Discussion

The present study describes different stages of infection of ulcerative disease in air-breathing fishes. The initial symptom of the disease was single or multiple red spots on the body. Ultimately ulcers developed in the affected areas. Occassionally ulcers became deep and haemorrhagic and some times necrotic. The outbreak of ulcerative disease of fishes occured in epizootic form for the first time in India in 1988. It caused heavy mortalities of various types of fishes cultured and wild and caused severe economic losses to fish farmers and fishermen. Present study also describes isolation and characterization of four types of bacteria, two fluorescent pseudomonads, one aeromonad and one micrococcus from ulcer tissues.

Prior to the recent outbreak of the disease in epizootic form several Indian workers reported ulcerative skin lesion in fishes (Gopalakrishnan, 1963; Monohar et al., 1976; Pal et al., 1978; Karunasagar et al., 1986; Kumar et al., 1986c and 1987a).

During late spring to early summer of 1971, an epizootic occured for the first time in pond cultured eel, Anguilla japonica of Shizuoka and Tokushima ;

prefectures in Japan. The characteristics of the disease were petechial haemorrhage in the area of mouth, opercula and ventral side of the body. The disease was named as 'Sekitenbyo' or red spot disease and the causative bacterium was identified as Pseudomonas anguilliseptica (Wakabayashi and Egusa, 1972). Muroga et al., (1973) described red spot disease in Japanese eel (A. japonica) in Tokushima prefecture of Japan. Jo et al., (1975) reported red spot disease in European eel Anguilla anguilla cultured in Tokushima prefecture and isolated P.anguilliseptica from diseased eel. Nakai et al.,(1986b) reported P. anguilliseptica infection in cultured ayu Plecoglossus altivelis in Shizuoka prefecture in Japan. Kuo and Kou (1978) isolated P. anguilliseptica from red spot disease of pond-cultured eel. A.japonica in Taiwan. In 1981 red spot disease was first recorded in European eel Anguilla anguilla in Scotland and causative factor was identified as P. anguilliseptica (Nakai and Muroga, 1982; Stewart et al. 1983). The disease caused by P. anguilliseptica in Salmonid fish was also reported from Finland and it was characterized by petechial haemorrhage in the ventral side of the skin, in the mouth region and in the anal region (Wiklund and Bylund, 1990).

Pseudomonas fluorescens has been reported as causative organism of haemorrhagic septicemia in European eel, Anguilla vulgaris (Andre et al., 1970), cyprinid fishes (Bullock and Mc Laughlin, 1970; Shiose et al., 1972), in yellow tail Seriola quinqueradiata (Kusuda, 1980); pond-cultured tilapia, Sarotherodon niloticus (Miyashita, 1984, Miyazaki et al., 1984). Sakai et al., (1989) isolated P. fluorescens from diseased rainbow trout Oncorhynchus mykiss in Iwate prefecture in Japan. The pathogenicity, of P. fluorescens was due to proteolytic enzyme (Bullock and Mc Laughlin, 1970).

Saeed et al., (1987) isolated P. putrefaciens from diseased rabbit fish Siganus rivulatus in the Red Sea. Rand and Wiles (1988) reported isolation of motile and non-motile gram negative bacteria such as Acinetobacter calcoaceticus, Aeromonas hydrophila, Pseudomonas sp. Vibrio parahaemolyticus from saddle shaped skin lesions commonly occuring in both wild and captive Bermudan reef silver side Atherina harringtonensia.

Aeromonas hydrophila is an important fish pathogen for various species of fresh water fishes (Bullock et al., 1971). Infection of this bacterium generally caused haemorrhage, exopthalmus and ulceration on the body surface and such bacterial infection was commonly known as Motile aeromonad septicemia (AFS 1975).

Disease caused by A. hydrophila was variously named by different workers e.g., red spot disease for European eel Anguilla anguilla (Schäperclaus, 1934), red disease of Japanese eel, A. japonica (Hoshina, 1962), red disease of carp, Cyprinus carpio (Egusa, 1978). In Japan Jo and Ōnishi (1980) isolated A. hydrophila from all diseased cultured ayu, Plecoglossus altivelis characterized with exopthalmus and subcutaneous ulceration.

An extensive work has been done on the Aeromonas disease of cyprinids clarifying the causes, infection, mechanism of attack and treatment by Takahashi (1984 b).

Other species of the genus Aeromonas also caused various types of disease in fishes such as furunculosis, carp erythrodermatitis, gold fish ulcer disease and head ulcer disease. Furunculosis is a disease of several fish species and has long been recognized (Mawdesley-Thomas and Jolly, 1968). The disease characterized with skin lesions on the body surface, superficial ulcerative lesion and often ulcer with exposed haemorrhagic muscle is caused by atypical Aeromonas salmonicida (Mc Carthy, 1975; Ellis et al.,1981; Hayasaka and Sullivan, 1981; Boomker et al., 1984; Ostland et al., 1987).

A. salmonicida caused carp erythrodermatitis in carp characterized with cutaneous ulcerative lesion (Fijan, 1972; Csaba et al., 1984), Gold fish ulcer disease (Mawdesley-Thomas 1969; Elliot and Shott, 1980; Whittington et al., 1987; Carson and Handlinger, 1988). Ohtsuka et al., (1984) reported the isolation of A. salmonicida from head ulcer of eel, Anguilla japonica characterized with ulcerative lesion on the head region. Kusuda and Takahashi (1970) isolated A. liquefaciens from scale protrusion disease in carp which occured in fish farm of Japan. But in all cases single bacterium is responsible for disease production.

The ulcerative fish disease affected not only air-breathing fishes (Pal and Pradhan, 1990b), but also various types of cultured and wild fishes in India causing heavy mortalities (Jhingran and Das, 1990; Kumar et al., 1991). During the last two decades the ulcerative fish disease has been reported often causing mass mortalities from different countries of the Indo-Pacific region by several workers (Rodgers and Burke, 1977, 1981, Anonymus, 1981, Haines, 1983; Tonguthai, 1985; Rahim et al., 1985, Lloberera and Gacutan, 1987; Whittington et al., 1987; Costa and Wijeyaratna 1989) Rahim et al., (1985) isolated A. hydrophila from the wounds of five species of fishes, Platosus anguillaris, Lates calcarifer, Epinephelus megachir, Labeo rohita and Serotherodon nilotica in Bangladesh.

Llobrera and Gacutan (1987) reported the presence of A. hydrophila associated with ulcerative disease epizootic in Phillippines. Costa and Wijeyaratne (1989) have reported that the primary cause of the ulcer disease among fresh water and estuarine fishes in Srilanka was unknown but A. hydrophila and an unidentified rhabodovirus have been associated with losses in fish in the affected region.

The carp fish farms in different European countries suffered the heaviest losses from haemorrhagic ulcer disease known as Infectious ascites, Infectious dropsy or Hydropes, Rubella, carp septicemia, viral Haemorrhagic septicemia (Schäperclaus, 1930, 1965; Volf and Havelka, 1965; Tomasec, 1963; Tomasec and Fijan, 1965; Goncharov, 1965; Kocylowski, 1965; Miaczynski, 1965; Bellet, 1965; Ghittino 1965). Opinion differed regarding the aetiology of the disease. At the conclusion of the first European Symposium on fish disease in Turin, Italy on October 20-24, 1962, a committee of experts opined that the disease should be named Viral Haemorrhagic Septicemia (VHS). Tomasec and 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 manifest itself in several forms. Fijan (1972) concluded that Infectious Dropsy in Carp (IDC) is a disease complex. The acute form of the disease is caused by a virus, Rhabdovirus carpio and is called the Spring Viremia of Carp (SVC) and the name Carp Erythrodermatitis (CE) is suggested for the disease previously known as chronic form of IDC. He also mentioned that in addition to SVC and CE, several other diseases are probably covered by the term of Infectious Dropsy in Carp.

Histological observations of the present investigation revealed various types of histopathological changes in the liver, kidney and spleen of infected fishes. Liver of naturally infected fish of A. testudineus, C. batrachus and H. fossilis showed necrosis, vaculation and cord like arrangement with enlarged sinusoids of hepatocytes. Similarly kidney showed tubular breakage, vaculation of tubular cells and necrosis. Besides, kidney of A. testudineus showed hyperplasia in some tubules. Vaculation, necrosis and cord like arrangement of splenocytes were noted in spleens of all the three species of fishes. These types of histopathological changes more or less resembled the histopathological changes caused by bacteria in the infected fishes reported by different authors. Miyazaki and Egusa (1977)

observed the hepatic edema and congestion in liver, cellular proliferation in the spleen, glomerulitis and atrophy of hematopoetic tissues in the kidney of Japanese eel, Anguilla japonica affected with red spot disease. Miyazaki (1980) found that A. hydrophila caused degeneration of hepatic cell, necrosis and degeneration of epithelia of renal tubules, atrophy of renal hematopoietic tissue, necrosis of sheathed arteries in spleen in Japanese eel A. japonica. Miyazaki, et al., (1984a) reported necrosis in liver and kidney, abcess and granulomatus changes in spleen of tilapia, Sarotherodon niloticus infected with P. fluorescens. Miyazaki and Jo (1985) also reported haemorrhage and congestion in liver, hemorrhage and destruction of tissues in spleen, necrosis in epithelial cells of renal tubules and haemorrhage in hematopoietic tissues in kidney of ayu, Plecoglossus altivelis affected with motile aeromonod disease. Hemosiderosis in the liver, spleen and kidney of crucian carp, carassius auratus affected with motile acromonad disease and deposition of hematoidin crystals in the spleen, kidney and blood vessels were observed by Miyazaki and Kaige (1985 b).

Wiklund and Bylund (1990) observed the changes in liver and kidney of salmonid fish in Finland infected with P. anguilliseptica. They found necrosis, Oedematous changes in the liver with disorganization of parenchymal cord structure and cloudy swelling of hepatic cell.

Oedematous changes of glomeruli and tubules, hyaline degeneration and accumulation of eosinophilic materials in tubules of kidney.

Many authors reported that the A. hydrophila produces toxic materials like hemolysin, protease and elastase (Schäperclaus, 1934; Hoshina 1962; Egusa,1978; Miyazaki,1980; Allan and Stevenson, 1981; Wakabayasi et al., 1981; Miyazaki and Jo 1985). The pathogenicity of A. hydrophila was due to these toxic substances.

Besides Pseudomonas and Aeromonas infection, several authors reported histopathological changes caused by other bacteria. Ross and Toth (1974) and Cone (1982) isolated Lactobacillus from rainbow trout which caused extensive degeneration and necrosis of liver, kidney and spleen. Histological changes of liver, spleen and kidney due to Streptococcus infection were also reported by Miyazaki, 1980, 1982; Miyazaki et al., 1984b; Rasheed et al.,1985. Histological changes of liver,spleen and kidney caused by Edwardsiella sp. were also described by several authors (Miyazaki,1980; Miyazaki and Kaige, 1985a; Blazer et al.,1985, Chen and Kumlin, 1989, Baxa et al., 1990).

The R_1 and R_2 bacteria in the present study were gram negative, straight, non-spore forming, motile, oxidative, catalase positive and produced yellowish green fluorescent pigment, so they belonged to the genus Pseudomonas (Stanier et al., 1966; Palleroni, 1984). 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 ke levan formation; it also satisfied two important characteristics (levan formation and denitrification inability) of biovar I of P. fluorescens (Palleroni, 1984), but it differed from biovar 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 to 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 and did not produce blue pigment (Pyocyanin) in

medium 'A' of King et al.,(1954). Ajellow and Hadley (1976) have reported a fluorescent pseudomonad capable of growth at 41°C but distinct from Pseudomonas aeruginosa. R_2 differed from P. chlororaphis on the basis of growth at 41°C, and it did not produce levan. These two bacteria R_1 and R_2 differed from P. putida on the basis of hydrolysis of gelatin, a character possessed by R_1 and R_2 and not by P. putida. Thus it is apparent that both R_1 and R_2 bacteria were fluorescent pseudomonads but they differed from each other. A decision about their taxonomic status and nomenclature must await further studies.

R_3 bacterium was gram negative, straight, non-spore forming, motile, fermentative, oxidase positive, catalase positive, nitrate reduction positive, indole positive. Thus morphological and physiological characteristics of R_3 bacterium satisfied the characteristics of the Genus Aeromonas. (Popoff, 1984). As it did not produce gas from glucose and as it was Voges-Proskauer reaction negative so it belonged to Aeromonas caviae (Popoff, 1984).

The sphere shaped bacterium, C, was gram positive, non motile, non spore-forming, catalase positive, indole negative, oxidase negative and oxidative, occuring singly, in pairs, in tetrad, in short chain or in irregular cluster (dividing in more than one plane). Colonies were yellow and small, smooth, convex. so it belonged to the genus Micrococcus (Kocur, 1986). It also satisfied the characteristics of the species Micrococcus varians (Kocur, 1986) e.g. oxidase negative, oxidative in metabolism, reduction of nitrate and nitrite, good growth between 25-37°C, and non pathogenic. It differed from Staphylococcus aureus in respect of carbohydrate metabolism. S. aureus was fermentative and the M. varians was oxidative. So it belonged to Micrococcus varians.

2. EXPERIMENTAL INDUCTION OF ULCER IN THE FISHES *Channa punctatus* AND *Anabas testudineus* BY BACTERIAL CULTURE

Materials and Methods

The culture of the four bacteria, two fluorescent pseudomonads (R_1 and R_2), *Aeromonas caviae* (R_3) and *Micrococcus varians* (C), isolated from ulcer tissues of air-breathing fishes were maintained routinely on agar slants and in nutrient broth. The bacteria were cultured in nutrient broth when required. Healthy fishes, *Channa punctatus* (25-30 gms), were acclimatized at laboratory condition for 10 days in glass aquaria measuring 90 x 35 x 35 cm in which the depth of static water was 20 cm. The fishes were fed with chopped earthworm and small fishes. Intramuscular injection of 0.1 ml ($6 - 8 \times 10^9$ cells/ml) of bacterial culture in mixed and in pure conditions were given at the trunk region. For each set of experiment ten fishes were taken and each experiment was repeated twice. The control fishes received only 0.1 ml of nutrient broth. Water temperature was maintained at $29 \pm 1^{\circ}C$. The gradual development of ulcers after injection was carefully observed.

Similarly intra-muscular injections of 0.1 ml ($6 - 8 \times 10^9$ cells/ml) of mixed culture and pure cultures of all the four 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 six times. Mortality was recorded upto 10 days after inoculation.

Observations and results

The control group of fishes, C. punctatus, and coccus treated group showed neither any development of ulcer nor any mortality (Figs. 41 and 42). Fishes 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 fishes died at this stage. After about 72 hours of inoculation the scales of the affected area fell down exposing an open sore. Ultimately the ulcer became deep and the muscle layers were also affected (Fig. 43). Some of the fishes also showed haemorrhages on other areas of the body surface. 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

Fig. 41. *C. punctatus* showing no ulcer formation after intramuscular injection of nutrient broth (control).

Fig. 42. *C. punctatus* showing no development of ulcer after intramuscular injection of pure culture of *Micrococcus varians* (C).

Fig. 43. *C. punctatus* showing severe ulcer formation after intramuscular injection of mixed culture of four bacteria (R_1, R_2, R_3 and C).

Fig. 44. *C. punctatus* showing ulcer formation after intramuscular injection of *Aeromonas caviae* (C).

Fig. 45. *C. punctatus* showing superficial ulcer formation after intramuscular injection of R_1 (fluorescent pseudomonad).

Fig. 46. *C. punctatus* showing superficial ulcer formation after intramuscular injection of R_2 (fluorescent pseudomonad).


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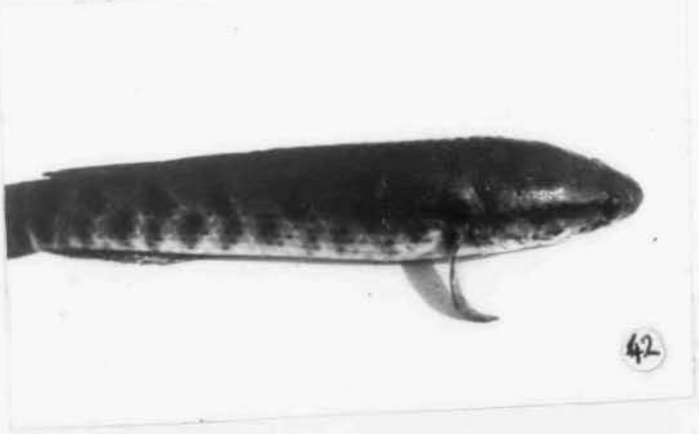
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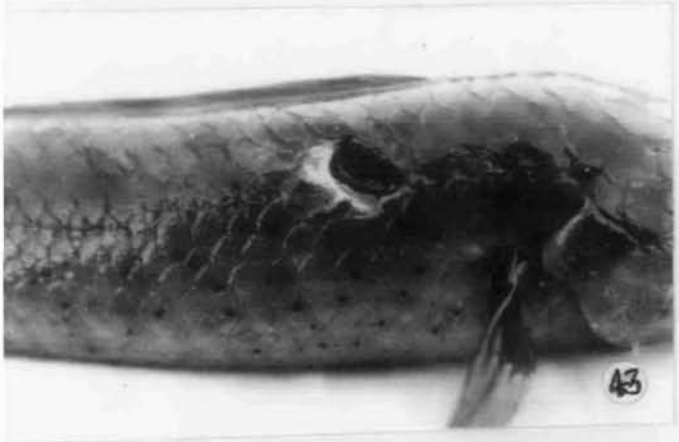
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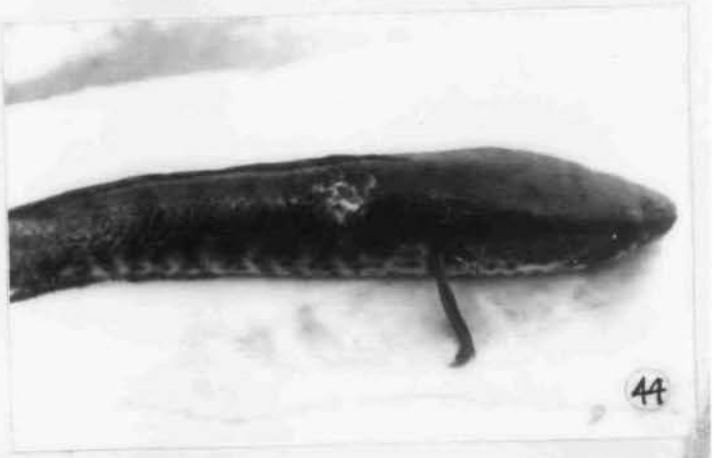
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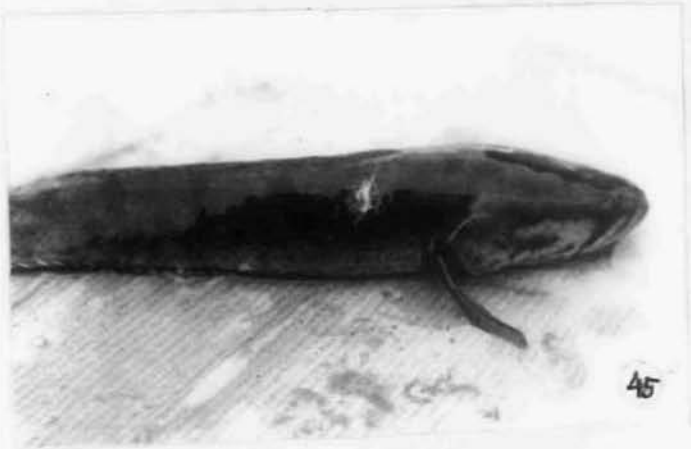
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Plate XV

Fig. 47. A. testudineus showing no ulcer formation after intramuscular injection of nutrient broth (control).

Fig. 48. A. testudineus showing no development of ulcer after intramuscular injection of pure culture of Micrococcus varians(C).

Fig. 49. A. testudineus showing superficial ulcer formation after intramuscular injection of pure culture of R_1 (fluorescent pseudomonad).


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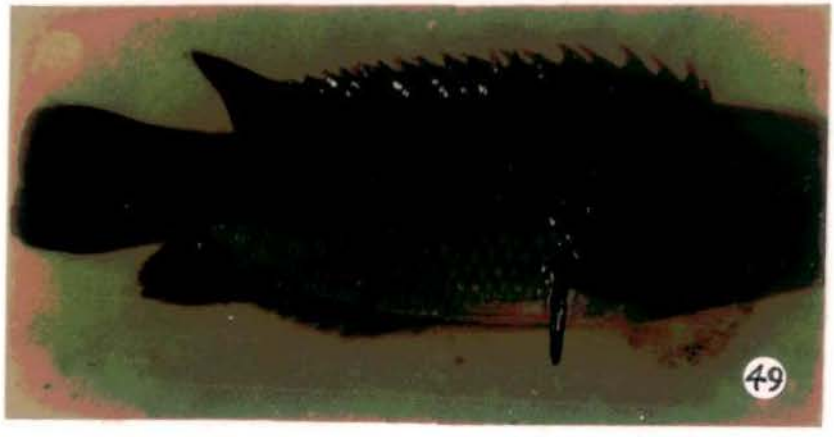
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Plate XVI

Fig. 50. A. testudineus showing superficial ulcer formation after intramuscular injection of pure culture of R_2 (fluorescent pseudomonad).

Fig. 51. A. testudineus showing ulcer after intramuscular injection of R_3 (Aeromonas caviae).

Fig. 52. A. testudineus showing severe ulcer formation after intramuscular injection of mixed culture of four bacteria (R_1, R_2 R_3 and C).


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depending on the nature of ulcer. The fish injected with the culture of R_3 (Aeromonas caviae) developed ulcer but was not as severe as induced by the mixed culture of the four bacteria (Fig. 44). The culture of R_1 and R_2 (pseudomonads) developed superficial ulcers at the point of injections (Figs. 45 and 46). The percent of mortalities of fishes were 70, 40, 25 and 35% in mixed culture, R_3, R_2 and R_1 treated groups, respectively (Table 3).

In Anabas, no ulcer formation was noticed in the control and C-treated groups of fishes (Figs. 47 and 48). Pure cultures of R_1, R_2 and R_3 bacteria induced superficial ulcer formation at the point of injection (Figs. 49-51). 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. 52). The percent of mortalities of fishes were 33.33, 13.33, 6.66 and 10.0% in mixed, R_3, R_2 and R_1 treated groups respectively and no mortalities were found in the C-treated and control groups of fishes (Table 4)

Discussion

The results of the experiments with C. punctatus and A. testudineus showed that the pure culture of two pseudomonads R_1 and R_2 induced superficial ulcer at the

Table 3: Percent of mortality and nature of ulcer formation in healthy fish C. punctatus by mixed and pure culture of R_3, R_2, R_1 and C bacteria. (a) Percent of mortality is given in the parenthesis.

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.

Table 4: Percent of mortality and nature of ulcer formation in healthy fish, A. testudineus by mixed and pure cultures of R_3, R_2, R_1 and C bacteria. (a) Percent of mortality is given in the parenthesis.

Parameters	No. of Fish injected	No. of fish died (a)	Nature of ulcer
Control	30	Nil	No ulcer
Mixed	30	10(33.33%)	Severe ulcer
R_3	30	4(13.33%)	Ulcer developed but not severe
R_2	30	2 (6.66%)	Superficial ulcer
R_1	30	3 (10.0%)	Superficial ulcer
C	30	Nil	No ulcer

point of injection and Micrococcus varians (C) induced no ulcer formation at all. Aeromonas caviae (R_3) and mixed culture of four bacteria induced ulcers but induction of ulcers by mixed culture were severe. Mortalities caused by the mixed culture of four bacteria were also high, 70% in C. punctatus and 33.33% in A. testudineus in comparison to that caused by the pure cultures of R_1, R_2 and R_3 bacteria; 35%, 25% and 40% respectively in C. punctatus and 10.0%, 6.66% and 13.33% respectively in Anabas testudineus. Micrococcus varians induced neither ulcer formation nor any mortality in both type of fishes.

Jhingran and Das (1990) reported that manifestation of ulcers took place within 72 hrs. after inoculation with Micrococcus sp.. Kumar et al., (1991) mentioned that possibly a virus was involved in the epizootic. Kusuda and Takahashi (1970) investigated the pathogenicity of Aeromonas liquefaciens isolated from scale protrusion disease of carp by inoculating into carp and gold fish, and found that percent of mortality of carp was higher than that of goldfish. Muroga and Nakajima (1981) reported artificial induction of red spot disease in eel, Anguilla japonica with Pseudomonas anguilliseptica. Pal and Pal (1986b) reported induction of ulcer in A. testudineus by mixed culture of two bacteria, one fluorescent

pseudomonad and another coccus, Micrococcus varians. Saeed et al.(1987) reported isolation of Pseudomonas putrefaciens from diseased rabbit fish Siganus rivulatus and successfully induced the same clinical sign as those observed during disease outbreak. Angelini and Seigneur (1988) isolated A. hydrophila and P. aeruginosa from South-American catfish, Rhamdia sapo fingerlings suffered from an epizootic which was similar to fin rot disease and the disease was experimentally reproduced by injecting the isolated bacteria in healthy R. sapo. Sakai et al., (1989) reported that P. fluorescens isolated from diseased rainbow trout was pathogenic to rainbow trout and tilapia, Sarotherodon niloticus.

From the present experiment it can be concluded that no one bacterium is responsible for induction of ulcer in C.punctatus and A. testudineus. The coccus, M. varians is nonpathogenic; two fluorescent pseudomonads, R_1 and R_2 are slightly pathogenic; the R_3, Aeromonas caviae is more pathogenic than R_1 and R_2 bacteria.