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TREATMENT OF SULFONAMIDE-RESISTANT FURUNCULOSIS IN TROUT AND DETERMINATION OF DRUG SENSITIVITY

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ABSTRACT

Treatment of furunculosis with sulfonamides was unsuccessful when the outbreak was caused by sulfonamide-resistant variants of the pathogen. The pathogen was sensitive to chloramphenicol and treatment with this antibiotic resulted in greatly reduced mortalities. Losses were also greatly affected by the strain-specific resistance or susceptibility of the experimental brook trout to furunculosis.

Sensitivity or resistance of *Aeromonas salmonicida* to sulfonamides and antibiotics was determined in vitro, using various liquid and solid bacteriological media. A simple method for the determination in vitro of sulfonamide- and antibiotic-sensitivity of *A. salmonicida* is described for field use. A revised method of treatment of furunculosis is also outlined.

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TREATMENT OF SULFONAMIDE-RESISTANT FURUNCULOSIS IN TROUT AND DETERMINATION OF DRUG SENSITIVITY

By S. F. SNIESZKO, *Bacteriologist*, and G. L. BULLOCK, *Fishery Aid*

During the past 2 years, the Microbiological Laboratory, at Leetown, W. Va.,[1] has received an increasing number of reports of outbreaks of furunculosis that failed to respond to treatment with sulfonamides. The problems resulting from furunculosis, a widespread, bacterial disease of salmonids, and control of the disease have been reviewed in detail by McCraw (1952) and Snieszko (1954a). The latter investigator recommended the use of antibiotics for diseased fish that failed to respond to treatment with sulfonamides. In some of the recent incidences of furunculosis at the Leetown station the disease did not respond to sulfonamide therapy, but control was effected with chloramphenicol. Failure of sulfonamides to control furunculosis suggested that the causal organism, *Aeromonas salmonicida*, was resistant to the drugs used.

Several methods are widely used in the routine determination in vitro of the sensitivity of bacteria to sulfonamides and antibiotics. The results obtained with antibiotics are generally accepted as reliable aids in the selection of the most promising treatment. Such, however, is not the case with sulfonamides, and "The relation of *in vitro* sensitivity test to clinical effectiveness is still controversial" (Burdette, Plank, and Clapper, 1955).

The experiments presented in this paper followed three lines of endeavor: (1) A comparison of the effectiveness of sulfonamide and antibiotic therapy in strains of trout which were either susceptible or resistant to furunculosis. (2) Comparison of the therapy of furunculosis with sulfonamides and chloramphenicol in trout which were suffering from furunculosis caused by sulfonamide-resistant, but chloramphenicol-sensitive, strains of *A. salmonicida*. (3) The development of a standard method for laboratory and field use of determining in vitro the sensitivity of *A. salmonicida* to sulfonamides and antibiotics.

[1] Post Office, Kearneysville, W. Va.

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Many factors are capable of affecting in-vitro tests of microbial sensitivity to sulfonamides; therefore, correct interpretation and application of the results of such tests will be reliable if a proved method of obtaining reproducible results is used.

As the result of the findings presented in this study and practical experience gained in the treatment of this disease in trout hatcheries over the entire country, a revised procedure has been evolved and described for the treatment of this disease.

The authors wish to express their thanks to Dr. K. E. Wolf for his assistance during the preparation of the manuscript and for furnishing the strains of *A. salmonicida* isolated in Iowa, Utah, Minnesota, and Wisconsin; and to Dr. R. E. Lennon for supplying the fingerling brook trout from Erwin, Tenn.

MATERIALS AND METHODS

Treatment with sulfonamides and chloramphenicol

Experiments on the therapy of furunculosis were carried out with two strains of fingerling brook trout (*Salvelinus fontinalis*) and a strain of brown trout (*Salmo trutta*). A strain of brook trout from Bellefonte, Pa., was selected because it was known to be resistant to furunculosis (Wolf 1954; Snieszko 1954b), and a strain from Erwin, Tenn., was used because it was suspected to be susceptible. The brown trout were from Cortland, N. Y. In general, brown trout are known to be more resistant to furunculosis than are most strains of brook trout (McCraw 1952; Wales and Berrian, 1937).

Equal weights of the trout were distributed among stainless steel troughs in which the water was maintained at temperatures of 12° to 13° C. (54°–55° F.). Infection of the fish was initiated by adding fresh cultures of *Aeromonas salmonicida* to the diet, and treatment was started when the first mortality due to furunculosis occurred.

Sulfonamides were administered at the rate of 200 milligrams per kilogram of trout per day, or 9 grams per 100 pounds of fish. Chloramphenicol[2] was given at the rate of 75 milligrams of pure antibiotic activity per kilogram of fish per day, or 3.4 grams per 100 pounds of fish. Treatment was continued to the end of the observations recorded in tables 1 to 3. Drugs were mixed with the quantities of food calculated from the Cortland Hatchery feeding charts (Deuel et al., 1952). A daily record of mortality was kept and most of the dead trout were examined bacteriologically.

Drug sensitivity tests in vitro

The drug sensitivity tests were performed with 84 cultures isolated from the experiment on therapy here described or procured during the past years from other experiments or received from other trout hatcheries (table 4). The routine qualitative sulfonamide and antibiotic sensitivity tests were run with commercial multiple sensitivity paper disks[3] on solid bacteriological media. Sensitivity to sulfonamides has also been determined in a quantitative manner by preparation of serial dilutions of these drugs in liquid bacteriological media.

The following solid bacteriological media were used with the paper disks:

1. Leetown standard furunculosis medium having the following composition:

Tryptic digest of casein	10 grams.
Yeast extract	5 grams.
Sodium chloride	2.5 grams.
Agar	15.0 grams.
Water	1 liter.
pH	6.8–7.0.

1A. The same, but buffered with 0.5 percent of secondary sodium phosphate and adjusted to pH 8.0.

2. Sensitivity Test medium C (Case Laboratories).

2A. The same, but buffered and adjusted as No. 2.

3. Mueller Hinton medium (Bacto).

4. Trypticase Soy agar (Baltimore Biological Laboratory).

[2] Racemic chloromycetin, Parke, Davis & Co., was used at a double rate, because the manufacturer advised that it has about 50 percent the therapeutic value of the d-rotatory isomer

[3] Multidisks, Case Laboratories, Chicago, Ill.

For the inoculation of the solid media, 24-hour-old cultures in nutrient broth were used. These were streaked with cotton swabs on 10-cm.-diameter petri plates, each containing 20 ml. of medium, and the disks were placed in position.

The quantitative sensitivity tests were run in liquid media. In order to select the most satisfactory substrate, the following media were tested:

Leetown standard furunculosis medium without agar.

Vitamin-free casamino acids (Difco), 1 percent.

Tryptic digest of casein, 1 percent.

Nutrient broth (Difco).

Nutrient broth (Difco) buffered with 0.5 percent of secondary sodium phosphate and adjusted to desired pH.

Proteose peptone No. 3 (Difco), 1 percent.

N-Z-Case (Sheffield Chemical Co.), 1 percent.

Nutrient broth buffered with sodium phosphate was selected as most promising for our purposes.

Because most of the sulfonamides are only slightly soluble in water, stock solutions of sodium salts were prepared by dissolving a known quantity of sulfonamide in diluted sodium hydroxide. Excess alkali over that needed for keeping sulfonamides in solution was neutralized with hydrochloric acid. Stock solutions were sterilized by filtration, stored under refrigeration, and used within a week. Serial dilutions of 1:25 or 1:50 stock solutions were carried out in 5 ml. of media. The first tube contained double-strength medium, and after the serial dilutions were made 5 ml. were discarded from the last tube. Inocula consisted of 0.1 ml. of a 24-hour-old culture diluted 1:5 with sterile water. All cultures were incubated at 20° C. and results were read as soon as abundant growth developed, usually within 24 to 48 hours.

RESULTS

Treatment with sulfonamides and chloramphenicol

The response to treatment with sulfonamides and chloramphenicol of experimental furunculosis in two strains of brook trout and one strain of brown trout is presented in tables 1 to 3. Sulfonamide treatment of experimental furunculosis in the Erwin brook trout failed completely (table 1). Losses were high because the trout were susceptible to furunculosis and the strain of *A. salmonicida* used to produce the disease developed resistance to sulfonamides. When the experiment

was repeated with the same strains of trout and bacteria, but with treatment with chloramphenicol, the response was rapid and favorable.

A similar experiment was run concurrently with a strain of brook trout from Bellefonte, Pa., that is known to be resistant to furunculosis. The fish failed to respond to sulfonamide treatment and bacteria isolated from dead trout were found also to be mostly resistant to sulfonamides. Trout surviving the sulfonamide treatment were given chloramphenicol and losses stopped within the first 5-day period (table 2). The overall fish losses were much lower in the resistant Bellefonte strain than in the susceptible Erwin strain.

The results with brown trout (table 3) were very similar to those obtained with Bellefonte brook trout since they are known to be more resistant to furunculosis than most of the strains of brook trout.

TABLE 1.—*Mortality of a strain of eastern brook trout (Erwin, Tenn.), susceptible to furunculosis, following treatment with sulfonamides and chloramphenicol*

A. TREATMENT WITH SULFONAMIDES [1]

[Initial weight of fish per lot (trough), 1,500 gm.; mean weight per fish, 19.8 gm.; mean number of fish per lot (trough), 76; fish infected orally July 29 and Aug. 1, 1955, and treatment started Aug. 8; mortality expressed as percent of fish at beginning of each period]

Period	Mortality under treatment with—				Mortality in controls	
	Sulfamerazine		Sulfisoxazole (Gantrisin [2])			
	Lot A	Lot B	Lot A	Lot B	Lot A	Lot B
Aug. 7–11	7.8	6.5	6.8	2.6	9.1	12.0
Aug. 12–16	12.6	16.6	22.0	39.1	26.0	55.0
Aug. 17–21	37.0	63.3	49.0	75.5	48.0	67.0
Aug. 22–24	33.3	82.0	48.1	63.6	41.0	80.0
Total mortality	66.2	95.0	81.0	94.7	79.2	97.3
Average, total mortality	80.6		87.9		88.2	

B. TREATMENT WITH CHLORAMPHENICOL

[Initial weight of fish per lot (trough), 1,500 gm.; mean weight per fish, 30 gm.; mean number of fish per lot (trough), 50; fish infected orally Sept. 8 and 9, 1955, and treatment started Sept. 16; mortality expressed as percent of fish at beginning of each period]

Period	Mortality under treatment in—		Mortality in controls	
	Lot A	Lot B	Lot A	Lot B
Sept. 16–20	4.1	14.0	9.1	19.1
Sept. 21–25	11.0	16.0	32.0	45.0
Sept. 26–30	0	0	38.2	67.0
Oct. 1–5	0	0	50.0	100.0
Total mortality	14.2	27.5	87.2	100.0
Average, total mortality	20.8		93.5	

[1] Strains of *A. salmonicida* isolated from dead trout were resistant to sulfonamides.

[2] Gantrisin used in these studies was supplied free of cost by Hoffmann La Roche, Nutley, N. J.

TABLE 2.—*Mortality of a strain of eastern brook trout (Bellefonte, Pa.), resistant to furunculosis, following treatment with sulfonamides and chloramphenicol*

A. TREATMENT WITH SULFONAMIDES [1]

[Initial weight of fish per lot (trough), 1,500 gm.; mean weight per fish, 27.6 gm.; mean number of fish per lot (trough), 54; fish infected orally July 29 and Aug. 1, 1955, and treatment started Aug. 19; mortality expressed as percent of fish at beginning of each period]

Period	Mortality under treatment with—				Mortality in controls	
	Sulfamerazine		Sulfisoxazole			
	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F
Aug. 17–21	0	1.8	0	4.0	3.6	0
Aug. 22–26	0	7.5	1.6	0	7.5	4.0
Aug. 27–31	3.5	4.0	1.6	0	8.1	4.1
Sept. 1–5	1.8	10.6	10.1	0	4.4	0
Total mortality	5.3	22.2	13.1	4.0	21.8	8.0
Average, total mortality	13.7		8.5		15.0	

B. TREATMENT WITH CHLORAMPHENICOL

[Fish surviving treatment with sulfonamides (2-A) used in this experiment; weight of fish per lot (trough), 2,000 gm.; mean weight per fish, 43 gm.; mean number of fish per lot (trough), 46; no additional infection, with treatment started Sept. 7, 1955; mortality expressed as percent of fish at beginning of each period]

Period	Mortality under treatment in—			Mortality in controls		
	Lot A	Lot C	Lot E	Lot B	Lot D	Lot F
Sept. 6–10	9.4	7.5	0	12.0	0	4.3
Sept. 11–15	0	0	0	10.8	0	0
Sept. 16–20	0	0	0	3.0	2.1	0
Sept. 21–25	0	0	0	3.1	0	2.2
Sept. 26–30	0	0	0	0	2.1	2.3
Total mortality	9.4	7.5	0	26.1	4.2	8.7
Average, total mortality	5.7			10.8		

[1] Strains of *A. salmonicida* isolated from dead fish were resistant to sulfonamides.

TABLE 3.—*Mortality of brown trout following treatment with sulfonamides and chloramphenicol*

A. TREATMENT WITH SULFONAMIDES [1]

[Initial weight of fish per lot (trough), 1,500 gm.; mean weight per fish, 22.0 gm.; mean number of fish per lot (trough), 68; fish infected orally July 29 and Aug. 1, 1955, and treatment started Aug. 12; mortality expressed as percent of fish at beginning of each period]

Period	Mortality under treatment with				Mortality in controls	
	Sulfamerazine with sulfaguanidine		Sulfisoxazole			
	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F
Aug. 12–16	3.1	3.0	1.3	1.7	1.4	6.6
Aug. 17–21	0	1.5	0	3.4	0	5.2
Aug. 22–26	4.8	3.1	2.7	0	1.4	13.0
Aug. 27–31	10.0	8.0	5.5	7.1	8.7	4.2
Sept. 1–5	5.6	10.3	8.8	3.8	4.8	2.2
Total mortality	22.0	25.7	17.3	17.5	17.8	29.0

[1] Strains of *A. salmonicida* isolated from dead fish were resistant to sulfonamides.

TABLE 3.—*Mortality of brown trout following treatment with sulfonamides and chloramphenicol*—Con.

B. TREATMENT WITH CHLORAMPHENICOL

[Fish surviving treatment with sulfonamides (3–A) used in this experiment; weight of fish per lot (trough), 1,500 gm.; mean weight per fish, 31 gm.; mean number of fish per lot (trough), 48; no additional infection, with treatment started Sept. 7, 1955; mortality expressed as percent of fish at beginning of each period]

Period	Mortality under treatment in—			Mortality in controls		
	Lot A	Lot C	Lot E	Lot B	Lot D	Lot F
Sept. 6–10	10.0	1.6	3.3	13.4	0	6.8
Sept. 11–15	2.2	0	0	8.9	6.1	7.3
Sept. 16–20	0	0	0	0	6.4	7.9
Sept. 21–25	0	1.6	0	0	0	5.7
Sept. 26–30	0	1.6	0	0	2.3	6.1
Total mortality	12.0	4.8	3.3	21.0	14.0	29.5
Average, total mortality	6.7			21.3		

While the results obtained in the treatment of furunculosis caused by sulfonamide-resistant but chloramphenicol-sensitive strains of *A. salmonicida* are of considerable interest in their own merit, they are presented in this paper chiefly as background information for the history of the majority of the strains of this bacterium which were used in the in-vitro sensitivity tests. Summary information on the cultures used for testing of the drug sensitivity is presented in table 4.

TABLE 4.—*Sensitivity of 84 strains of* A. salmonicida *to sulfonamides tested with sodium sulfadiazine in buffered nutrient broth*

Date and source of culture	Place of isolation	Number of strains that were—			
		Sensitive [1]	Intermediate	Resistant [2]	Total
1955:					
Brook trout:					
Not treated	Leetown	0	2	12	14
Treated with sulfonamides.	do	0	1	9	10
Treated with chloramphenicol.	do	0	0	5	5
Brown trout:					
Not treated	do	1	5	1	7
Treated with several sulfonamides.	do	2	0	9	11
Total		3	8	36	47
1956:					
Brown trout: Not treated	Leetown	13	2	2	17
Rainbow trout: Not treated	Iowa	2	0	0	2
Do	Utah	0	0	2	2
Brown and brook trout: Treatment (?).	Minnesota	4	0	0	4
Brook trout: Treatment (?).	Wisconsin	3	0	0	3
Total		22	2	4	28
Before 1955:					
Miscellaneous stock cultures stored in lyophilized form since 1952.	Leetown	4	2	3	9
Grand total		29	12	43	84

[1] Cultures in which there was no growth within 24 hours in buffered nutrient broth having a pH of 8.0 and containing sodium sulfadiazine in the concentration of 1:1,600.
[2] Cultures in which there was undiminished growth in nutrient broth containing sodium sulfadiazine in the concentration of 1:100.

Sensitivity tests with antibiotics

Tests of the sensitivity of *Aeromonas salmonicida* to various antibiotics were performed with 47 strains of the pathogen that were isolated during the summer of 1955 from fingerling brook trout known to be either susceptible or resistant to furunculosis and from fingerling brown trout. Trout from which the bacteria were isolated had been treated with sulfonamides or chloramphenicol, or had served as controls (table 4). Cultures of these strains were grown on the Leetown standard furunculosis medium on which 6-tipped sensitivity disks containing various antibiotics had been placed (table 5 and fig. 1). All strains of *A. salmonicida* that were tested were found to be uniform in their response to disks containing antibiotics. No correlation between the sensitivity to antibiotics and sulfonamides was apparent.

TABLE 5.—*Sensitivity of* Aeromonas salmonicida *to selected antibiotics as determined with Multidisks*

Antibiotic	Quantity of antibiotic in disk	Width of zones of growth inhibition (mm.)
Chloromycetin [1]	10 μg	14–19
Terramycin [2]	do	12–14
Tetracycline	do	10–13
Aureomycin [3]	do	10–12
Erythromycin	do	5– 8
Streptomycin	do	4– 6
Neomycin	do	1– 2
Penicillin	1.5 units	0– 1
Carbomycin	5 μg	0
Bacitracin	5 units	0
Polymyxin B	10 units	0
Viomycin	10 μg	0
Furadantin [4]	50 μg	8–10

[1] Chloramphenicol.
[2] Oxytetracycline.
[3] Chlortetracycline.
[4] Nitrofurantoin; related to furacin, which has possibilities in the treatment of furunculosis (Gutsell 1948).

Sensitivity tests with sulfonamides

Tests were run in liquid and solid media. The first liquid substrate tested was the standard furunculosis medium containing serial dilutions of sodium sulfamerazine. The pH of the medium was 6.8 to 7.0 and the sulfonamide concentrations ranged from 1:100 to 1:10,000. Thirty resistant and 10 sensitive strains of *A. salmonicida* were used. The results were not satisfactory because sensitive strains of *A. salmonicida* grew as well as the resistant strains. Crystals of sulfamerazine appeared in test tubes with the higher drug concentrations. Therefore, other liquid media such

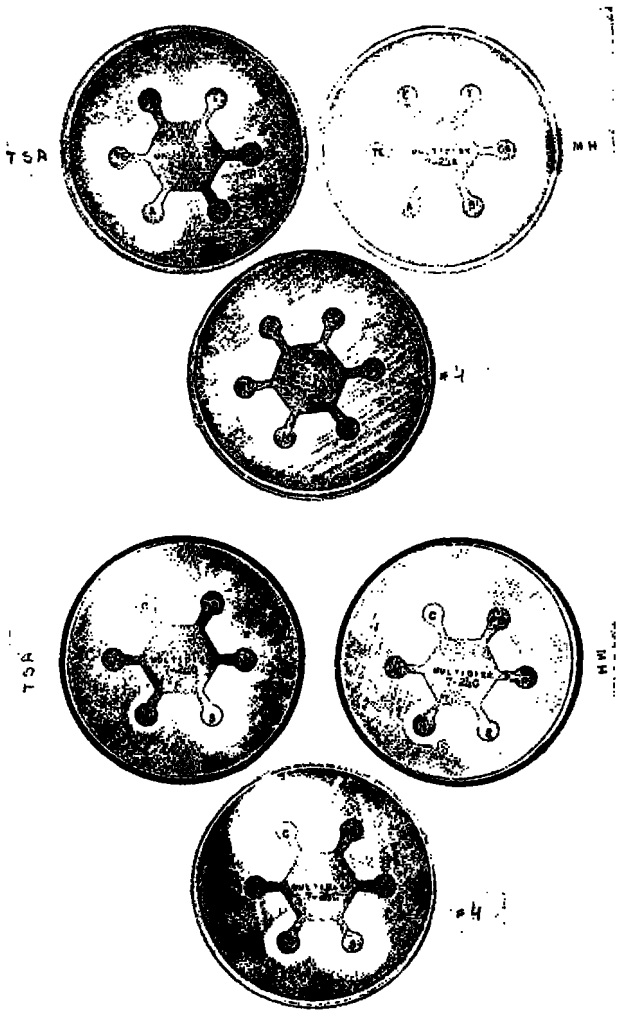

FIGURE 1.—Sensitivity of *Aeromonas salmonicida* in vitro to various antibiotics as determined with three media: Trypticase Soy agar (TSA), Mueller Hinton agar (MH), and standard furunculosis agar (#4). The antibiotic and quantity used on Multidisks, reading clockwise, were as follows: Upper: Tetracycline (TE), 10μg.; erythromycin (E), 10 μg.; terramycin (T), 10 μg.; carbomycin (CA), 5 μg.; bacitracin (B), 5 units; aureomycin (A), 10 μg. Lower: Viomycin (V), 10 μg.; chloromycetin (C), 10 μg.; polymyxin B_1 (PB), 10 units; penicillin (P), 1.5 units; streptomycin (dihydro) (S), 10 μg.; neomycin (N), 10 μg. The presence of a clear zone indicates sensitivity.

as proteose peptone #3, vitamin-free casamino acids, nutrient broth, and N-Z-Case were tested. Surprisingly, the most promising results were obtained with nutrient broth, and this medium was used in all subsequent experiments unless otherwise indicated. Since the sodium salts of sulfonamides give alkaline solutions and their solubility and ionization decreases with decreasing alkalinity of the medium, nutrient broth was buffered by adding 0.5 percent of secondary sodium phosphate and the pH of the medium adjusted as required. In the unbuffered nutrient broth (original pH, 6.8) sodium sulfamerazine precipitated if added in concentration up to 1:250. In such a medium the sulfa-sensitive and sulfa-resistant strains of *A. salmonicida* grew equally well.

Since Griffin et al. (1953) found that *A. salmonicida* has a wide pH tolerance, the buffered nutrient broth was adjusted to pH 7.0, 7.5, 8.0, 8.5, and 9.0. The more alkaline the medium, the more striking were the differences noted between the sulfa-sensitive and sulfa-resistant strains. The all-round best results were obtained at pH 8.0 to 8.5; therefore, all subsequent tests were run within this pH range unless otherwise specified.

According to Northey (1948), "Frequently it is found that once an organism has become resistant to one of the potent sulfonamide drugs it is resistant to all the commonly used sulfa drugs." Therefore it should be advantageous to select for a routine in-vitro test with *A. salmonicida* a sulfonamide, or sulfonamides, which would be the most convenient to work with. With this in mind, several commonly used sulfonamides were tested with the two resistant and two sensitive strains of *A. salmonicida*. Sodium salts prepared in the laboratory were serially diluted in the buffered nutrient broth adjusted to pH 8.0 to 8.5. The results are presented in table 6. Results for sulfaguanidine are omitted from the table because a soluble sodium salt could not be prepared within the desired pH range.

On the basis of these results, all available strains (84) of *A. salmonicida* were tested with sulfadiazine, though sulfamerazine or sulfisoxazole could be used equally well. Results are presented in table 4. It is evident that the largest number of resistant cultures were isolated at Leetown in 1955 from treated or control trout during a period when treatments with sulfonamides were being carried out. Cultures isolated at Leetown in 1956 before any treatments started were predominantly sensitive. There were some intermediate strains which produced a scanty growth in the medium with sodium sulfadiazine.

The highest dilutions of sulfonamides arresting growth of *A. salmonicida* in a buffered nutrient

TABLE 6.—*Growth of sulfonamide-resistant and sulfonamide-sensitive strains of* Aeromonas salmonicida *in buffered, nutrient broth with various sulfonamides*

[Question mark (?) indicates reliable reading could not be made because of precipitate in medium]

Strain number and sulfonamide dilution	Sulfonamides (sodium salt)							
	Sulfadiazine	Sulfamerazine	Sulfamethazine	Sulfathiazole	Sulfisoxazole	Sulfaquinoxaline	Sulfathialidine	Sulfanilamide
Sensitive #1:								
1:50	?	?	?	?	−	?	?	?
1:100	−	−	?	?	−	?	+++	?
1:200	−	−	+	−	−	?	+++	?
1:400	−	−	+++	+	−	−	+++	?
1:800	−	−	+++	+++	−	−	+++	+
1:1600	−	−	+++	+++	−	−	+++	++
Control	+++	+++	+++	+++	+++	+++	+++	+++
Sensitive #2:								
1:50	?	?	?	?	−	−	?	?
1:100	−	−	?	?	−	+	+++	?
1:200	−	−	−	−	−	+	+++	?
1:400	−	−	−	+	−	+	+++	?
1:800	−	−	−	+	−	+	+++	+
1:1600	−	−	−	+	−	+	+++	++
Control	+++	+++	+++	+++	+++	+++	+++	+++
Resistant #1:								
1:50	?	?	?	?	−	−	?	?
1:100	++	−	?	?	−	−	+++	?
1:200	+++	+++	+	−	+	−	+++	?
1:400	+++	+++	+++	+	+++	+	+++	?
1:800	+++	+++	+++	+++	+++	+++	+++	+
1:1600	+++	+++	+++	+++	+++	+++	+++	++
Control	+++	+++	+++	+++	+++	+++	+++	+++
Resistant #2:								
1:50	?	?	?	?	−	−	?	?
1:100	+++	−	+	?	+	−	+++	?
1:200	+++	+++	+++	−	+++	+	+++	?
1:400	+++	+++	+++	+	+++	+++	+++	?
1:800	+++	+++	+++	+++	+++	+++	+++	+
1:1600	+++	+++	+++	+++	+++	+++	+++	++
Control	+++	+++	+++	+++	+++	+++	+++	+++

broth were determined with laboratory-prepared sodium salts of sulfadiazine, sulfamerazine, sulfamethazine, and sulfisoxazole. Ten strains of sulfonamide-sensitive and 10 resistant cultures were used. Serial dilutions of sulfonamides ranged from 1:100 to 1:102,400. Control test tubes contained medium without sulfonamides. After 24 hours of incubation the resistant strains grew well in a sulfonamide concentration of 1:100 (1:200 with sulfamerazine). Growth of the sensitive strains was absent in the following concentrations and in all lower dilutions:

Sulfadiazine	1:12,800
Sulfamerazine	1:6,400
Sulfamethazine	1:6,400
Sulfisoxazole	1:6,400

The amount of growth was much reduced up to the highest dilution of the sulfonamides tested (1:102,400, or 10 μg. per ml.). After 48 hours the results differed very little except in the medium with sulfamethazine which showed abundant growth up to 1:200 and scanty at 1:100 (the highest concentration tested).

The final series of experiments was conducted for the purpose of selecting a reliable method of determining the drug sensitivity of *A. salmonicida* under either laboratory or field conditions.

In a preliminary test, the optimum pH range for the appearance of growth inhibition zones on an agar medium was determined. Filter paper disks (6 mm. in diameter) [4] were impregnated with 1 mg. of filter-sterilized sodium sulfadiazine dissolved in 0.02 ml. of water and air dried at 37° C. Standard furunculosis medium (buffered, adjusted to pH 7.0, 7.5, 8.0, 8.5, and 9.0, and sterilized) was distributed in 20-ml. quantities in 10 petri plates (10-cm. in diameter), the medium was inoculated by swabbing the surface with a 24-hour culture of *A. salmonicida*, and disks were positioned. Results were recorded after 24 and 48 hours of incubation at 20° C. Zones were distinctly visible after 24 hours, but the width of the zones could be more conveniently determined after 48 hours. The widths of the zones with sulfonamide-sensitive strains at various pH levels were as follows:

pH 7.0	11 mm.
pH 7.5–8.0	15 mm.
pH 8.5	16 mm.
pH 9.0	17 mm.

There were no clear zones with sulfa-resistant strains. The best results, based on the width of

[4] No. 740–E., C. Schleicher & Schuell Co.

the zones and abundance and rapidity of growth, was in the medium with pH 8.0.

As a result of this preliminary test to determine the optimum pH range, another experiment was carried out with 6-tipped commercial multiple disks and single disks prepared in the laboratory. On standard furunculosis medium, zones of partial growth inhibition appeared around disks with 1 milligram of sodium sulfisoxazole, 1.0 and 0.5 milligram of sodium sulfadiazine, and 0.1 milligram of thiosulfil.[5] No zones appeared around disks containing 0.1 milligram of sulfisoxazole, sulfadiazine, elkosin, sulfamerazine, or triple sulfa.[6] The same test was repeated with (1) Case Laboratory Sensitivity Test medium "C," as prepared by the manufacturer, and (2) the same medium buffered and alkalized to pH 8.0. Results are presented in table 7.

Growth of *A. salmonicida* was strikingly less abundant on nonbuffered "Case" medium, probably because of the presence of dextrose. To test the effect of dextrose, a standard furunculosis medium buffered and adjusted to pH 8.0 was enriched with 0.5 percent of this sugar. On such a medium and with multiple disks containing as much as 0.5 milligram of sulfonamides per tip, no clear zones appeared with sensitive cultures.

TABLE 7.—*Sensitivity of sulfa-sensitive strains of* A. salmonicida *to sulfonamides cultured on Sensitivity Test medium "C" (Case)*

[L=disks prepared at Leetown; PZ=zones of partial growth inhibition]

Quantity of sulfonamide used	Average zone width (in mm.) on medium—	
	Not modified	Buffered and alkalized
Sodium sulfadiazine:		
0.1 milligram	1.3	2.8
0.1 milligram (L)	1.8(PZ)	8.4(PZ)
0.5 milligram (L)	6.9(PZ)	15.0
1.0 milligram (L)	9.7(PZ)	17.5
Sodium sulfisoxazole:		
0.1 milligram	6.8(PZ)	6.1
1.0 milligram (L)	13.5(PZ)	20.3
Sulfamerazine: 0.1 milligram	0	3.6
Thiosulfil: 0.1 milligram	4.5	3.6
Triple sulfa: 0.1 milligram	0	2.1

A similar test was performed with commercial Mueller Hinton and Trypticase Soy agar media and 7-tipped Multidisks containing 0.1 or 0.5 milligram of sulfonamides per disk. The width of the zones could not be recorded because the clear zones were so wide that practically no growth appeared on the 10-cm. petri plates. Therefore the test was repeated with single disks, prepared at the Leetown laboratory, which contained 0.1, 0.25, and 0.5 milligram of sodium sulfadiazine.

The results presented in table 8 show that the lack of growth on 10-cm. petri plates using multiple disks was due to the great width of zones produced by disks containing more than 0.1 milligram of sulfonamide. Therefore use of multiple disks with higher concentrations of sulfonamides and with Mueller Hinton and Trypticase Soy agar media in 10-cm. petri plates is not advisable.

TABLE 8.—*Determining sulfonamide sensitivity of* A. salmonicida *using two media and single disks*

Quantity of sodium sulfadiazine per disk	Width of clear zone (mm.) on—	
	Mueller Hinton medium (Difco)	Trypticase Soy agar (BBL)
	Mm.	*Mm.*
0.1 milligram	12	7-8
0.25 milligram	13-15	10
0.5 milligram	15-16	12-13

The sulfonamide-sensitivity test in which filter paper disks containing drugs were employed could be much more conveniently performed under field conditions if petri plates were replaced by test tubes. Therefore, the buffered and alkalized standard furunculosis medium, Mueller Hinton medium (Difco), and Trypticase Soy agar (BBL) were used as agar slants. Media were inoculated by making longitudinal streaks with a loop containing a suspension of *A. salmonicida*, and disks were deposited in the center of the slant. Excellent clear zones appeared (fig. 2) with sulfa-sensitive strains.

Another approach to the performance of the sulfonamide-sensitivity test under simulated field conditions was made with agar media containing sodium sulfadiazine added to the buffered and alkalized standard furunculosis medium. These solutions were sterilized by filtration or were incorporated in the medium before sterilization in the autoclave. In all cases, clear differences were noticed in the growth of sulfonamide-sensitive and sulfonamide-resistant strains of *A. salmonicida*. The optimum concentrations of sodium sulfadiazine in the medium were 1:500 and 1:1,000.

It is expected that extensive field trials based on the results presented here will permit selection

[5] Ayerst Laboratories.
[6] A mixture of sulfamerazine, sulfamethazine, and sulfadiazine.

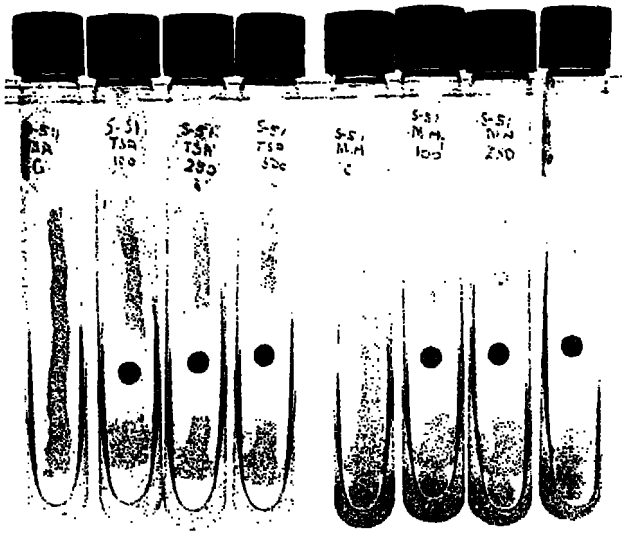

FIGURE 2.—Sulfonamide-sensitivity test performed with a sensitive culture of *A. salmonicida* using Trypticase Soy agar (left) and Mueller Hinton agar (right). Laboratory-prepared disks contained 0.1, 0.25, and 0.5 milligram of sodium sulfadiazine.

of the most convenient method for the determination of the sensitivity of fish-pathogenic bacteria to sulfonamides, antibiotics, and perhaps, to other therapeutic agents.

DISCUSSION

Micro-organisms appear to be capable of developing resistance to the majority of drugs which are employed clinically. In view of the cross-resistance shown between different groups of drugs, it is obviously important that the clinician should have information as to which groups of drugs may be usefully tried in infections with resistant strains (Work and Work, 1948).

It has been found, as could be expected, that with the wide use of sulfonamides for treatment of fish diseases, there is an increasing number of outbreaks of furunculosis which are refractory to treatment with these drugs. Since "once resistance is highly developed it is apparently permanent" (Northey 1948), it is increasingly important to have means of rapid and reliable determination of such resistance.

In this paper, observations are described on the treatment of experimentally induced furunculosis employing strains of trout which are susceptible or resistant to this disease. The outbreaks were caused by strains of the pathogen which were found to be either sensitive or resistant to sulfonamides. When it was found that the disease was refractory to treatment with sulfonamides, chloramphenicol was used with good results (tables 1 to 3). Oxytetracycline (terramycin), which is also effective in the treatment of furunculosis and ulcer disease (Snieszko et al., 1952), was not used in this investigation because strains of *A. salmonicida* resistant to this antibiotic have recently been isolated.

It is evident from the results described here that in addition to chloramphenicol and oxytetracycline other antibiotics and sulfonamides arrest the growth of *A. salmonicida* in vitro. The results of these tests cannot be applied indiscriminately for therapeutic use. While some of the sulfonamides gave promising results in vitro, their use in the treatment of fish diseases may be limited by their toxicity to some species of fish or by the arresting of the fish's growth during treatment. Other drugs may not be absorbed from the intestinal tract and are therefore useless in systemic infections by micro-organisms sensitive to them in vitro (Johnson and Brice, 1953; Snieszko and Wood, 1955; Snieszko and Friddle, 1951; Snieszko and Griffin, 1955). It is interesting that chlortetracycline (aureomycin) which has been found to be effective in vitro against *A. salmonicida* and *Hemophilus piscium* is entirely ineffective in the treatment of trout suffering from furunculosis and ulcer disease (Snieszko, Griffin, and Friddle, 1952). Therefore, the in-vitro tests, while very convenient because of the speed and ease with which they can be performed, must be supplemented by exact treatment trials with several species of fishes, before the therapeutic value of drugs can be determined.

The greatest value of the in-vitro test is in the determination of the acquired drug resistance of the pathogen concerned. This can be explained best by using an example. It is known that furunculosis usually can be effectively treated with sulfonamides. It has also been shown in this paper that the growth of sensitive strains of *A. salmonicida* can be arrested in vitro with these drugs. Therefore, if it is found that a particular strain of *A. salmonicida* is resistant to sulfonamides in vitro, an outbreak of furunculosis caused by that strain will most likely be refractory to a treatment with sulfonamides. It does not mean, however, that if the organism is found to be sensitive to a certain drug in vitro, the treatment with that drug of fish infected with such an organism will also be effec-

tive. The experience with chlortetracycline previously cited may serve as an example. It is well to remember the limitations of the in-vitro test for a correct interpretation of the results.

The results of our studies show that in the in-vitro test sulfadiazine, sulfamerazine, and sulfisoxazole, and probably other sulfonamides, can be used equally well to determine sulfonamide sensitivity of *A. salmonicida*. In the therapeutic and feeding experiments, however, sulfadiazine has been found to be more toxic to the fish than has sulfamerazine, and sulfisoxazole has been found to be free from any detectable untoward effects even to brown trout (Snieszko and Wood, 1955). Unfortunately, the experiment on the use of sulfisoxazole for the treatment of furunculosis was unsuccessful because the strains of *A. salmonicida* isolated from the outbreak were resistant to sulfonamides [7] (tables 1 to 3).

Testing of the sensitivity of *A. salmonicida* to sulfonamides and antibiotics can be carried out in several ways. The most practical method seems to be the use of drug-containing, filter-paper disks. Selection of a proper medium is very important when testing the sensitivity of the organism to sulfonamides. Trypticase Soy agar, special furunculosis agar medium buffered and alkalized, and Mueller Hinton agar, are especially recommended. In the first two media, the brown pigment so characteristic of *A. salmonicida* is produced within 48 hours and the paraphenylenediamine test of Griffin (1952) can be performed as soon as growth is visible to the unaided eye. With the first two media, disks should contain 0.5 milligram of the drug; with Mueller Hinton medium, only 0.1 milligram. The test can be equally well performed using either petri plates or agar slants—the latter are more suitable for fieldwork. Also media in which sulfonamides have been incorporated may be used provided that the control medium is free of sulfonamides and that both sulfonamide-resistant and sulfonamide-sensitive strains of *A. salmonicida* are available for controls.

RECOMMENDATIONS

On the basis of the results reported in this paper and past experience in the treatment of furunculosis, we wish to make the following recommendations:

1. As soon as a disease suspected to be furunculosis breaks out, start treatment with sulfonamides. Sulfamerazine is still considered the drug of choice, but sulfamethazine or a combination of sulfamerazine with sulfaguanidine can also be used (Snieszko 1954a). Sulfisoxazole is a drug of great promise, but it still has to be evaluated experimentally.

2. The diagnosis of furunculosis should be confirmed as soon as possible by bacteriological examination. This should include determination in vitro of the sensitivity of the pathogen to the drug.

3. The dosage of sulfonamides is 8 to 10 grams per 100 pounds of fish per day. Treatment should last for 10 to 20 days and should result in a complete stoppage of mortalities due to furunculosis. Recurrences are particularly likely in disease-susceptible strains of trout.

4. Sulfonamides should never be used at lower levels or treatments repeated at short intervals. Such practice is the surest way to produce sulfa-resistant strains of the pathogen.

5. If the response to the treatment with sulfonamides is not rapid, if recent experience has shown that treatment with sulfonamides is not effective, or if the results of laboratory examination show that the organism is sulfa-resistant but sensitive to oxytetracycline or chloramphenicol, either antibiotic should be used at a rate of 2.5 to 3.0 grams (of the antibiotic activity) per 100 pounds of fish per day. Chloramphenicol is somewhat better than oxytetracycline.

6. One should never rely on any drug for a permanent control of furunculosis, or of any other disease. There is no drug in existence which would permit elimination of any animal or plant disease. The best a drug can do is to reduce losses temporarily. Long-lasting control of furunculosis, or any other infectious disease, is only possible by elimination of the source of infection, good sanitation, and introduction or development by selective breeding, of a strain of fish with greater disease resistance. Good hatchery practices, avoidance of crowding, and balanced nutrition are very important factors in keeping fish healthy.

SUMMARY

Therapeutic studies were performed with three sulfonamides and one antibiotic (chloramphenicol) with two strains of fingerling brook trout—one

[7] One experiment of this series with sulfisoxazole was performed at Leetown in 1955 by Bo Svenonius, a visiting fishery biologist from Sweden.

susceptible to furunculosis; the other resistant to this disease—and one strain of brown trout. Since it was found that the disease was caused by a sulfa-resistant type of the pathogen, only the treatment with the antibiotic was effective.

Testing for drug resistance has shown that many strains of *A. salmonicida* were resistant to sulfonamides, but sensitive to antibiotics. Therefore, studies were made in order to develop a rapid and reliable field method for the determination of drug sensitivity of *A. salmonicida*.

Of all media tested, only the Trypticase Soy agar and the modified Leetown standard furunculosis medium can be used for the determination of sensitivity of *A. salmonicida* to sulfonamides and antibiotics and at the same time for the rapid presumptive test of Griffin. Mueller Hinton agar is excellent for the determination of drug sensitivity, but not for the presumptive test of Griffin involving the production of brown pigment.

A method of the determination of drug sensitivity employing media in the form of agar slants and single disks containing the drug is recommended for field use.

A revised and up-to-date method of treatment and control of furunculosis is described.

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