APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1995, p. 2335–2340 Vol. 61, No. 6
0099-2240/95/$04.00+0

Effect of Oxytetracycline-Medicated Feed on Antibiotic Resistance of Gram-Negative Bacteria in Catfish Ponds

ANGELO DEPAOLA,[1]* JAMES T. PEELER,[2] AND GARY E. RODRICK[3]

Gulf Coast Seafood Laboratory, U.S. Food and Drug Administration, Dauphin Island, Alabama 36528[1]; Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration, Washington, D.C. 20204[2]; and Department of Food Science and Human Nutrition, University of Florida, Gainesville, Florida 32611[3]

Received 2 December 1994/Accepted 12 April 1995

The effect of oxytetracycline-medicated feeds on antibiotic resistance in gram-negative bacteria from fish intestines and water in catfish ponds was investigated. In experiments in the fall and spring, using ponds with no previous history of antibiotic usage, percentages of tetracycline-resistant bacteria in catfish intestines obtained from medicated ponds increased significantly after 10 days of treatment. In the fall, resistance of the intestinal and aquatic bacteria returned to pretreatment levels within 21 days after treatment. In the spring, resistance declined after treatment but remained higher than pretreatment levels for at least 21 days in intestinal bacteria and for 5 months in aquatic bacteria. *Plesiomonas shigelloides*, *Aeromonas hydrophila*, and *Citrobacter freundii* were isolated frequently in both spring and fall; *Escherichia coli*, *Klebsiella pneumoniae*, *Edwardsiella tarda*, and *Enterobacter* spp. were isolated primarily in the spring. Oxytetracycline treatment did not affect the distribution of bacterial species in the fall but may have accelerated a shift toward greater prevalence of members of the family *Enterobacteriaceae* in the spring. Multiple antibiotic resistance did not appear to be elicited by oxytetracycline treatment.

The widespread use of antimicrobial agents for treating bacterial diseases in aquaculture (15) has been associated with increased antibiotic resistance in *Aeromonas hydrophila* (1, 6), *A. salmonicida* (7, 9), *Edwardsiella tarda* (5, 33), *E. ictaluri* (34), *Vibrio anguillarum* (10), *V. salmonicida* (22), *Pasteurella piscicida* (8), and *Yersinia ruckeri* (17). However, the effects of chemotherapeutics on the bacterial ecology of fish and fish-rearing waters have received relatively limited attention. Although bacteriological surveys of cultured ayu (4) and channel catfish (26) have associated increased antibiotic resistance with antimicrobial agent usage, those studies lacked complete antibiotic usage history data and systematic sampling.

Controlled studies are needed to determine the effect of antimicrobial therapy on the microbial ecology of catfish ponds. The objectives of this study, therefore, were to determine the effect of oxytetracycline (OTC) on (i) densities of gram-negative bacteria and the prevalence of tetracycline resistance (Tc^r) in catfish ponds during and after administration of OTC; (ii) the composition of bacterial taxa; and (iii) resistance to unrelated antibiotics.

MATERIALS AND METHODS

Ecosystem. Elevated levee ponds (0.02 to 0.04 ha) at the U.S. Fish and Wildlife Service Southeastern Fish Cultural Center (Marion, Ala.) were used in feeding experiments in the fall of 1989 and spring of 1990. Ponds were filled with well water (70 m) at the beginning of each experiment and maintained to a depth of 1.0 m. According to U.S. Fish and Wildlife Service records, antibiotic-medicated feeds had never been used in these ponds and consistently low levels of Tc^r bacteria (<4%) were observed in the water and intestinal contents of catfish from these ponds before this study (unpublished results).

Channel catfish were seined from U.S. Fish and Wildlife Service ponds, weighed, and stocked in the experimental ponds at a rate of 3,360 kg/ha (3,000 lb/acre). Mean fish weight was 120 g, and the range (80 to 1,500 g) represented the sizes of fish found in commercial ponds during various stages of growth.

Commercially prepared catfish feeds (100 to 900 kg) were obtained from Purina Feed Mill (Montgomery, Ala.) and stored for 3 months at 3°C. OTC was not found at levels of <0.3 mg/kg in any of the experimental unmedicated feeds by methods of the Association of Official Analytical Chemists International (12). Medicated feeds contained 2.2 g/kg, as stated on the label.

Acclimation and feeding protocols were slightly different in the fall and spring experiments. In the fall, fish were placed in ponds 3 to 5 days after filling. During a 4-week acclimation and bacterial stabilization period, beginning 1 week after stocking, fish were fed at a typical production rate (2.5% of body weight per day). This was followed by a 10-day period of treatment with OTC administered orally at 50 mg/kg of fish per day, which is the approved usage of OTC for channel catfish (32). During treatment, two ponds received medicated feed and two control ponds received unmedicated feed. After treatment, all ponds received unmedicated feed at production level for 21 days, the legally required time for sale of channel catfish after OTC medication. Fish were fed at a maintenance level (2.5% of body weight on Monday, Wednesday, and Friday) for the remaining 2 weeks of the experiment. In the spring, fish were stocked 2 weeks after ponds (one control and two treatment ponds) were filled; maintenance feeding was initiated 2 weeks after stocking and continued for 5 weeks. A production feeding period of 6 weeks preceded the 10-day treatment. All ponds received production feed for 8 weeks after treatment and then maintenance feed for the remainder of the study. Pond water temperature and dissolved-oxygen concentration were monitored daily with a YSI 58 dissolved-oxygen meter (Yellow Springs Instrument Co., Yellow Springs, Ohio).

Temperature and dissolved oxygen varied little between ponds. In the fall, water temperature was generally between 20 and 25°C, until a sharp decrease to 10°C occurred in mid-October, and was 8 to 18°C through November. In the spring, the temperature was about 15°C in March, but it increased to >20°C by mid-May and was about 25°C in June and July. Dissolved oxygen was usually between 6 and 12 ppm in both experiments.

Sampling procedures. Surface waters were collected aseptically in sterile 500-ml wide-mouth polyethylene bottles (2). Specimens were collected weekly (more frequently during treatment) at the deep and shallow ends of each pond. Bottles were immediately placed in an ice chest with bagged ice and transported to the Food and Drug Administration Gulf Coast Seafood Laboratory (Dauphin Island, Ala.) for microbiological analysis. Analysis of all samples was generally completed within 5 h of collection. Duplicate aliquots from each bottle were analyzed.

Catfish intestinal contents were sampled immediately before treatment with medicated feed, 1 day after the 10-day medication period, and 21 days after the medication period. Five fish were collected by hook and line from each pond. The fish were placed individually in plastic bags, covered with ice, and transported to the laboratory. The external abdominal surface was disinfected by swabbing with 70% ethanol, and an incision was made over the peritoneal cavity. The intestines were severed slightly anterior to the pyloric valve and the anus, and the contents were aseptically extruded into a sterile 50-ml polypropylene tube.

Bacteriological methods. Serial tenfold dilutions of water and catfish intestinal

* Corresponding author. Mailing address: FDA Gulf Coast Seafood Laboratory, P.O. Box 158, Dauphin Island, AL 36528. Phone: (205) 694-4480. Fax: (205) 694-4477.

TABLE 1. Overall recovery of Tc^r gram-negative bacteria in pond water and catfish intestines by direct and induction methods in the fall experiment

Specimen source	No. of specimens tested	CFU/g or ml[a]	Mean % Tc^r[b]	
			Direct method	Induction method
Intestines	59	1.3×10^7	10.3*	44.5†
Water	92	1.4×10^3	9.8*	29.3†

[a] Gram-negative bacterial counts were determined by the spread plate technique on Mac.

[b] Values followed by different symbols in a row are significantly different at $\alpha = 0.05$. Percent recovery means are shown; however, the nonparametric test of significance (27) was performed on the ranks.

contents were prepared in 0.85% NaCl, and 0.1-ml aliquots were spread plated onto MacConkey (Mac) agar plates (Difco Laboratories, Detroit, Mich.) containing 2.0% agar. The plates were incubated overnight at 35°C. The proportion of Tc^r colonies was determined by two replica plating methods on Mac plates containing 25 to 250 colonies. The direct method consisted of replica plating (Replipad; FMC Inc., Rockland, Maine) the colonies onto Mac plates containing 30 μg of tetracycline (TC) per ml, which were incubated overnight at 35°C (26). With the induction method (19), the Mac growth was first replica plated onto tryptic soy agar (Difco) containing 1 μg of TC per ml. After 3 to 4 h at 35°C, the growth on the tryptic soy agar containing 1 μg of TC per ml was replica plated onto Mac plates containing 30 μg of TC per ml, which were incubated overnight at 35°C. The number of colonies on the Mac plates containing 30 μg of TC per ml, determined by direct and induction methods, was divided by the number on the corresponding Mac plates to determine the Tc^r proportion.

Five representative Tc^r colonies were selected for each of five test portions of intestinal contents and two of corresponding waters. All colonies were selected from Mac plates containing 30 μg of TC per ml (induction method) and streaked for purification on tryptic soy agar plates containing 1 μg of TC per ml. Cultures were stored at room temperature on tryptic soy agar slants covered with sterile mineral oil. The API 20E system (Analytab, Plainview, N.Y.) was used to identify isolates. Antibiotic resistance profiles were determined by the Uniscept KB system (Analytab).

Three batches of unmedicated feed and one batch of medicated feed were used in each experiment. Feeds (100 g) were blended dry for 1 min to a fine powder. Serial tenfold dilutions were prepared in 0.85% NaCl; total aerobic plate counts were determined in plate count agar (Difco) after incubation at 35°C for 48 h. Additionally, 10 g of each blended feed was placed in 100 ml of sterile distilled water and incubated overnight at 35°C. A loopful of the overnight enrichment was then streaked onto Mac agar, which was incubated at 35°C overnight. Representative colonies (two to seven) were identified and tested for Tc^r by the induction method and confirmed by the Uniscept KB system.

Statistical analyses. Counts of gram-negative bacteria were transformed to $\log_{10}$ (count per milliliter or gram) to assume normality. Percentage estimates of Tc^r were also computed for the direct and induction recovery procedures on the basis of untransformed counts of gram-negative bacteria from Mac agar. Analyses of variance were performed as described by Ostle and Mensing (27), and these were applied to the log-transformed data. A nonparametric comparison of mean ranks (21) was used to test the hypothesis that percentage estimates of Tc^r were equal. All tests were performed at the $\alpha = 0.05$ level.

RESULTS

Tc^r. Overall mean Tc^r in the fall was significantly greater ($\alpha = 0.05$) in pond water and catfish intestines with the induction method than with the direct method (Table 1). However, the direct method provided closer agreement between the estimates of Tc^r in water (9.8%) and intestines (10.3%). Bacterial densities in the catfish intestinal contents (10^7/g) were about 4 logs higher than those in pond water (10^3/ml) by weight-to-volume comparisons.

The percent Tc^r in aquatic bacteria as determined by the direct method was plotted against time after stocking in the fall experiment (Fig. 1). Tc^r was relatively constant (5 to 20%) during pretreatment, when fish in all four experimental ponds received production levels of the unmedicated ration. Resistance increased steadily in the medicated ponds but remained constant in the control ponds throughout treatment. During the 21-day posttreatment period, Tc^r declined to pretreatment

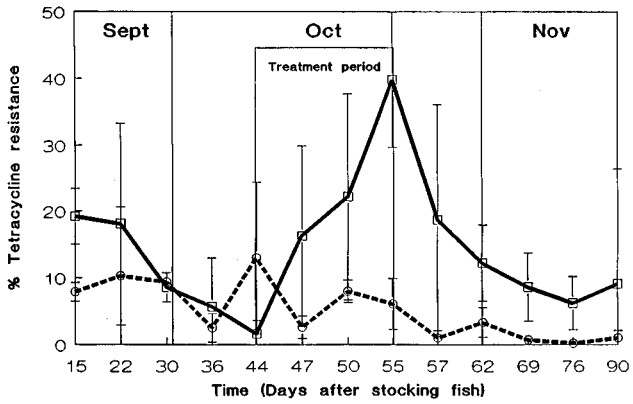

FIG. 1. Effect of OTC-medicated feed on the development and persistence of TC-resistant bacteria (direct method) in pond water during the fall experiment. Symbols: □, medicated; ○, control.

levels in the medicated ponds and decreased slightly in the control ponds.

In the fall experiment, the effect of OTC on the Tc^r of intestinal bacteria was similar to that on the Tc^r of aquatic bacteria (Table 2). Intestinal bacteria from the medicated ponds were Tc^r after treatment in significantly higher percentages ($\alpha = 0.05$) than were those from the pre- and posttreatment periods, as determined by the direct and induction meth-

TABLE 2. Tc^r gram-negative bacteria from intestinal contents of catfish from control and OTC-medicated ponds in the fall and spring

Treatment (season) and variable	Period[a]		
	Pretreatment	During treatment	Posttreatment
No-medication control (fall)			
No. of samples	10	10	10
CFU/g[b] of Mac	6.8×10^7*	3.8×10^6†	2.2×10^7*†
Direct %[c]	8.2[d]	5.4	0.5
Induction %[c]	35.2[d]	43.1	31.7
OTC (fall)			
No. of samples	9	9	9
CFU/g of Mac	1.5×10^7[d]	2.6×10^6	2.2×10^7
Direct %	0.3*	48.4†	3.1*
Induction %	34.3*	84.0†	42.4*
No-medication control (spring)			
No. of samples	5	5	5
CFU/g of Mac	2.5×10^8[d]	5.0×10^8	5.0×10^7
Direct %	4.0*	19.4†	5.8*
Induction %	14.5[d]	27.3	25.8
OTC (spring)			
No. of samples	10	10	10
CFU/g of Mac	2.5×10^8[d]	2.5×10^7	2.5×10^8
Direct %	3.9*	81.7‡	41.9†
Induction %	22.7†	88.8*	72.3*

[a] Catfish intestinal contents were sampled immediately before treatment with medicated feed (pretreatment), 1 day after the 10-day medication period (during treatment), and 21 days after the medication period (posttreatment). Values followed by different symbols in a row are significantly different at $\alpha = 0.05$. Geometric means and percent recoveries are shown; however, the nonparametric test of significance (27) was performed on the ranks.

[b] Geometric mean of gram-negative bacterial count on Mac.

[c] Mean percent Tc^r bacteria determined by the respective method.

[d] Values in this row do not differ significantly at $\alpha = 0.05$.

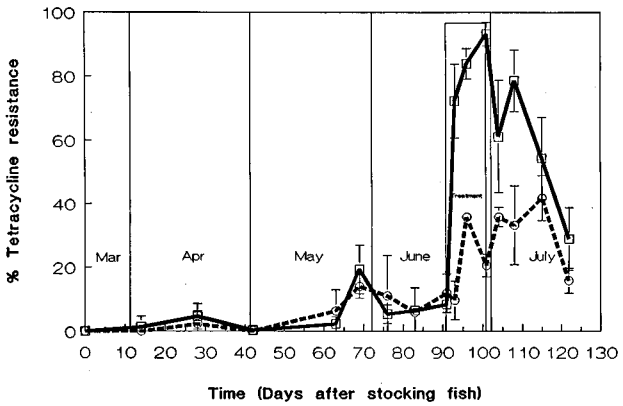

FIG. 2. Effect of OTC-medicated feed on the development and persistence of TC-resistant bacteria (direct method) in pond water during the spring experiment. Symbols: □, medicated; ○, control.

TABLE 3. Percentages of Tc^r bacterial species in catfish intestinal contents and pond water samples[a]

Species[b]	% Tc^r isolates from:	
	Intestines	Water
P. shigelloides	55.0	22.4
A. hydrophila	21.7	45.7
C. freundii	13.3	14.8
E. coli	3.8	3.3
E. tarda	4.0	1.9
K. pneumoniae	1.5	5.7
Enterobacter spp.	0	1.9
Serratia spp.	0	1.0
Unknown	0.6	2.9

[a] 525 intestinal and 210 water isolates were tested.
[b] Identified by API 20E system.

ods. Tc^r percentages in pre- and posttreatment specimens did not differ significantly. Bacterial densities (Mac agar) were similar for intestinal specimens from medicated ponds at each treatment phase. No significant differences ($\alpha = 0.05$) in Tc^r were observed in the control ponds with either method.

Data from the spring experiment are presented in Fig. 2 and Table 2. No Tc^r bacteria were found in pond water before fish were stocked (day 14 after the pond was filled), and their percentage was slight before feeding (day 28). Tc^r increased initially with production feeding (day 63), but resistance stabilized before treatment (days 91 to 101). Tc^r increased rapidly in aquatic bacteria of medicated ponds and exceeded 90% by the end of treatment. In the control pond, Tc^r levels fluctuated during treatment but were well below that of medicated ponds. During posttreatment, Tc^r decreased in the medicated ponds. After 21 days posttreatment (day 122), Tc^r percentages were similar in both treated and control ponds but fluctuated considerably in control (10 to 60%) and treated (15 to 50%) ponds through November, when sampling was terminated (data not shown).

In the spring experiment, Tc^r of the catfish intestinal bacteria was highest in medicated ponds immediately after treatment and significantly higher 21 days posttreatment than immediately before treatment (Table 2). In catfish intestinal microflora from the control pond, Tc^r was significantly higher after treatment, as determined by the direct method but not as determined by the induction method. Bacterial densities of intestinal contents were relatively constant in treated and control ponds.

Feed bacteriology. Aerobic plate counts in feed were 2.3 to 4.8 $\log_{10}$ CFU/g. Tc^r bacteria were recovered from 75% of feeds (four of four and two of four batches in the fall and spring, respectively). Of the 40 isolates tested, 55% were Tc^r. Most isolates were members of the family *Enterobacteriaceae*; no representatives of the family *Vibrionaceae* were recovered. Tc^r was prevalent in *Enterobacter agglomerans* (seven of seven isolates), *Klebsiella pneumoniae* (six of nine isolates), Centers for Disease Control and Prevention group 41 (three of four isolates), and *Citrobacter* spp. (three of three isolates).

Bacterial species. The compositions of Tc^r bacteria in catfish intestines and pond waters during the two experiments were compared (Table 3). Although *Plesiomonas shigelloides*, *A. hydrophila* and *Citrobacter freundii* were prevalent in both experiments, representatives of the family *Enterobacteriaceae*, including *C. freundii*, *Escherichia coli*, *E. tarda*, and *K. pneumoniae*, were isolated more frequently in the spring. These species were similarly prevalent in pond water and catfish intestines, except that *P. shigelloides* and *A. hydrophila* were most abundant in catfish intestines and pond water, respectively.

The distribution of bacterial species fluctuated considerably during the fall; however, no prevalent trends were associated with OTC treatment (Table 4). In the spring, OTC treatment may have affected the bacterial composition of catfish ponds. During the treatment period, *P. shigelloides* was isolated three times more frequently from control than from medicated ponds, where *E. coli* and *K. pneumoniae* accounted for approximately 50% of the isolates. These two species were not found in the control pond until the posttreatment period, when microbial diversity was greatest and little difference was observed between control and medicated ponds.

Resistance to other antibiotics. Resistance of the frequently isolated bacteria to the 18 antibiotics of the Uniscept KB system is shown in Table 5. Data from all treatment periods from both the fall and spring experiments were combined, since no apparent trends in resistance patterns were observed. High Tc^r (>95% of all species) was consistent with replica plating results, as found by the induction method. Isolates of *P. shigelloides* were almost uniformly resistant to ampicillin and ticarcillin, most were also resistant to piperacillin and mezlocillin, and all were sensitive to other β-lactam drugs, except a few that were resistant to cefoperazone. Greater aminoglycoside resistance was observed in *P. shigelloides* isolates in the fall than in the spring, when trimethoprim-sulfamethoxazole resistance was higher. Most *A. hydrophila* isolates were resistant to ampicillin and ticarcillin and generally sensitive to other drugs. Most *C. freundii* isolates were resistant to cefazolin, cephalothin, and cefoxitin, and about one-third were resistant to ampicillin. *C. freundii* was sensitive to most drugs but showed the highest resistance to nitrofurantoin (11.4% in spring). Aminoglycoside resistance in *E. coli*, which was isolated only in the spring, was higher (44.4%) than in other species. Tc^r *E. tarda* isolates were sensitive to all other Uniscept KB drugs. Few isolates of any species were resistant to chloramphenicol.

Multiple antibiotic resistance, as determined by the mean number of resistances to 18 individual or six classes of antibiotics in the Uniscept KB system, was compared in bacterial isolates from control and medicated ponds for each of the treatment periods during the fall and spring (data not shown). In the fall, bacterial isolates were resistant to about four individual drugs and two drug classes regardless of type or length of treatment. Resistance fluctuated more in the spring, and no apparent trend was associated with OTC treatment.

TABLE 4. Effect of OTC-medicated feed on isolation percentages of bacterial taxa from catfish ponds during fall and spring experiments[a]

Season and species	% of total isolated					
	Pretreatment		During treatment		Posttreatment	
	Medicated	Control	Medicated	Control	Medicated	Control
Fall						
P. shigelloides	54.3	32.9	38.6	45.7	30.0	48.6
A. hydrophila	40.0	35.7	18.6	37.1	60.0	47.1
C. freundii	4.3	24.3	28.6	17.1	8.6	4.3
Other	1.4	7.1	7.1	0	1.4	0
Spring						
P. shigelloides	78.6	85.7	20.0	65.7	31.4	51.4
A. hydrophila	20.0	5.7	8.6	11.4	18.6	11.4
C. freundii	1.4	5.7	20.0	14.3	14.3	8.6
E. coli	0	0	30.0	0	5.7	5.7
E. tarda	0	0	0	0	22.9	17.1
K. pneumoniae	0	0	21.4	0	2.9	0
Other	0	2.9	0	8.6	4.3	5.7

[a] In the fall, 70 medicated and 70 control isolates were tested in each period. In the spring, 70 medicated and 35 control isolates were tested in each period.

DISCUSSION

The effect of antibiotic-supplemented feed on the antimicrobial resistance of the gram-negative bacteria of catfish ponds studied in separate fall and spring experiments demonstrated a rapid increase of Tc^r in the water and catfish intestinal bacteria of ponds that received OTC-medicated feed. Resistance decreased to levels similar to those of control ponds within 21 days after the last dose of OTC. The OTC treatment did not appear to affect the distribution of bacterial taxa in the fall but may have temporarily altered the bacterial flora in spring. Resistance to drugs other than TC did not increase in bacteria of OTC-treated ponds.

In previous aquarium experiments with rainbow trout (14) and goldfish (31), Tc^r increased in response to orally administered OTC. However, those studies were not conducted in a pond ecosystem and did not address long-term persistence of Tc^r after OTC administration.

Aerobic incubation of Mac agar at 35°C was used to isolate gram-negative enteric bacteria of potential public health concern. Although the predominant gram-negative species (*A. hydrophila*, *P. shigelloides*, *C. freundii*, *E. coli*, *K. pneumoniae*, and *E. tarda*) are opportunistic human pathogens (30), they rarely cause infections except in chronically ill individuals. However, they can survive in or colonize human intestines, potentially transferring antibiotic resistance to the normal microflora or to ingested pathogens.

Estimates of Tc^r by the induction method, confirmed by Uniscept KB, were significantly higher than those determined by the direct method. These findings support previous work (19) indicating that most Tc^r *A. hydrophila* isolates from catfish ponds required an induction step. Isolates of the present study were evaluated for various classes of Tc^r determinants (manuscript in preparation), and results of the direct method were compared with those of earlier work (26, 29).

The bacterial density of pond water fluctuated considerably among sampling times, different ponds, and pond locations but

TABLE 5. Antibiotic resistance in catfish pond bacteria during spring experiment

Antibiotic(s)	% of isolates with resistance[a]				
	P. shigelloides (n = 338)	*A. hydrophila* (n = 184)	*C. freundii* (n = 96)	*E. coli* (n = 27)	*E. tarda* (n = 22)
Trimethoprim-sulfamethoxazole	18.6	4.9	0	0	0
Nitrofurantoin	0	0.5	5.2	0	0
Ampicillin	97.3	97.3	33.3	11.1	0
Ticarcillin	83.4	88.6	8.3	3.7	0
Piperacillin	92.0	0	0	3.7	0
Mezlocillin	79.3	1.6	0	3.7	0
Cefazolin	0	7.1	93.8	11.1	0
Cefamandole	0	1.1	5.2	0	0
Cephalothin	0	5.4	41.7	11.1	0
Cefoxitin	0	2.7	84.40	0	0
Cefotaxime	0	0	2.1	0	0
Cefoperazone	5.3	0	0	0	0
Amikacin	15.7	0.5	0	0	0
Gentamicin	7.4	1.1	0	44.4	0
Tobramycin	5.6	12.5	0	44.4	0
Netilmicin	0.9	1.6	0	44.4	0
Chloramphenicol	0.3	0	2.1	0	0
TC	9	96.7	100	100	100

[a] Antibiotic resistance was determined by the Uniscept KB system.

was unaffected by addition of catfish, feed, or OTC. The species composition frequencies of Tc^r bacteria in catfish intestines and pond water were remarkably similar. The most prevalent species were *A. hydrophila* in water and *P. shigelloides* in catfish intestines (26). The microbiological characteristics of pond water were indicative of those in catfish intestines, and samples were also simpler to collect, process, and analyze.

Bacterial taxa in this study were not substantially altered by oral administration of OTC, as observed in goldfish (31) and rainbow trout (14). In the spring, members of the family *Enterobacteriaceae* (other than *C. freundii*) accounted for <3% of isolates until the water temperature increased to 25°C in June, which corresponded to the OTC treatment period. Members of the family *Enterobacteriaceae* were recovered more frequently from treated ponds than those of the family *Vibrionaceae* in July, after the medication period, but did not become prevalent in control ponds until July, 21 days after OTC treatment, supporting the observation by MacMillan and Santucci (25) of a seasonal effect on the composition of catfish bacterial taxa. OTC may have accelerated a seasonal shift in the microbial population by sharply reducing existing microflora through the selection process. This point merits further investigation, since *Enterobacteriaceae* family members, especially *E. coli*, are normal microflora of the human intestine that transfer antibiotic resistance under various conditions. Data from control ponds (Fig. 1 and 2) suggest that a seasonal trend in Tc^r is related to a shift in bacterial taxa. A previous survey of catfish ponds (25) reported higher Tc^r in members of the family *Enterobacteriaceae* (e.g., *E. coli*) than in *A. hydrophila* and *P. shigelloides*. Tc^r bacteria may also be transferred from treated ponds to control ponds by frogs, snakes, turtles, birds, or various mammals that are active in warm weather.

Although OTC was not found in the unmedicated control feed used in these experiments, OTC contamination (>300 μg/g) and elevated antibiotic resistance in catfish bacteria resulted when other unmedicated feed was used in aquarium studies (18). Catfish feeds may also have been a source of antibiotic-resistant bacteria or Tc^r determinants in the present study, since Tc^r gram-negative bacteria were prevalent in both medicated and unmedicated feeds. Although the predominant pond bacteria (*P. shigelloides* and *A. hydrophila*) were not found in the feeds, *C. freundii* and *K. pneumoniae* were isolated from both feed and pond specimens.

The antibiotic resistance profiles of *A. hydrophila* and *P. shigelloides*, other than the high resistance to TC, were similar to those of human clinical isolates (16, 20, 28). Resistance to drugs other than TC may have resulted from intrinsic factors unrelated to selection; e.g., *A. hydrophila* is generally resistant to certain β-lactam drugs, such as ampicillin (7).

A major concern of antibiotic usage is acquisition of multiple-antibiotic resistance (5–7, 23, 24, 33, 34). The Uniscept KB system was chosen for this study because of its extensive use in clinical microbiology to test for resistance to antibiotics that are of greatest importance in human medicine. Results of Uniscept KB testing in the present study indicated that multiple-antibiotic resistance did not occur to any appreciable degree. Multiple-antibiotic resistance has been reported in fish pathogens and bacteria from aquaculture environments associated with a variety of drugs or an uncertain antibiotic usage history (3–5, 11, 13, 26). In studies of rainbow trout, OTC resistance increased but resistance to unrelated drugs remained constant throughout a 10-day treatment period (14). Although the relatively low incidence of resistance to drugs, including TC, may be attributed to the absence of antibiotics in these ponds before this study, in controlled experiments (23, 24) oral administration of OTC resulted in an increase in long-term Tc^r and in multiple-antibiotic-resistant strains in the feces of chickens not previously exposed to antibiotics. Repeated use of medicated feeds may establish antibiotic-resistant strains in catfish ponds, but this possibility was not investigated in this study.

The implications of this study are important for the pond-raised channel catfish industry and for human health. The increase of Tc^r during the OTC medication period may limit the effectiveness of the treatment, since many of the resistant bacteria were potential fish pathogens. Higher resistance was also noted in potential human pathogens for which TC is the drug of choice; thus, aquafarm workers may be at increased risk of wound infections from resistant strains of bacteria during antimicrobial treatments. However, as shown in this study, Tc^r declined after the treatment period and resistance was similar to that of unmedicated ponds within 21 days. Current regulations for prevention of tissue residues prohibit the sale of catfish for 21 days after OTC treatment has been terminated. Thus, oral administration of OTC to catfish by the recommended protocol does not appear to pose an increased risk of antibiotic-resistant bacterial infection to consumers of catfish.

ACKNOWLEDGMENTS

We thank R. P. Bates, G. Bitton, D. E. Duggan, R. Francis-Floyd, and W. S. Otwell, University of Florida, for assisting with the experimental design and manuscript review; the FDA Denver Regional Laboratory personnel for examining catfish feeds for OTC residues; and L. Torrans, W. Deavors, and J. C. Jones (U.S. Fish and Wildlife Service, Southeastern Fish Cultural Laboratory) for providing ponds, taking care of fish, and maintaining records.

REFERENCES

1. **Akashi, A., and T. Aoki.** 1986. Characterization of transferable R plasmids from *Aeromonas hydrophila*. Bull. Jpn. Soc. Sci. Fish. **52:**649–655.
2. **American Public Health Association.** 1970. Recommended procedures for the examination of seawater and shellfish, 4th ed. American Public Health Association, Washington, D.C.
3. **Aoki, T.** 1974. Studies of drug-resistant bacteria isolated from water of carp-ponds and intestinal tracts of carp. Bull. Jpn. Soc. Sci. Fish. **40:**247–254.
4. **Aoki, T.** 1975. Effects of chemotherapeutics on bacterial ecology in the water of ponds and the intestinal tracts of cultured fish, ayu (*Plecoglossus altivelis*). Jpn. J. Microbiol. **19:**7–12.
5. **Aoki, T., T. Arai, and S. Egusa.** 1977. Detection of R plasmids in naturally occurring fish pathogenic bacteria, *Edwardsiella tarda*. Microbiol. Immunol. **21:**77–83.
6. **Aoki, T., and S. Equasa.** 1971. Drug sensitivity of *Aeromonas liquifaciens* isolated from freshwater fishes. Bull. Jpn. Soc. Sci. Fish. **37:**19–28.
7. **Aoki, T., S. Equasa, T. Kimura, and T. Watanabe.** 1971. Detection of R factors in naturally occurring *Aeromonas salmonicida* strains. Appl. Microbiol. **22:**716–717.
8. **Aoki, T., and T. Kitao.** 1985. Detection of transferable R plasmids in strains of the fish-pathogenic bacterium, *Pasteurella piscicida*. J. Fish Dis. **8:**345–350.
9. **Aoki, T., Y. Mitoma, and J. H. Crosa.** 1986. The characterization of a conjugative R-plasmid isolated from *Aeromonas salmonicida*. Plasmid **16:** 213–218.
10. **Aoki, T., T. Satoh, and T. Kitao.** 1987. New tetracycline resistance determinant on R plasmid from *Vibrio anguillarum*. Antimicrob. Agents Chemother. **31:**1446–1449.
11. **Aoki, T., and T. Watanabe.** 1973. Studies of drug-resistant bacteria isolated from eel-pond water and intestinal tracts of the eel (*Anguilla japonica* and *Anguilla anguilla*). Bull. Jpn. Soc. Sci. Fish. **39:**121–130.
12. **Association of Official Analytical Chemists.** 1990. Oxytetracycline in feeds, p. 126–127. *In* K. Helrich (ed.), Official methods of analysis, 15th ed. Association of Official Analytical Chemists, Arlington, Va.
13. **Austin, B.** 1985. Antibiotic pollution from fish farms: effects on aquatic microflora. Microbiol. Sci. **2:**113–117.
14. **Austin, B., and A. M. J. Al-Zahrani.** 1988. The effect of antimicrobial compounds on the gastrointestinal microflora of rainbow trout, *Salmo gairdneri* Richardson. J. Fish Biol. **33:**1–14.
15. **Austin, B., and D. A. Austin.** 1987. Bacterial fish pathogens: disease in farmed and wild fish. Ellis Horwood, Chichester, United Kingdom.
16. **Chang, B. J., and S. M. Bolton.** 1987. Plasmids and resistance of antimicrobial agents in *Aeromonas sobria* and *Aeromonas hydrophila* clinical isolates.

Antimicrob. Agents Chemother. **31:**1281–1282.
17. **DeGrandis, S. A., and R. M. W. Stevenson.** 1985. Antimicrobial susceptibility patterns and R plasmid-mediated resistance of the fish pathogen *Yersinia ruckeri*. Antimicrob. Agents Chemother. **27:**938–942.
18. **DePaola, A.** 1995. Tetracycline resistance by bacteria in response to oxytetracycline-contaminated catfish feed. J. Aquat. Anim. Health **7:**155–160.
19. **DePaola, A., P. A. Flynn, R. M. McPhearson, and S. B. Levy.** 1988. Phenotypic and genotypic characterization of tetracycline- and oxytetracycline-resistant *Aeromonas hydrophila* from cultured catfish (*Ictalurus punctatus*) and their environments. Appl. Environ. Microbiol. **54:**1861–1863.
20. **Fass, R. J., and J. Barnishan.** 1981. In vitro susceptibilities of *Aeromonas hydrophila* to 32 antimicrobial agents. Antimicrob. Agents Chemother. **19:** 357–358.
21. **Hettmansperger, T. P.** 1984. Statistical inference based on ranks, p. 178–194. John Wiley & Sons, Inc., New York.
22. **Husevag, B., B. T. Lunestad, P. J. Johannessen, O. Enger, and O. B. Samuelsen.** 1991. Simultaneous occurrence of *Vibrio salmonicida* and antibiotic-resistant bacteria in sediments at abandoned aquaculture sites. J. Fish Dis. **14:**631–650.
23. **Levy, S. B., G. B. Fitzgerald, and A. B. Macone.** 1976. Spread of antibiotic resistance from chicken to chicken and chicken to man. Nature (London) **260:**40–42.
24. **Levy, S. B., G. B. Fitzgerald, and A. B. Macone.** 1976. Changes in the intestinal flora of farm personnel after introduction of a tetracycline supplemented feed on a farm. N. Engl. J. Med. **195:**583–588.
25. **MacMillan, J. R., and T. Santucci.** 1990. Seasonal trends in intestinal bacterial flora of farm-raised channel catfish. J. Aquat. Anim. Health **2:**217–222.
26. **McPhearson, R. M., A. DePaola, S. R. Zywno, M. L. Motes, Jr., and A. M. Guarino.** 1991. Antibiotic resistance in gram-negative-bacteria from cultured catfish and aquaculture ponds. Aquaculture **99:**203–211.
27. **Ostle, B., and R. W. Mensing.** 1975. Statistics in research, 3rd ed., p. 289–374. The Iowa State University Press, Ames, Iowa.
28. **Reinhardt, J. F., and W. L. George.** 1985. Comparative in vitro activities of selected antimicrobial agents against *Aeromonas* species and *Plesiomonas shigelloides*. Antimicrob. Agents Chemother. **27:**643–645.
29. **Rolland, R. M., G. Hausfater, B. Marshall, and S. B. Levy.** 1985. Antibiotic-resistant bacteria in wild primates: increased prevalence in baboons feeding on human refuse. Appl. Environ. Microbiol. **49:**791–794.
30. **Shotts, E. B., Jr.** 1987. Bacterial diseases of fish associated with human health. Vet. Clin. North Am. Small Anim. Pract. **17:**241–247.
31. **Sugita, H., M. Fukumoto, H. Koyama, and Y. Deguchi.** 1988. Changes in the fecal microflora of goldfish *Carassius auratus* with the oral administration of oxytetracycline. Nippon Suisan Gakkaishi **541:**2181–2187.
32. **U.S. Government Printing Office.** 1986. Oxytetracycline. Title 21, parts 586–589. *In* Code of Federal regulations. U.S. Government Printing Office, Washington, D.C.
33. **Waltman, W. D., and E. B. Shotts.** 1986. Antimicrobial susceptibility of *Edwardsiella tarda* from the United States and Taiwan. Vet. Microbiol. **12:**277–282.
34. **Waltman, W. D., and E. B. Shotts.** 1986. Antimicrobial susceptibility of *Edwardsiella ictaluri*. J. Wildl. Dis. **22:**173–177.