PART II

SCIENTIFIC AND TECHNICAL

BEHAVIOUR OF MARINE MICROFLORA TOWARDS ANTIBIOTICS
I. SENSITIVITY TOWARDS CHLORTETRACYCLINE (CTC)

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The sensitivity of 61 cultures of bacteria isolated from fish towards chlortetracycline (CTC) at 5 ppm and 20 ppm levels has been determined on two solid media, viz; sea water agar (SWA) and a distilled water based medium consisting of peptone, beef extract, glucose and NaCl (PBGA). The cultures employed consisted of (i) gram-negative rods of marine origin viz; *Achromobacter, Pseudomonas, Vibrio* and *Flavobacterium* and (ii) gram positive organisms viz; *Micrococci* and *Corynebacterium.* Depending on the inhibition zone diameter, the order of CTC sensitivity was found to be *Pseudomonas* < *Vibrio* < *Achromobacter* as determined on PBGA at CTC levels of 5 and 20 ppm. The SWA enhanced the development of resistance in majority of the cultures as compared to PBGA. PBG broth containing CTC at less than 10 ppm exerted a bacteriostatic effect only while that containing 10 ppm or more exerted a bactericidal effect on *Achromobacter, Pseudomonas* and *Micrococci* cultures. The significance of these results in fish preservation by CTC is discussed.

INTRODUCTION

It is well known that the effect of an antibiotic as a preservative for sea foods depends on its action on the microbial flora present on the latter. Of the many antibiotics known, only CTC has been approved for use in preserving fish (Tarr *et al*, 1952). Being a broad spectrum antibiotic, CTC acts on a great many different species of bacteria. The object of the present study was to determine the sensitivity of different species belonging to the major genera of bacteria usually present on the body of fresh ocean caught fish. The flora of fish caught off Cochin has been found to consist of *Achromobacter, Pseudomonas, Vibrio* and smaller proportions of *Corynebacterium* and *Micrococci* (Karthiayani and Iyer, 1967). It was felt that a knowledge of

the individual sensitivity towards CTC of these different genera would also help in deciding optimum concentration of CTC in a dip or in the ice used to pack the fish.

Single culture studies with bacteria from sea water and spoiling fish have shown that most of these are sensitive to a few parts per million of CTC (Velankar, 1958; Novak *et al*, 1960; Horie *et al*, 1961). Velankar (1958) tested about 50 marine bacteria and found that excepting 4 strains, all were sensitive to 2-5 ppm CTC. The major species from shrimp and oysters were inhibited at 5 to 22 ppm of CTC over a period of 7 days at 28°C (Novak *et al.*, 1960). In determining the sensitivity of the cultures in solid media, it had already been observed that the cultures were less sensitive to CTC on SWA plates than on nutrient agar plates (Southcott and Tarr, 1961). Hence in our present studies, comparison was made on both SWA and nutrient agar.

Materials and Methods

Well isolated colonies were picked from plates poured out of fresh sardines and prawns caught in Cochin waters using SWA as plating medium. They were classified into different groups according to Karthiayani and Iyer (*loc. cit.*) and used for the study. The stock consisted of 10 cultures of *Pseudomonas*, 12 of *Achromobacter*, 30 of *Vibrio*, 3 of *Flavobacterium*, 3 of *Micrococci* and 3 of *Corynebacterium*.

In addition to ordinary SWA, a solid nutrient medium of the following composition was also used.

Peptone	—	1 g.
Beef extract	—	0.3 ,,
Sodium chloride	—	2.3 ,,
Glucose	—	0.1 ,,
Agar	—	1.5 ,,
Distilled water	—	100 ml.
pH	—	6.8

This medium is referred to as PBGA or PBG broth as the case may be. A stock solution of CTC (Aureomycin, Cyanamide India Ltd.) was prepared in sterile distilled water. This was diluted to appropriate concentrations (ppm) of CTC for the experiments. Sensitivity was determined by the cylinder plate technique in general and by turbidimetric method for a restricted number of cultures.

From the stock culture, the strains were initially grown in PBG broth (9 ml) at room temperature (28°±2°C) for 24 to 48 hours. 1 ml of the suitably diluted growth was pipetted into sterile petridishes and melted PBGA (*ca* 20 ml) cooled to about 40°C was poured into each dish and mixed thoroughly with the inoculum. When the medium had set well, sterile steel cylinders (10 mm height × 7.5 mm dia) were placed on the seeded agar (2 in each petridish). 0.04 ml of the appropriate CTC solution (5 ppm and 20 ppm) was pipetted into each steel cylinder. The plates were then kept in a refrigerator (8°C) for 2 hours, after which they were incubated at room temperature for 18 to 20 hours. The diameter of the zone of inhibition was measured. Simultaneously the same experiment was conducted using SWA also. In this case bacterial cells cultivated in sea water peptone were used for seeding the agar.

In the case of the turbidity method, graded levels of CTC (0, 1.25, 2.5, 5 and 10 ppm) were incorporated into a series of PBG broth (9 ml). 0.2 ml of the 24 hours old culture was inoculated into each tube. The tubes were incubated at room temperature and the time (in hrs or days) required for the development of visual turbidity was noted.

On the basis of the diameter of the zone of inhibition, the cultures were divided into three groups.

(i) Highly sensitive — zone diameter, above 15 mm

(ii) Medium sensitive — „ „ between 10 & 15 mm

(iii) Insensitive (or resistant) —„ „ Nil or below 10 mm

In the turbidity method, if no turbidity developed even after 8 days of incubation, the CTC concentration in that tube was read as the bactericidal concentration for the particular culture.

Results and Discussion

The comparative evaluation on the two solid media showed that the cultures, in general, were less sensitive to CTC at the 5 ppm and 20 ppm levels when tested on SWA, ie, the zone diameters on SWA were always less than those on PBGA. This is in accordance with the observation of Southcott and Tarr (1961) who found that the presence of Mg^{++} (and not Na^{+} or Fe^{+++}) in sea water contributed to the increased resistance to the tetracycline. This is also supported by our finding that CTC is rapidly destroyed by sea water at room temperature (Table IV). Hence the effective concentration of CTC in the test medium tends to become less, thus allowing an apparent increase in the resistance of the cultures tested in the sea water based media.

Tables I and II give the percentages of different cultures, coming under different genera, as highly sensitive, medium sensitive and insensitive according to the definitions for these three categories mentioned earlier.

Pseudomonas were found to be less sensitive to CTC at both the levels employed in these experiments. In PBGA, 75% of the cultures tested were insensitive to 5 ppm and 50% to 20 ppm CTC. 100% of

Table I Percentage of each genus of bacteria grouped as insensitive, medium sensitive & highly sensitive to 5 ppm CTC on PBGA & SWA.

Genus	No of cultures	PBAG			SWA		
		Insensitive	Medium sensitive	Highly sensitive	Insensitive	Medium sensitive	Highly sensitive
Achromobacter	12	44	28	28	72	28	Nil
Pseudomonas	10	75	Nil	25	100	Nil	„
Vibrio	30	69	31	Nil	69	31	„
Flavobacterium	3	Nil	Nil	100	100	Nil	„
Micrococci	3	„	100	Nil	„	„	„
Corynebacterium	3	100	Nil	„	„	„	„

Table II Percentage of each genus of bacteria grouped as insensitive, medium sensitive & highly sensitive to 20 ppm CTC on PBGA and SWA

Genus	No of cultures	PBGA			SWA		
		Insensitive	Medium sensitive	Highly sensitive	Insensitive	Medium sensitive	Highly sensitive
Achromobacter	12	44	28	28	56	42	Nil
Pseudomonas	10	50	50	Nil	100	Nil	„
Vibrio	30	60	40	„	69	31	„
Flavobacterium	3	Nil	Nil	100	100	Nil	„
Micrococci	3	„	100	Nil	„	„	„
Corynebacterium	3	100	Nil	„	„	„	„

these cultures were insensitive at both the levels of CTC on SWA wherein the effective CTC concentration in the medium was insufficient to exert a bacteriostatic effect. *Vibrios* were next to *Pseudomonas* in decreasing order of resistance to CTC followed by *Achromobacter* and *Flavobacterium*. In the case of *Achromobacter*, the percentages of the three categories are similar at both the levels on PBGA showing that 5 ppm is as effective as 20 ppm but then, on SWA a greater proportion is resistant at 5 ppm level than at 20 ppm, obviously due to the higher effective CTC concentration available in 20 ppm plates after allowance for destruction of the antibiotic by sea water. The *Micrococci* were medium sensitive at the two levels of the antibiotic in PBGA and 100% resistant on SWA at both levels of CTC. *Corynebacterium* were quite insensitive to CTC at both levels on the two media.

Table III presents the duration required by three select genera for the development of visual turbidity in graded concentrations of CTC in PBG broth. The results corroborate with those with solid media. *Achromobacter*, being least resistant, took 5.3 days for the development of turbidity at 1.25 ppm, the more sensitive *Micrococci* took 7 days and the least sensitive *Pseudomonas* took only 44 hours. In this case the turbidity in the CTC-incorporated broth appeared simultaneously as with the control containing no antibiotic. The 10 ppm concentration of the antibiotic, in which no growth was observed for all three groups even after 9 days, was taken as bactericidal under the experimental conditions employed.

The data clearly show that *Pseudomonas* are the least sensitive to the antibiotic at levels tried in these experiments. This observation may have a due significance when we consider the use of CTC as a fish preservative especially when it is accepted that *Pseudomonas* are the most active spoilers as far as fish are concerned (Shewan, 1961). Some experimental data (unpublished) have, however, been collected which tend to incriminate members of the *Achromobacter* group as being spoilers in the case of marine shrimp held in ice storage. The present experiments showing that *Achromobacter* cultures are sensitive to CTC offer promise in the preservation of this class of seafood by the antibiotic. Since most of the *Vibrio* cultures were found to be destroyed during storage in ice (un-published data), their apparently

Table III Time for the development of visual turbidity in presence of graded concentration of CTC in PBG broth (Times given are averages for ten *Achromobacter* strains, four of *Pseudomonas* and three of *Micrococci*. Individual strains of each genus showed only slight variations in the time taken for development of turbidity).

Genus	CTC concentration in ppm. 0	1.25	2.5	5.0	10.0
Achromobacter	28 hr	5.3 days	6.3 days	8.3 days	No growth even after 9 days
Pseudomonas	44 „	44 hr	3 „	8 „	„
Micrococci	46 „	7 days	7 „	No growth even after 9 days	—

Table-IV Destruction of CTC in sea water at room temperature.

Time in hrs	Residual concn. of CTC μg/ml.	% destruction of CTC
0	5.31	0.0
2	2.50	52.1
3	1.50	71.7
24	0.68	87.3
9 days	0.21	96.1

higher resistance to CTC (than *Achromobacter*) is likely to be offset by their rapid reduction in number under cold storage.

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