Drug Sensitivity of *Aeromonas liquefaciens* Isolated from Freshwater Fishes

Takashi AOKI and Syuzo EGUSA*

(Received September 7, 1970)

Two hundred and fifty strains of *Aeromonas liquefaciens* isolated from fishes and soft-shelled turtles were studied for their sensitivity to 9 sorts of chemotherapeutics using agar-plate dilution method. One hundred and ten of the 250 strains were additionally tested for sensitivity to 3 other drugs.

Most of the strains tested were highly sensitive to chloramphenicol, chlortetracycline, tetracycline, relatively high sensitive to nifuriprinol, sulfamonomethoxine, and sulfoxazole and moderately sensitive to fradiomycin, furazolidone, nitrofurazone, streptomycin and kanamycin and resistant to aminobenzyl penicillin.

Criteria for defining resistant strains to the drugs were tentatively determined. According to the criteria out of the 250 strains 28 were one-drug-resistant, 38 were two-drug-resistant, 4 were three-drug-resistant and 8 were four-drug-resistant. Tetracycline-sulfanilamide resistance was a characteristic resistance pattern of drug-resistant *A. liquefaciens*.

In recent years a variety of chemotherapeutics have been largely used as feed supplement by fish-culturists in Japan for the prevention or treatment of bacterial diseases of fishes. It is not seldom that drugs are applied directly to fish ponds. In view of the fact that the use of antibiotics and other chemotherapeutics as feed additives for livestock and poultry has resulted in the increase of drug-resistant enteric bacteria in these animals,¹⁾ it seems likely that the wide use of chemotherapeutics for fishes has caused the increase of drug-resistant fish-pathogenic bacteria. In fact, we have often heard of reports, though without scientific data, of outbreaks of bacterial fish-diseases that failed to respond to chemotherapy. SNIESZKO and BULLOCK published two reports on the appearance of drug-resistant *Aeromonas salmonicida* in 1957^{2,3)}, but since then no systematic work has been done on this problem either in Japan or abroad.

The present authors have studied the sensitivity of a number of strains of *Aeromonas liquefaciens* isolated from fishes in fish-farm ponds in various parts of the country, and found that considerable portion of the strains are resistant to any or some of the drugs tested, though the majority were highly or moderately sensitive to most of the drugs. The present paper reports the results of the study.

Materials and Methods

- 1) Strains of *A. liquefaciens* studied: Use was made of 250 strains isolated from

* Department of Fisheries, Faculty of Agriculture, University of Tokyo, Tokyo, Japan (青木宙・江草周三: 東京大学農学部)

188 eels (*Anguilla japonica*), 27 carp (*Cyprinus carpio*), 12 ayu (*Plecoglossus altivelis*), 21 goldfishes (*Carassius auratus*), and 2 soft-shelled turtles (*Trionyx sinensis japonicus*) during the period from March, 1963 to August, 1969. All the animals from which the strains were isolated were collected from farm-ponds in various parts of Japan, except 6 wild ayu (Table 1). Of the 250 strains, 154 were isolated from diseased individuals and the remaining 96 were from apparently healthy ones.

The identification of *A. liquefaciens* was based on BULLOCK's scheme⁴⁾. According to SCHUBERT's scheme in 1967⁵⁾, almost all the strains are identified as *A. hydrophila* and a few as *A. punctata*.

2) Chemotherapeutics used: The chemotherapeutics used were tetracycline hydrochloride (Lederle), chloramphenicol (Sankyo), dihydrostreptomycin sulfate (Sankyo),

Table 1. Sources of the 250 strains of *Aeromonas liquefaciens* used for sensitivity tests.

			Eel	Carp	Ayu	Goldfish	Soft-shelled turtle
Location of fish ponds	Shizuoka	Yaizu	30(8)*				
		Yoshida**	82(3)	1			
		Hamana***	17		1		2
	Aichi	Toyohashi	16				
		Atsumi	11		1		
		Ishiki	12	2			
	Nagano	Shioda		21			
	Shiga	Hikone			3(1)		
	Kochi	Nangoku		3			
	Mie	Tsu	1		1		
Year of collection	Tokyo	Edogawa				21(3)	
		Tokyo Univ.****	19(2)				
5 rivers*****							
Year of collection	1963		1				
	1964		4				
	1965			1			
	1966		21(3)				
	1967		8		12(2)	8(3)	
	1968		52	2		3	
	1969		102(2)	24		10	2
Total			188	27	12	21	2

* Figures in parentheses indicate the number of strains which were lost before sensitivity tests with aminobenzyl penicillin, fradiomycin and kanamycin.

** Including Oigawa.

*** Including Shinohara, Maisaka, Bentenjima, Arai, and Kosai.

**** Experimental tanks in the campus of Tokyo Univ. into which eels were transferred several times from farm-ponds at Yoshida or Yaizu.

***** Naka R. (Ibaragi Pref.), Kuma R. (Kumamoto Pref.), Chikugo R. (Fukuoka Pref.), Toyo (Aichi Pref.).

kanamycin sulfate (Sankyo), fradiomycin sulfate (Meiji), aminobenzyl penicillin (Meiji), furazolidone (Takeda), nifuriprinol (Dainippon), and sulfamonomethoxine (Daiichi).

Of these 9 drugs, fradiomycin, kanamycin and aminobenzyl penicillin were tested on 232 of the 250 strains, because 18 strains had been lost before performance of tests with these drugs. Chlortetracycline hydrochloride (Lederle), nitrofurazone (Dainippon), and sulfisoxazole (Yamanouchi) were used for additional sensitivity tests using 110 strains obtained before 1968.

3) Procedures used for sensitivity test: The serial dilution method in agar proposed by the Japan Society of Chemotherapy⁶⁾ was employed, except for incubation temperature which was 28°C in the present study. Heart-infusion agar (Eiken) was used as a medium for sensitivity tests with antibiotics and nitrofur derivatives. N,N-dimethylformamide was used as a solvent of nitrofur derivatives. Ten milligrams of the drug was first dissolved in 5 ml of the solvent and then the solution was diluted with the same amount of distilled water to make a stock solution. Twenty-four-hour-old cultures at 28°C in Tryptosoy broth (Eiken) were used as inocula. While in sensitivity tests with sulfa drugs use was made of KNIGHT's semisynthetic medium to which was added agar to give 1.5% concentration and all inocula were grown at 28°C for 24 hours in the same medium without agar. Each organism was tested at least twice for sulfa drugs. Controls were always run using *A. liquefaciens* Y-62.

The highest dilution, or the minimum concentration, of a drug inhibitory to a test strain was taken as the MIC of the drug to the strain.

Results

The results of the sensitivity tests performed are shown in Table 2 and those of the additional tests in Table 3. From these tables and Table 4 which gives a comparison of inhibitory potencies of drugs, it may be said that most strains are highly sensitive to tetracycline, chloramphenicol, and chlortetracycline, moderately sensitive to the remaining drugs, except aminobenzyl penicillin to which they are highly resistant.

The distribution curves of sensitivities of the strains to the nine drugs are given in Fig. 1. As a curve at the top on the left of the figure shows, the 250 strains studied showed a sensitivity distribution curve bearing two peaks on the sensitive and resistant sides against tetracycline. This suggests that the strains, except three moderately sensitive strains showing MIC values between 3.1 and 12.5 mcg/ml, may fall into two groups depending on their sensitivities to the drug; a large group consisting of highly sensitive strains showing MIC values from 0.2 to 1.6 mcg/ml and a small group of resistant strains situated within a MIC range from 25 to 250 mcg/ml.

The sensitivity distribution curves against chloramphenicol and streptomycin have

Table 2. Drug sensitivities of 250 strains of *Aeromonas liquefaciens* isolated from fishes (I).

MIC mcg/ml		Number of strains															Total
Drug	0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100	200	500	1.000	>1.000	
Tetracycline			3	124	49	6	1	1	1	16	17	28	4				250
Chloramphenicol				148	82	4	1	1	3	7	4						250
Streptomycin							2	103	106	19	4	1	3	6		6	250
Furazolidone				10	19	49	30	38	79	24	1						250
Nifuriprinol	1	9	22	40	40	33	40	47	15	1		2					250
Sulfamonomethoxine		3	13	20	68	66	22	4		1			1		1	51	250
Kanamycin								73	125	31	3						232
Fradiomycin						19	127	63	22	1							232
Aminobenzyl penicillin						1			1	2	1		2	6	25	194	232

Table 3. Drug sensitivities of 110 strains of *Aeromonas liquefaciens* isolated from fishes (II. Results of additional sensitivity tests).

MIC mcg/ml	Number of strains														Total
Drug	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50	100	200	500	1.000	>1.000	
Chlortetracycline	9	57	13	1	1	8	10	10	1						110
Nitrofurazone				3	16	26	35	29		1					110
Sulfisoxazole		6	15	11	38	16	6							18	110

Fig. 1. Sensitivity distribution curves of *Aeromonas liquefaciens* strains against nine drugs.

Ordinate; percentage frequency

Abscissa; MIC levels

Abbreviations: TC; tetracycline, CP; chloramphenicol, SM; streptomycin, NF; furazolidone, P-7138; nifuriprinol, SMM; sulfamonomethoxine, FRM; fradiomycin, KM; kanamycin, ABPC; aminobenzyl penicillin.

two and three peaks, respectively, though the peaks on the resistant side are all very small, suggesting the existence of a small number of strains resistant to these drugs. A curve at the top on the right of Fig. 1 clearly shows that there are a number of strains highly resistant to sulfamonomethoxine, though the majority of the strains are highly sensitive to the drugs.

On the contrary the strains showed a distribution curve of sensitivity having one peak against kanamycin and fradiomycin. The ranges of MIC of these drugs were narrow and there was no strain being far beyond the ranges and consequently it may be said that no strains were found resistant to these drugs.

The sensitivity distribution curves against two sorts of nitrofurans derivatives, so

Table 4. Comparison of the inhibitory potencies of 12 drugs tested against *Aeromonas liquefaciens*.

	Minimum concentrations of drugs inhibitory to (mcg/ml)	
	50% of the tested strains	70% of the tested strains
Chloramphenicol Tetracycline Chlortetracycline	0.4	0.8
Sulfamonomethoxine Sulfisoxazole Nifuriprinol	1.6	3.1
Fradiomycin	3.1	6.2
Furazolidone	6.2	12.5
Streptomycin Kanamycin Nitrofurazone	12.5	12.5
Aminibenzyl penicillin	> 1.000	> 1.000

far as the appearances go, had two peaks respectively, but it seems unreasonable to divide the strains into two groups, sensitive and resistant, either to furazolidone or to nifuriprinol, though there were 2 strains which were apparently resistant to nifuriprinol (Fig. 1, Table 2). The organism was highly resistant to aminobenzyl penicillin, but it is interesting to note that there were some strains which were sensitive to this drug.

There was found a correlation between the sensitivity of the organism to chemically

Table 5. Relation between the sensitivities of 110 strains of *Aeromonas liquefaciens* to tetracycline and chlortetracycline.

MIC mcg/ml		Chlortetracycline								
		0.2	0.4	0.8	1.6	3.1	6.2	12.5	25	50
Tetracycline	0.2									
	0.4	7*	30	2						
	0.8	2	27	8						
	1.6			2	1					
	3.1			1						
	6.2									
	12.5							1		
	25					1	7	4		
	50						1	2	3	
	100							3	4	1
	200								3	

* Number of strains.

Table 6. Relation between the sensitivities of 110 strains of *Aeromonas liquefaciens* to sulfanilamide derivatives.

MIC		Sulfisoxazole						
mcg/ml		0.4	0.8	1.6	3.1	6.2	12.5	>1.000
Sulfamono- methoxine	0.1	1*	1					
	0.2	5	6					
	0.4		3	3	1			
	0.8		5	7	8			
	1.6			1	22	6		
	3.1				7	10	2	
	6.2						4	
	...							
	200							1
	500							
	1.000							1
	>1.000							16

* Number of strains.

related drugs; that is, between tetracycline and chlortetracycline (Table 5), sulfamonomethoxine and sulfisoxazole (Table 6), and furazolidone, nitrofurazone and nifuriprinol (Table 7).

Discussion

There are several reports on the drug sensitivities of fish-pathogenic *A. liquefaciens* (synonymous to *A. punctata*; *A. hydrophila*, *Pseudomonas punctata*)⁷⁻¹⁶. The sensitivities of the organism to various drugs reported by various authors are practically equal to those of our strains which are sensitive to respective drugs, though the numbers of strains they used were very limited and their methods of sensitivity test were different from one another and from ours.

In the foregoing chapter the present authors used the term 'resistant', but it is not necessarily easy to rigidly define 'resistant' strains. For example, on the basis of sensitivity distribution of the strains shown in Fig. 1, a group of strains showing MIC levels over 25 mcg/ml of tetracycline can be reasonably defined as resistant to tetracycline, but the question whether or not the 3 intermediately sensitive strains are also resistant has puzzled us. The same may be said of cases with chloramphenicol, streptomycin and sulfamonomethoxine. So it is inevitable to determine somewhat arbitrarily critical MIC levels for defining resistant *A. liquefaciens*.

In the present study critical MIC levels for defining resistant *A. liquefaciens* are tentatively determined as shown in Table 8, by being chiefly based on the results of the present study and, in addition, by taking account of the standard MIC levels proposed

Table 7. Relation among the sensitivities of 110 strains of *Aeromonas liquefaciens* to furazolidone, nitrofurazone and nifuriprinol.

MIC		Nifuriprinol										Nitrofurazone									
mcg/ml		0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5	25		0.8	1.6	3.1	6.2	12.5	25	50	100		
Furazolidone	0.4	3*												1	2						
	0.8	2	2	1	1								1	5							
	1.6		4	14	5								1	7	11	4					
	3.1		1	5	9	2	1							2	11	5					
	6.2			3		7	5									2	12	1			
	12.5				1	9	11	8	6	1					2	12	22				
	25						1	4	3								2	6			
	50				1															1	

* Number of strains.

Table 8. The MIC levels for defining drug-resistant *Aeromonas liquefaciens* and the number of resistant strains.

	Criteria of drug resistance (MIC levels defining drug-resistant <i>A. liquefaciens</i>) (mcg/ml)	No. of resistant strains No. of studied strains	Criteria for drug resistance (mcg/ml)	
			<i>Shigella</i> ¹⁸⁾	Gram-negative bacteria ¹⁹⁾
Chloramphenicol	25	11/250	25	25—50
Tetracycline	25	65/250	25	12.5—100
Streptomycin	100	16/250	12.5	25—50
Furazolidone	50	1/250	12.5	—
Nifuriprinol	50	2/250	—	—
Sulfamonomethoxine	100	53/250	50	200—800
Fradiomycin	100	0/232	12.5	—
Kanamycin	100	0/232	12.5	12.5—50
Aminobenzyl penicillin	100	227/232	25	50—400

by the Research Committee of the Shigella Strains in Japan for defining resistant *Shigella*¹⁸⁾, and those by the Research Committee of Gram-negative Bacterial Infections to define various resistant gram-negative bacteria¹⁹⁾. The number of drug-resistant strains of *A. liquefaciens* thus defined are given in the second column from the left of Table 8.

As shown in Table 8, out of the 232 strains studied 227 (97.8%) were highly resistant to aminobenzyl penicillin, and so it seemed appropriate to exclude aminobenzyl penicillin resistance from the discussion here of resistance patterns of *A. liquefaciens* strains.

Patterns of drug resistance of drug-resistant strains of *A. liquefaciens* are given in Table 9, which shows that 38 strains were two-drug-resistant, four strains three-drug-resistant, and 8 strains four-drug-resistant.

Table 9. Drug-resistance patterns of drug-resistant strains of *Aeromonas liquefaciens* isolated from fishes.

	No. of strains	Resistance pattern*	No of strains
One-drug-resistant	28	CP	3
		TC	16
		SM	2
		NF (p-7138)	2
		SA	5
Two-drug-resistant	38	TC, SA	36
		TC, SM	1
		SM, NF (Furazolidone)	1
Three-drug-resistant	4	TC, SM, SA	4
Four-drug-resistant	8	CP, TC, SM, SA	8

* Abbreviations. TC: tetracycline, CP: chloramphenicol, SM: streptomycin, NF: nitrofurazone derivatives, SA: sulfanilamide derivatives.

It is well worth of notice that 36 of the 38 two-drug-resistant strains had TC, SA-resistance (tetracycline-sulfanilamide resistance) and the 4 three-drug-resistant strains and the 8 four-drug-resistant strains were all resistant to TC and SA. Accordingly it may be said that TC, SA-resistance is a characteristic resistance pattern of drug-resistant *A. liquefaciens*.

It is interesting to note that no drug-resistant strain was found among 6 strains isolated from wild diseased ayu. The MIC values of drugs to those strains were as follows: tetracycline, chlortetracycline, chloramphenicol, and nifurprinol, <0.8 mcg/ml; fradiomycin and furazolidone, <3.1 mcg/ml; sulfamonomethoxine, <6.2 mcg/ml; streptomycin, nitrofurazone, and sulfisoxazole, <12.5 mcg/ml; kanamycin, <25 mcg/ml; aminobenzyl penicillin, 1000 mcg/ml. It seems, however, to be too early to discuss whether or not this is due to the fact that wild ayu have no chance of coming into contact with chemotherapeutics.

References

- 1) ANDERSON, E. S.: *Ann. Rev. Microbiol.*, **22**, 131-180 (1968).
- 2) SNIESZKO, S. F. and G. L. BULLOCK: *Prog. Fish-Cult.*, **19**, 99-107 (1957).
- 3) SNIESZKO, S. F. and G. L. BULLOCK: *U.S.D.I., Fish and Wildl. Serv., Fish Bull.*, **125**, 555-564 (1957).
- 4) G. L. BULLOCK: *Develop. Appl. Microbiol.*, **5**, 101-108 (1964).
- 5) R. W. H. SCHUBERT: *Int. J. Systematic Bacteriol.*, **17**, 23-37 (1967).
- 6) JAPAN SOCIETY OF CHEMOTHERAPY SUB COMMITTEE ON MIC: *Chemotherapy*, **16**, 1, 98-99 (1968).
- 7) W. SCHÄPERCLAUS: *Z. Fischerei*, **5**, 1-60 (1956).
- 8) W. SCHÄPERCLAUS: *ibid.*, **7**, 509-628 (1958).
- 9) T. HOSHINA: *J. Tokyo Univ. Fish.*, **6**, 1-105 (1962).
- 10) E. KARASEK: *Zbl. Vet. Med.*, **14**, 663-670 (1967).
- 11) M. SHIMIZU and Y. TAKASE: *This Bull.*, **33**, 544-554 (1967).
- 12) M. SHIMIZU and Y. TAKASE: *Fish Path.*, **2**, 59-67 (1967).
- 13) K. MUROGA and S. EGUSA: *ibid.*, **2**, 141-147 (1968).
- 14) G. L. BULLOCK and D. COLLIS: *Bureau of Sport Fisheries and Wildlife Technical Paper*, **32**, 9 pp. (1969).
- 15) J. A. ROBINSON *et al.*: *ibid.*, **35**, 7 pp. (1969).
- 16) R. KUSUDA and Y. TAKAHASHI: *Fish Path.*, **4**, 87-97 (1970).
- 17) K. SHIRAKI *et al.*: *ibid.*, **4**, 130-137 (1970).
- 18) T. EZAKI *et al.*: *Chemotherapy*, **17**, 447-451 (1969).
- 19) S. ISHIYAMA *et al.*: *ibid.*, **17**, 42-46 (1969).