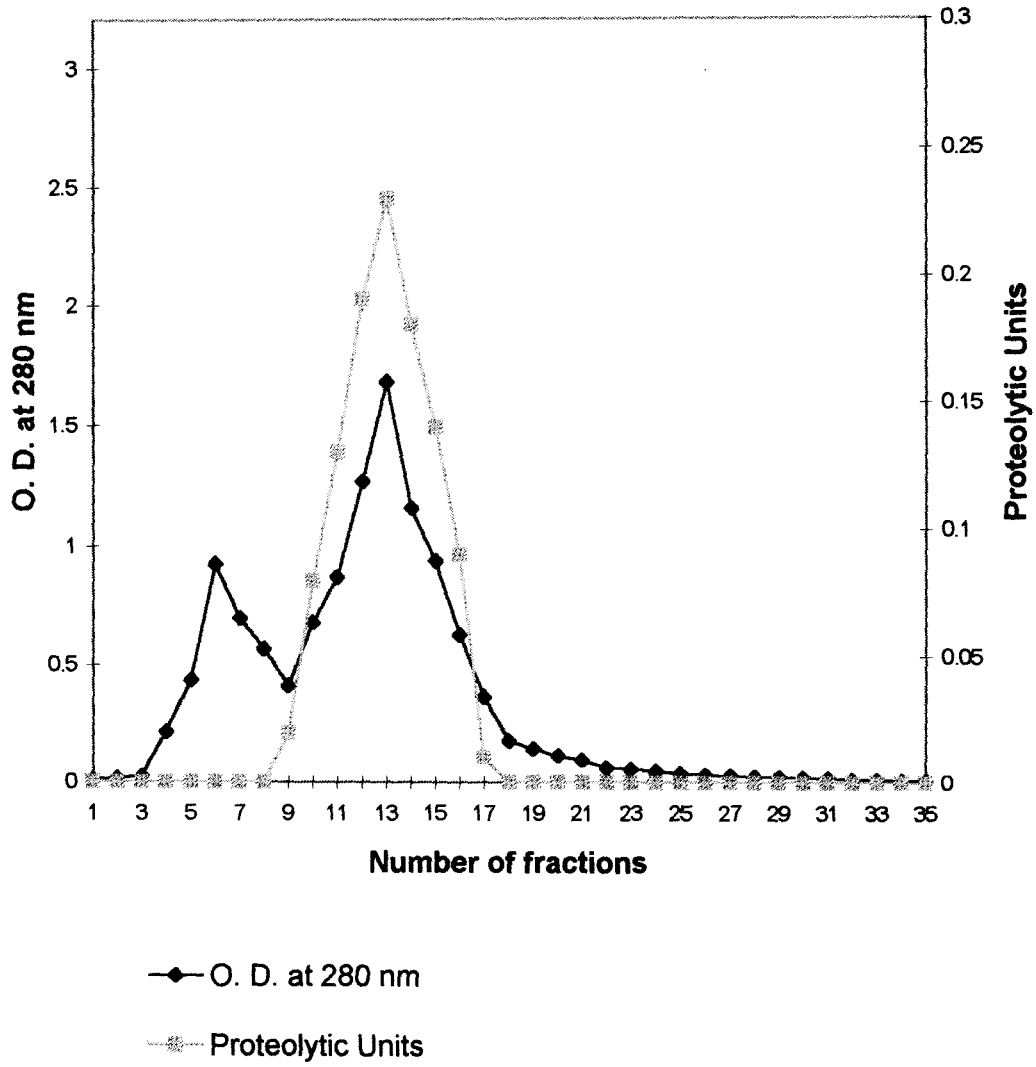
4.1.5.2. Partial purification of extracellular virulence factors

The culture filtrate containing the ECP was subject to precipitation with ammonium sulphate (solid (80%)) and gel filtration (ppt. dissolved in 0·1 (M) Tris-HCl buffer, pH-7·4) and results of the elution (Figs. 25, 26 and 27) showed presence of two major peaks in the culture filtrates of all the three bacteria. In case of R1 and R2, the first peak showed neither haemolytic nor proteolytic activity nor toxic activity. In case of R3, the first peak showed very low proteolytic activity and no haemolytic activity or toxic activity. The second peak of all the three bacteria showed proteolytic activity, however, the activity was higher in the *Aeromonas* (R3) than the two Pseudomonads (R1 and R2). Among the Pseudomonads, R1 had a lower proteolytic activity than R2. The haemolytic activity was associated totally with the second peak of all the three bacteria. R1 had lower haemolytic activity in comparison to R2 and R3 where the activities were almost similar. Pooled fractions of the second peak of all the three bacteria were concentrated by polyethylene glycol and injected intramuscularlly to healthy *A. testudineus*. It was found to be toxic as it produced

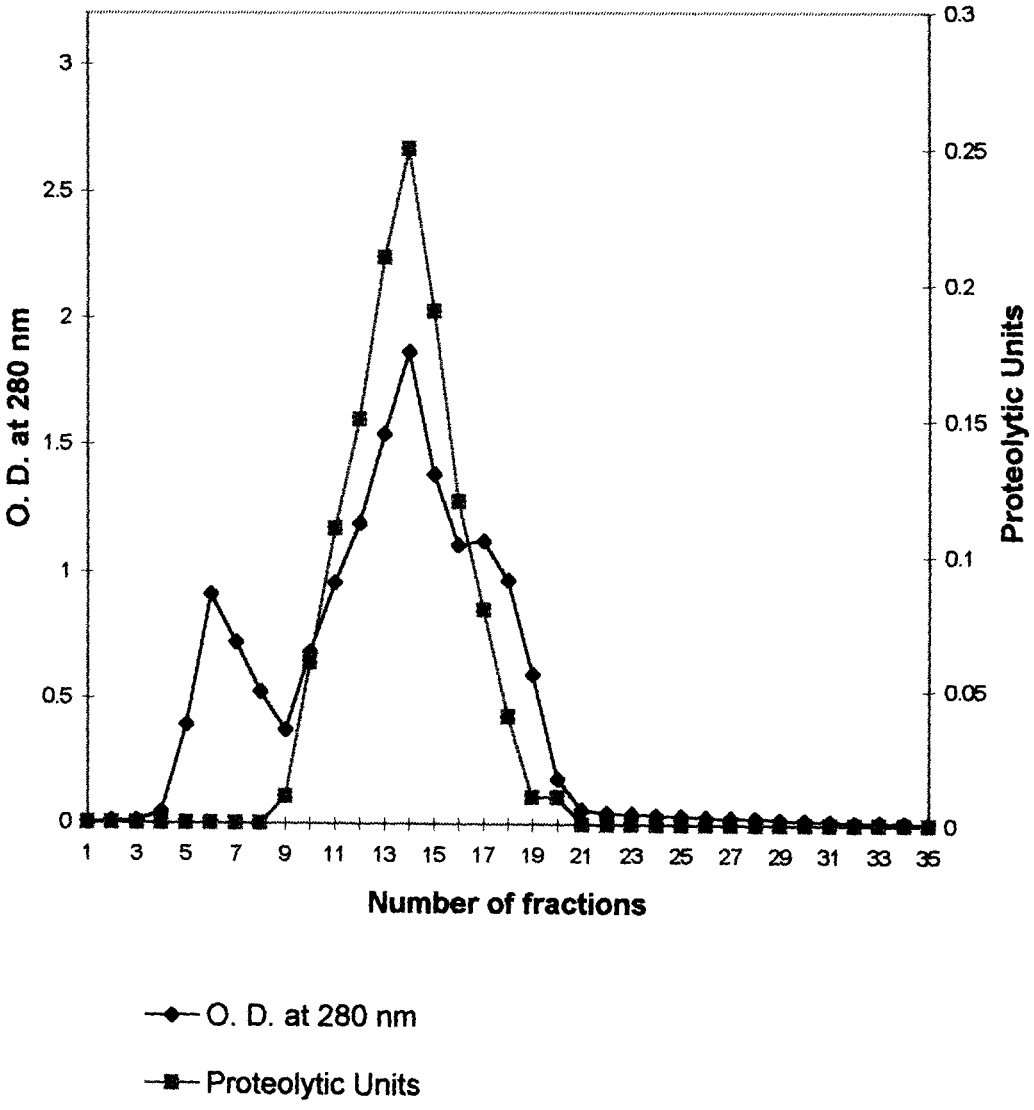
Sephadex G 200 filtration of extracellular products of the pathogenic bacteria, R1 (fluorescent Pseudomonad)



Sephadex G 200 filtration of extracellular products of the pathogenic bacteria R2 (fluorescent Pseudomonad)



Sephadex G 200 filtration of extracellular products of the pathogenic bacteria R3 (*Aeromonas caviae*)



reddish swellings with haemorrhages at the site of injection. The purification procedure gave about 12 to 15% recovery of haemolytic activity and 35 to 39% recovery of proteolytic activity.

4.2. Studies on fishes affected by epizootic ulcerative syndrome obtained from natural sources and isolation of microorganisms present in the lesion.

The three pathogenic bacteria R1, R2 and R3 were isolated from affected air breathing fishes in the northern districts of West Bengal in 1990 (Pal and Pradhan, 1990). At that time fungus or any other pathogenic organism were not found to be present in the ulcer region of the affected fishes. However, during the course of this study, there were some reports of fungus involvement in the disease causing secondary infections in EUS affected fishes from Indian waters (Mohan and Shankar, 1994). Besides, there was a heavy outbreak of the disease in the southern districts of West Bengal affecting mainly *Channa* spp., *Puntius* spp. and *Mystus* spp. Pal (1996, 1997) reported outbreaks of the disease in the northern districts every year since its first occurrence in 1988. Under these circumstances it was considered worthwhile to study the bacterial and fungal species that may be present at the site of the ulcerative lesion of affected fishes in both North and South Bengal regions.

4.2.1. Observations on the external pathological condition of the diseased fishes

EUS affected fishes, *Clarias batrachus*, *Channa punctatus*, *Anabas testudineus*, *Heteropneustes fossilis* and *Colisa fasciata* were obtained from parts of Darjeeling, Jalpaiguri and Coochbehar districts in North Bengal as stated under materials and methods. All fishes showed lesions on their body surface (Figs. 28-34). Some fishes (*C. batrachus* and *A. testudineus*) were kept in glass aquaria meassuring 90 x 35 x 35 cms in which the depth of static water was 20 to 25 cm. Water temperature was maintained at 28-30^{0}C. The fishes which died were removed immediately and preserved in formalin solution.

The affected fishes could be classified into three stages. In the first stage, the fish showed small red spots on the skin, measuring about 3 to 4 mm

in diameter in one or two areas with discolouration of the skin around those spots. In fish with scales only the mucous layer was affected in the first stage and the scales were found to be almost intact (Figs. 29).

In the second stage, the red spots grew in size, took a circular to oval shape measuring 5 to 20 mm in diameter. In the scaly fishes, the scales of the affected area were lost at this stage. The skin at the affected site was eroded along with a portion of the underlying muscle (Figs. 30 and 31). A change in the normal swimming pattern was noticed at this stage. The fishes became sluggish with irregular opercular movement .

In the third stage, the lesions systematically grew bigger and became deep and haemorrhagic. These advanced lesions were large (20 to 40 mm in diameter), edematous, necrotic open ulcers exposing the underlying skeletal musculature (Figs. 28, 32, 33 and 34). The tails were sometimes affected and in severe cases, the peduncle portion was totally eroded. The fish at this stage showed very little movement. They mainly remained motionless near the surface making 45^0 to 90^0 angle of their body to the surface of water with their head directed upward.

Altogether 52 *A. testudineus* and 35 *C. batrachus* were brought to the laboratory and kept in glass aquaria for further observation. Of these, only 7 *A. testudineus* and 3 *C. batrachus* survived after 15 days of observation.

4.2.2. Histopathological studies of the EUS affected fishes

Some of the naturally affected fishes of the four air breathing species were sacrificed and portions of ulcer, liver and kidney were processed for histopathoogical observation. Blood was also collected and smear preparations were stained and observed under the microscope. In all the infected fishes various histopathological changes in the ulcer, liver and kidney were detected.

Ulcer

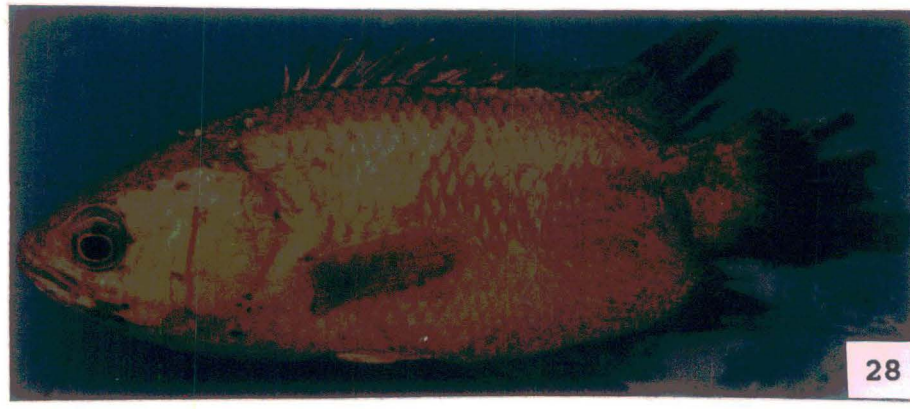
Histological sections of the severe ulcers showed total loss of the epidermis and scales. The dermis was lost in very severe cases. In most cases the dermis became highly fibrous with inflammatory infiltration and necrosis in the affected areas. In the muscle fibres, a necrotic granulomatous response was

Plate XI

Fig. 28. Naturally infected *Anabas testudineus* showing skin lesion with affected tail fins.

Fig. 29. Naturally infected *Channa punctatus* showing small red spots (initial superficial ulcers) on the body.

Fig. 30. Naturally infected *Clarias batrachus* showing ulcers near the tail region.



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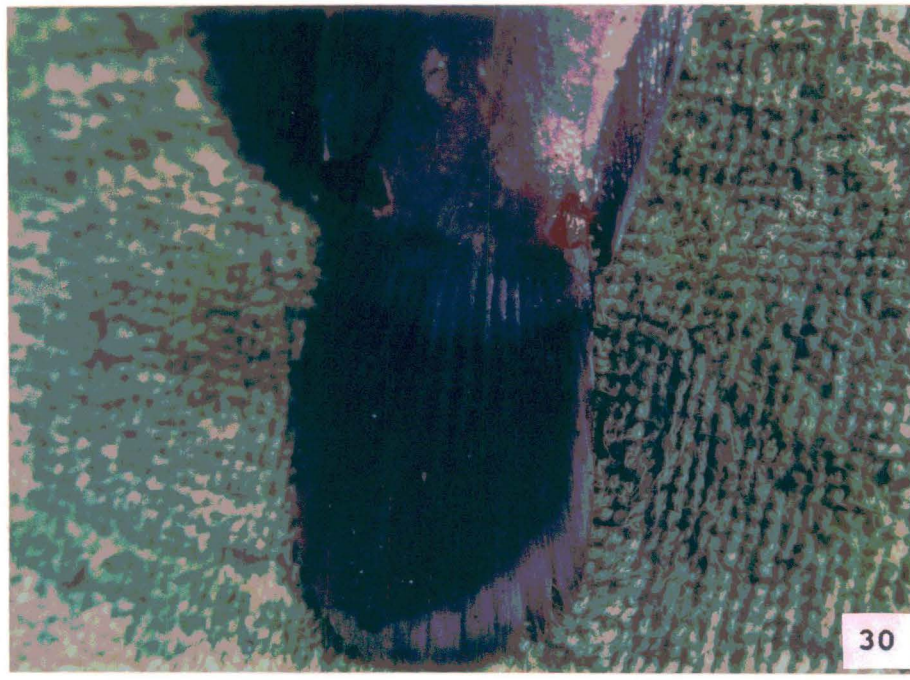

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Plate XII

Fig. 31. Naturally infected *Colisa fasciata* showing ulcer near the eye.

Fig. 32. Naturally infected *Heteropneustes fossilis* showing infection near the tail region.

Fig. 33. Naturally infected *Heteropneustes fossilis* showing infection near the tail region.

Fig. 34. Naturally infected *Channa punctatus* showing ulcer in the body.


31


32


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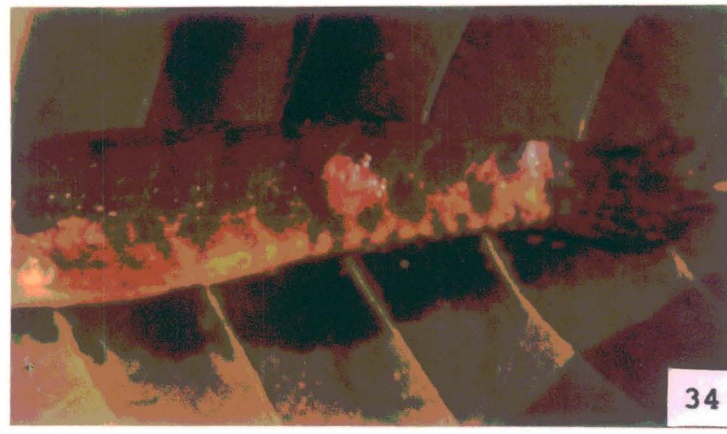

34

the major change (Fig. 35a-35e). Giemsa stained sections showed presence of bacteria in the muscle layer beneath the skin.

Liver

Histological observation of the liver showed that the normal architechture of the liver was lost in some regions. There was vacuolation in the hepatocytes (Fig. 36a) and in some regions, the hepatocytes were arranged in a chord like arrangement with enlarged sinusoids. Infiltration of blood capillaries were observed in some regions (Fig. 36b). Giemsa stained sections showed presence of bacteria in the liver of the affected fishes.

Kidney

Microscopic observation of the kidney sections of naturally infected fishes revealed severe changes in the renal tubules. Tubular breakage, tubular necrosis and vacuolation of tubular cells were the most frequent changes (Fig. 37). Accumulation of haemosiderin laden macrophages and necrosis was seen in the haematopoietic regions. Haemorrhages in some regions were also detected. Sections stained with giemsa showed presence of bacteria in the kidney of affected fishes.

Blood

Smear preparations of blood from infected fishes showed rounded cells with vacuolation in the cytoplasm. Perinuclear hallow was found in some cells. Nuclear shadows of erythrocytes (disintegrated erythrocytes) were found to be present in large numbers (Fig. 38). Presence of bacteria was detected in some regions.

4.2.3. Isolation of bacteria and their characterization

Only bacteria were isolated from all fishes from Midnapur district of West Bengal. Fungus or any other agent was not detected in the smear preparation of the lesions of the affected *C. punctatus*, *Mystus* sp. and *Puntius* sp. obtained from this region. Presence of fungus was detected only in one fish (*C.*

Plate XIII

Fig. 35 a, b. Sections through ulcer showing highly granulomatous and necrotic changes in the muscle of naturally infected *Heteropneustes fossilis*. (x200) (H-E).

Fig. 35 c. Necrotic and degenerative changes in the subdermal and muscle layers of the naturally infected *H. fossilis*. (x200) (H-E).

Fig. 35 d. Showing degenerative changes in the muscle of naturally affected *H. fossilis*. (x400) (H-E).

Fig. 35 e. Showing necrotic changes in the muscle of naturally affected *H. fossilis*. (x400) (H-E).

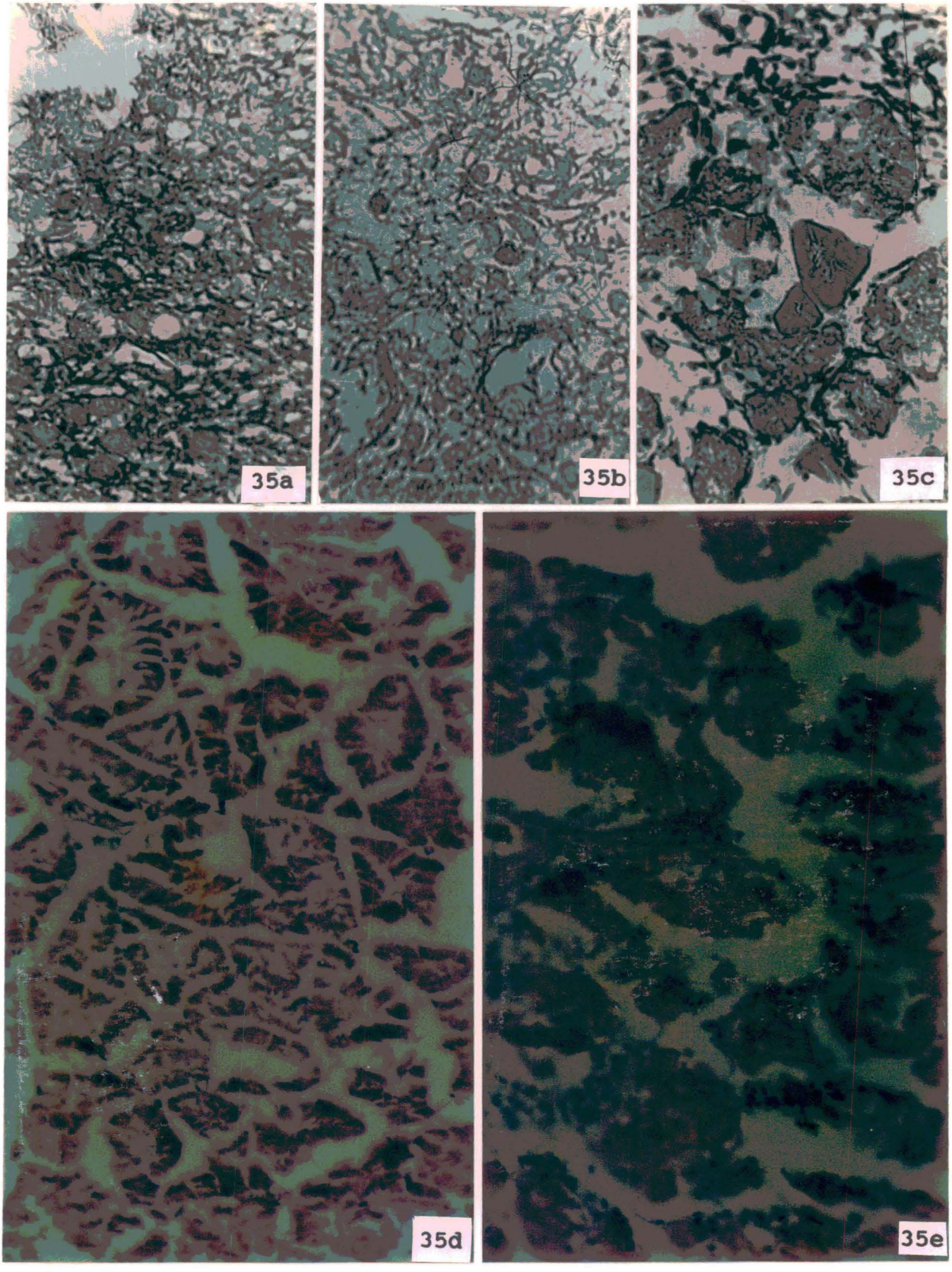

35a
35b
35c
35d
35e

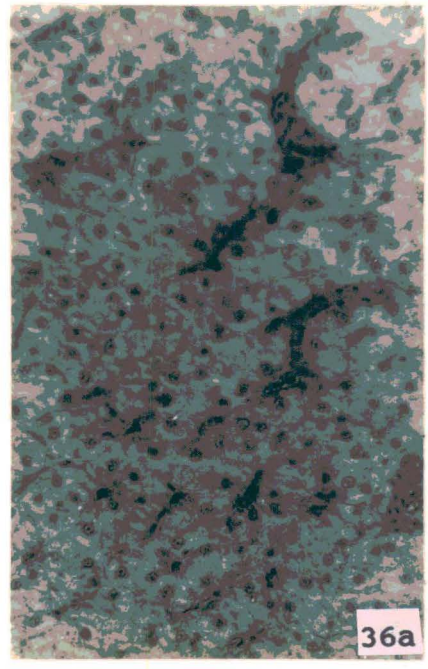
Plate XIV

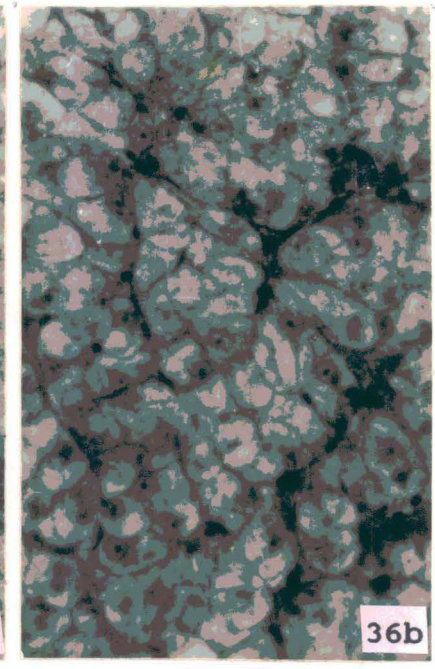
Fig. 36. a. Section of liver of naturally infected *H. fossilis* showing infiltration of blood cappillaries (x200) (H-E).

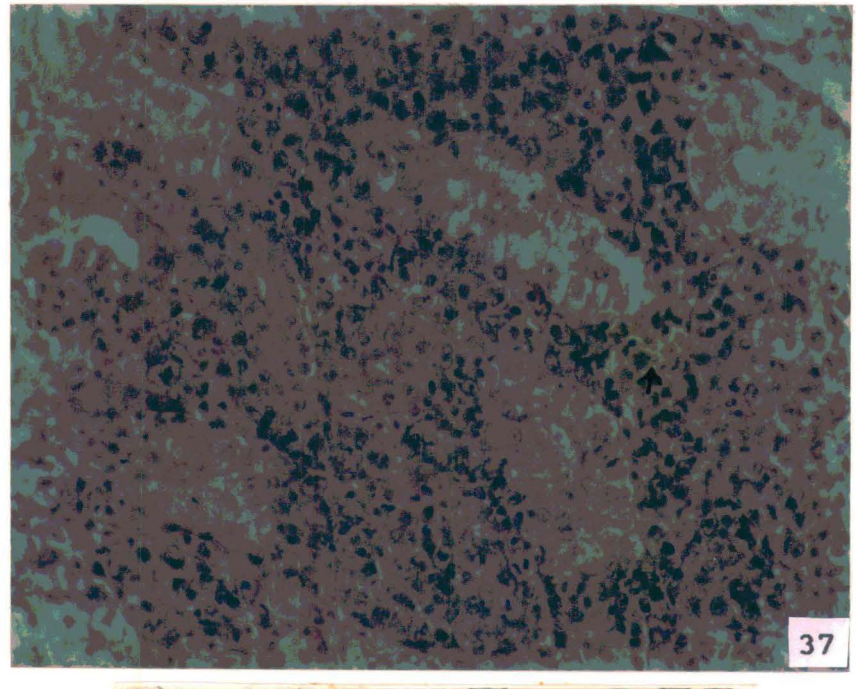
Fig. 36 b. Section of liver of naturally infected *H. fossilis* showing vacuolation of hepatocytes (x200) (H-E).

Fig. 37. Section from kidney of naturally infected *H. fossilis* showing haemosiderin laden macrophages (arrow) and tubular necrosis. (x400) (H-E).

Fig. 38. Blood smear from naturally infected *H. fossilis* showing degeneration of erythrocytes (x200) (H-E).


36a


36b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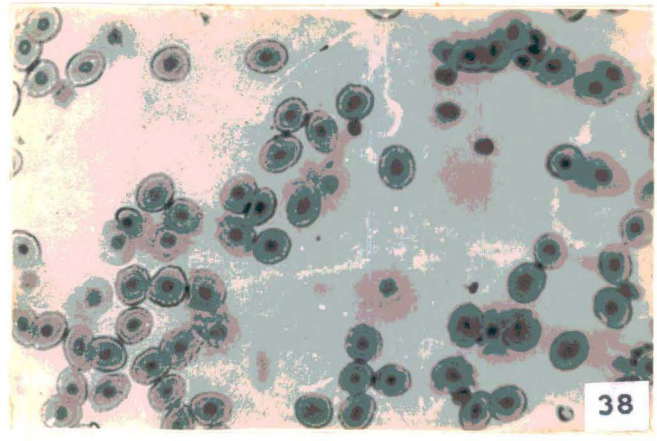

38

batrachus) obtained from the Sonapur region in the Darjeeling district of North Bengal.

The bacteria isolated from the lesions of the affected fishes were characterized by various biochemical tests which are described in details under materials and methods. R1, R2 and R3 were included as control strains. Six types of bacteria were isolated from the skin lesions of *C. punctatus* (Table 13 and 14), five types of bacteria were isolated from the skin lesions of *Mystus* sp. (Table 15 and 16) and five types of bacteria were isolated from the ulcer of *Puntius* sp. (Table 17 and 18). Results of the morphological observations and biochemical tests of R1, R2 and R3 are shown in Table 11 and 12.

Among the isolates of *C. punctatus*, four belonged to the genus *Pseudomonas* and among the rest two, one belonged to the genus *Bacillus* and the other belonged to the genus *Aeromonas*. Among the isolates of *Mystus* sp. three belonged to the genus *Aeromonas*, one was found to be *Moraxella* sp. and the remaining one belonged to the genus *Pseudomonas*. Among the isolates of *Puntius* sp. one belonged to the genus *Micrococcus*, another one belong to the genus *Pseudomonas* and the rest were identified as motile *Aeromonas* sp. The results of some biochemical tests are shown in Figs. 39a, b and c. A total study on the bacterial isolates reveales that *Pseudomonas* and *Aeromonas* were the most common bacteria present in the ulcers (Table 19, Figs. 40 a, b and c).

Table 11. Morphological characteristics of the three bacteria R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*) isolated from ulcers of epizootic ulcerative syndrome affected air breathing fishes.

	Bacterial isolates		
	R1	**R2**	**R3**
Shape	Rod	Rod	Rod
Occurrence	Singles, pairs, chains	Singles, pairs, chains	Singles, pairs, chains
Size	2.2 - 2.7 x 0.68 - 0.75 μ m	1.8 - 2.2 x 0.68 - 0.72 μ m	1.8 - 2.7 x 0.65 - 0.68 μ m
Spore	-*	-	-
Agar colonies	Circular, smooth, slightly convex	Circular, smooth, slightly convex	Circular, smooth, convex
Culture in NB	Turbid with pellicle and sediments	Turbid with pellicle and sediments	Turbid

* - = absent

Table 12. Biochemical characteristics of the three bacteria R1, R2 (fluorescent pseudomonads) and R3 (*Aeromonas caviae*) isolated from ulcers of epizootic ulcerative syndrome affected air breathing fishes.

	Bacterial isolates		
	R1	**R2**	**R3**
Gram reaction	-	-	-
Motility	+	+	+
Growth at :			
25°C	m	m	m
30°C	g	g	g
37°C	g	g	g
42°C	n	m	n
Indole production	-	-	-
M-R	-	-	-
V-P	-	-	-
Nitrate	-	+	+
Gas from glucose	-	-	-
Oxidase	+	+	+
Catalase	+	+	+
Gelatin hydrolysis	+	+	+
O-F test	O	O	F

Contd.

Table 12 (Contd.)

	Bacterial isolates		
	R1	**R2**	**R3**
Acid from :			
glucose	+	+	+
Fructose	+	+	+
L-Arabinose	-	+	+
Sorbitol	-	-	-
Sucrose	+	-	+
m-inositol	+	-	-
Mannitol	+	+	+
Adonitol	-	-	-
Levan from sucrose	+	-	-
Arginine hydrolysis	+	+	+
H_2S from cystein	-	-	-
Citrate utilization	+	+	+
Pigment formation	+[a]	+[a]	–

* : + = positive; - = negetive; g = good growth; m = moderate growth; n = no growth; O = Oxidative; F = Fermentative

[a] greenish yellow diffusible pigment produced in King's B medium

Table 13. Morphological characteristics of bacteria isolated from ulcers of *Channa punctatus**

	Bacterial isolates					
	A01	**B01**	**P01**	**P02**	**P03**	**P04**
Shape	Rod	Rod	Rod	Rod	Rod	Rod
Occurrence	Mostly in singles	Singles, pairs, chains	Singles, pairs, chains	Singles, pairs, chains	Singles, pairs, chains	Singles, pairs, chains
Size		2.7-3.5 x 0.75-0.8 μm	2.0-2.5 x 0.75-0.8 μm	2.0-2.5 x 0.75-0.8 μm	2.5-3 x 0.7-0.8 μm	2.2-0.3 x 0.7-0.8 μm
Spore	-	+	-	-	-	-
Agar colonies	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth flat	Circular, smooth flat	Circular, smooth flat	Circular, smooth flat
Culture in NB	Turbid	Turbid with pellicle and sediments	Turbid with pellicle	Turbid with pellicle	Turbid with pellicle	Turbid with pellicle

* : + = present; - = absent

Table 14 : Biochemical characteristics of bacteria isolated from ulcers of *Channa punctatus**

	Bacterial isolates					
	A01	**B01**	**P01**	**P02**	**P03**	**P04**
Gram reaction	-	+	-	-	-	-
Motility	+	+	+	+	+	+
Growth at :						
25°C	m	m	m	m	m	m
30°C	g	g	g	g	g	g
37°C	g	g	g	g	g	g
42°C	n	m	n	n	n	n
Indole production	+	-	-	-	-	-
M-R	+	-	-	-	-	-
V-P	+	-	-	-	-	-
Nitrate	+	+	+	+	-	-
Gas from glucose	+	-	-	-	-	-
Oxidase	+	+	+	+	+	+
Catalase	+	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+	+
O-F test	F	O	O	O	O	O

Contd.

Table 14 (Contd.)

	Bacterial isolates					
	A01	**B01**	**P01**	**P02**	**P03**	**P04**
Acid from :						
glucose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+
Sorbitol	-	-	+	-	+	+
Sucrose	+	+	+	+	+	+
m-inositol	-	-	+	-	-	+
Mannitol	+	+	+	+	+	+
Adonitol	-	-	-	-	-	-
Levan from sucrose	-	-	-	-	+	+
Arginine hydrolysis	+	-	+	+	+	+
H_2S from cystein	+	-	-	-	-	-
Citrate utilization	+	-	+	+	+	+
Pigment formation	-	-	+[a]	+[b]	+[b]	+[b]

* : + = positive; - = negetive; g = good growth; m = moderate growth; n = no growth; O = Oxidative; F = Fermentative

[a] reddish brown diffusible pigment produced in King's B medium

[b] greenish yellow diffusible pigment produced in King's B medium

Table 15. Morphological characteristics of bacteria isolated from ulcers of *Puntius* sp.*

	Bacterial isolates				
	A02	**A03**	**A04**	**C01**	**P05**
Shape	Rod	Rod	Rod	Sphere	Rod
Occurrence	Mostly in singles	Mostly in singles	Mostly in singles	Singles, pairs, tetrads or in irregular clusters	Singles, pairs or in chains
Size	2.5-3.2 x 0.7-0.8 mm	2.5-3.0 x 0.65-0.8 mm	2.8-3.2 x 0.75-0.8 mm	1.2-1.6 m diameter	2.5-3.0 x 0.5-0.6 mm
Spore	-	-	-	-	-
Agar colonies	Circular, smooth, convex	Circular, smooth, convex	Circular, smooth convex	Circular, smooth convex	Circular, smooth slightly convex
Culture in NB	Turbid	Turbid	Turbid	Turbid with pellicle and sediments	Turbid with pellicle and sediments

* - = absent

Table 16. Biochemical characteristics of bacteria isolated from ulcers of *Puntius* sp.*

	Bacterial isolates				
	A02	**A03**	**A04**	**C01**	**P05**
Gram reaction	-	-	-	+	-
Motility	+	+	+	-	+
Growth at :					
25°C	m	m	m	m	m
30°C	g	g	g	g	g
37°C	g	g	g	g	g
42°C	n	n	n	n	m
Indole production	+	+	+	-	-
M-R	w	+	+	-	-
V-P	-	+	+	-	-
Nitrate	+	+	+	w	+
Gas from glucose	+	+	+	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Gelatin hydrolysis	+	+	+	+	+
O-F test	F	F	F	O	O

Contd.

Table -16. (Contd.)

	Bacterial isolates				
	A02	**A03**	**A04**	**C01**	**P05**
Acid from :					
glucose	+	+	+	+	+
Fructose	+	+	+	+	+
L-Arabinose	-	+	+	-	+
Sorbitol	-	-	-	+	-
Sucrose	+	+	+	+	-
m-inositol	-	-	-	+	-
Mannitol	+	+	+	+	+
Adonitol	-	-	-	-	-
Levan from sucrose	-	-	-	-	-
Arginine hydrolysis	-	+	+	-	+
H_2S from cystein	+	+	+	-	-
Citrate utilization	+	+	+	+	+
Pigment formation	-	-	-	bright yellow colonies	yellowish green in King's B medium

* : + = positive; - = negetive; g = good growth; m = moderate growth; n = no growth; O = Oxidative; F = Fermentative ; w = weak

Table 17. Morphological characteristics of bacteria isolated from ulcers of *Mystus* sp.*

	Bacterial isolates				
	V01	**A05**	**A06**	**M01**	**P06**
Shape	Rod	Rod	Rod	Rod	Rod
Occurrence	Mostly in singles	Singles, pairs or in chains	Singles, pairs or in chains	Singles, pairs or in chains	Singles, pairs or in chains
Size	2.6-3.0 x 0.68-0.75 μm	2.8-3.3 x 0.7-0.75 μm	2.5-3.0 x 0.7-0.8 μm	1.5-1.7 x 0.9-1.2 μm	2.7-3.5 x 0.75-0.8 μm
Spore	-	-	-	-	-
Agar colonies	Circular, smooth, convex	Circular, smooth convex	Circular, smooth convex	Circular, smooth convex	Circular, smooth, slightly convex
Culture in NB	Turbid with sediments	Turbid	Turbid	Turbid with sediments	Turbid with pellicle and sediments

* - = absent

Table 18. Biochemical characteristics of bacteria isolated from ulcers of *Mystus* sp.*

	Bacterial isolates				
	V01	**A05**	**A06**	**M01**	**P06**
Gram reaction	-	-	-	-	-
Motility	+	+	+	+	+
Growth at :					
25°C	m	m	m	m	m
30°C	g	g	g	g	g
37°C	g	g	g	g	g
42°C	n	n	n	n	n
Indole production	-	+	+	-	-
M-R	-	+	+	-	-
V-P	-	+	+	-	-
Nitrate	+	+	-	-	+
Gas from glucose	-	+	+	-	-
Oxidase	+	+	+	+	+
Catalase	+	+	+	+	+
Gelatin hydrolysis	+	+	+	-	+
O-F test	F	F	F	O	O

Contd.

Table -18. (Contd.)

	Bactrerial isolates				
	V01	**A05**	**A06**	**M01**	**P06**
Acid from :					
glucose	+	+	+	–	+
Fructose	+	+	+	–	+
L-Arabinose	+	+	–	–	+
Sorbitol	–	–	–	–	+
Sucrose	+	+	+	–	+
m-inositol	–	–	–	–	+
Mannitol	+	+	+	–	+
Adonitol	–	–	–	–	–
Levan from sucrose	-	-	-	-	-
Arginine hydrolysis	+	+	+	+	+
H_2S from cystein	-	+	-	-	-
Citrate utilization	+	+	+	-	+
Pigment formation	-	-	-	-	yellowish green in King's B medium

* : + = positive; - = negetive; g = good growth; m = moderate growth; n = no growth; O = Oxidative; F = Fermentative

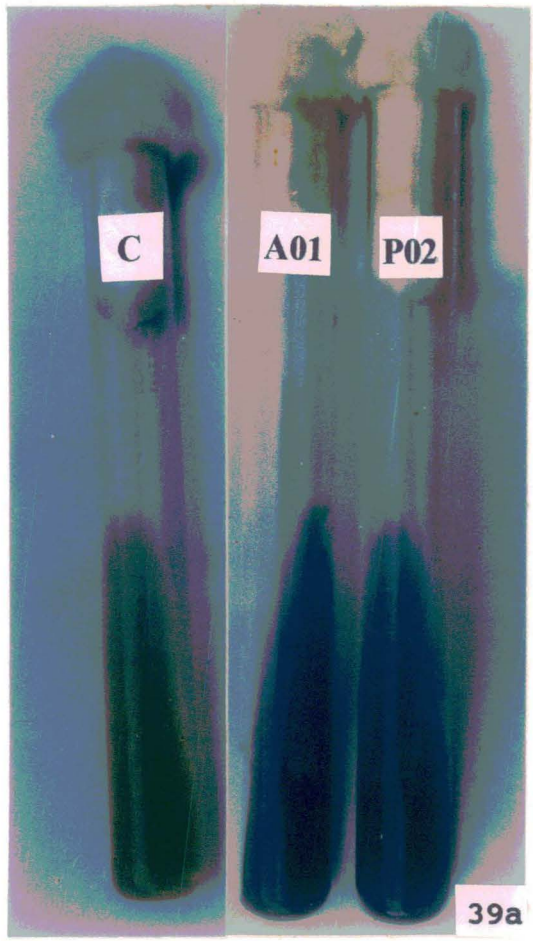
Plate XV

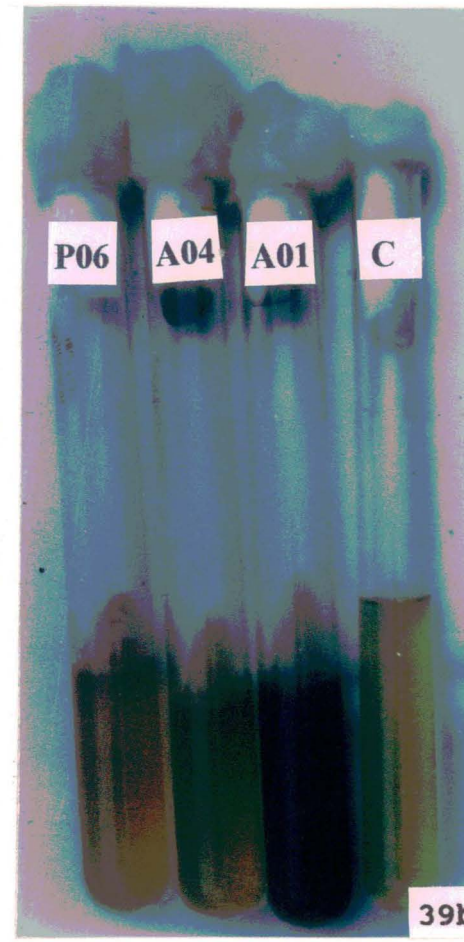
Fig. 39. Characterization of bacterial isolates by various biochemical tests :

a. Utilization of citrate by isolates A01 (*Aeromonas hydrophila*) and P02 (*Pseudomonas* sp.) indicated by change of colour of Simmon's Citrate medium from green to blue (C= Control).

b. Hydrogen Sulphide production by isolates A01 (*Aermonas hydrophila*) and A04 (*Aeromonas hydrophila*) indicated by blackening of lead acetate paper placed below the cotton plug in Hajna's modified motility medium containing cystein. Isolate P06 (*Pseudomonas* sp.) showed negetive result (C= Control).

c. Formation of reddish brown pigment by isolate P01 (*Pseudomonas* sp.) in King's B medium (C= Control).


C
A01
P02
39a


P06
A04
A01
C
39b

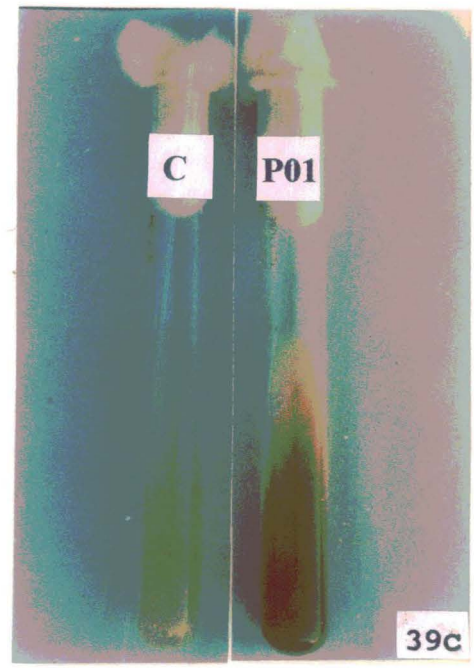

C
P01
39c

Table 19. Bacteria isolated from surface ulcers of naturally infected fishes.

Type of isolated bacteria	Source fish	No. of strains isolated	Isolate numbers	Total No. of strains of each type of bacteria
Bacillus	*C. punctatus*	1	B01	1
Aeromonas	*C. punctatus*	1	A01	6
	Puntius sp.	3	A02, A03, A04	
	Mystus sp.	2	A05, A06	
Moraxella	*Mystus* sp.	1	M01	1
Pseudomonas	*C. punctatus*	4	P01, P02, P03, P04	6
	Puntius sp.	1	P05	
	Mystus sp	1	P06	
Micrococcus	*Puntius* sp.	1	C01	1
Vibrio	*Mystus* sp.	1	V01	1

4.2.4. Isolation of fungus

Fungus could be detected in the smear preparation of only one fish *C. batrachus*. The fungus was isolated and its morphological features were examined after staining with cotton blue. It was very slow growing in both GPYA and PDA with very thin growing hyphae. The fungus was grown at 15 to 17^{0}C. Considerable difficulty was faced during maintenance of the fungus because continuous growth was very unpredictable and sometimes the culture died

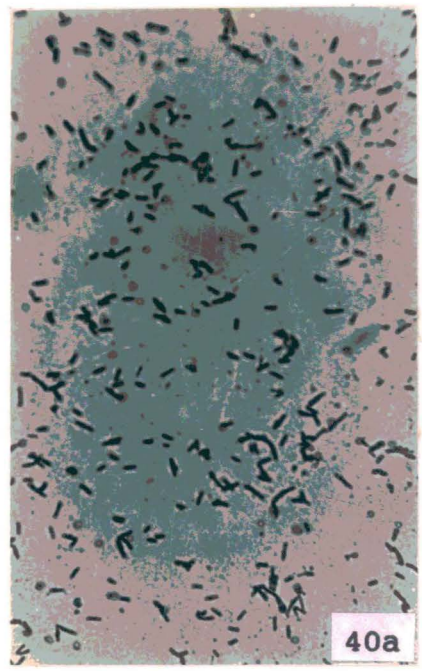
Plate XVI

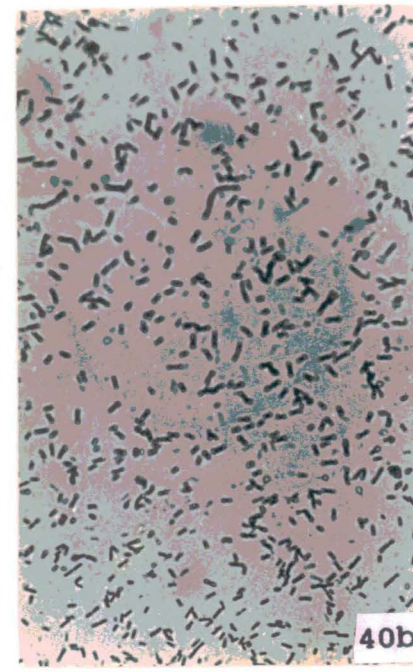
Fig. 40.a. Phase contrast micrograph of isolate A01 (*Aeromonas hydrophila*). (x200)

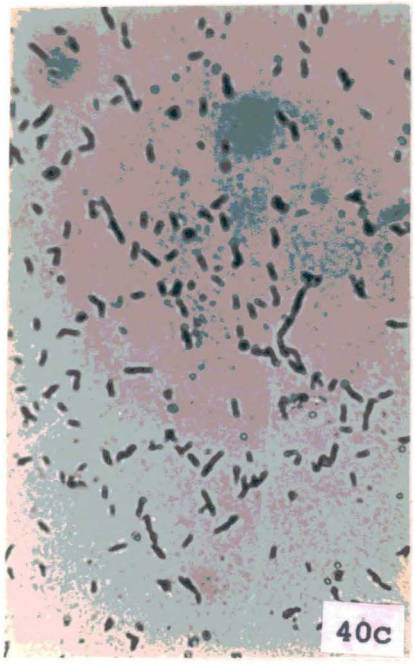
Fig. 40 b. Phase contrast micrograph of isolate A06 (*Aeromonas sobria*). (x200)

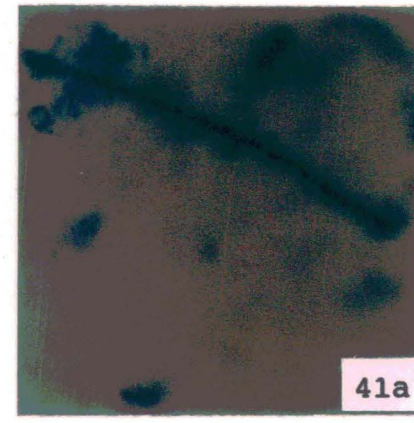
Fig. 40 c. Phase contrast micrograph of isolate P06 (*Pseudomonas* sp.). (x200).

Fig.41. a,b. Showing fungal mycelia isolated from ulcers of *Clarias batrachus*. (x200) (cotton blue).


40a


40b


40c


41a


41b

without any apparant reason. Extreme precaution was taken in maintaining the fungal cultures and it was subcultured very often so that the culture was not lost.

Under the microscope, the fungus from the fish tissue was aseptate and moderately wide with branchings (Figs. 41a and 41b). However, in culture it became much narrow particularly at the growing ends. In the fungus isolated from the fish tissue, the spores were arranged in a single row inside the zoosporangia. The zoospores were circular and were encysted.

4.2.5. Pathogenicity test of the isolated bacteria in *C. punctatus* and *H. fossilis* and a comparison with the pathogenicity of R1, R2 and R3.

Experimental induction of ulcer by the isolated bacteria was tried in *C. punctatus* and *H. fossilis* in comparison to R1, R2 and R3. Cell suspension of $1x10^7$ cells / ml of each isolate and R1, R2 and R3 in 0.85% saline were injected intramuscularlly to healthy *C. punctatus* and *H. fossilis*. Each bacteria were injected to 10 fishes. The fishes were observed for 15 days and mortalities if any, were recorded.

It was found that among the 20 isolates, four *Aeromonas* strains and two *Pseudomonas* strains were able to induce ulcer in healthy fish, while the other isolates were nonpathogenic. (Table 20 and 21). However, the percentage mortality data revealed that the virulence of the pathogenic isolates were lower in comparison to R1, R2 and R3 (Figs. 42 and 43).

The nature of ulcers produced by the pathogenic bacteria were graded as described in section 4.1.1.2.1. Accordingly, ulcers produced by isolate A07 was recorded to be moderate while the rest of the isolates produced only superficial ulcers (Fig. 44a, b and c). The percentage mortality in *C. punctatus* induced by isolates A01, A02, A03 and A06 were 10%, 5%, 10%, 15%, respectively while that induced by P02 and P06 were 10%, and 0% respectively. The percentage mortality in *H. fossilis* induced by isolates A01, A02, A03 and A06 were 15%, 5%, 10%, 20%, respectively while that induced by P02 and P06

were 10%, and 5% respectively. No mortality was recorded in the rest of the inoculated fishes in both species even after 15 days of observation

Table 20 : Percentage mortality and Nature of ulcer formation in *Channa punctatus* induced by the bacteria isolated from ulcers of *C. punctatus*, *Mystus* sp. and *Puntius* sp.

Bacterial isolates	Number of fishes inoculated	Number of fishes dead[a]	Nature of ulcer	Percentage mortality
Control[b]	20	0	Nil	-
A01	20	2	superficial	10%
A02	20	1	superficial	5%
A03	20	2	superficial	10%
A06	20	3	superficial	15%
P02	20	2	superficial	10%
P06	20	0	superficial	0%

[a] Total number of fishes dead after 15 days of inoculation with bacteria

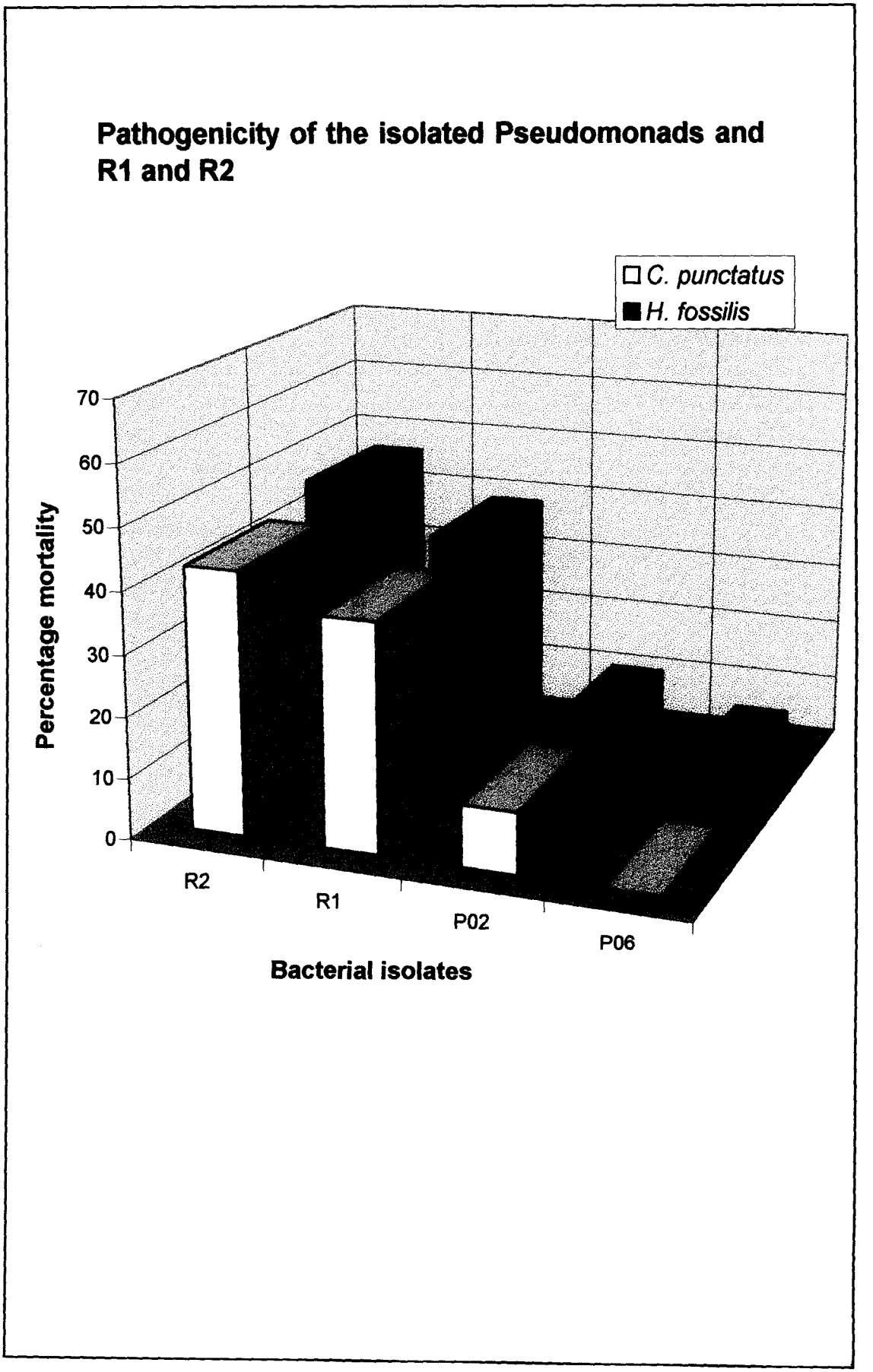
[b] Control set received intramuscular injection with 0.5 ml / 100 gm body weight of sterile saline (0.85%NaCl) solution

Table 21 : Percentage mortality and Nature of ulcer formation in *Heteropneustes fossilis* induced by the bacteria isolated from ulcers of *C. punctatuus*, *Mystus* sp. and *Puntius* sp.

Bacterial isolates	Number of fishes inoculated	Number of fishes dead[a]	Nature of ulcer	Percentage mortality
Control[b]	20	0	Nil	-
A01	20	3	superficial	15%
A02	20	1	superficial	5%
A03	20	2	superficial	10%
A06	20	4	superficial	20%
P02	20	2	superficial	10%
P06	20	1	superficial	5%

[a] Total number of fishes dead after 15 days of inoculation with bacteria

[b] Control set received intramuscular injection with 0.5 ml / 100 gm body weight of sterile saline (0.85%NaCl) solution


Pathogenicity of the isolated Pseudomonads and R1 and R2
C. punctatus
H. fossilis
Percentage mortality
70
60
50
40
30
20
10
0
R2
R1
P02
P06
Bacterial isolates

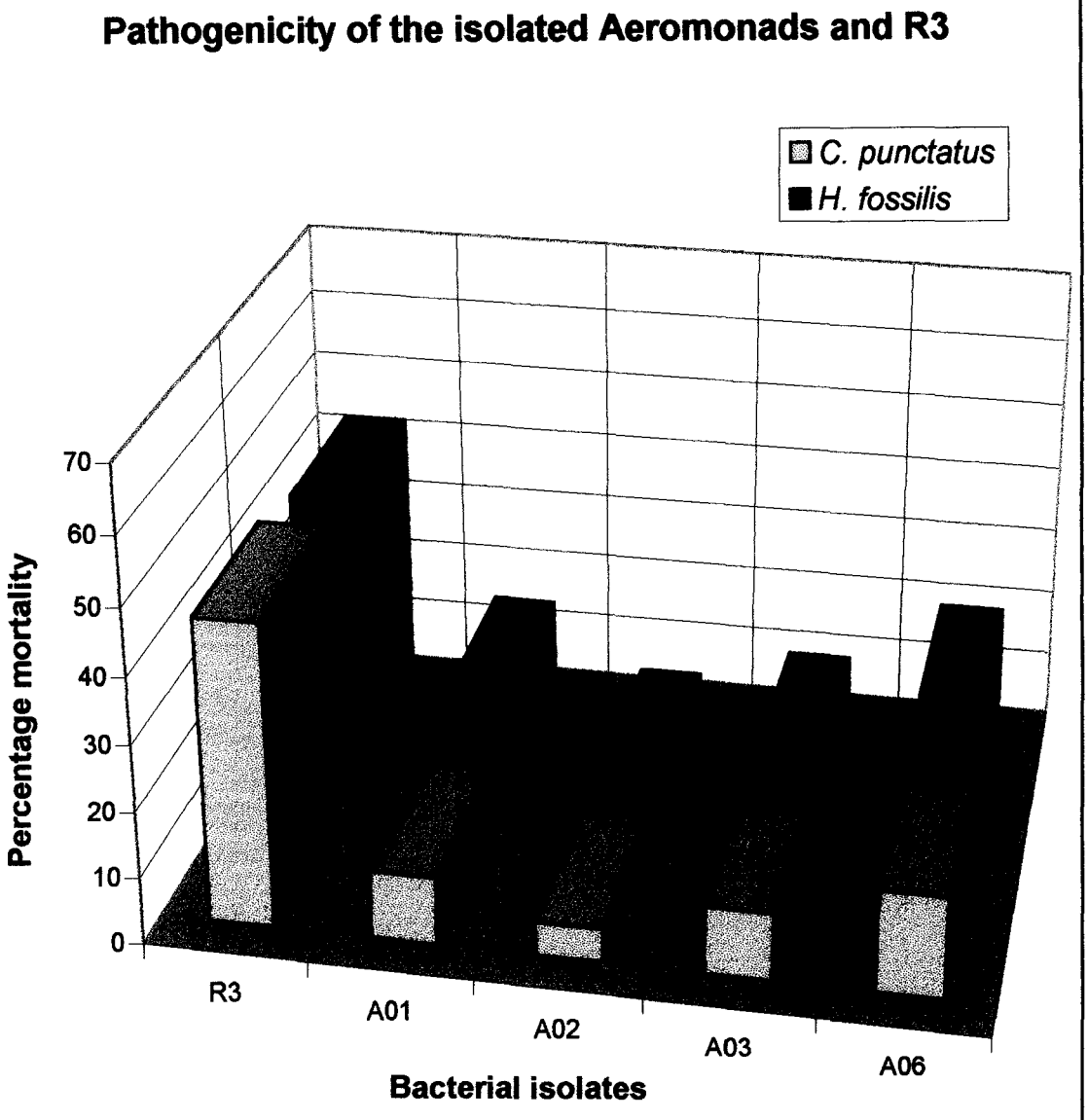

Pathogenicity of the isolated Aeromonads and R3
C. punctatus
H. fossilis
Percentage mortality
70
60
50
40
30
20
10
0
R3
A01
A02
A03
A06
Bacterial isolates

Plate XVII

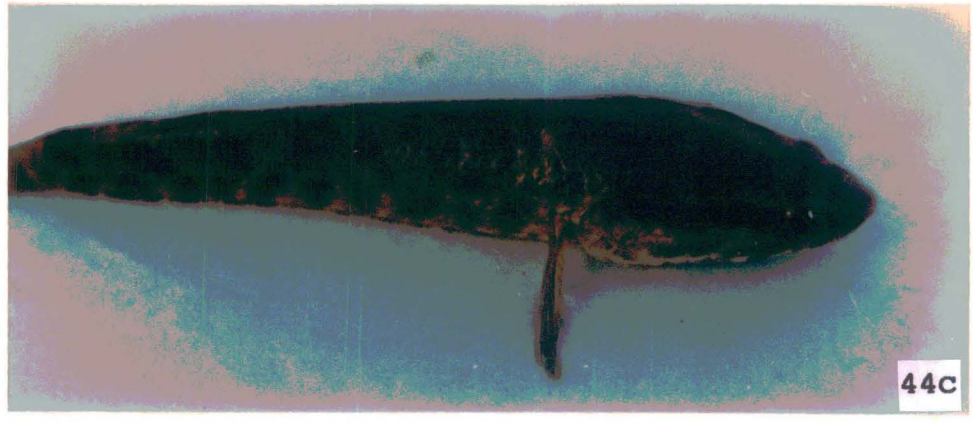
Fig. 44 a. *Channa punctatus* showing superficial ulcer formation 72 hours after intramuscular injection with isolate A01 (*Aeromonas hydrophila*).

Fig. 44 b. *Channa punctatus* showing local haemorrhage 72 hours after intramuscular injection with isolate A06 (*Aeromonas sobria*).

Fig. 44 c. *Channa punctatus* showing superficial ulcer formation 72 hours after intramuscular injection with isolate P06 (*Pseudomonas* sp.)

44a

44b



44c

4.2.6. *In vitro* Antibiotic susceptibilities of nineteen bacterial isolates from EUS affected fish.

The antimicrobial susceptibility of all bacteria isolated during the present study from skin ulcers of diseased fishes and R1, R2 and R3 were measured by the drug disc diffusion method in Mueller Hinton agar plates as described under materials and methods. The antibiotic sensitivity profile of bacterial isolates from *Channa punctatus*, *Puntius* sp. and *Mystus* sp. and that of R1, R2 and R3 are listed in tables 22, 23, 24 and 25 respectively.

The results showed that all Pseudomonads and Aeromonads were resistant to penicillin and ampicillin (Figs. 45 and 46). *Bacillus* sp. was resistant to ampicillin only (Fig. 47) while *Micrococcus* sp. was resistant to norfloxacin and nalidixic acid (Fig. 48) and *Moraxella* sp. was resistant to streptomycin and chloramphenicol. *Vibrio* sp. was susceptible to all the antibiotics tested.

Among the pathogenic bacteria, R1, R2 (fluorescent Pseudomonads) and R3 (*Aermonas caviae*) were found to be resistant to ampicillin and penicillin. In addition R2 was resistant to co-trimoxazole and R3 was resistant to co-trimoxazole and erythromycin (Table 26). The antibiotic resistance patterns of the pathogenic bacteria isolated from skin ulcers of *C. punctatus*, *Puntius* sp. and *Mystus* sp. show that all were resistant to penicillin and ampicillin. In addition the Aeromonads were resistant to erythromycin and P02 (*Pseudomonas* sp.) was resistant to Co-trimoxazole. Percentage resistance data showed that 84.21% of all the bacteria tested (both pathogenic and non pathogenic) were resistant to ampicillin, 78.95% were resistant to penicillin, 52.63% were resistant to erythromycin and 36.84% were resistant to co-trimoxazole (Table 27). Thus penicillin, ampicillin, erythromycin and co-trimoxazole cannot be used as chemotherapeutic agents in treating fish ulcers. One non pathogenic Aeromonad (A04) isolated from ulcers of *Puntius* sp. showed multiple resistance to seven antibiotics including oxytetracycline (Fig. 49). However, no other bacteria was resistant to oxytetracycline and it showed 94.74% sensitivity among the isolates. Other effective antibiotics included kanamycin (100% sensitivity), chloramphenicol (94.74% sensitivity), norfloxacin

(94.74% sensitivity), amoxicillin (89.48% sensitivity), nalidixic acid (94.74% sensitivity) and streptomycin (94.74% sensitivity) (Table 27). A comparative data of the inhibition zones of the pathogenic Pseudomonads (Fig. 50) and Aeromonads (Fig. 51) against 5 commonly used antibiotics showed that oxytetracycline was particularly effective against the Pseudomonads. In case of Aeromonads, it was one of the most effective antibiotics. The minimum inhibitory concentrations of R1 and R2 were 3.2 μg/ml and that of R3 was 6.4 μg/ml.

Table 22 : Antibiotic sensitivity[a] of the bacteria isolated from *Channa punctatus*

Antibiotics tested [b]	*Bacterial Strains*					
	A01	**B01**	**P01**	**P02**	**P03**	**P04**
Amoxicillin (30)	R	S	S	S	HS	HS
Ampicillin (10)	R	R	R	R	R	R
Chloramphenicol (30)	HS	S	S	S	S	HS
Co-trimoxazole (25)	S	HS	R	R	S	S
Erythromycin (15)	R	S	R	S	R	S
Gentamycin (10)	R	HS	HS	HS	HS	HS
Kanamycin (30)	S	HS	HS	HS	HS	HS
Nalidixic acid (30)	S	S	S	S	S	HS
Norfloxacin (10)	S	HS	S	HS	HS	HS
Oxytetracycline (30)	HS	HS	S	HS	HS	HS
Penicillin (10)	R	S	R	R	R	R
Streptomycin (10)	S	HS	HS	HS	HS	S

[a] Sensitivity tested by antibiotic disc diffusion in Mueller Hinton agar plates. S = Sensitive; HS = Highly Sensitive; R = Resistant.

[b] Concentration of the antibiotic in μg / ml is given in the parenthesis.

Table 23 : Antibiotic sensitivity[a] of the bacteria isolated from *Puntius* sp.

Antibiotics tested[b]	*Bacterial Strains*				
	A02	**A03**	**A04**	**C01**	**P05**
Amoxicillin (30)	S	S	R	HS	S
Ampicillin (10)	R	R	R	HS	R
Chloramphenicol (30)	HS	HS	S	HS	S
Co-trimoxazole (25)	S	S	R	HS	R
Erythromycin (15)	R	R	R	S	R
Gentamycin (10)	R	S	R	HS	HS
Kanamycin (30)	S	S	S	S	HS
Nalidixic acid (30)	S	HS	S	R	S
Norfloxacin (10)	S	HS	S	R	S
Oxytetracycline (30)	HS	HS	R	HS	HS
Penicillin (10)	R	R	R	S	R
Streptomycin (10)	S	S	S	S	HS

[a] Sensitivity tested by antibiotic disc diffusion in Mueller Hinton agar plates. S = Sensitive; HS = Highly Sensitive; R = Resistant.

[b] Concentration of the antibiotic in μg / ml is given in the parenthesis.

Table 24 : Antibiotic sensitivity[a] of the bacteria isolated from *Mystus* sp.

Antibiotics tested [b]	*Bacterial Strains*				
	V01	**A05**	**A06**	**M01**	**P06**
Amoxicillin (30)	S	S	S	S	HS
Ampicillin (10)	S	R	R	S	R
Chloramphenicol (30)	HS	HS	HS	R	S
Co-trimoxazole (25)	S	R	S	S	S
Erythromycin (15)	S	R	R	S	S
Gentamycin (10)	HS	R	S	HS	HS
Kanamycin (30)	S	S	S	S	HS
Nalidixic acid (30)	S	S	S	S	S
Norfloxacin (10)	S	S	HS	HS	S
Oxytetracycline (30)	HS	S	S	S	S
Penicillin (10)	S	R	R	HS	R
Streptomycin (10)	S	S	S	R	S

[a] Sensitivity tested by antibiotic disc diffusion in Mueller Hinton agar plates. S = Sensitive; HS = Highly Sensitive; R = Resistant.

[b] Concentration of the antibiotic in μg / ml is given in the parenthesis.

Table 25 : Antibiotic sensitivity[a] of R1, R2 (fluorescent Pseudomonads) and R3 (*Aeromonas caviae*) isolated from epizootic ulcerative syndrome affected air-breathing fishes.

Antibiotics tested[b]	*Bacterial isolates*		
	R1	R2	R3
Amoxicillin (30)	HS	S	S
Ampicillin (10)	R	R	R
Chloramphenicol (30)	S	S	S
Co-trimoxazole (25)	S	R	R
Erythromycin (15)	S	S	R
Gentamycin (10)	HS	HS	S
Kanamycin (30)	HS	HS	S
Nalidixic acid (30)	S	S	S
Norfloxacin (10)	HS	HS	S
Oxytetracycline (30)	HS	HS	HS
Penicillin (10)	R	R	R
Streptomycin (10)	S	HS	S

[a] Sensitivity tested by antibiotic disc diffusion in Mueller Hinton agar plates. S = Sensitive; HS = Highly Sensitive; R = Resistant.

[b] Concentration of the antibiotic in μg / ml is given in the parenthesis.

Plate XVIII

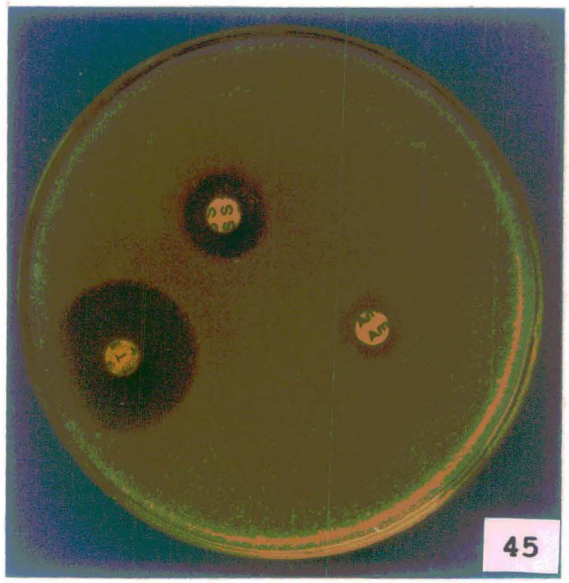
Fig.45. Antimicrobial sensitivity test with isolate A01(*Aeromonas hydrophila*) showing resistance to amoxicillin (Am), sensitivity to streptomycin (S) and high sensitivity to oxytetracycline (T).

Fig.46. Antimicrobial sensitivity test with the pathogenic isolate R3 (*Aeromonas caviae*) showing resistance to penicillin (P), sensitivity to norfloxacin (Nx) and high sensitivity to oxytetracycline (T).

Fig.47. Antimicrobial sensitivity test with isolate B01 (*Bacillus* sp.) showing resistance to ampicillin (A), sensitivity to erythromycin (E) and high sensitivity to co-trimoxazole (Co).

Fig.48. Antimicrobial sensitivity test with isolate C01 (*Micrococcus* sp.) showing sensitivity to erythromycin and high sensitivity to ampicillin (A) and co-trimoxazole (Co).

Fig. 49. Antimicrobial sensitivity test with isolate A04 (*Aeromonas hydrophila*) showing multiple resistance towards penicillin (P), erythromycin (E) and oxytetracycline (O).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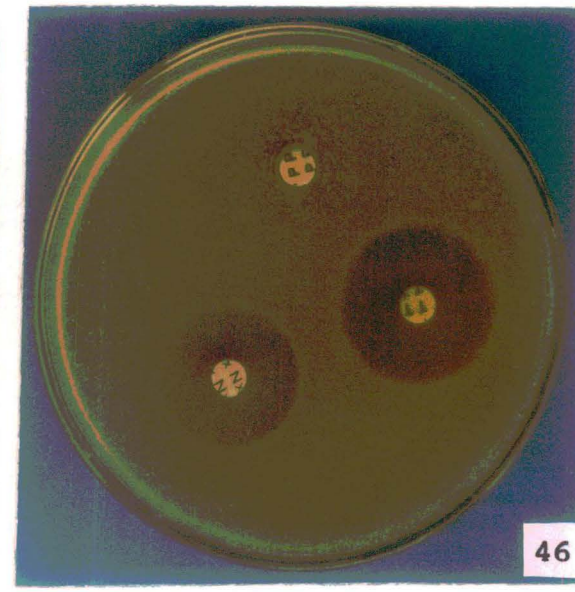
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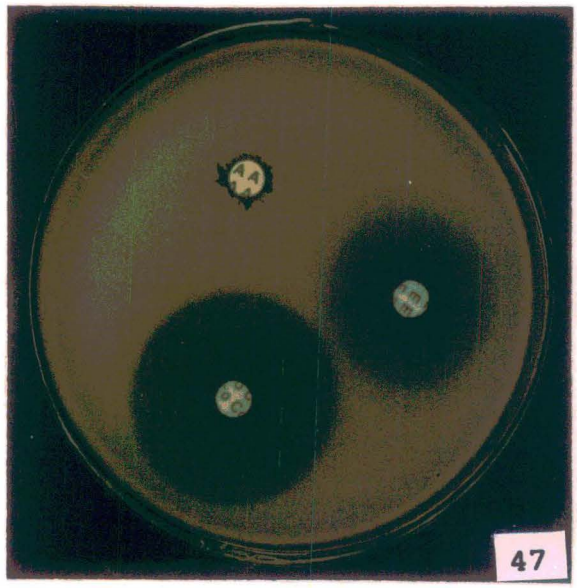
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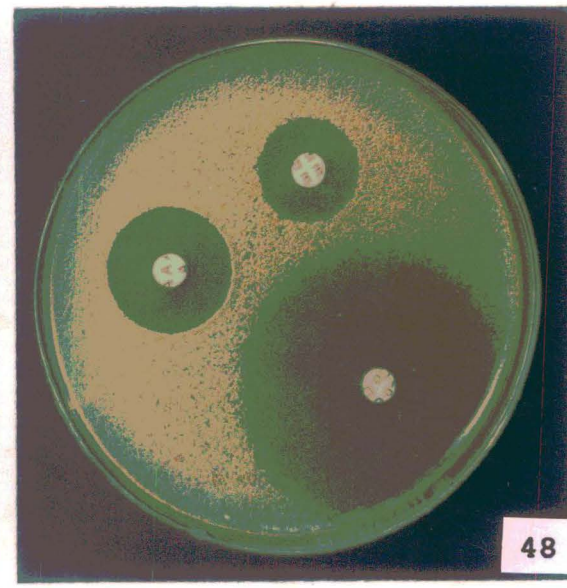
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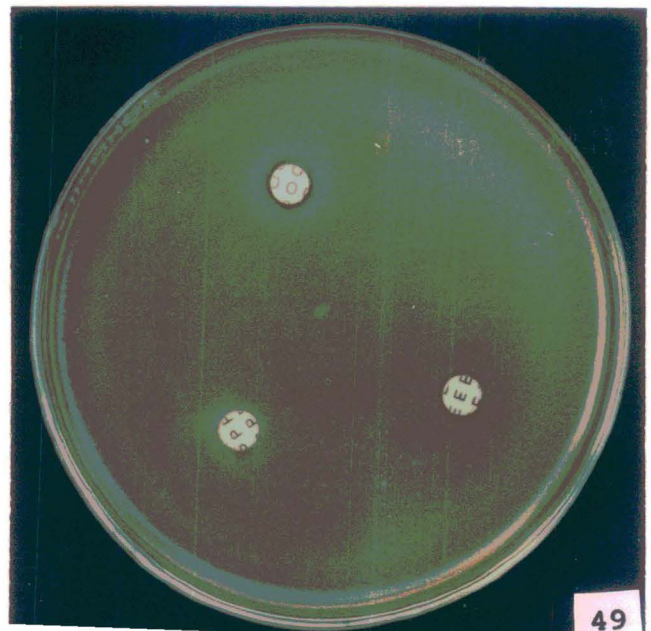
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Table 26 : Drug resistance patterns of bacteria isolated from epizootic ulcerative syndrome affected fish[a]

Isolate Number	Isolate identity	Resistance markers
* A01	*Aeromonas hydrophila*	Am Ax Er Ge Pe
* A02	*Aeromonas sobria*	Am Er Ge Pe
* A03	*Aeromonas hydrophila*	Am Er Pe
A04	*Aeromonas hydrophila*	Am Ax Ct Er Ge Otc Pe
A05	*Aeromonas hydrophila*	Am Ct Er Pe
* A06	*Aeromonas sobria*	Am Er Pe
B01	*Bacillus* sp.	Am
C01	*Micrococcus* sp.	Na Nf
M01	*Moraxella* sp.	Cl Sm
P01	*Pseudomonas* sp.	Am Ct Er Pe
* P02	*Pseudomonas* sp.	Am Ct Pe
P03	*Pseudomonas* sp.	Am Er Pe
P04	*Pseudomonas* sp.	Am Pe
P05	*Pseudomonas* sp.	Am Ct Er Pe
* P06	*Pseudomonas* sp.	Am Pe
* R1	*Pseudomonas* sp.	Am Pe
* R2	*Pseudomonas* sp.	Am Ct Pe
* R3	*Aeromonas caviae*	Am Ct Er Pe
V01	*Vibrio* sp.	- [b]

[a] Am = Ampicillin; Ax = Amoxacillin; Cl = Chloramphenicol; Ct = Co-trimoxazole; Ge = Gentamycin; Na = Nalidixic acid; Nf = Norfloxacin; Otc = Oxytetracycline; Pe = Penicillin; Sm = Streptomycin

[b] Susceptible to all antibiotics tested

* Pathogenic isolates

Table 27 : Percentage distribution* of bacterial sensitivity to the chemotherapeutic agents

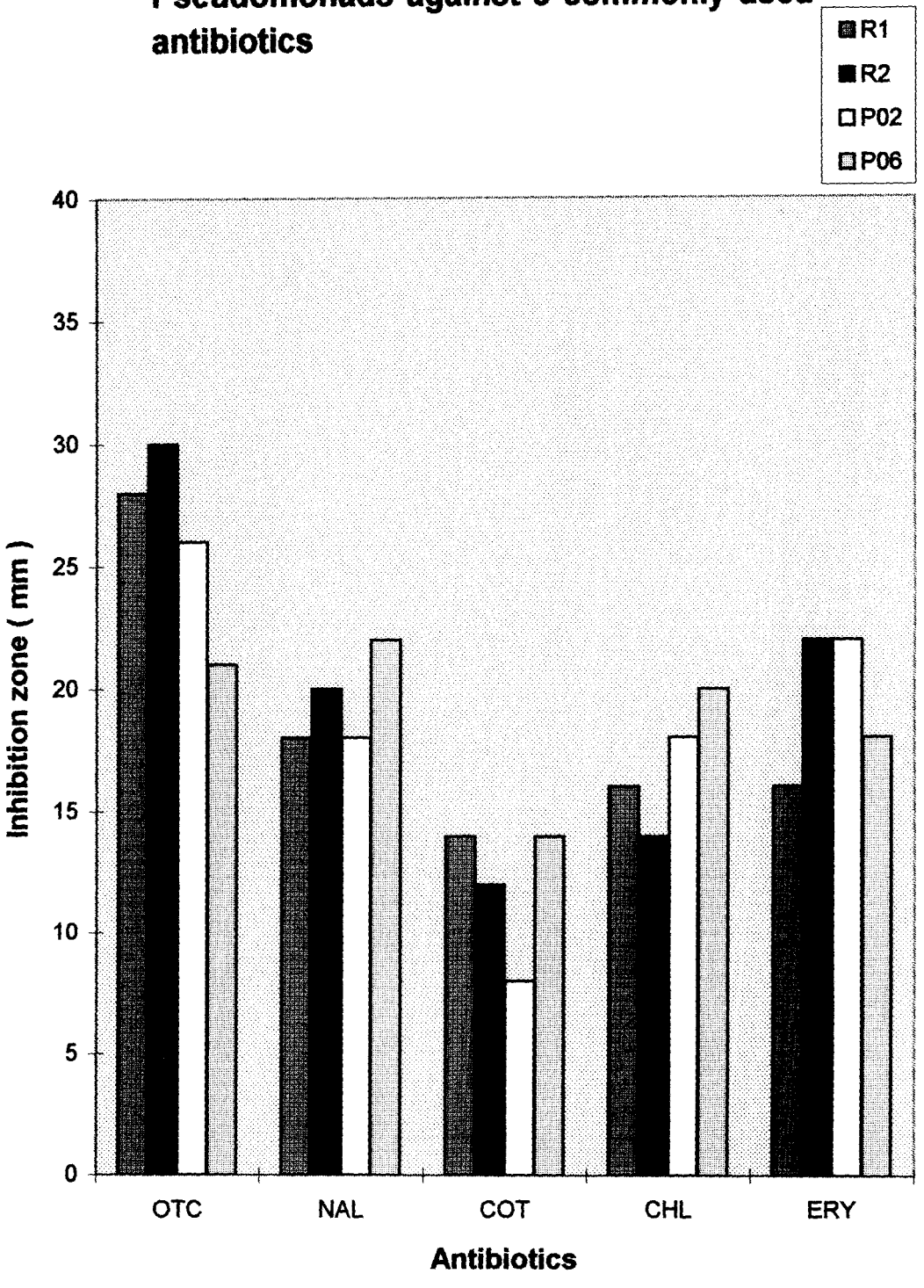
Antibiotics tested	Concentrations used	Percentage of bacteria sensitive	Percentage of bacteria resistant
Amoxicillin	30 µg/ml	89.48	10.52
Ampicillin	10 µg/ml	15.79	84.21
Chloramphenicol	30 µg/ml	94.74	5.26
Co-trimoxazole	25 µg/ml	63.16	36.84
Erythromycin	15 µg/ml	47.37	52.63
Gentamycin	10 µg/ml	78.95	21.05
Kanamycin	30 µg/ml	100	0
Nalidixic acid	30 µg/ml	94.74	5.26
Norfloxacin	10 µg/ml	94.74	5.26
Oxytetracycline	30 µg/ml	94.74	5.26
Penicillin	10 units	21.05	78.95
Streptomycin	10 µg/ml	94.74	5.26

* Percentage calculated on the basis of 19 isolates

4.2.7. Antibiotic treatment of ulcers caused by experimental induction of bacteria.

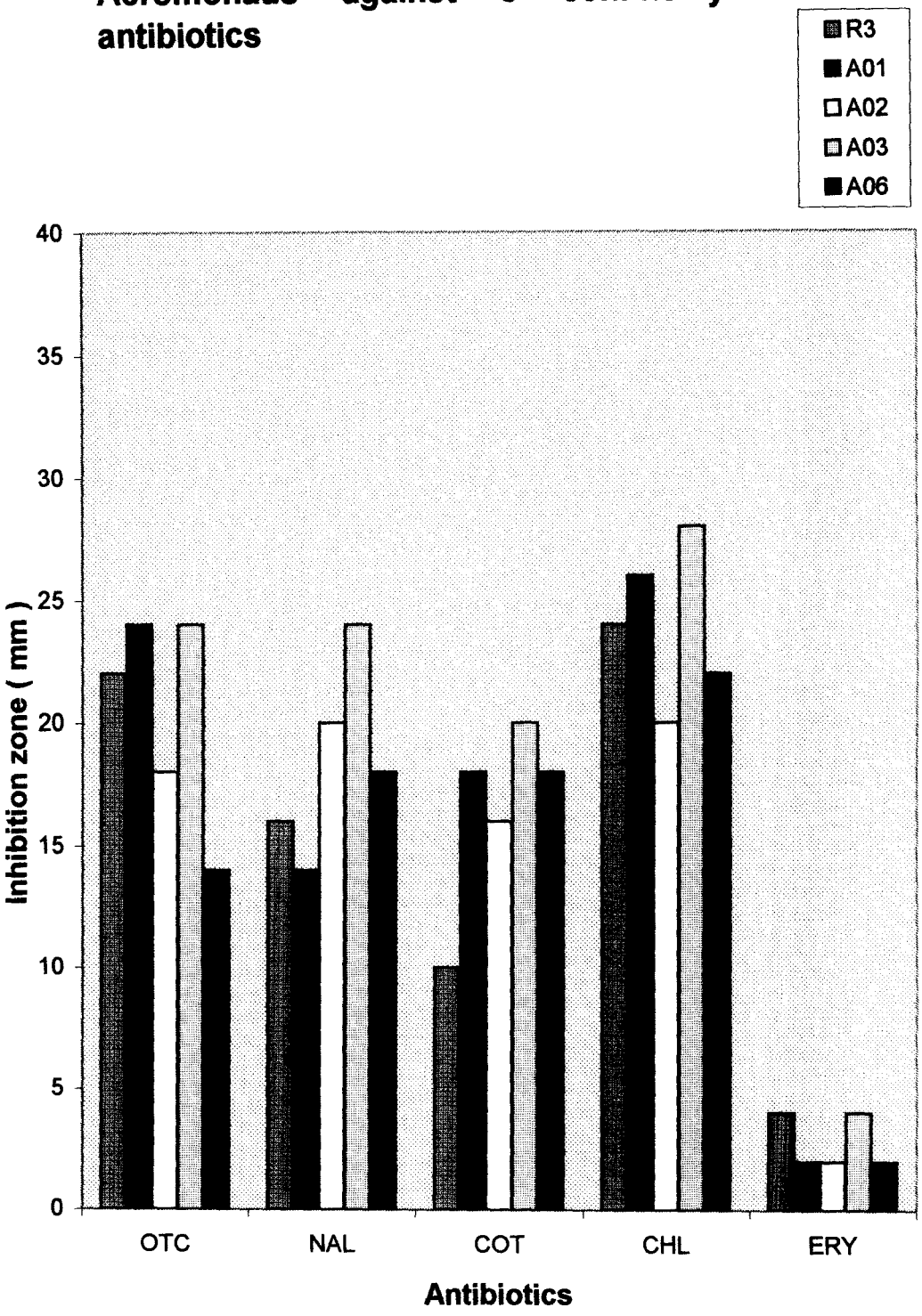
Bacterial suspensions of R1, R2 and R3 were injected intramuscularlly to healthy fishes (*Channa punctatus*) in pure and mixed condition. Each bacterial suspension and the mixed suspension were injected to 80 fishes. The methods of inoculation are described under materials and methods. Most of the fishes manifested external signs of the disease within 24 hours of inoculation. The area around the injection site turned reddish with swelling. After 24 hours, 10 fishes at a time, which were showing signs of ulcer formation, were netted out

Sensitivity of the isolated pathogenic Pseudomonads against 5 commonly used antibiotics



OTC=Oxytetracycline, NAL=Nalidixic acid, COT=Co-trimoxazole, CHL=Chloramphenicol, ERY=Erythromycin

Antibiotic sensitivity of the isolated pathogenic Aeromonads against 5 commonly used antibiotics



OTC=Oxytetracycline, NAL=Nalidixic acid, COT=Co-trimoxazole, CHL=Chloramphenicol, ERY=Erythromycin

from each aquarium and bath treatment was done by exposing the fishes to oxytetracycline solutions for periods of 5 min, 10 min, 30 min and 1 hr. A set of 10 fishes were not exposed to oxytetracycline and kept as untreated control. All fishes were kept under observation for 15 days and mortalities, if any, were recorded. The whole experiment was repeated thrice and the mean mortality rates were calculated.

The fishes unexposed to oxytetracycline showed progressive ulcer development. The small reddish swellings grew in size until the scales fell off and the surface layer of the skin was eroded. This type of moderate ulcers were noted in the fishes injected with pure bacterial suspensions. In the fishes treated with mixed bacterial suspension, the ulcers became deep and necrotic exposing an open sore. The fishes died with severe ulcers on their body. The mortality rates of unexposed fishes were 80% in case of fishes injected with a mixed bacterial suspension, 50% in case of fishes injected with R2 and R3 and 40% in case of fishes injected with R1. Comparison between antibiotic treatment of fishes for different time periods showed that the mortality rates of fishes exposed for longer periods (30 min, 1hr) was clearly lower than fishes exposed for only 5 or 10 min (Tables 28-31). The mortality also decreased with increasing concentration of the antibiotic. In fishes treated with 500 μg/ml of oxytetracycline for 1 hr, there was no significant difference in the condition of the fishes in the first 48 hrs after treatment. The ulcers did not grow in size. No mortality was recorded within 72 hours of treatment. Only 3.3% mortality was recorded in fishes which were injected with R1, 6.6% mortality was recorded in fishes which were injected with R2 and R3 and 26.6% mortality was recorded in fishes which were injected with a mixed bacterial suspension of R1, R2 and R3. After 4 days, the ulcers showed signs of healing. The ulcers of all the surviving fishes completely healed within the observation period of 15 days. On the other hand, almost no difference could be observed externally between fishes treated with 50 μg/ml oxytetracycline for 5 min and the untreated fishes. The mortality rates also were similar though there was a drop in the mortality rates in the first 48 hours after treatment. The cumulative mortalities are graphically represented in Figs 52-67.

Table 28 : Percentage mortality of bacteria infected fishes after treatment with 50 μg/ml oxytetracycline

Bacterial isolate	*Time of exposure*			
	5 min	10 min	30 min	60 min
R1	36.6	33.3	30	23.3
R2	50	43.3	36.6	26.6
R3	46.6	46.6	40	30
M	76.6	73.3	66.6	60

[a] $1x10^7$ cfu / ml of bacterial suspension in 0.85% saline administered intramuscularlly

Table 29 : Percentage mortality of bacteria infected fishes after treatment with 100 μg/ml oxytetracycline

Bacterial isolates	*Time of exposure*			
	5 min	10 min	30 min	60 min
R1	36.6	30	23.3	20
R2	46.6	40	30	26.6
R3	43.3	40	33.3	26.6
M	70	66.6	60	53.3

[a] $1x10^7$ cfu / ml of bacterial suspension in 0.85% saline administered intramuscularlly

Table 30 : Percentage mortality of bacteria infected fishes[a] after treatment with 200 μg/ml oxytetracycline

Bacterial isolate	*Time of exposure*			
	5 min	10 min	30 min	60 min
R1	33.3	33.3	23.3	13.3
R2	46.6	43.3	33.3	20
R3	43.3	36.6	26.6	20
M	73.3	66.6	53.3	43.3

[a] $1x10^7$ cfu / ml of bacterial suspension in 0.85% saline administered intramuscularlly

Table 31 : Percentage mortality of bacteria infected fishes after treatment with 500 μg/ml oxytetracycline

Bacterial isolate	*Time of exposure*			
	5 min	10 min	30 min	60 min
R1	33.3	26.6	16.6	3.3
R2	36.6	30	20	6.6
R3	40	33.3	16.6	6.6
M	66.6	60	40	26.6

[a] $1x10^7$ cfu / ml of bacterial suspension in 0.85% saline administered intramuscularlly

Cumulative mortalities of R1 injected fishes after treatment with 50μg/ml oxytetracycline

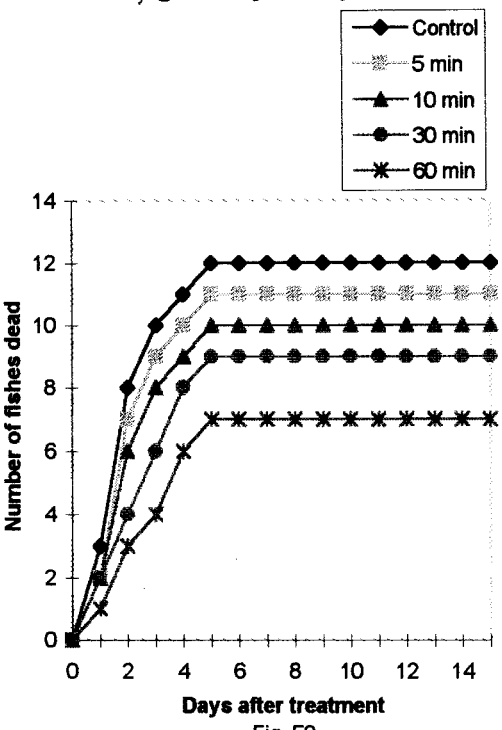


Fig. 52

Cumulative mortalities of R2 injected fishes after treatment with 50μg/ml oxytetracycline

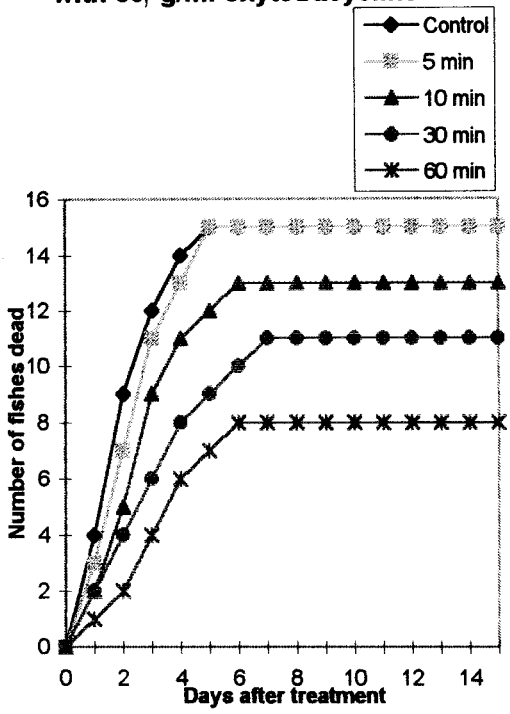


Fig. 53

Cumulative mortalities of R3 injected fishes after treatment with 50μg/ml oxytetracycline

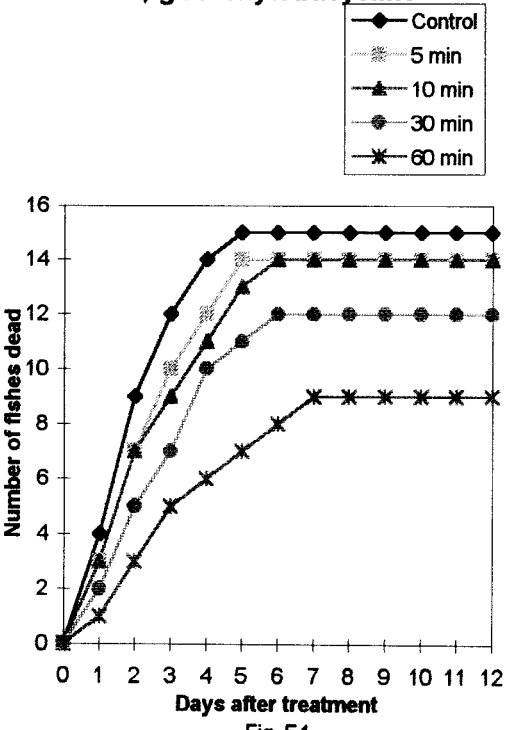


Fig. 54

Cumulative mortalities of mixed bacteria injected fishes after treatment with 50μg/ml oxytetracycline

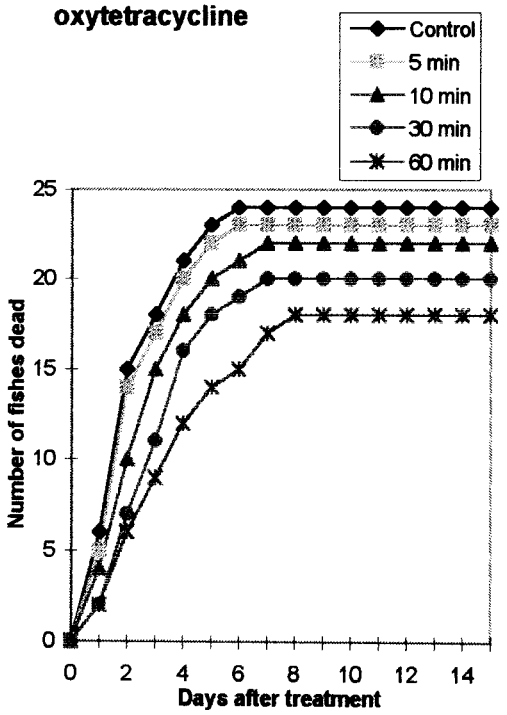


Fig. 55

Cumulative mortalities of R1 injected fishes after treatment with 100 µg/ml oxytetracycline

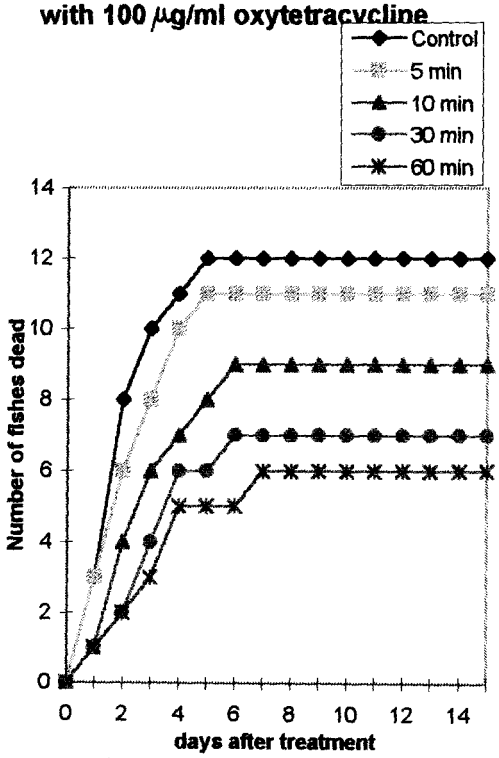


Fig. 56

Cumulative mortalities of R2 injected fishes after treatment with 100 µg/ml oxytetracycline

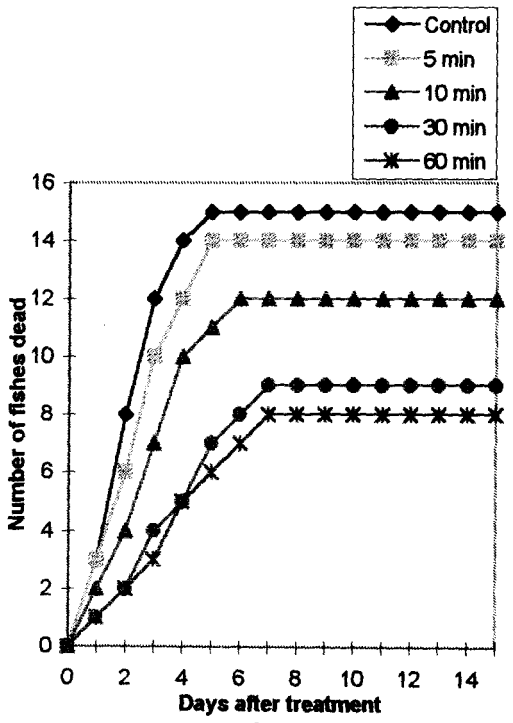


Fig. 57

Cumulative mortalities of R3 injected fishes after treatment with 100 µg/ml oxytetracycline

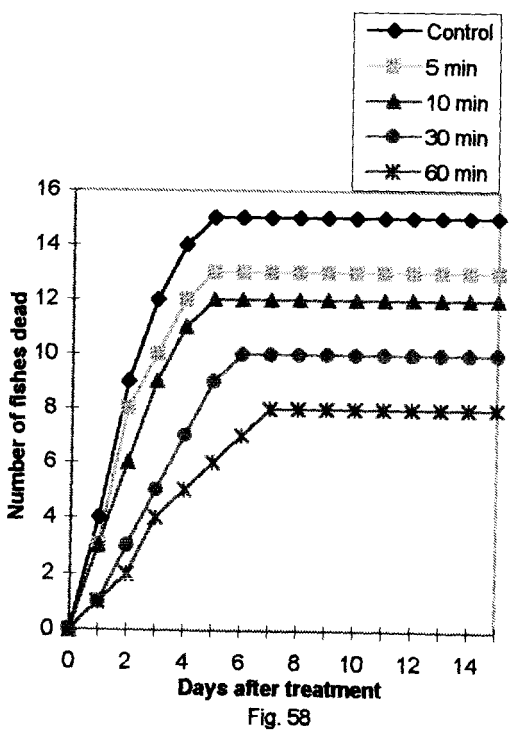


Fig. 58

Cumulative mortalities of mixed bacteria injected fishes after treatment with 100 µg/ml oxytetracycline

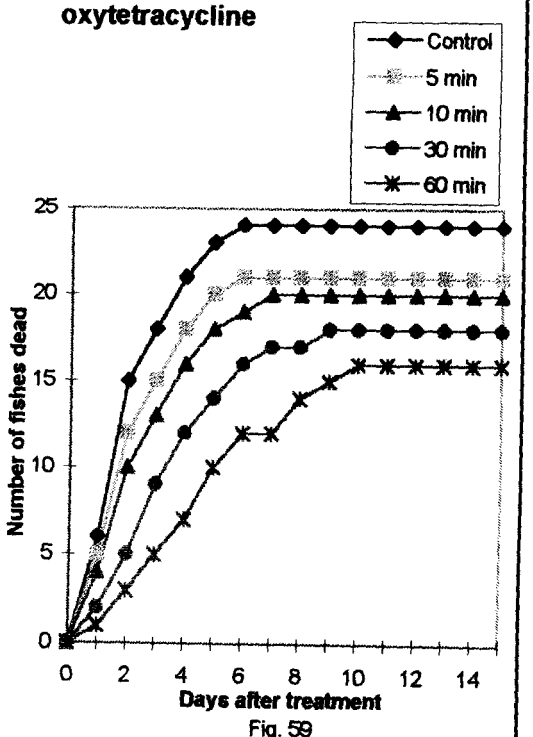


Fig. 59

Cumulative mortalities of R1 injected fishes after treatment with 200 µg/ml oxytetracycline

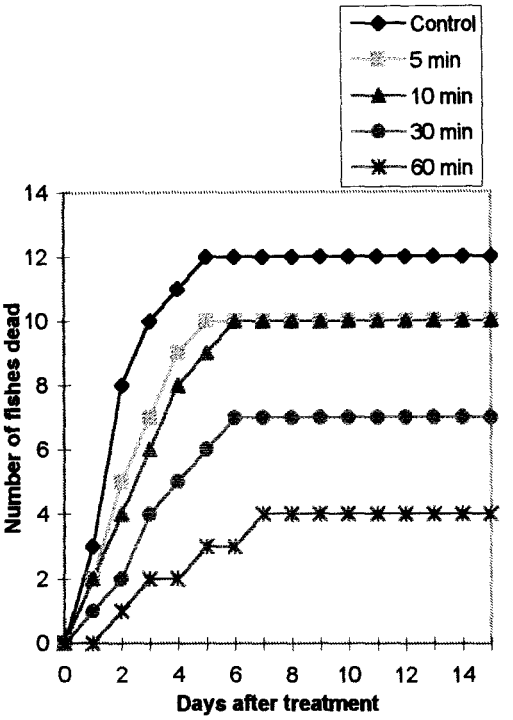


Fig. 60

Cumulative mortalities of R2 injected fishes after treatment with 200 µg/ml oxytetracycline

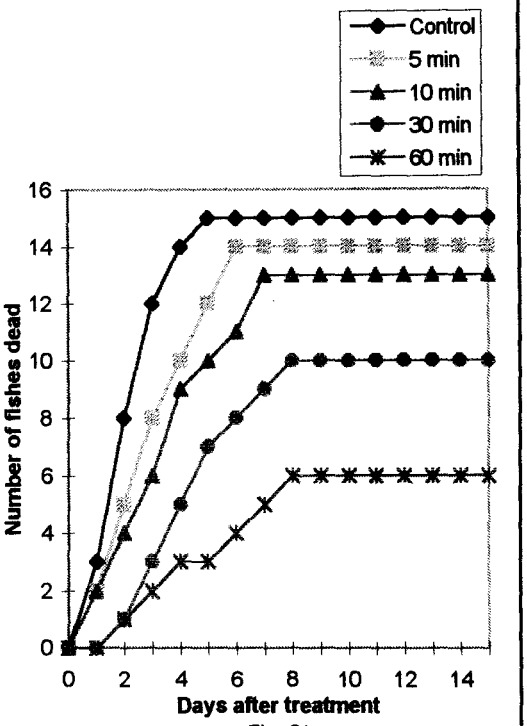


Fig. 61

Cumulative mortalities of R3 injected fishes after treatment with 200 µg/ml oxytetracycline

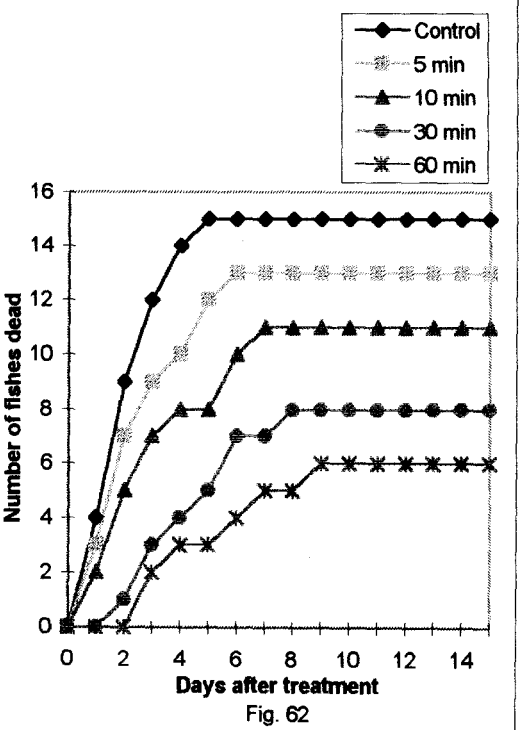


Fig. 62

Cumulative mortalities of mixed bacteria injected fishes after treatment with 200 µg/ml oxytetracycline

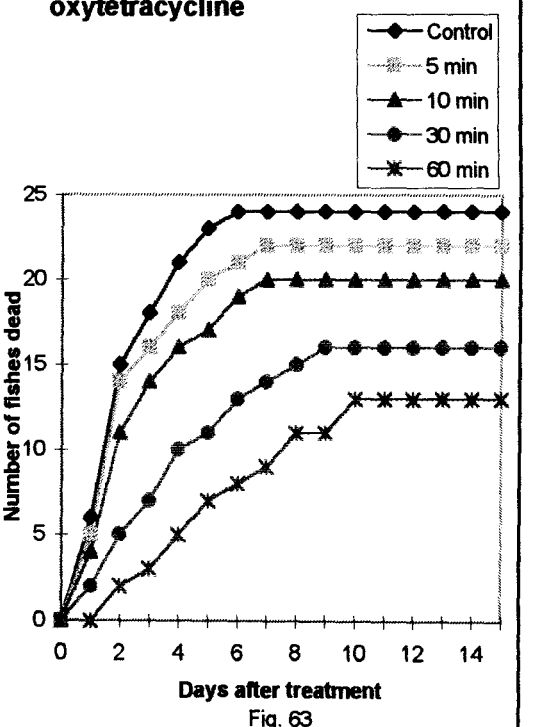


Fig. 63

Cumulative mortalities of R1 injected fishes after treatment with 500 μg/ml oxytetracycline

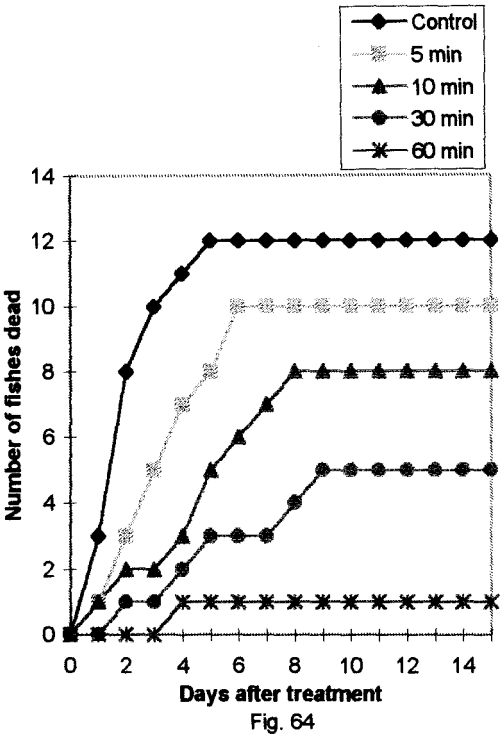


Fig. 64

Cumulative mortalities of R2 injected fishes after treatment with 500 μg/ml oxytetracycline

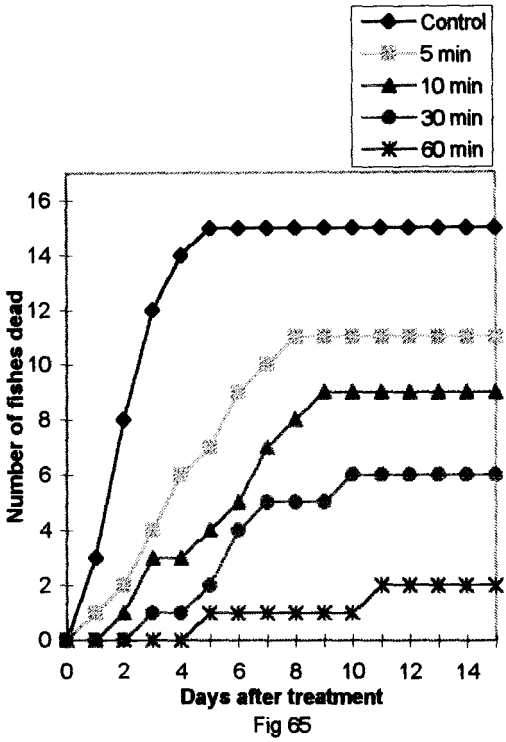


Fig 65

Cumulative mortalities of R3 injected fishes after treatment with 500 μg/ml oxytetracycline

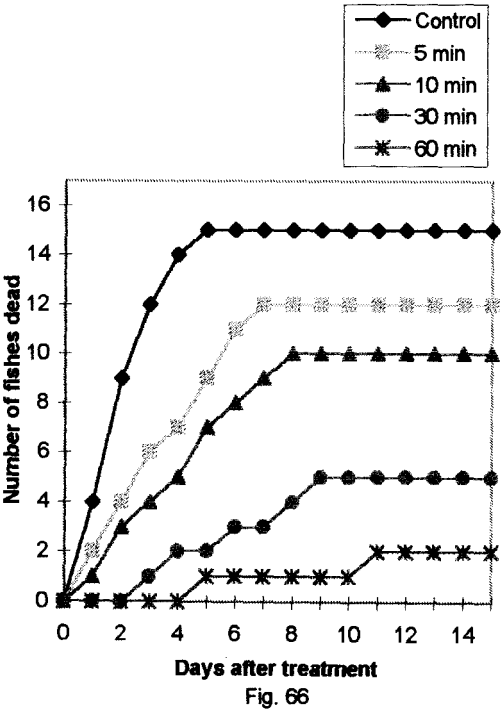


Fig. 66

Cumulative mortalities of mixed bacteria injected fishes after treatment with 500 μg/ml oxytetracycline

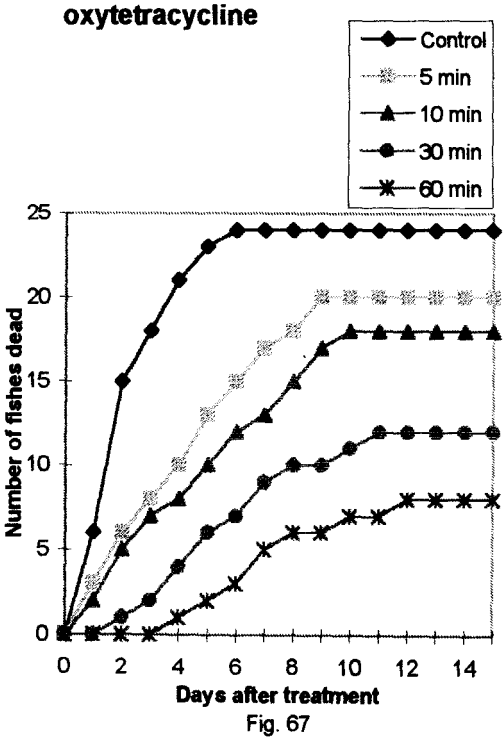


Fig. 67