6. DRUG SENSITIVITY TESTING AND PRELIMINARY OBSERVATION ON VACCINATION BY FORMALIN - KILLED BACTERIA IN MIXED CONDITION IN FISH *Anabas testudineus.*

Materials and Methods

Two fluorescent pseudomonads (R_1 and R_2) one aeromonad (*Aeromonas caviae*, R_3) and one coccus (*Micrococcus varians*, C) isolated from ulcer tissues of air-breathing fishes were tested for their susceptibility against five drugs, such as Streptomycin, Oxytetracycline, Chloramphenicol, Trimethoprim/Sulphamethoxazole (1/5) and Penicillin by agar diffusion method. First 15 ml of sterile molten nutrient agar was poured on a sterile petridish and was allowed to solidify it. 1 cc of 18 hrs. grown pure culture of the test bacterium was inoculated in a conical flask containing 20 ml molten nutrient agar at 40-45°C and shaken gently. Then it was poured over the previously solidified agar layer and was again allowed to solidify. Six cylindrical cups were made in the upper layer of the agar plate with the help of a sterile

metallic cork borer of 0.5 mm diameter. Drug sensitivity of the isolated bacteria (R_1, R_2, R_3 and C) was determined by using following concentration of the drugs per cup; streptomycin (10 µg), oxytetracycline (30 µg), chloramphenicol (30 µg) trimethoprim/ sulphamethoxazole, 1/5 (30 µg) and penicillin G(10 U). Centrally made cup was filled with sterile distilled water as a control cup. The plates were incubated at 30°C for 12 hrs. Appearance of clear zone surrounding the cup indicated sensitivity to the test drug. The test was repeated five times.

For vaccine preparation three bacteria (R_1, R_2 and R_3) in mixed condition were grown in nutrient broth at 30°C for 24 hrs. Bacteria were centrifuged and washed three times in saline solution and resuspended in 10 ml. sterile physiological saline solution containing 6 - 8 x 10^9 cells/ ml and formalin was added to a final concentration of 0.5% (v/v). Then the suspension was kept at 37°C for 2 days. Formalin-inactivated cells were collected by centrifugation and washed three times with saline solution and finally resuspended in 10 ml sterile saline solution and then stored at 5°C. Fifty A. testudineus (20 - 30 gm) were vaccinated by intraperitoneal injection of formalin-inactivated bacteria at a dose of 0.1ml. cell suspension. They were kept in

glass aquarium measuring 90 x 35 x 35 cm. The fishes were fed with chopped earthworm and small fishes. After 7 days of first vaccination a booster vaccination were carried out by the same procedure. Water temperature varied from 28 to 30°C. After two, four, six, eight and ten weeks of booster vaccination ten vaccinated fishes, for each experiment, were challenged intra-peritoneally with 24 hrs. culture of mixed bacteria (R_1, R_2 and R_3) at a dose of 0.2 ml (6-8 x 10^9 cells/ml). Parallely, ten non-vaccinated fishes were challenged with 0.2 ml (6-8 x 10^9 cells/ml) of mixed culture of three bacteria (R_1, R_2 and R_3) for each experiment which served as control. Both vaccinated and non-vaccinated challenged fishes were kept separately in glass aquaria measuring 50 x 24 x 24 cm. at a depth of 16 cm. of static water and were routinely maintained. The mortality was noted upto 10 days.

Observations and Results

Result of drug sensitivity testing was shown in Table-16. It was found that two pseudomonads (R_1 and R_2) and A. caviae (R_3) were highly sensitive (++) to streptomycin and M. varians (C) was sensitive (+) to streptomycin. M. varians (C) was highly sensitive(++)

Table 10: Drug sensitivity of the isolated bacteria from air-breathing fishes.

Drug	Strain			
	R_1	R_2	R_3	C
Streptomycin (10 µg)	++	++	++	+
Oxytetracycline (30 µg)	+	+	+	++
Chloramphenicol (30 µg)	+	+	+	+
Trimethoprim/sulphamethoxazole 1/5 (30 µg)	++	++	++	++
Penicillin G (10 U)	-	-	-	++

++, Highly sensitive; + sensitive;

- resistant.

to oxytetracycline and the rods (R_1, R_2 and R_3) were sensetive (+) to oxytetracycline. All four bacteria (R_1, R_2, R_3 and C) were sensetive (+) to chloramphenicol. The drug Trimethoprim/sulphamethoxazole (1/5) was found to be highlyeffective (++) against all the four types of bacteria (R_1, R_2, R_3 and C). Penicillin was highly effective (++) only against coccus (C) bacteria and it showed no effect against three rods (R_1, R_2 and R_3).

Mortalities of vaccinated and non-vaccinated group of fishes were shown in Table.11. The percent of mortalities of vaccinated group of fishes were 20%, 20%, 20%, 40% and 40% after challenges at 2nd, 4th, 6th, 8th and 10th weeks of booster vaccination respectively. In case of non-vaccinated (control) fishes mortalities were 50%, 70%, 60%, 70%, and 60% after challenges at 2nd, 4th, 6th, 8th and 10th weeks from the date of booster vaccination.

Discussion

All three rod shaped bacteria (R_1, R_2 and R_3) and coccus (C) were highly sensitive (++) to Trimethoprim/sulphamethoxazole (1/5). Streptomycin was also

Table 11: Mortality of vaccinated and control non-vaccinated fish *A. testudineus*

Week	Vaccinated fish		Control fish	
	No. of fish challenged	No. of fish died(%)	No. of fish challenged	No. of fish died(%)
2	10	2(20)	10	5(50)
4	10	2(20)	10	7(70)
6	10	2(20)	10	6(60)
8	10	4(40)	10	7(70)
10	10	4(40)	10	6(60)

highly effective (++) against three rod shaped bacteria (R_1, R_2 and R_3) and effective (+) against coccus (C). All the bacteria showed their sensitivity (+) against oxytetracycline and chloramphenicol. Penicillin G was highly effective only against coccus (++).

Several workers tested the antimicrobial drug sensitivity against fish bacteria (Schäperclaus, 1965; Volf and Havelka, 1965; Wakabayashi and Egusa, 1972; Wiklund and Bylund, 1990). Use of antimicrobial agent may prove to be useful control of bacterial fish disease in the field but misuse of such antibiotics may increase the bacterial drug resistance. Volf and Havelka (1965) on the basis of antimicrobial drug sensitivity used chloramphenicol for control of infections by Aeromonas punctata ascitae and also used streptomycin in mixed infections (A. punctata ascitae and Pseudomonas fluorescens) . They concluded that with full use of this treatment for five years in carp culture, the losses of fish were reduced from 20 percent to 5 percent. Jo (1978) reported that oxolinic acid, nalidixic acid and piromidic acid administered by bathing or orally were found to be effective to control red spot disease of eel. Takahashi (1984 b) found that dipping 20 ppm. of chloramphenicol or

oxytetracycline for 30 minutes and oral administration 100 mg/kg b.w/day of sulfamonomethoxine or 10 mg/kg /day of oxolinic acid for 5 days were effective against Aeromonas disease of carps.

Wiklund and Bylund (1990) reported from drug sensitivity testing that ampicillin and trimethoprim/ sulphametoxazole were most active against Pseudomonas anguilliseptica

Several Indian workers suggested antibiotics and drugs such as erythromycin, nalidixic acid, oxytetracycline, terramycin, sulphamethoxazole, bactrim for controlling the ulcer disease (Jhingran, 1990; Jain, 1990; Saha et al., 1990). Kumar et al. (1991) used terramycin for controlling the disease but did not find any effective results.

The results of the percent drug sensitivity testing indicated that the drug Trimethopmim/sulphamethoxizole (1/5) and antibiotic streptomycin could be effective against the disease. But the main difficulty in using drugs/antibiotics as a control measure is the vast area and varied water bodies effected with the disease.

Upto 6th. week after booster vaccination the mortalities of the vaccinated fish were 20% and the mortalities of the control fishes were 50-70% for the same period. Results indicated slight effectiveness of the vaccine. Nothing can be predicted from this preliminary work on vaccination. To develop effective vaccine against ulcerative disease details work to be done on the cell mediated immunity, antibody mediated immunity and on the effectiveness of different method of immunization such as formalin - inactivated vaccination, Heat-inactivated vaccination and immersion vaccination.