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Title: IN VITRO AND IN VIVO EVALUATION OF ANTIMICROBIALS AGAINST BACTERIAL FISH PATHOGENS

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A series of antimicrobial drug screening procedures were performed using selected bacterial fish pathogens. Yersinia ruckeri (enteric redmouth), Aeromonas salmonicida (furunculosis), Vibrio anguillarum (vibriosis), Mycobacterium sp. (fish mycobacteriosis) and bacterial kidney disease, were isolated from fish, pure-cultured, and tested for sensitivities to aminoglycosides, sulfonamides, anti-tuberculars and other antibiotics. In vitro measurements of sensitivity included Kirby-Bauer antimicrobial disc sensitivity zone of inhibition and minimal inhibitory concentration.

In vivo measurements were performed in juvenile coho salmon (Oncorhynchus kisutch) challenged with Y. ruckeri and two antimicrobials which were active in vitro. Sulfamethoxazole-trimethoprim (SMX-TMP) demonstrated a significant reduction in mortality at intraperitoneal (ip) doses of 5 mg/kg SMX and 1 mg/kg TMP daily for 11 days post ip bacterial challenge. A further reduction in mortality was demonstrated at 25 mg/kg SMX and

5 mg/kg TMP. Tobramycin (TBM), which demonstrated in vitro activity against Y. ruckeri, was toxic, especially to the kidney, at subtherapeutic doses. Twelve daily doses of TBM of 5 mg/kg produced cumulative mortality of 50 and 100 per cent at 17 and 22 days, respectively. This mortality was significantly higher than that seen in fish challenged with Y. ruckeri only (4.6×10^7 organisms/20 g fish). The highest mortality was demonstrated in fish receiving both TBM and Y. ruckeri.

The use of aminoglycoside antibiotics for treatment of fish diseases appears to be contraindicated due to their toxicity. Further in vitro and in vivo testing of developed antimicrobial agents against fish bacterial pathogens is proposed.

In Vitro and in Vivo Evaluation of Antimicrobials Against Bacterial Fish Pathogens

by

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Typed by researcher for Steven Richard Schneider

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INTRODUCTION

The increased use of antimicrobial agents to combat bacterial diseases in fish has not been paralled by increased research into the efficacy of these agents against the diseases for which they are employed. Oxytetracycline and sulfamerazine, currently the antimicrobial agents approved for use in the United States, do not provide a wide enough spectrum and/or are ineffective against a variety of bacterial fish pathogens. As the use of these two drugs continues, resistant strains of bacteria continue to emerge. Other antimicrobial drugs are being tested and may soon be registered (Meyer and Schnick, 1976; Schnick and Meyer, 1979).

The five bacterial fish pathogens chosen for this study constitute a representative cross-section of organisms that cause economically significant morbidity and mortality in the Pacific Northwest. Aeromonas salmonicida, the causative agent of fish furunculosis, has been widespread throughout Europe and North America in this century. Sulfonamides had been effective in treatment of furunculosis, but their increased usage has produced resistant strains.[1] Other antimicrobials have been tried with varying degrees of success (Herman, 1968). Vibrio anguillarum,

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[1]Personal communication, J. L. Fryer, Oregon State University.

the causative agent of vibriosis in fish, remains a widespread bacterial pathogen despite intense research with various immunological defense mechanisms (Fryer, Nelson and Garrison, 1972). As with A. salmonicida, resistant strains have emerged with the continuous use of sulfonamides.[2] Yersinia ruckeri, the causative agent of enteric redmouth in fish, has recently been extending its geographic range (Bullock, Stuckey and Shotts, 1978). The first epizootic in Canada occurred in 1973, where a combination of sulfamerazine, chloramphenicol and oxytetracycline proved effective (Wobeser, 1973). Bacterial kidney disease (Corynebacterium salmonius sp. nov., Sanders and Fryer, 1978) produces an intracellular chronic bacteremia, making it the most difficult of the bacterial fish diseases to treat. Numerous attempts at antimicrobial therapy have shown few successes, the most notable with erythromycin (Bullock, Stuckey and Wolf, 1975). Hoskins (1976) recommended destruction of infected stock and tank disinfection rather than attempting antimicrobial therapy. Mycobacterium sp., the causative agent of fish mycobacteriosis, is a frequent problem in fresh water aquaria (Sniesko, 1978). Chemotherapeutic agents which are active against human tuberculosis (Mycobacterium tuberculosis) have shown some activity (Conroy and Solarolo, 1965) as have sulfonamide combinations (Kelly, 1976). Thus, these five organisms continue to be a biologic and economic

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[2]Personal communication, J. L. Fryer, Oregon State University.

problem for the fisheries industry.

The goal of this study was to determine the activity of selected antimicrobials against the five pathogens discussed above, including agents which have not been evaluated previously for fish diseases. Of the agents evaluated, two which showed high in vitro activity were evaluated for efficacy in vivo against Y. ruckeri, strain HI-70x. The toxicity of one of these agents, tobramycin, was evaluated in detail.

MATERIALS AND METHODS

Sources of Bacterial Isolates and Antimicrobials

Bacterial strains were obtained from the Fish Disease Laboratory, Oregon State University, Corvallis. *Aeromonas salmonicida* (strain SS-70) was originally isolated in 1970 from the South Santiam River, Oregon, from a chinook salmon (*Oncorhynchus tshawytscha*). *Vibrio anguillarum* (LS-1-74) was originally isolated in 1974 from Lint Slough near Newport, Oregon from a chinook salmon. *Yersinia ruckeri* (HI-70) was originally isolated in 1970 in Hagerman, Idaho from a rainbow trout (*Salmo gairdneri*) and a second strain (TSI-78) was isolated in 1978 from a rainbow trout at Thousand Springs, Idaho. The bacterial kidney disease organism (BKD), strain 1-MK-78-KD, was isolated in 1978 from a chinook salmon at the McKenzie River Hatchery, Oregon. *Mycobacterium* sp. was isolated in 1978 from the kidney of a silver white cloud fish (*Tanichthys albonubes*) in a fresh water aquarium.

The sources of antimicrobial agents and discs were: amikacin (Bristol laboratories); capreomycin, succinyl-sulfathiazole, sulfadiazine, sulfaguanidine, sulfamerazine, sulfanilamide and sulfaniyl-acetamide (School of Pharmacy, Oregon State University); chloramphenicol, erythromycin, kanamycin, neomycin, novobiocin, penicillin G, streptomycin and tetracycline

(Difco Dispens-o-Disc Sensitivity discs); cycloserine and tobramycin (Eli Lilly laboratories); ethambutol (American Cyanamide [Lederle] laboratories); erythromycin, unformulated crystals (Abbott laboratories); gentamicin (Schering laboratories); rifampin (Ciba-Geigy and Dow Chemical laboratories); sulfamethoxazole and trimethoprim (Burroughs-Wellcome laboratories).

Determination of Viable Count versus Optical Density

Determination of viable count versus optical density for A. salmonicida, V. anguillarum and Y. ruckeri was determined by the method of O'Leary (1977). Side arm flasks with 30 ml of Trypticase Soy Broth^{R} (TSB; Difco) were inoculated with 1.0 ml of an overnight broth culture and incubated at 25°C. At periodic intervals, plate counts were performed in duplicate and the optical density at 525 nm was measured in a Bausch and Lomb Spectronic 20 colorimeter using TSB as a reference (Figure 1).

Disc Sensitivities

Zones of inhibition to antimicrobial discs were determined by the method of Bauer, Kirby et al. (1966). Broth cultures of A. salmonicida, V. anguillarum and Y. ruckeri were grown to standardized turbidity (A = .43 at 525 nm). Petri plates (9 cm) were filled with Mueller-Hinton agar adjusted to pH = 7.3 with 0.1 M KOH and inoculated with 0.2 ml of broth cultrue. Antimicrobial discs (6 mm) were placed four to a plate, four discs for each antimicrobial, and inhibitory zones recorded at 36 h. Mycobacterium sp. was

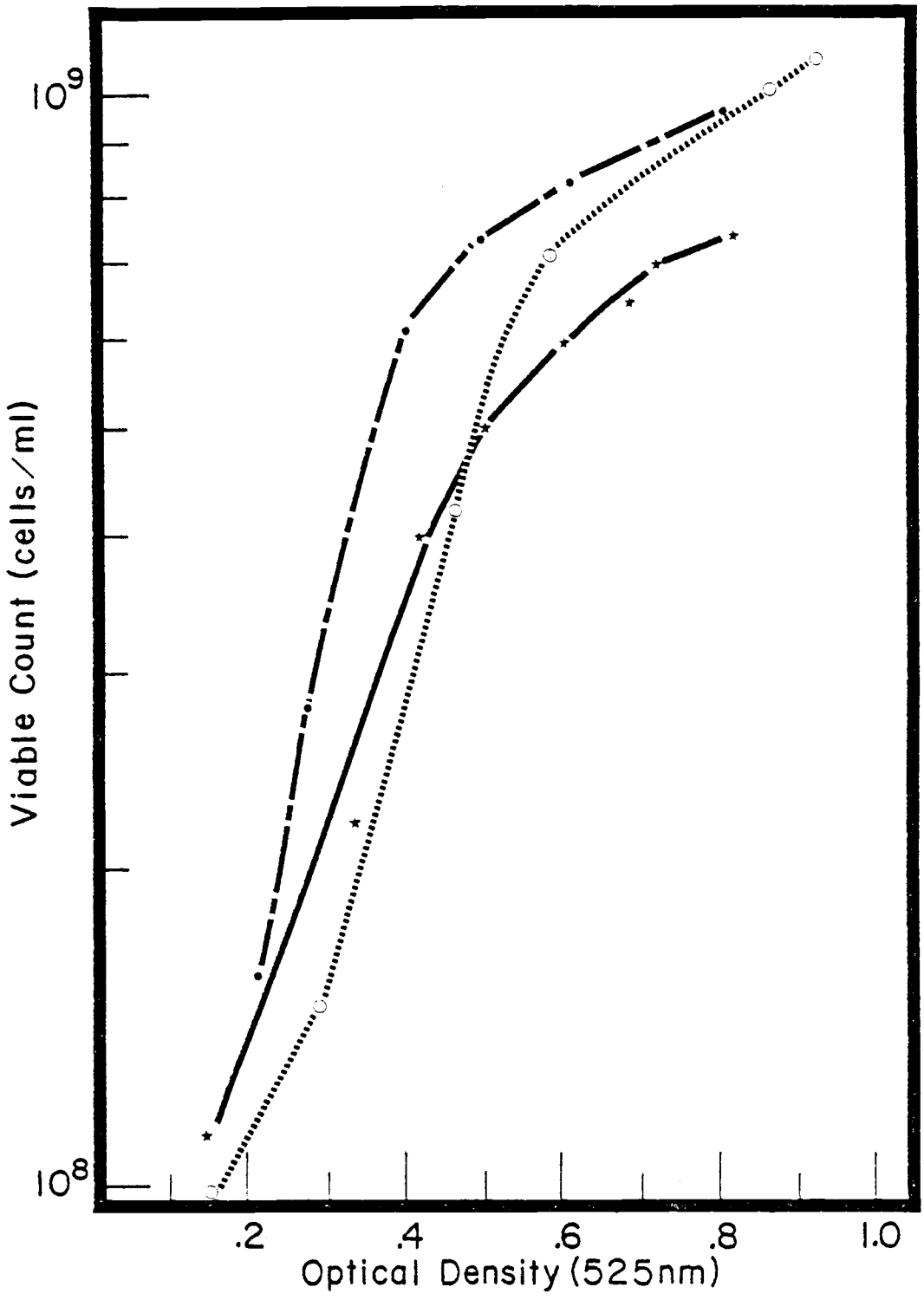

Figure 1. Determination of viable count versus optical density. ○ = A. salmonicida; ● = V. anguillarum; ★ = Y. ruckeri.

grown on modified Petragnani medium (Difco)[3] on slants in 15 x 100 mm test tubes. Each slant contained 10 ml of media and was inoculated with 3×10^9 organisms in 0.1 ml, as determined by optical density at 525 nm. One antimicrobial disc was placed per slant, in duplicate, and inhibitory zones read in seven days.

Minimal Inhibitory Concentration (MIC)

Minimal inhibitory concentrations (MIC's) were determined by the method of Washington and Barry (1974) for A. salmonicida, V. anguillarum and both strains of Y. ruckeri. Overnight broth cultures were diluted to 5×10^6 organisms/ml, 0.1 ml of this added to 4.8 ml TSB and 0.1 ml of antimicrobial solution. Three antimicrobial concentrations were chosen per drug as determined from MIC's for human Gram negative bacterial pathogens (Weinstein, 1975). Absorbance at 525 nm was measured for the test cultures at 24 and 48 h.

Minimal inhibitory concentrations for BKD were determined by incorporation of antimicrobials into the agar medium, since broth culture of the organism proved unsuccessful. Kidney Disease Medium 2 was prepared by the method of Evelyn (1977) and added 2.4 ml in 5 x 50 mm test tubes with 0.1 ml antimicrobial solution and slanted. Pure cultures of BKD confirmed by Gram stain (positive), lack of motility and agglutination of specific antiserum[4],

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[3]Modified by the method of K. Pilcher, Oregon State University.

[4]Bacterial kidney disease antiserum obtained from the Fish Disease Laboratory, Oregon State University, as prepared by the method of Banowetz (1974).

were diluted 1:100 with sterile phosphate buffered saline (PBS)-peptone and 0.025 ml added to each experimental slant tube. After 30 days, the tubes were examined for the presence or absence of growth.

Production of Y. ruckeri (HI-70x)

Phosphate buffered saline was added to lyophilized vials of Y. ruckeri (HI-70) and the resulting suspension was streaked for isolation on Trypticase Soy Agar^{R} (TSA; Difco). Six colonies were identified as Y. ruckeri by Gram stain (negative), motility at 25°C, but not at 37°C, lack of formation of oxidase and agglutination by specific antiserum[5]. Injection of 0.1 ml of a TSB culture of Y. ruckeri in fish was fatal in four days. The culture was passed through a series of six transfers in juvenile coho salmon (O. kisutch) by ip injection of a suspension of kidney tissue from the fish infected in the previous manner. Kidney tissue from the last fish in the series was cultured on TSA with resulting colonies positively identified as Y. ruckeri. Centrifugation of TSB culture grown from these colonies produced packed cells which were mixed with sterile bovine serum, the resulting suspension lyophilized and labeled HI-70x.

Antimicrobial Efficacy Studies

Experiments were conducted in duplicate and were similar to

[5]HI-70 antiserum obtained from the Fish Disease Laboratory, Oregon State University, as prepared by the method of O'Leary (1977).

those employed with A. salmonicida (Groberg et al., 1978). Juvenile coho salmon were tested at 18°C, 25 fish per tank, with varying antimicrobial concentrations both with and without bacterial challenge. All fish were between 15 and 25 g and were provided by the Fish Disease Laboratory, Oregon State University, Corvallis.

Experimental fish were divided into five and seven duplicate groups of 25 for efficacy studies of tobramycin (TBM) and sulfamethoxazole-trimethoprim (SMX-TMP), respectively (Table 1). The five groups common to both were positive control, low dose, high dose control and negative control. Two additional groups utilized in the SMX-TMP study were prophylactic dose and low dose control.

After 24 hours of adaptation to the 18°C tanks, the fish were infected by ip injection of 0.1 ml of centrifuged and washed broth cultures of Y. ruckeri. Challenged fish in the TBM study received 4.1×10^7 organisms/fish and those in the SMX-TMP study received 7.5×10^7 organisms/fish. Fish were injected after anesthetization in a 50 mg/l benzocaine bath. Appropriate control groups received identical sham challenge of 0.1 ml PBS. Antimicrobial treatment was begun at 0 h-(SMX-TMP: challenged prophylactic dose), 12 h-(TBM: challenged and control groups for low and high dose), and 24 h-(TBM: challenged low and high dose, control high dose) post-challenge by ip injection. Surviving fish were injected daily with the group's respective antimicrobial dosage.

Tobramycin for injection was prepared by dilution in sterile water from unformulated TBM crystals (powder) obtained from Eli

Table 1. Dosage and initial time of injection of antimicrobial for tobramycin(TBM)- and sulfamethoxazole-trimethoprim(SMX-TMP)-treated fish.

Group	Bacterial Challenge	Drug Challenge Dosage (mg/kg) and Time of Initial Dosage post-Bacterial or Sham Challenge (hours)	
		TBM	SMX + TMP
Positive Control	+	0	0
Negative Control	-	0	0
Low Dose	+	2 (24 h)	5 (12 h) 1
High Dose	+	5 (24 h)	25 (12 h) 5
High Dose Control	-	5 (24 h)	25 (12 h) 5
Low Dose Control	-		5 (12 h) 1
Prophylactic Dose	+		25 (0 h) 5

Lilly laboratories. Solutions were prepared at 0.5 mg/ml and 1.25 mg/ml, giving 2 mg/kg and 5 mg/kg dosages per fish, respectively, when 0.1 ml was administered to 20 g fish. These dosages were chosen above and below standard human dosage of 3 mg/kg (Weinstein, 1975).

Sulfamethoxazole-trimethoprim for injection was prepared by a 1:1 dilution in sterile water and propylene glycol (v/v) from unformulated SMX and TMP crystals (powder) obtained from Burroughs-Wellcome laboratories. Sulfamethoxazole (125 mg) and TMP (25 mg) were added to a 25 ml volumetric flask, diluted in 12.5 ml of sterile propylene glycol, to which 12.5 ml sterile water was added. This solution, 5 mg/ml SMX and 1 mg/ml TMP, when injected into a 20 g fish, gave a SMX-TMP dosage of 25 and 5 mg/kg, respectively. Dilution of 5 ml of the above solution to 25 ml gave 1 mg/ml SMX and 0.2 mg/ml TMP, which when injected into a 20 g fish, gave a SMX-TMP dosage of 5 and 1 mg/kg, respectively. These dosages were based on trial dosages of SMX-TMP which proved efficacious.

Infections of experimental fish were confirmed at necropsy by streaking sections of kidney onto TSA. Yersinia ruckeri was confirmed by methods previously mentioned.

Tobramycin Toxicity Studies

Determination of TBM toxicity was performed by ip injection in coho salmon of aqueous suspensions of the drug. Six groups of

20 g fish (5 per group) were given the following dosages: 7.5, 15 or 30 mg/kg/d or 7.5, 15 or 30 mg/kg/2d. Fish surviving through day five were killed by immersion in 50 mg/l benzocaine bath and sectioned for histological examination. Brain, kidney, liver, muscle and skin sections were fixed in Bouin's fluid, sectioned at 6 μm, stained with hematoxylin and eosin and examined by light microscopy.

RESULTS

IN VITRO

Disc Sensitivity

Diameters of zones of inhibition for antimicrobials against A. salmonicida, V. anguillarum and Y. ruckeri are summarized in Table 2. Susceptibility to antimicrobials was generally similar across chemical classes. Among seven aminoglycosides, the order of decreasing sensitivity was gentamicin > tobramycin > kanamycin = amikacin > neomycin > streptomycin > paromomycin. Among the sulfonamides evaluated, only SMX showed significant inhibition. Sulfamerazine, the sulfonamide currently registered for use in fishes, was without significant effect. Trimethoprim and a combination of SMX-TMP demonstrated significant inhibition against V. anguillarum and Y. ruckeri (HI-70x). Trimethoprim was ineffective against A. salmonicida and SMX-TMP was ineffective against Y. ruckeri (TSI-78). Among the three anti-tubercular agents, only rifampin showed activity and only against A. salmonicida and V. anguillarum. Erythromycin was intermediately active against all except A. salmonicida, which was highly susceptible. Tetracycline, the 5-dehydroxy derivative of oxytetracycline, which is approved for use in fishes, showed activity against A. salmonicida and V. anguillarum and was intermediately active against Y. ruckeri. Of the 18 antimicrobials which demonstrated activity against Y. ruckeri,

Table 2. Zones of inhibition obtained with *A. salmonicida*, *V. anguillarum*, and *Y. ruckeri*[a]

Antimicrobial	Disc Content	Bacterium: *A. salmonicida* SS-70	*V. anguillarum* LS1-74	*Y. ruckeri* HI-70x	*Y. ruckeri* TSI-78	Interpretive Standards[b] R	I	S
amikacin	10µg	18	14	22	16	≤11	12-13	≥14
capreomycin	30µg	ϕ	ϕ	ϕ	ϕ			
chloramphenicol	30µg	39	31	23	23	≤12	13-17	≥18
cycloserine	30µg	ϕ	ϕ	ϕ	ϕ			
erythromycin	15µg	28	16	16	16	≤13	14-17	≥18
ethambutol	20µg	ϕ	ϕ	ϕ	ϕ			
gentamicin	30µg	25	22	29	26	≤12	-	≥13
kanamycin	30µg	26	18	28	25	≤13	14-17	≥18
neomycin	30µg	24	20	25	21	≤12	13-16	≥17
novobiocin	30µg	10	21	8	10			
paromomycin	30µg	18	14	18	19			
penicillin G	10 U	ϕ	ϕ	ϕ	ϕ	≤11	12-21	≥22
rifampin (Ciba)	10µg	19	19	ϕ	ϕ			
rifampin (Dow)	10µg	21	20	8	8			
streptomycin	10µg	13	16	21	20	≤11	12-14	≥15
succinyl-sulfathiazole	250µg	ϕ	ϕ	ϕ	ϕ	≤12	13-16	≥17
sulfadiazine	250µg	ϕ	ϕ	10	7	≤12	13-16	≥17
sulfaguanidine	250µg	ϕ	ϕ	ϕ	ϕ	≤12	13-16	≥17
sulfamerazine	250µg	ϕ	ϕ	13	7	≤12	13-16	≥17
sulfamethazine	250µg	ϕ	ϕ	9	7	≤12	13-16	≥17
sulfamethoxazole	250µg	21	16	21	8	≤12	13-16	≥17
sulfamethoxazole-trimethoprim[c]	25µg	27	25	34	ϕ	≤10	11-15	≥16
sulfanilamide	250µg	ϕ	ϕ	ϕ	ϕ	≤12	13-16	≥17
sulfaniyl-acetamide	250µg	ϕ	ϕ	ϕ	ϕ	≤12	13-16	≥17
tetracycline	30µg	24	22	17	15	≤14	15-18	≥19
tobramycin	30ug	24	20	27	23	≤11	12-13	≥14
trimethoprim	10µg	ϕ	35	35	38			

[a] inhibition measured in mm from a 6mm disc; ϕ = no inhibition

[b] values based on human bacterial pathogens from Barry (1977); R = resistant, I = intermediate, S = susceptible

[c] 23.75 µg SMX and 1.25 µg TMP

12 were less active against strain TSI-78 than against strain HI-70x. The most dramatic difference was demonstrated with SMX-TMP, which showed significant inhibition of strain HI-70 but was ineffective against strain TSI-78.

Among the antimicrobials tested against Mycobacterium sp., activity in decreasing order is kanamycin > rifampin > streptomycin > ethambutol > tobramycin = gentamicin > capreomycin = paromomycin. Cycloserine, amikacin and SMX-TMP were without effect.

Minimal Inhibitory Concentration

Minimal inhibitory concentrations were calculated as relative optical density (ROD) units from the following formula:

$$\text{Relative Optical Density} = \frac{OD_{control} - OD_{treated}}{OD_{control}}$$

Thus, for an antimicrobial which showed complete inhibition of growth, ROD = 1.0, and for an antimicrobial which showed no activity, ROD = 0.0. ROD was plotted as a function of log dose of antimicrobial in µg/ml by a method similar to Sabath and Matsen (1974). The data are summarized as follows: sulfonamides and trimethoprim, Figure 2; anti-tuberculars, Figure 3; tobramycin, gentamicin and amikacin, Figure 4; paromomycin and erythromycin, Figure 5.

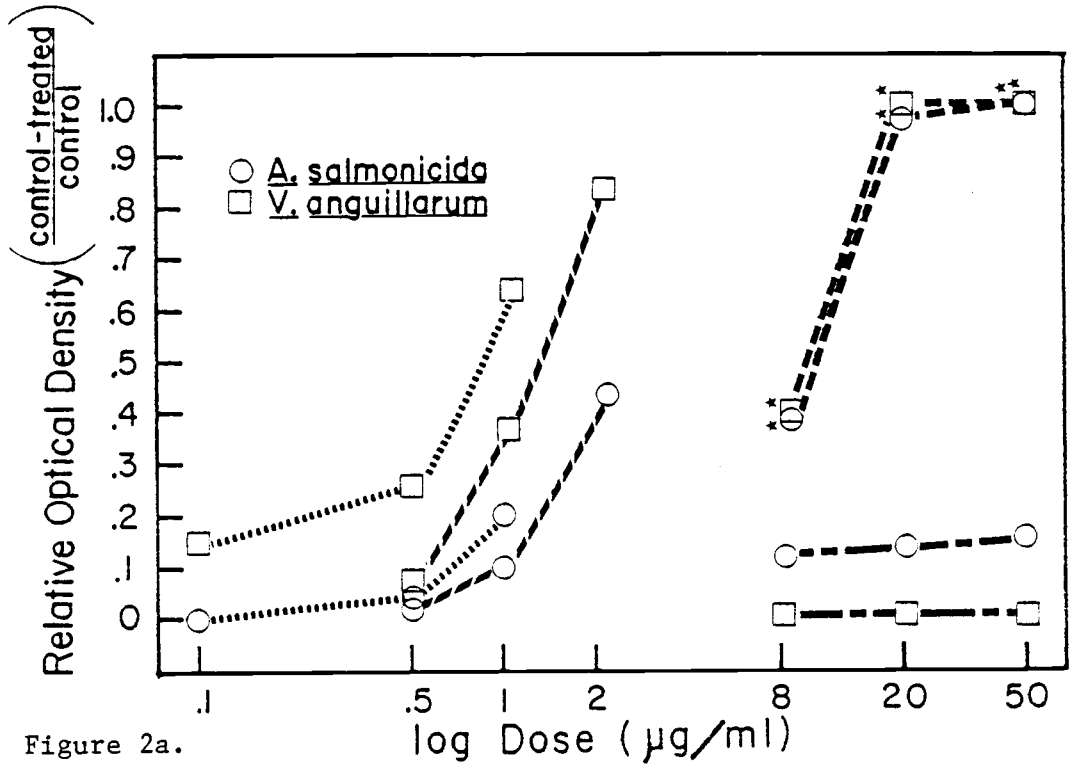

Figure 2a.

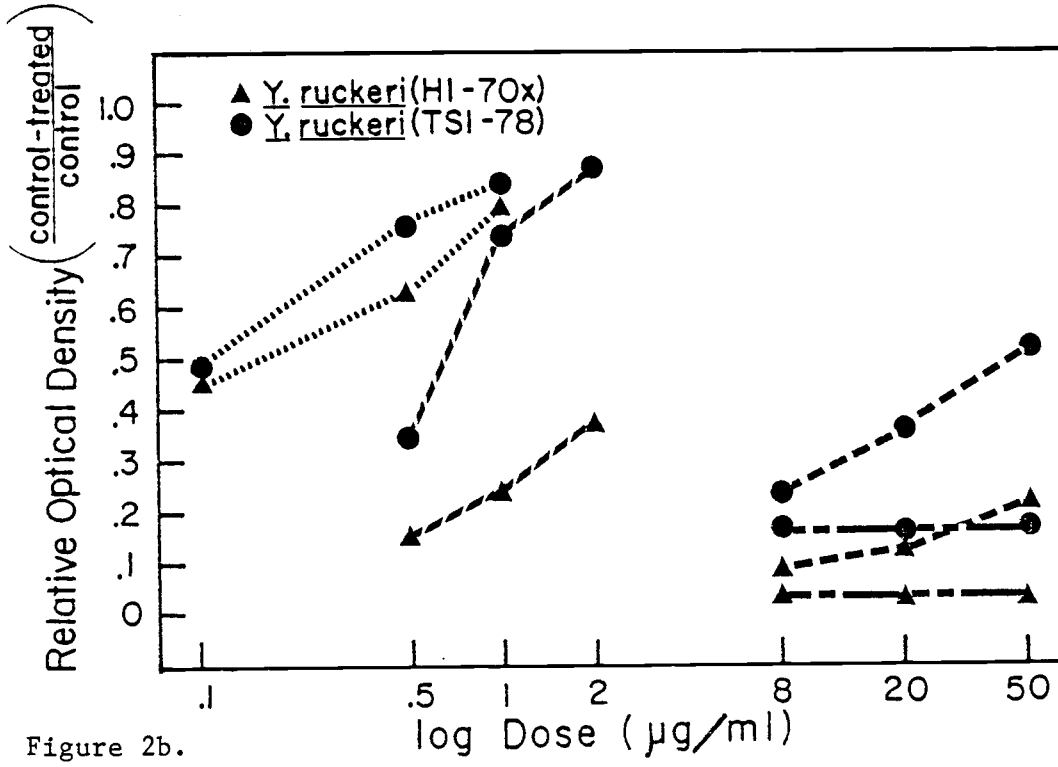

Figure 2b.

Figure 2a and 2b. MIC represented as relative optical density (525 nm) at 24 h versus log dose. ▮▮▮▮▮ = trimethoprim; ▰▰▰ = sulfamethoxazole-trimethoprim; ▬ ▬ = sulfamethoxazole; ▬▬ ▬ = sulfamerazine; ★ = inhibition remaining at 48 h.

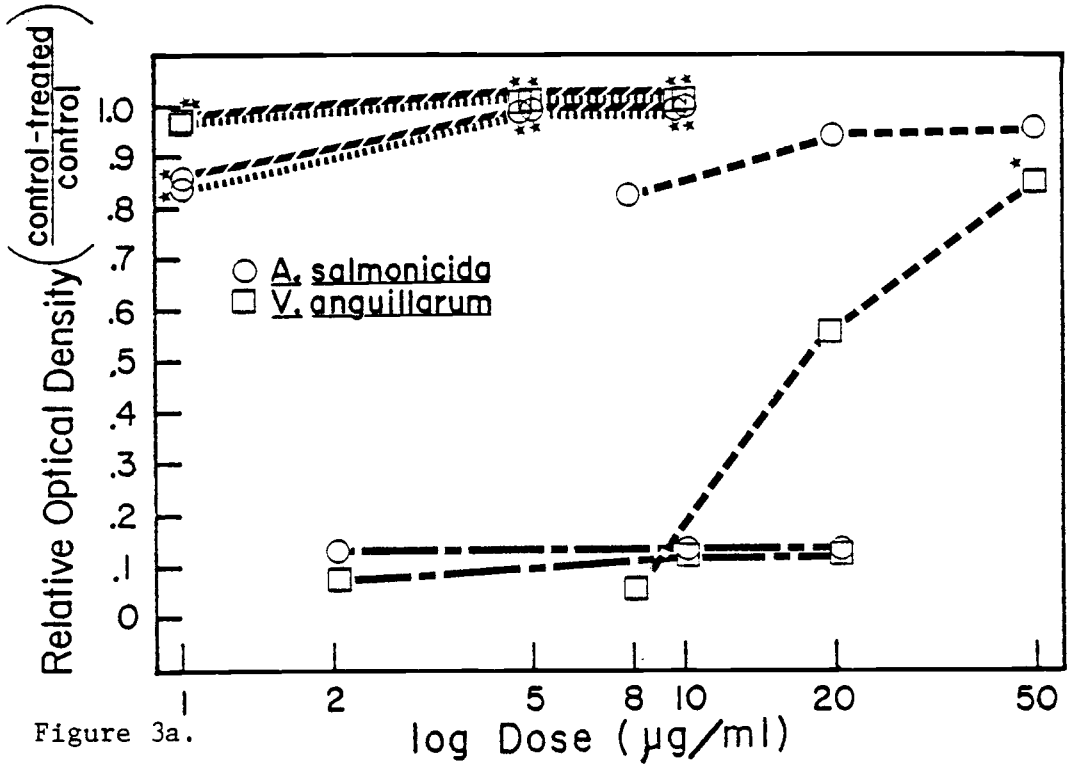

Figure 3a.

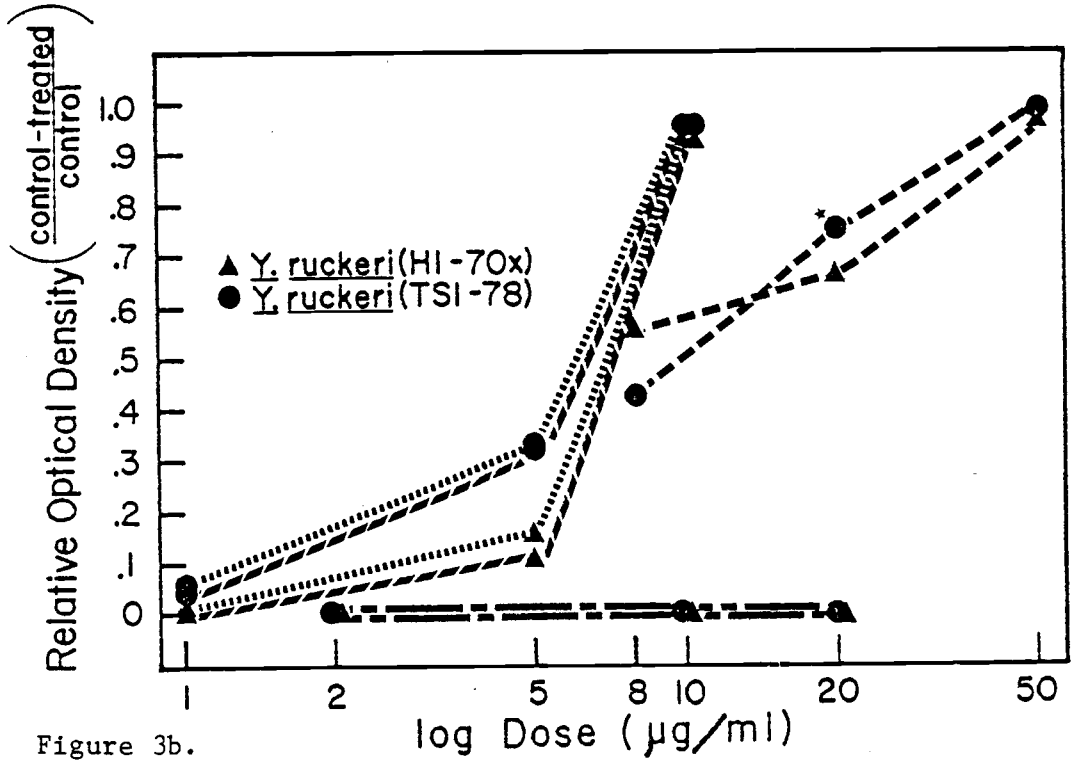

Figure 3b.

Figure 3a and 3b. MIC represented as relative optical density (525 nm) at 24 h versus log dose. ▮▮▮▮▮ = rifampin (Ciba); ▰▰ = rifampin (Dow); ▮ ▬ ▮ = cycloserine; ▬▬ ▬ ▬ = ethambutol; ★ = inhibition remaining at 48 h.

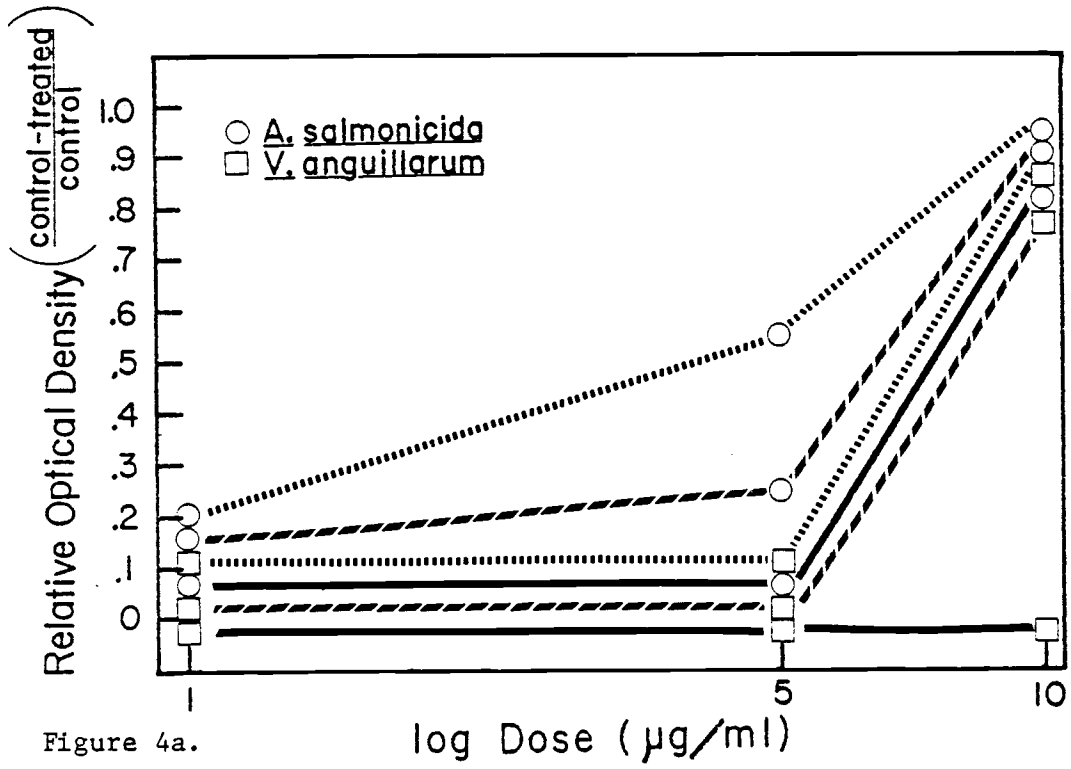

Figure 4a.

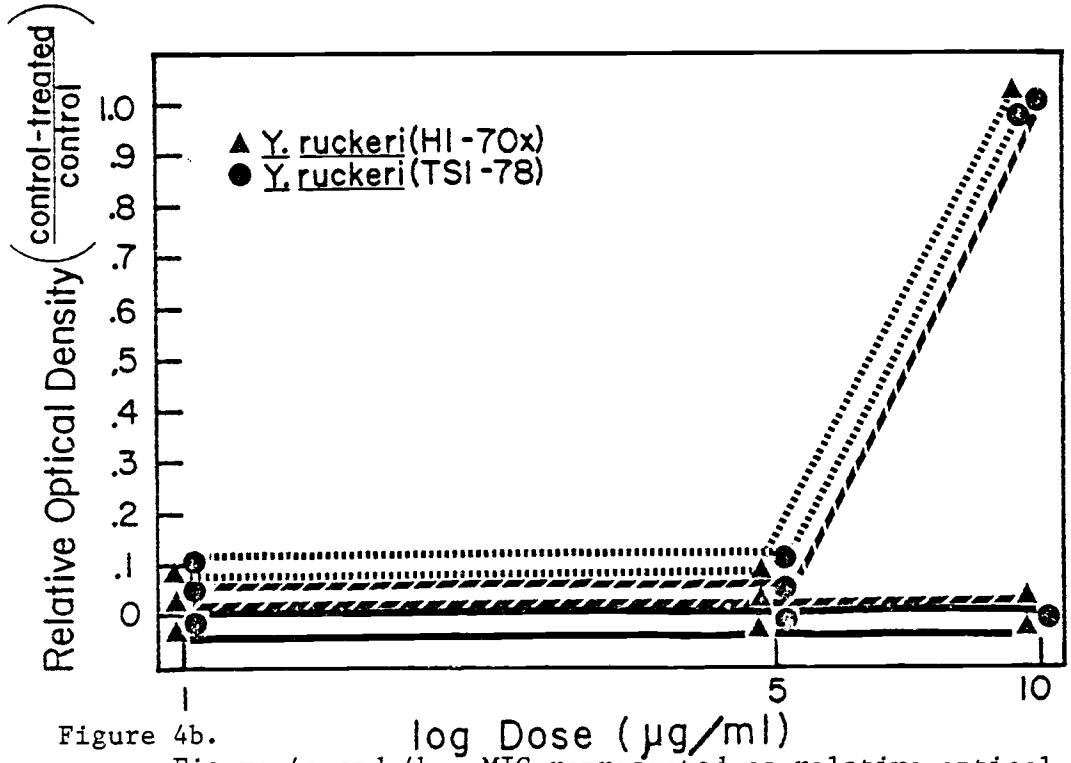

Figure 4b.

Figure 4a and 4b. MIC represented as relative optical density (525 nm) at 24 h versus log dose. ▪▪▪▪▪▪▪▪ = tobramycin; ▰▰▰▰ = gentamicin; ▬▬▬▬ = amikacin.

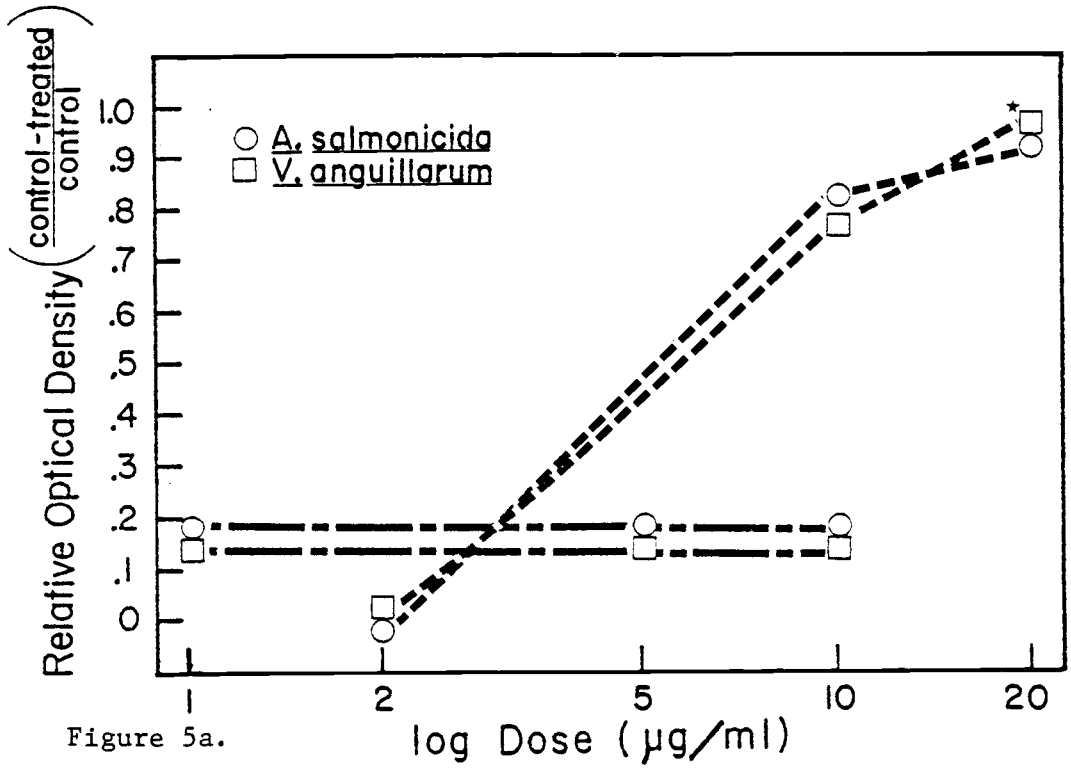

Figure 5a.

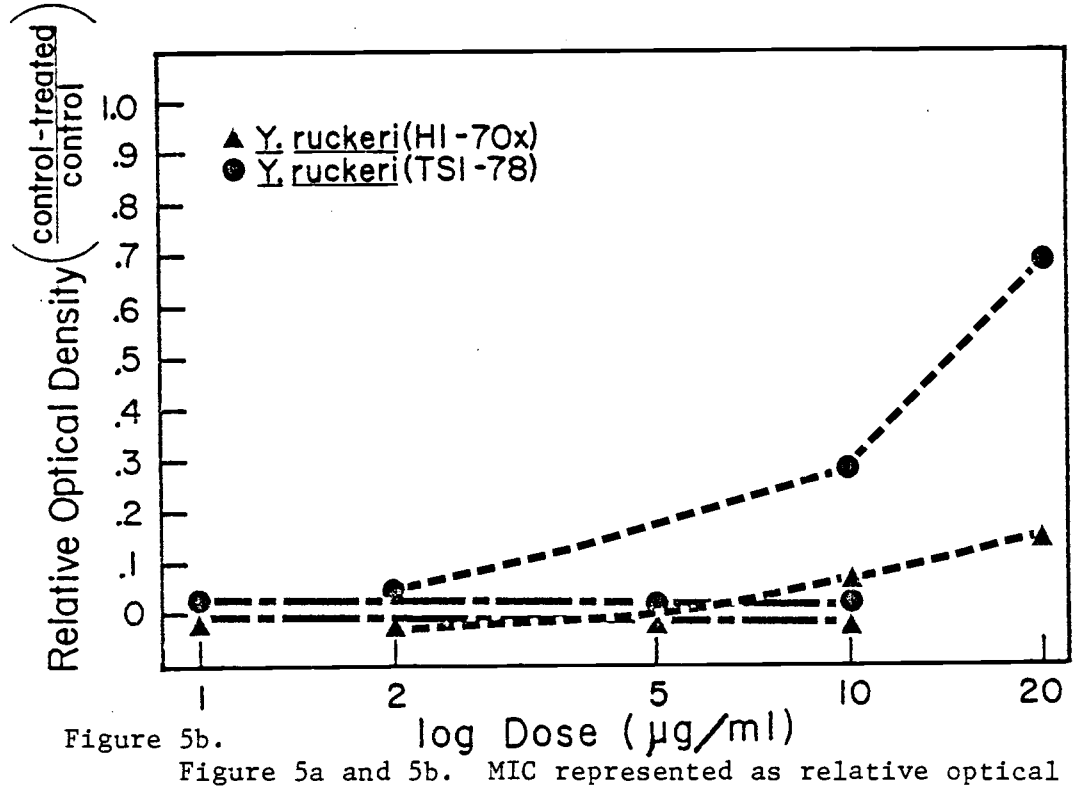

Figure 5b.

Figure 5a and 5b. MIC represented as relative optical density (525 nm) at 24 h versus log dose. ▬▬ ▬ ▬= paromomycin; ▬ ▬ ▬ ▬= erythromycin; ★ = inhibition remaining at 48 h.

Variable activities were observed when the test organisms were evaluated with the sulfonamides and TMP (Figure 2). Sulfamethoxazole demonstrated complete inhibition of A. salmonicida and V. anguillarum at 20 µg/ml, yet was only moderately inhibitory against both strains of Y. ruckeri even at 50 µg/ml. Trimethoprim demonstrated good inhibition of both strains of Y. ruckeri at 0.1 µg/ml, yet showed poor inhibition of A. salmonicida and V. anguillarum at 0.5 g/ml. Sulfamethoxazole-trimethoprim, dosed at a ratio of 19:1 (w/w), demonstrated an activity series reflecting a combination of the single agent resulst: Y. ruckeri (TSI-78) > V. anguillarum > Y. ruckeri (HI-70x) > A. salmonicida. Sulfamerazine demonstrated slight activity against Y. ruckeri (HI-70x) at 50 µg/ml, but was without activity against the other test organisms.

Among the three anti-tuberculars tested, the order of decreasing activity was rifampin > cycloserine > ethambutol (Figure 3). Rifampin demonstrated complete, lasting inhibition of A. salmonicida and V. anguillarum at 1 µg/ml. Nearly complete inhibition of both strains of Y. ruckeri was demonstrated at 10 µg/ml. Cycloserine was most active against A. salmonicida, less active against both strains of Y. ruckeri, and least active against V. anguillarum. Complete inhibition was demonstrated against all organisms at 50 µg/ml. Ethambutol was without effect at doses up to 20 µg/ml.

Among the aminoglycosides, only tobramycin demonstrated complete inhibition against all test organisms, that occurring at 10 µg/ml (Figure 4). Gentamicin demonstrated nearly complete

inhibition at 10 µg/ml for all organisms except Y. ruckeri (HI-70x). Amikacin was without effect at 10 µg/ml.

The MIC data for paromomycin and erythromycin are summarized in Figure 5. The macrolide erythromycin demonstrated complete, lasting inhibition against V. anguillarum, slightly less against A. salmonicida, little against Y. ruckeri (HI-70x) and none against Y. ruckeri (TSI-78). Paromomycin was without effect at 10 µg/ml.

The MIC data for antimicrobials tested against BKD, using an antimicrobial dilution in Kidney Disease Medium 2 (see methods), are summarized in Table 3. Among the aminoglycosides, the order of decreasing sensitivity was paromomycin > tobramycin = gentamicin > amikacin. Among the anti-tuberculars, rifampin showed complete inhibition at 5 µg/ml; cycloserine and ethambutol were without effect. Among the sulfonamides tested, similar inhibition was demonstrated for sulfamerazine and SMX at 50 and 20 µg/ml, respectively. Trimethoprim, without effect at 2 µg/ml, did contribute an effect when combined with SMX at a ratio of 2/0.1 µg/ml SMX/TMP. Erythromycin demonstrated slight activity at the highest dose tested, 40 µg/ml.

IN VIVO

Statistical Evaluation

Cumulative mortality (%) was plotted as a function of time (days) for all groups of fish (Table 1). Linear multiple regres-

Table 3. Minimal inhibitory concentration's for selected antimicrobials against bacterial kidney disease.

Antimicrobial	µg/ml				
	1	5	10	20	50
amikacin	+	+	+	-	-
gentamicin	+	+	±	-	-
paromomycin	+	±	-	-	-
tobramycin	+	+	±	-	-
rifampin (Dow)	±	-	-	-	-
rifampin (Ciba)	±	-	-	-	-

	8	20	50	100
cycloserine	+	+	+	+
sulfamerazine	+	+	-	-
sulfamethoxazole	±	-	-	-

	2	10	20	40
erythromycin	+	+	+	±
ethambutol	+	+	+	+

	0.1	0.5	1	2
trimethoprim	+	+	+	+

	0.5/.025	1/.05	2/0.1	4/0.2	20/1
sulfamethoxazole/ trimethoprim	+	±	-	-	-

+ = heavy growth
± = intermediate or light growth
- = no growth

sion equations were calculated from the discrete sets of points, utilizing day, log day, and day^{-1} as independent variables. The "best" single linear regression model was chosen separately for TMB data and for SMX-TMP data, according to R^2 values, mean square error and residuals (Neter and Wasserman, 1974). Regression lines were compared for similar slope and intercept, with p values calculated by t-test statistics.

Tobramycin Efficacy

The "best" single linear regression model for the in vivo TBM experiment was

$$\text{cumulative mortality} = b_0 + b_1 \text{time}.$$

Figure 6 contains the data for the five groups tested and includes the regression equations with R^2 values.

The calculated p values for similarity between slopes and between intercepts for selected pairs of groups are presented in Table 4.

Demonstration of Y. ruckeri infection was proven at necropsy for all mortalities from day 1-5 from kidney streaking. The presence of Y. ruckeri as demonstrated by similar methods was variable for mortalities during days 6-12: positive and negative control, 0/0; high dose control, 0/9; low dose, 7/8; high dose, 9/13.

Fish in the negative control and high dose control tanks were monitored through day 22 to investigate cumulative mortality due

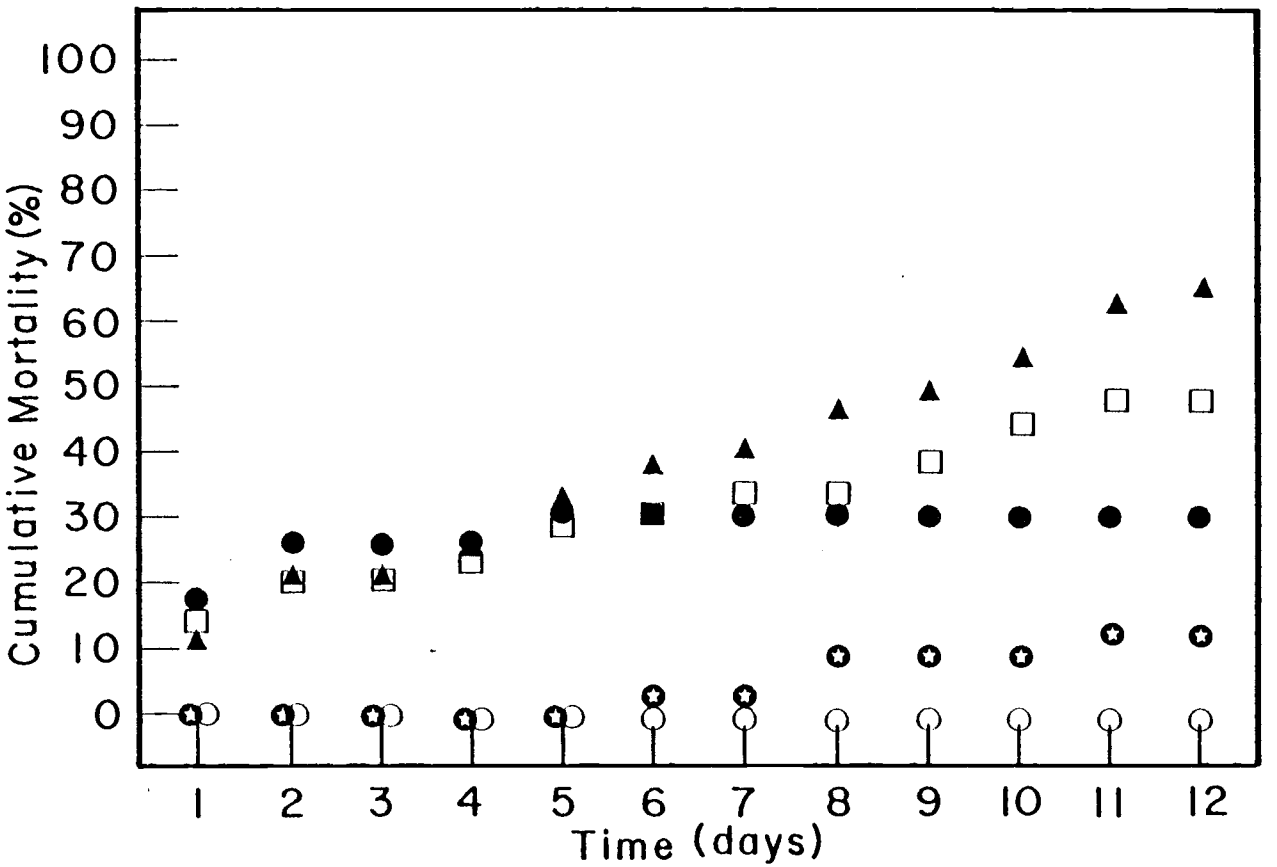

Figure 6. Mortality curves for various groups of fish in the tobramycin efficacy studies.

Group	R^2	Equation	Group	R^2	Equation
▲ = High Dose	.965	Y = 9.9 + 5.1X	✪ = High Dose Control (days 5-12)	.836	Y = -3.0 + 2.5X
□ = Low Dose	.902	Y = 11.9 + 3.4X	High Dose Control (days 5-22)	.933	Y = -2.1 + 6.2X
● = Positive Control	.400	Y = 19.4 + 1.5X	○ = Negative Control	1.000	Y = 0

Table 4. p values for similarity between slopes and between intercepts for selected pairs of regression lines from groups in the tobramycin study.

Group			
1	2	Slope	Intercept
Positive Control	Low Dose	.008	.16
Positive Control	High Dose	.0001	.07
Low Dose	High Dose	.0008	.76
High Dose	High Dose Control	.26[a]	<.00001[a]

[a]Data from days 5 to 12 (high dose) and days 5 to 22 (high dose control.

to TBM. Lethality steadily increased from 18% at day 12, to 50% at day 17 and 100% at day 22.

Examination of fish at necropsy showed striking differences between groups. Mortalities from day 1-5 were characterized by redness and inflamation around the mouth, opercula, base of the rayed fins and vent. Upon internal examination, generalized edema, hemorrhaging and inflamation were characteristic. Mortalities from the high dose control were all characterized by yellowing of the dorsal area from the brain to dorsal fin, severe discoloration of the viscera, extreme loss of kidney tissue and significant weight gain. An increase in water weight as much as 100% of body weight was evident in some fish, their ventral surfaces greatly distended due to increased abdominal fluid.

Sulfamethoxazole-Trimethoprim Efficacy

The "best" single linear regression model for the in vivo SMX-TMP experiment was

$$\text{cumulative mortality} = b_0 + b_1 \text{time}^{-1}.$$

The data for the seven groups tested including the regression equations with R^2 values are given in Figure 7.

The calculated p values for similarity between slopes and between intercepts for selected pairs of groups are given in Table 5.

Table 5. p values for similarity between slopes and between intercepts for selected pairs of regression lines from groups in the sulfamethoxazole-trimethoprim study.

Group			
1	2	Slope	Intercept
Positive Control	Low Dose	.006	<.0001
Low Dose	High Dose	.008	<.0001
High Dose	Prophylactic Dose	.0005	<.0001
Prophylactic Dose	Negative Control	.0001	.0001

Demonstration of Y. ruckeri infection was proven at necropsy for all mortalities. The pathology of these fish was similar to that described for deaths due to TBM during days 1-5.

Fish in the negative control, low and high dose control tanks

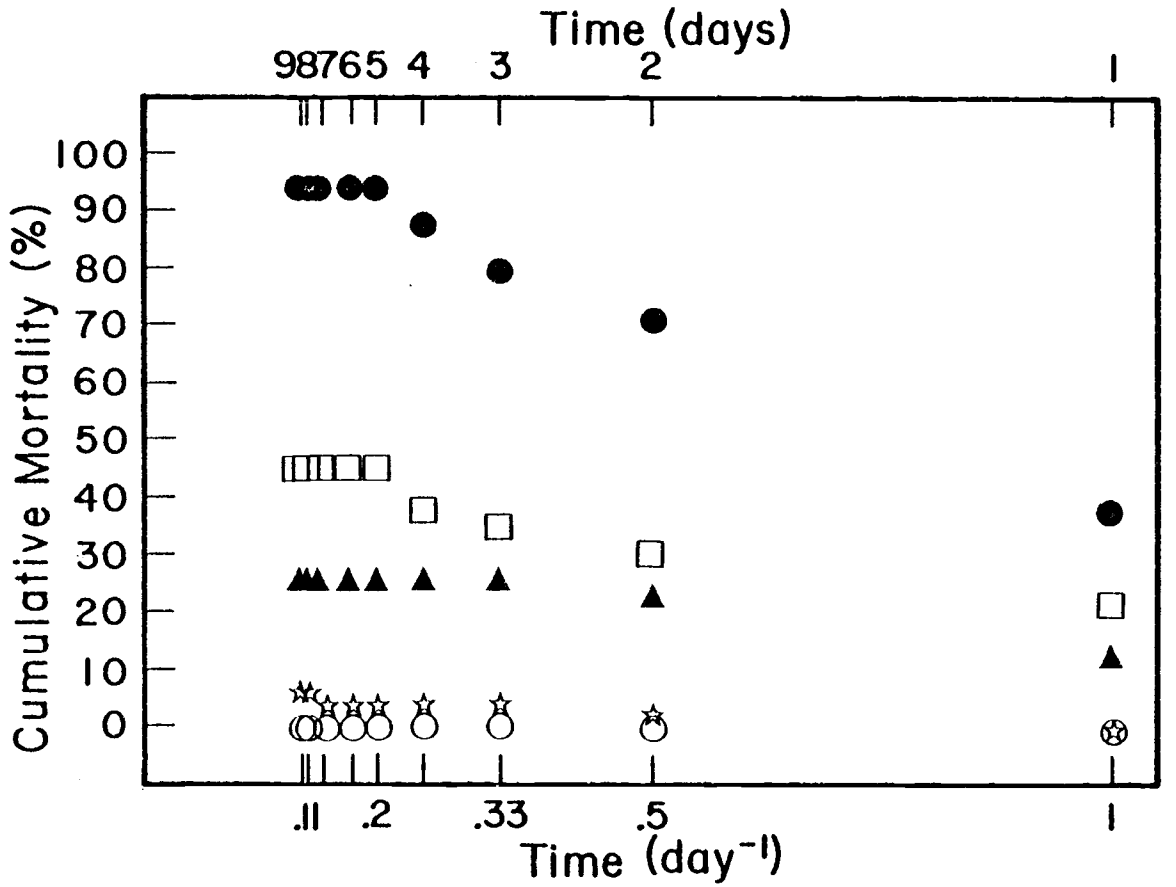

Figure 7. Mortality curves for various groups of fish in the sulfamethoxazole-trimethoprim efficacy study.

Group	R^2	Equation
▲ = High Dose	.928	Y = 28.8 - 15.7 X
□ = Low Dose	.958	Y = 44.8 - 24.0 X
● = Positive Control	.987	Y = 103.5 - 64.3 X

Group	R^2	Equation
☆ = Prophylactic Dose	.830	Y = 5.6 - 5.9 X
○ = High Dose Control Low Dose Control Negative Control	1.000	Y = 0

were monitored through day 40 to investigate cumulative mortality due to SMX-TMP. One death occurred in each of these groups.

Tobramycin Toxicity

Dosing schedule and selection of animals for histological section are summarized in Table 6. The histological findings for the five fish examined are summarized in Table 7.

Damage to the kidney was significant in all treated fish. Lethal kidney damage was demonstrated even at the lowest dosage, 7.5 mg/kg/2d for 5 days (fish #5). Compared to controls (Figure 8), a fish at this dosage was characterized by degenerative changes in the first (PI) and second (PII) proximal convoluted tubules (Figure 9). Both PI and PII segments were intact, but epithelial cell changes, nuclear changes and loss of the microvilli brush-border were evident. Distal tubules were noted to have changed from cuboid (normal) to squamous (abnormal). Necrotized luminal casts, being sloughed through the nephron, were observed in these distal segments.

Kidney damage to a fish receiving 15 mg/kg/2d for 5 days was severe. Intact PI and PII segments could not be located in a search of all microscopic fields (Figure 10). All proximal segments were damaged either extensively or totally. Damage to the distal convoluted tubule was extensive. All distal tubules examined were squamous-shaped and all contained sloughed luminal casts.

Table 6. Cumulative mortality of fish after tobramycin challenge.

Dosage[a]		Day[b]			Fish Selected for Histological Study[c]
mg/kg	frequency	3	4	5	Fish #
saline	qd	0/5	0/5	0/5	
7.5	q2d	0/5	0/5	1/5	5
7.5	qd	0/5	0/5	1/5	4
15.0	q2d	0/5	0/5	2/5	1,2,3
15.0	qd	1/5	3/5	5/5	
30.0	q2d	1/5	2/5	5/5	
30.0	qd	1/5	5/5		

[a]qd = every day; q2d = every other day (i.e.,day 0,2,4).

[b]no mortalities were found until day 3.

[c]histological findings reported in Table 7 and in results section.

Table 7. Histological findings from selected tissues of fish dosed with tobramycin.[a]

	Fish # and Dosage[b]				
	15 mg/kg/2d			7.5 mg/kg/d	7.5 mg/kg/2d
Tissue Damage	1	2	3	4	5
Liver necrosis	moderate	moderate	none	hydropic degeneration around central vein	none
Skin and Muscle Necrosis	none	none	none	none	none
Brain Alterations	moderate	-	-	-	moderate
Kidney Necrosis					
Proximal I	complete	extensive	moderate	moderate	moderate
Proximal II	complete	extensive	moderate	moderate	moderate
Distal	extensive	moderate	moderate	moderate	moderate
Sloughing	extensive	moderate	moderate	moderate	moderate

[a]terms apply to number of cells affected: complete = 90-100 %; extensive = 50-90 %; moderate = 0-50 %; none = 0%.

[b]refer to Table 6

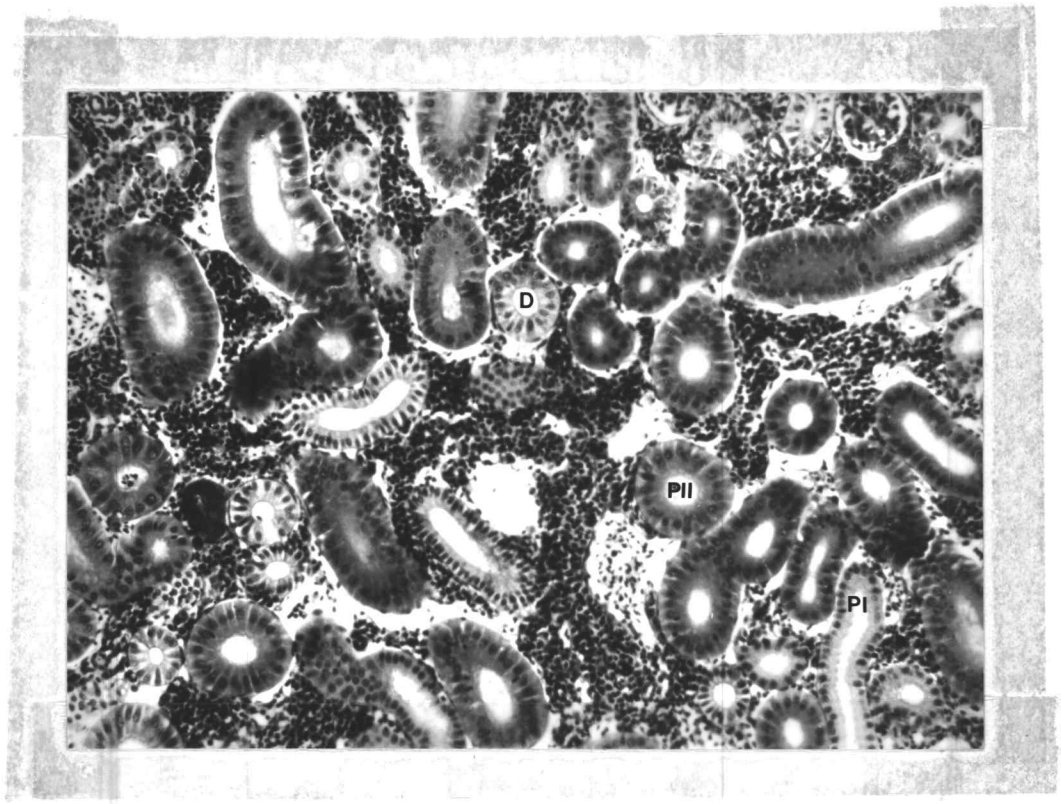

Figure 8. Photomicrograph of kidney tissue from a control coho salmon. All nephron segments, first proximal (PI), second proximal (PII) and distal (D) are present and normal. Hematoxylin and eosin, x 512.

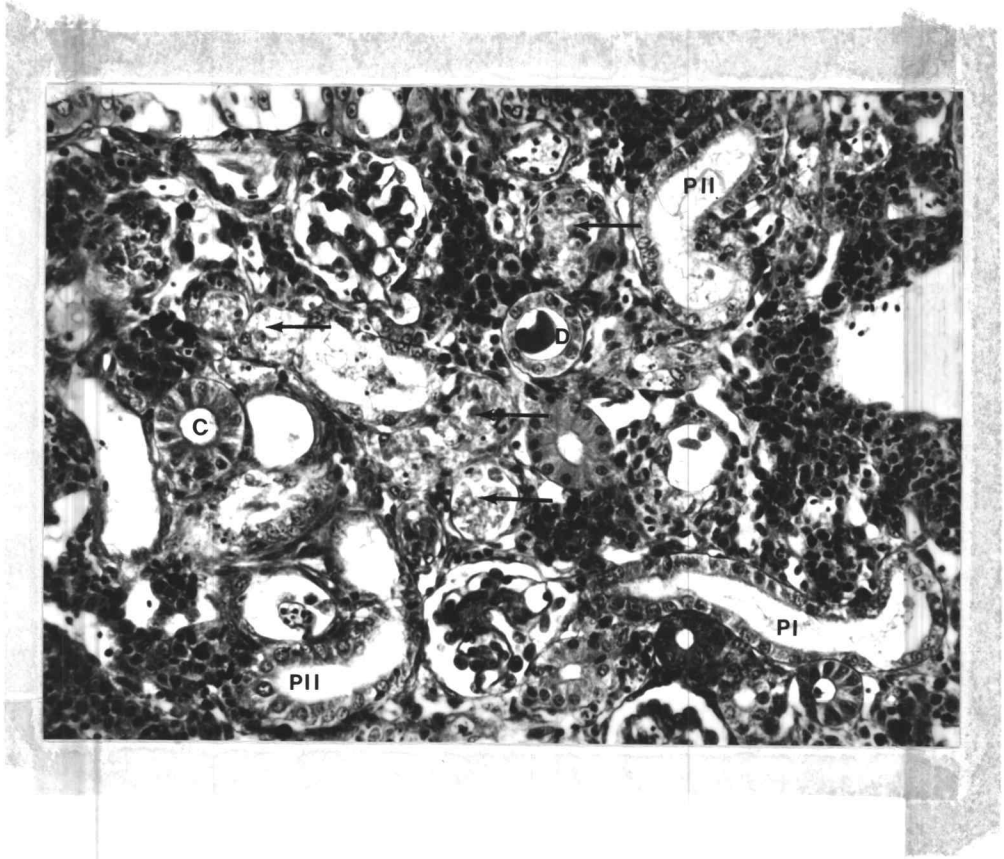

Figure 9. Photomicrograph of kidney tissue from a coho salmon that received 7.5 mg/kg/2d tobramycin for 5 days. The first (PI) and second (PII) proximal segments of some nephrons are still intact, although degenerative changes have occurred. Proximal segments from other nephrons are totally necrotic (arrows). A distal convoluted tubule (D) in the center contains a luminal cast. Collecting ducts (C) appear normal. Hematoxylin and eosin, x 512.

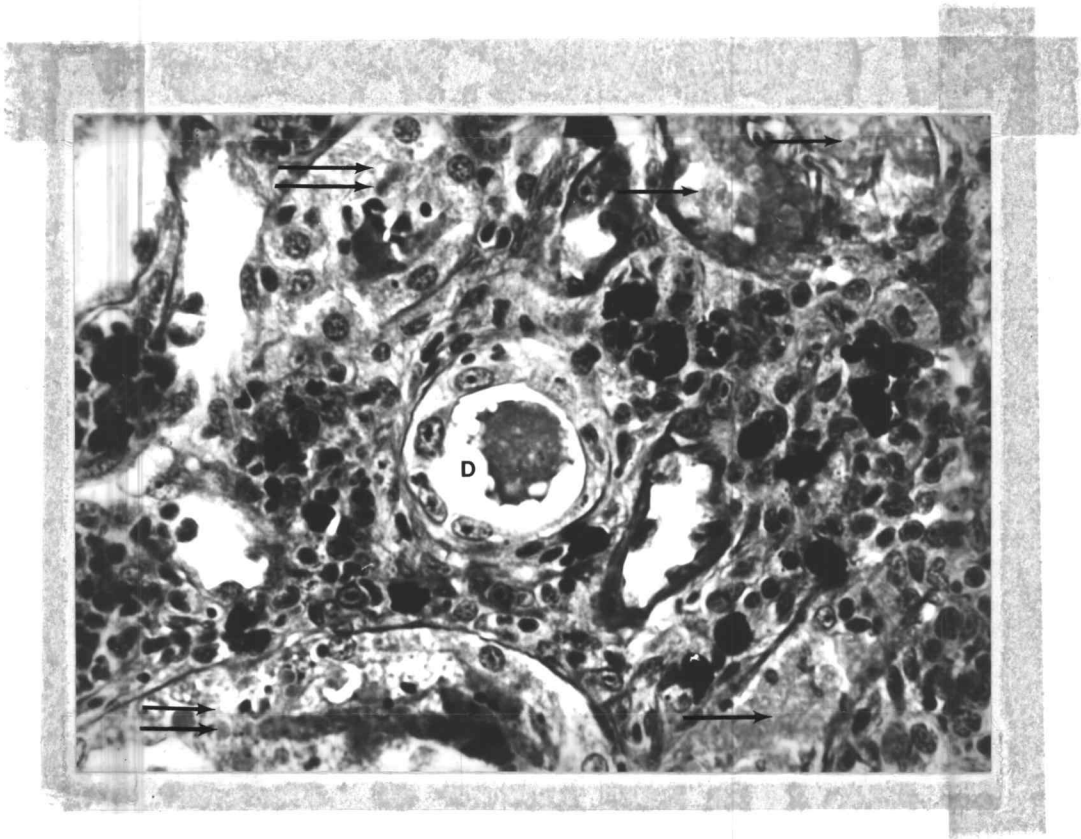

Figure 10. Photomicrograph of kidney tissue from a coho salmon that received 15 mg/kg/2d tobramycin for 5 days. Total necrosis of several proximal segments (arrows) is evident, along with extensive necrosis of two other proximal segments (double arrows) and a distal tubule (D). Hematoxylin and eosin, x 1280.

Brain damage was also evident in treated fish. Edematous or hyperplastic meninges and cytoplasm-free cells of the outer molecular layer of the optic lobes were evident in the fish receiving even the lowest dose.

DISCUSSION

Disc Sensitivity and Minimal Inhibitory Concentration

The in vitro results for disc sensitivity and MIC correlated well for all of the antimicrobials, with the exceptions of SMX-TMP on *Y. ruckeri*, cycloserine on all organisms and amikacin on *A. salmonicida* (Table 2, Figures 2-5). Sulfamethoxazole-trimethoprim demonstrated good activity against *Y. ruckeri* (TSI-78) in the MIC study yet no activity in the disc study. The difference in activity was probably due to the different ratios of the drugs in the two methods (Disc, SMX/TMP = 19/1; MIC, SMX/TMP = 5/1). These ratios differ due to the relatively high levels of SMX that occur in the blood. The relatively higher amount of TMP in the MIC study accounts for the greater activity, since TMP alone was highly active in both systems. An unexplainable comparison of the in vitro methods exists for SMX, which was relatively more active against *Y. ruckeri* (TSI-78) than *Y. ruckeri* (HI-70x) in the MIC study, yet relatively less active in the disc study. Cycloserine was active in the MIC study but inactive in the disc study. Increasing the disc concentration beyond 30 µg probably would have produced inhibition in the disc study. Amikacin showed moderate to good activity against all organisms in the disc study, yet was active only against *A. salmonicida* in the MIC study.

Yersinia ruckeri

The in vitro data demonstrate a wide variey of antimicrobials which are active against Y. ruckeri (HI-70x), but fewer against Y. ruckeri (TSI-78). In contrast, a previous report with a closely related species, Y. enterocolotica, documented that there existed little sensitivity difference among 150 strains (Nilehn, 1969). A possible explanation for the variable sensitivities of the Y. ruckeri strains (HI-70x) and (TSI-78) may be due to the treatment of salmonids with a variety of antimicrobials, including sulfamethazine, chloramphenicol, oxytetracycline, chlortetracycline and sulfamerazine, as reported by Rucker (1966), McDaniel (1971), Wobeser (1973) and Dunlin (1978).

Yersinia ruckeri (HI-70x) produced vastly different in vivo results for the TBM group and for the SMX-TMP group. Both TBM and SMX-TMP were chosen for in vivo studies because of their excellent activity in vitro. However, while SMX-TMP produced a significant reduction in mortality, TBM proved too toxic to be efficacious.

Statistical analysis of the TBM data shows that slopes from the regression lines were significantly different between positive control, low dose and high dose (Figure 6 and Table 4). Tobramycin dosage and death were significantly positively correlated, thus antimicrobial effects of the drug were not observed in the mortality study. Yersinia ruckeri (HI-70x) isolated from mortalities did provide some evidence of an antimicrobial effect by TBM. Four of

the mortalities in the high dose group failed to show Y. ruckeri cultures from the kidneys, thus TBM, while positively contributing to the mortality rate, did produce an antimicrobial effect. A comparison of slope and intercept for high dose (days 5-12) and high dose control (days 5-22) shows no significant difference in slope ($p = .26$) Thus, death to the two groups past day 5 may be occurring by a similar mechanism, that of death due to TBM toxicity. Since deaths from day 1-5 in the high dose group demonstrated similar pathology to deaths in the positive controls, one can conclude that both TBM and infection contributed to death in the high dose group.

Statistical analysis of the SMX-TMP data shows that all slopes and intercepts from the regression equations were significantly different for positive control, low dose, high dose and prophylactic dose (Figure 7 and Table 5). Since no deaths occurred in the high dose control, low dose control and negative control, the slopes and intercepts of these groups were each significantly different from those in the positive control, low dose and high dose. Sulfamethoxazole-trimethoprim dosage and death were significantly negatively correlated, since the low dose group, which received 20% the dose of the high dose group, had about twice the cumulative mortality in 9 days. Timing of the initial dose was critical, as is shown by comparing the prophylactic dose group to the high dose group. Co-administration of drug and inoculum produced less mortality than administration of drug 12 hours post-inoculation.

Sulfamethoxazole-trimethoprim appears to be relatively non-toxic, since 31 days after therapy was stopped (day 9), mortality in the high dose control and low dose control groups did not differ significantly from negative controls.

Aeromonas salmonicida

The in vitro data demonstrate that chloramphenicol, tetracycline, sulfamethoxazole, rifampin, tobramycin, gentamicin, amikacin, kanamycin, streptomycin and paromomycin are active against A. salmonicida. Previous workers have reported activity with sulfisoxazole (Gutsell, 1948; Snieszko and Wood, 1955), furazolidone (Post and Keiss, 1962) and sulfadimethoxine-ormetoprim (Bullock et al., 1974).

Vibrio anguillarum

The in vitro data demonstrate that TMP, SMX-TMP, chloramphenicol, rifampin, tetracycline, tobramycin, gentamicin, amikacin, kanamycin, streptomycin and paromomycin are active against V. anguillarum. Previous workers have reported activity with chloramphenicol and streptomycin (Kusuda, 1966), chloramphenicol alone (Almeida et al., 1967), oxytetracycline or sulfamerazine (Wood, 1968) and nitrofurazone (Kubota and Hagita, 1963). Recently, nifurpirinol demonstrated significant in vivo activity against V. anguillarum (Egidius and Andersen, 1979).

Bacterial Kidney Disease

The MIC data for BKD demonstrate good activity for paromomycin, sulfamethoxazole, rifampin, and SMX-TMP, intermediate activity for gentamicin, tobramycin and sulfamerazine, and relative inactivity for amikacin, cycloserine, erythromycin, ethambutol and TMP. Literature searches indicate that this is the first time many of these agents have been evaluated against BKD (Rucker et al., 1951; Wolf and Dunbar, 1959; DeCew, 1972; Bullock et al., 1975).

The relative inactivity demonstrated by erythromucin contradicts previous results with other strains (Bullock et al., 1975). Two explanations may clarify this finding. BKD (1-MK-78-KD) may be a strain which has become insensitive to erythromycin, or the MIC assay system utilized may not be appropriate for erythromycin. Further testing will be necessary to confirm these conflicting results.

Mycobacterium sp.

The disc sensitivity data for Mycobacterium sp. generally agree with the work of Conroy and Solardo (1965). The present study supports their finding that kanamycin, rifampin and streptomycin demonstrate the best in vitro activity of antimicrobials tested. Ethambutol, gentamicin and tobramycin were active, but cycloserine and amikacin were inactive. Sulfamethoxazole-trimethoprim was without effect, which contradicts the work of Kelly (1976) who reports successful treatment of Mycobacterium marium with SMX-TMP.

Tobramycin Toxicity

The tobramycin toxicity study demonstrated 100% lethality to coho salmon at dosages 6 times less than the "no toxic effect" dosage to rats (Table 6; Welles et al., 1973). Fish given 7.5 mg/kg/2d for 5 days exhibited marked kidney damage (Table 7). Fish given 5 mg/kg/d began dying on day 6, with 100% cumulative mortality on day 22 (Figure 6). Fish which received 2 mg/kg/d for 12 days post-ip challenge of Y. ruckeri (HI-70x) also died due to TBM toxicity (see p values, Table 4). Therefore, a "no toxic effect" level for TBM in coho salmon must be significantly less than 2 mg/kg/d; such a dose level would be significantly below the recommended human dosage of 3 mg/kg/d (Weinstein, 1975). The toxicology of TBM and the other aminoglycosides has been well documented (Barza and Scheife, 1977; Appel and Neu, 1977). The nephrotoxicity of TBM and the other aminoglycosides has been greatly studied in mammals, where doses as high as 15 mg/kg/d for 30 days produced no damage to rat kidney (Welles et al., 1973).

Antimicrobial toxicity to fishes has been reported by several other researchers. Wood (1957) reported that 10 g/100 lb fish of sulfamethazine in the feed produced significant mortality, but was unable to duplicate the results. In further experimentation, Wood (1957) found that 300 g/100 lb fish of sulfamethazine in the feed produced less than 1% mortality. Piper (1961) reported that erythromycin thiocyanate at 100 mg/kg/d for 6 days, adminis-

tered in feed, produced choking, spasms and histologically-demonstrated kidney tubule damage. Warren (1963) reported that it required 500 mg/kg/d for 4 days to produce such effects. DeCew (1972) found that a mixture of penicillin G procaine (22,000 IU/kg), dihydrostreptomycin sulfate (28 mg/kg) and oxytetracycline (5 mg/kg) injected subcutaneously twice a year for three years produced no toxicity to adult chinook salmon, but was teratogenic to 0-15% of the progeny. Mandible and fin anomalies were the most common defects. McBride et al. (1975) demonstrated degeneration of the tubular epithelium of the kidney in adult rainbow trout with kanamycin at 20 mg/kg/week for 5 weeks.

The present study is the first to document the increased sensitivity of a fish species to the nephrotoxic potential of TBM. The coho salmon and other fishes may be useful for comparative toxicology of potentially nephrotoxic human drugs.

CONCLUSION

Antimicrobials continue to be of value in the management of fish diseases. However, as in human antibacterial chemotherapy, new agents must continually be developed to combat the increasing virulence of resistant strains and reduce toxicity to the treated host. One logical avenue of development involves engineering antimicrobial agents specifically for use in fishes.

The present study indicates that the development of a sulfonamide-pyrimidine combination like SMX-TMP is warranted. One such agent, sulfadimethoxine-ormetoprim (Ro5-0037) has been evaluated as efficacious against A. salmonicida (Bullock et al., 1974) and is being investigated for registration (Schnick and Meyer, 1979).

The development of an aminoglycoside for use in Gram negative bacterial fish pathogens may be contraindicated due to toxicity problems. Though these agents continue to be developed for treatment of Gram negative bacterial human pathogens, the development of an aminoglycoside for use in fish diseases has not occurred. Two aminoglycoside-like compounds, Cinodine^{R}HCl and Iprocinodine^{R} HCl, have been proposed for veterinary use (USAN, 1979). The toxicology of these and other such agents need to be fully examined before their use in fishes is indicated.

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