OCCURRENCE AND SURVIVAL OF SALMONELLAE IN THE ALIMENTARY TRACT OF SOME FRESH WATER FISHES

by

WILLIAM T. MARTIN

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Approved by:

V. D. Foltz

Major Professor

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INTRODUCTION

The isolation of salmonellae from the alimentary tracts of fish has been rarely reported. The first instance recorded was the isolation by Gibbons in 1934 of an Eberthella (Salmonella typhi) from a marine fish taken off the Eastern Coast of Canada. This was also the only reported isolation of a salmonellae from fish of the North American Continent. Workers in foreign countries subsequently also found salmonellae in the alimentary tracts of fish taken from polluted waters.

Investigators working in this country have neglected this area although there is reason to believe that organisms of the genus Salmonella might be found in the alimentary tract of fish taken from our polluted waters. First, there are reports which indicate that the bacterial flora of the fish gut is directly influenced by the organisms in the food and water taken in by the fish, and it is also recognized that a sewage pollution problem exists in this country. Secondly, turtles which inhabit the same water are frequently infected with salmonellae, and they too are cold blooded creatures.

The demonstration that fresh water fish do harbor salmonellae would indicate that fish when used for human food would be a potential source of infection. This information would be of value to the rapidly developing industry of fish culture in the United States.

The objectives of this study were to survey the extent of natural salmonella contamination of the alimentary tract of fresh water fish taken from two locations on a river, to determine the source of the salmonella in this river, and to determine experimentally the survival time of these organisms in the fish alimentary tract.

LITERATURE REVIEW

Most studies of the intestinal flora of marine and fresh water fish have been concerned with either the predominant natural flora or with the absence or presence of Escherichia coli. A few investigators have looked specifically for enteric pathogens in the alimentary tract of fish with surprising success.

In general, it has been concluded that the flora of the fish gut is dependent on the bacteria in the water and in the food which supported the fish (11,12,13,18). Fish taken from the open sea had no E. coli, but fish taken from littoral waters and polluted rivers commonly had coliforms in their alimentary tracts (1,3,5,10,11,12,13,15,16,18,32).

Guelen exposed marine fish taken from the open sea to a culture of E. coli, and was able to demonstrate the survival of these bacteria in the gut of the fish for only seven days (15). He concluded that enteric bacteria were not capable of survival for extended periods of time in the gut of the marine fish.

Similar studies in fresh water using trout, bluegills, and carp, showed that coliforms which had been ingested via food or water disappeared from the gut within 1-14 days (10,13). Margolis concluded that trout and bullheads lost their intestinal flora as a result of fasting (22).

There have been only a few instances of salmonellae and shigellae being isolated from the intestinal tract of fishes, excluding shellfish. Coincidental isolations of enteric pathogens have been made during normal flora studies. Gibbons isolated an Eberthella (S. typhi) from one of forty-three marine fish taken off the Eastern Coast of Canada (12); Gohar isolated a shigella from one of 150 fish from the Red Sea (14); Arcisz isolated

S. enteritidis from one of 29 Caribbean fish (2); Thjotta and Somme isolated both salmonella and shigella from fish (29); and Van der Brock isolated a salmonella from an eel kept in a polluted harbour (31). On the other hand, those investigators who looked specifically for salmonellae and shigellae in fish were very successful. In 1949 Trawiniska reported the isolation of 13 strains of salmonellae from 80 fish taken from a pond in Poland (30). In 1950 Leiguarda found 19.6 percent of 97 fish from Plata River in Argentina carrying salmonellae (21). From the Nile River in 1954, Floyd and Jones found 44 of 376 pools of fish (3 fish per pool) or 11.1 percent of their pools to contain salmonellae, shigellae, or both (5). In 1956 Gulasekharan found that 39 of 629 fish from the Colombo River in Ceylon contained salmonellae (17). In 1957 Jadin found 2 percent of the fish from the Great Lakes of Central Africa to harbor salmonellae (19). Also in 1957 Gaugusz found 6 percent of the fresh water crayfish in Polish rivers to be infected with salmonellae (9). In every case the source of these salmonellae and shigellae was either known or suspected to be the sewage which polluted the water from which the fish were taken.

Geldrisch and Clarke conducted extensive studies on fecal streptococci and coliform bacteria found in the alimentary tract of fish taken from the Little Miami River in Ohio (10). No mention was made of isolating enteric pathogens. However, they demonstrated the growth of both salmonellae and shigellae in fecal material obtained from blue gills at 20 C. This material was sterilized with ethylene oxide before inoculation. Under similar circumstances fecal material from carp was toxic to the test organisms at both 10 and 20 C.

MATERIALS AND METHODS

General Materials and Methods

Time Span of Studies: The different phases of this study were conducted more or less simultaneously and without regard to the results from any other part of the study. The experimental work was begun in the middle of June 1966 and was completed the end of July 1966.

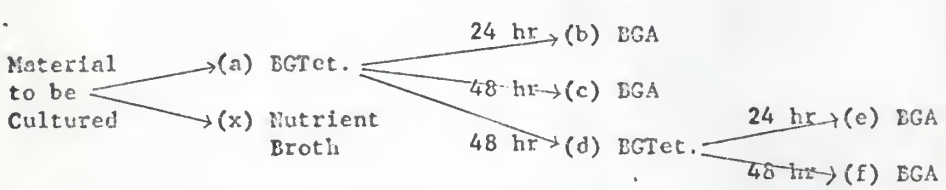
Media: The media used was the dehydrated commercial products of the Digestive Ferments Company (Difco). All media was prepared in accordance with the manufacturer's specifications, with the following exceptions:

Tetrathionate Broth (BGTet.): 1/100,000 wgt./vol. of brilliant green dye (certified for use in bacteriological media) as modified by Kaufmann and described in Salmonellae in Foods was used throughout this study (7).

Brilliant Green Agar (BGA): For the purpose of plating sewer swab enrichments, 8 mg. of Sodium Sulfadiazine (Lederle) was added per 100 ml of brilliant green agar and mixed well prior to pouring plates (7,8). No sulfadiazine was used in the BGA plates used in the other phases of this investigation because overgrowth with pseudomonas and other organisms was not a significant problem.

Isolation and Identification:

General Culture Scheme



Alimentary Tracts of River Fish: Steps (a), (b), (c), (d), (e), (f), were used unless an early step produced positive results.

Sewer Swabs: Steps (a), (d), (e), (f), were used (sulfadiazine was used in the BGA to help overcome the massive numbers of other bacteria present).

Qualitative Cultures of Experimental Fish: Steps (a), (b), (c).

Quantitative Cultures "MPN's" of Pooled Fish: Steps (a), (b), (c) in multiples of 5 specimens for each ten-fold dilution.

Feed Samples: Those specimens cultured in duplicate were processed by steps (a), (b), (c), and (x), (d), (e), (f).

All specimens were streaked from BGTet. selective enrichment broth after incubation at 37 C to BGA plates. Following incubation of the plates, suspicious salmonella-like colonies were picked to Triple Sugar Iron Agar (TSI) slants. Those slants having salmonella reactions of: alkaline (red) slant, acid (yellow) butt, gas (separation of agar from glass tube), and with or without hydrogen sulfide (formation of black color in agar) were tested with polyvalent "H" salmonella antiserum by the slide technique. Positive cultures were subsequently streaked to MacConkey Agar and a single colony picked to a nutrient agar slant which was sealed (screw capped tube) and held for serotyping.

The definitive serotyping of all cultures was done at one time by the author in the laboratories of the Epidemic Aid Laboratory Section, Epidemiology Branch of the Communicable Disease Center. The methodology used was the standard serological procedures described by Edwards and Ewing (4). All cultures were typed without knowledge of their individual relationship to the study.

Survey Materials and Methods

To determine the occurrence of salmonellae in the alimentary tract of fish, and to find a source of pollution for the two rivers to be studied, a culture survey of fish and sewage was undertaken.

Collection of Fish From the Republican River (7/14/66) Kansas State Fisheries Biologists collected the fish for this study and at the same time made a total fish population survey of the Republican River below the Junction City, Kansas sewage treatment plant. This plant had only primary treatment facilities and processed between one and two million gallons of sewage each day. The river was low with water flowing swiftly in the main channel which had an average depth of about 3 feet. Many sandbars were exposed by the low water level despite the fact that a large volume of water was flowing in this river.

Rotenone was added to the river at the point where the sewage plant effluent enters the channel and at intervals downstream. Because very few fish were available, collections continued downstream for at least 1.5 miles below the sewer outfall. The fish were taken from the river by hand or with dip nets and were placed in an insulated picnic cooler. Immediately on leaving the river, ice was poured over the fish, and they were then transported 20 miles to the laboratory in Manhattan, Kansas.

Handling of Fish in Laboratory: The fish were sorted according to size and species; their weights and lengths were recorded, and scales or spurs were collected from representative fish of each size group for determining the ages of the fish.

Two fish were cultured individually, one because of its size (30 pounds), the other was small (0.5 pound), the only fish of that species. All other fish were cultured in pools of from two to ten fish each, depending on the size and species of the fish. Large and small fish were not pooled together, thus as many as ten small fish of a species may have been put together while

only 2 larger fish were pooled. With the exception of the 30 pound catfish mentioned above all fish weighed under one pound, and many of the channel catfish weighed less than one ounce.

Each fish was placed on its back on a stainless steel surface and was supported on either side with alcohol soaked cotton. The entire abdomen was sponged off with cotton soaked with 95 percent ethyl alcohol. Scissors sterilized by alcohol flaming were used to cut through the abdominal wall just anterior to the anus; holding the back ventral fin with fingers or forceps (depending on size of fish) the abdominal wall was cut forward on both sides to the gill plate. The flap of abdominal tissue was then either folded back over the head of the fish or was cut off and discarded leaving the viscera of the fish fully exposed.

Flamed forceps and scissors were used to remove the stomach and intestine from each fish and to mince these organs into sterile pint jars.

Culture Methods: Brilliant green tetrathionate broth was poured over these pools of tissue in an amount approximately three times the sample volume. These jars were then incubated at 37 C and were streaked after 24 and 48 hours to BGA plates. The BGA plates were incubated 24 hours at 37 C and observed for suspicious, salmonella-like colonies. Two or three suspicious colonies were picked to TSI agar slants which were incubated overnight at 37 C. From those original samples which did not yield salmonellae after 24 or 48 hours incubation, 0.2 ml was transferred to a fresh 10 ml tube of BGTet. broth. This tube was also streaked after incubation at 37 C for 24 and 48 hours to BGA. When suspicious colonies were first evident, subsequent steps as described above were not carried out for that sample.

All samples were kept, however, in the event that the suspicious colonies were not salmonellae. All TSI's were observed for typical salmonellae reactions.

The growth from slants fitting this description was tested with polyvalent "H" salmonella serum (Difco) by slide agglutination. All positive cultures were then streaked to MacConkey agar, and a single colony was picked to insure purity and to stab into a nutrient agar butt as a stock culture for future reference and serotyping.

Collection of Fish From the Kansas River (7/18/66) Fish were collected from the Kansas River below the sewage treatment plant in Manhattan, Kansas. This plant provided primary treatment for approximately a million gallons of sewage each day. The Kansas River was low at the time of fish collection but probably had a flow of 3 to 5 times as much water as the Republican River previously described. The Kansas River is a continuation of the Republican River.

Rotenone was added in a line across the river at the sewage outfall. No further Rotenone was used because the results were adequate. Fish were collected as far as one mile downstream, although most of the fish taken probably originated within a few hundred yards below the sewage outfall and were carried further downstream by the current.

Many large fish were taken (5-25 pounds), and due to their size, they were thrown on the dry floor of the boat where they remained until they were transferred to galvanized tubs to be carried to the laboratory. Smaller fish were put in picnic chests with crushed ice as they were collected.

Processing of Fish: These fish were cultured on the day of collection (7/18) and the day after collection (7/19). The fish were sorted, weighed, measured, and scales and spurs collected for the population survey by the Fisheries Biologist. All fish were refrigerated until they were processed for culture.

Handling of fish for culture was the same as described for the fish taken from the Republican River except the stomachs of these fish were not cultured. It was noted that the stomachs of the channel catfish were gorged with minnows which had been more rapidly affected by the Rotenone than the catfish. To prevent this from possibly affecting the numbers of salmonellae recovered, only the intestine was cultured of all fish taken from the Kansas River.

Sewer Cultures: In an effort to demonstrate a source of salmonellae which could contaminate the Republican and Kansas Rivers, the sewage from the towns of Manhattan, and Junction City, Kansas were cultured from 7/2 to 7/14/66.

Sanitary napkins were stapled two or three times along the midline with an ordinary office stapler; the napkin was then folded, the gauze ends tied together and a length of string attached. This was then put into a widemouth jar with the end of the string protruding from under the lid and sterilized. These sanitary napkins henceforth referred to as "Sewer Swabs" were used to culture the sewage (23).

On each of six days a "sewer swab" was placed into: (a) the raw sewage as it entered the plant; (b) the treated effluent as it left the treatment plant; (c) and in the river just below the outfall. This was done at the sewage treatment plants of both Manhattan and Junction City.

These swabs were set by holding the exposed end of the string and shaking the sterile "sewer swab" out of the jar into the area to be cultured. The string was adjusted to the right length and secured to some stable object. These "sewer swabs" were exposed to the sewage for 48 hours at which time new "swabs" were set, and the exposed ones were collected and put into empty sterile widemouth pint jars. These jars were then taken directly to the laboratory.

Culturing Sewer Swabs: Brilliant green tetrathionate broth was added in an amount equal to approximately 3 times the volume of the "sewer swab", and the jars were placed in an incubator at 37 C. In some instances, the "sewer swab" was divided in two, only one-half being cultured. Because of the fluctuating velocity of the sewage flow and the abrasiveness of the sides of the sewage conduit it often happened that only the gauze cover of the "sewer swab" was recovered.

The first "sewer swab" enrichments were plated at 24 and 48 hours on BGA with poor results. Approximately 0.2 ml of the original enrichment was then transferred to 10 ml of fresh BGTet. broth, incubated 24 hours at 37 C and streaked to BGA with sulfadiazine. This procedure prevented the overgrowth of salmonellae by other sewage organisms. All subsequent "sewer swabs" were transferred to fresh enrichment at 48 hours, and this sub-enrichment was streaked to BGA with sulfadiazine at 24 and 48 hours. The original enrichment was not plated. The sub-enrichments were held until suspicious colonies had been picked to TSI and those slants showing typical salmonellae reactions had been tested with salmonella polyvalent "H" serum. These cultures were then streaked to MacConkey agar for purity and were stocked for future reference and serotyping.

Experimental Materials and Methods

In an attempt to determine the survival time of salmonellae in the alimentary tract of experimentally infected channel catfish, a group of fish were inoculated with a culture of salmonellae and placed in tanks of water without food. At appropriate intervals, groups of these fish were sacrificed and the number of salmonellae still remaining in the alimentary tract were cultured quantitatively and qualitatively.

Two, 150 gallon galvanized steel cattle tanks, each equipped with a stand pipe to regulate the water level and an aerator to maintain sufficient oxygen in the water, were used to keep the fish alive for this experiment. The continuous flow of water into these tanks was passed through an activated charcoal filter which removed the chlorine from the city water. The water flow was approximately 0.5 gallon per minute per tank. The water temperature in the tanks throughout the study was 65 F.

Ninety-five channel catfish were seined from a farm pond which had been stocked the previous year. The fish were approximately the same size and averaged about 0.5 pound each. They had been receiving a supplemental ration of a pelleted fish feed prepared by the Department of Flour and Feed Milling Industries of Kansas State University for experimental purposes. When the fish were put in the experimental tanks, they regurgitated the contents of their stomach due to handling and the change in the water temperature. A seine was stretched over the tanks to prevent any fish from jumping over the sides. The fish were left in the tanks (approximately one-half of the fish in each tank) for 3 days undisturbed. Four fish died during this time and 2 others died early in the experiment and were discarded.

Control Fish: To determine whether or not these fish might have salmonellae in their alimentary tracts as a result of eating a ration which may have been contaminated, twenty fish (10 from each tank) were removed with a dip net, placed in an empty (no water) picnic cooler, and transported to the laboratory where they were cultured for salmonellae as described under the section on culturing of fish from the Republican River. In this case each fish was cultured individually.

Fish Feed: Samples of commercial fish feed were cultured for salmonellae. Duplicate 30 gm samples were cultured from each specimen: one sample was flooded with brilliant green tetrathionate broth, the other with nutrient broth which served as a pre-enrichment from which 0.2 ml was subsequently transferred at 48 hours to a tube containing 10 ml of BGTet. broth. The tetrathionate broths were each streaked to BGA after 24 and 48 hours of incubation at 37 C and processed as previously described.

The ingredients which were put into the fish feed compounded by the Kansas State Mill were also cultured for salmonellae using direct BGTet. enrichment.

Culture Used to Inoculate Fish: Cultures of S. thompson, S. muenchen, and S. typhi-murium grown for 18 hours at 37 C in trypticase soy broth were mixed in equal proportions in a 6 oz prescription bottle. After thorough mixing, an aliquot was immediately refrigerated and the remaining mixture was used to inoculate the experimental fish. After inoculating the fish, plate counts in triplicate were made on both the portion used for inoculating and the refrigerated aliquot. The average of the combined plate counts was 1.02×10^9 per ml. The salmonella cultures used were isolated from pet turtles (6).

Inoculation of Fish: Each fish was inoculated individually and released into a second tank. Following inoculation, half of the fish were transferred back to the first tank.

Each fish was caught with a dip net wrapped in a wet chamois which stuck to the slick skin and provided protection from the sharp spurs. While the fish was held in a mouth-up position, a pipette containing 5 ml of the salmonella culture was inserted into its mouth and down the esophagus into the stomach where 1 ml of the culture (1.02×10^9 salmonellae) was delivered. Two fish were observed to regurgitate this inoculation, but they were not reinoculated. There was no way of determining what happened to the inoculum after the fish were released.

Sampling Schedule: Fish were cultured quantitatively in pools of 5 fish each at 4 day intervals starting at 24 hours after inoculation. A decreasing number of pools was done on each sample date because of the unexpected length of the survival time of the bacteria. The last pool of this experiment, consisting of only 4 fish, was cultured eight days after the preceding pool in an effort to reach an end point. On each sample date a water sample was also collected from the fish tank and cultured quantitatively for salmonellae.

The fish were dipped from the tanks (equal number from each tank) on the first and second sample dates at which time all the remaining fish were placed into one tank. The fish were carried to the laboratory for culture in a picnic cooler which contained no water, and were still alive when cultured.

Quantitative and Qualitative Culture: Each fish was cultured qualitatively and also incorporated into a pool which was cultured by the Most Probable Number (MPN) technique of enumeration (26).

Each fish was struck on the head with a pair of rib cutters to kill and immobilize it. The abdomen of the fish was swabbed with 95 percent ethyl alcohol and was opened as previously described in culturing the river fish. The intestinal tracts from approximately 1/4 inch below the stomach to 1/4 inch above the anus were removed and placed individually in sterile petri dishes and refrigerated until ground. Each intestine was placed in a separate sterile mortar and pestle, and sterile sand was added to each to facilitate grinding. To each mortar 10 ml of diluent (0.15 percent peptone saline) (27) was added and the grinding was completed. The liquid from each of five specimens was pooled into a 20 x 150 mm sterile screw capped tube. The tissue and sand left in the mortar was washed with 20 ml of EGTet. broth, which was poured back into the tube for a qualitative culture of that individual fish. The pooled mixture of the liquid from five fish was distributed into EGTet. broth as follows: (a) 5 ml into each of 5 tubes containing 20 ml of enrichment, (b) 1 ml into each of 5 tubes containing 10 ml of enrichment, (c) appropriate 10-fold dilutions from 1×10^{-1} to 1×10^{-5} were transferred into 5 tubes per 10-fold dilution containing 10 ml of enrichment broth.

At the same time the fish were taken from the tanks, a "MPN" of salmonellae in the tank water was either set up by pipetting directly from the tank, or in some instances, a sample of water was collected in a sterile container and taken to the laboratory to be inoculated into EGTet. enrichment broth.

The water MPN consisted of the following: (a) five, 50 ml water samples each inoculated into a bottle containing 100 ml of enrichment, (b) five, 10 ml samples each inoculated into a tube containing 20 ml of

enrichment, (c) five, 1 ml samples each inoculated into a 10 ml tube of enrichment, (d) five, one-tenth ml samples each inoculated into a 10 ml tube of enrichment.

All water samples were taken from the same tank throughout the study. This tank was the one in which all inoculated fish were first released and into which the fish were re-combined on the fifth day of the experiment.

On the fifth day post inoculation, a cotton swab was inserted into the lumen of the stomachs of five fish and streaked directly to BGA. Four of these five plates produced salmonellae. On the ninth day post inoculation, the bottom of each stomach (from all 15 fish cultured on that date) was cut off with alcohol flamed scissors and dropped into a tube of BGTet. broth. All these samples yielded salmonellae and from the next sample date onward, the stomachs were removed and cultured quantitatively and qualitatively in a manner identical to that described for the culture of the fish intestine.

All quantitative enrichment cultures were streaked to BGA plates after 24 and 48 hours of incubation at 37 C. Colonies from the five highest "MPN" dilutions were picked to TSI agar slants. Those TSI's giving typical salmonella reactions were tested with salmonella polyvalent "H" antiserum and in some cases these slants were also tested with salmonella "O" sera of Groups B, C_1, and C_2. These "O" groups corresponded to those of the three serotypes inoculated into the experimental fish. Complete serotyping of these cultures was not carried out except for one isolation of each of the representative "O" groups (B, C_1, and C_2) which were isolated from the last group of fish sacrificed on the 29 day of the experiment.

RESULTS

It should be noted that the "sewer swabs" were set on 7/2, 7/4, 7/6, 7/8, 7/10, and 7/12 at both sewage treatment plants and were correspondingly collected two days after being set. This immediately preceded the collection of fish from the Republican River at Junction City on 7/14. The fish from the Kansas River at Manhattan were collected on 7/18/66 which was four days after the last sewer swabs were collected.

Survey of River Fish: Of the two fish from the Republican River (Table 1) which were cultured individually, one was positive for salmonella (S. cubana). Twelve of fourteen pools (85.7 percent) with from 2 to 10 fish per pool were positive for salmonellae. Overall 13 of 16 (81.3 percent) of the total specimens were positive with eight different serotypes being isolated. The frequency of isolation is as follows: S. enteritidis (4 isolations), S. berta (2), S. infantis (2), and 1 each S. senftenberg, S. blockley, S. panama, S. lomita, and S. cubana.

Twelve fish of 5 pounds and over taken from the Kansas River (Table 2) were cultured individually, and 6 (50 percent) were positive for salmonellae. These fish were of two species, bullheads and suckers of which 1 of 5 (20 percent) and 5 of 7 (70 percent) were positive respectively. Seventeen (94.4 percent) of 18 pools of 2 or more fish each were positive for salmonellae. Salmonella enteritidis was again the most common serotype with 8 isolations. Additional isolations were S. blockley (6), S. anatum (4), S. typhi-murium and S. thompson, 3 each; S. bredeney and S. tennessee, 2 each; and S. newport, S. montevideo, S. oranienburg, and S. muenchen were isolated once each.

Table 1

Salmonellae Isolated from Stomach and Intestine of Fish Taken from Republican River Below the Junction City, Kansas Sewage Treatment Plant, 7/14/66

Species of Fish	No. Samples	No. Fish Each Sample	No. Positive Samples	Serotype
Bullhead Catfish (*Ameiuris* sp.)	1	1	1	*S. cubana*
" " "	1	5	0	
Channel Catfish (*Ictalurus* sp.)	1	2	0	
" " "	3	5	3	*S. infantis* (2), *S. lomita* (1)
" " "	3	10	3	*S. enteritidis* (1), *S. blockley* (1), *S. panama* (1)
Carp sucker (*Catostomus* sp.)	2	4	2	*S. senftenberg* (1), *S. berta* (1)
" " "	1	5	1	*S. enteritidis* (1)
" " "	1	6	1	*S. berta* (1)
Drum (*Carpiodes* sp.)	1	5	1	*S. enteritidis* (1)
White Bass (*Roccus* sp.)	1	3	1	*S. enteritidis* (1)
Gar (*Lepidosteus* sp.)	1	1	0	
Total	16		13	

Table 2

Salmonellae Isolated from Intestine of Fish Taken from the Kansas River Below the Manhattan, Kansas Sewage Treatment Plant 7/18/66

Species of Fish	No. Samples	No. Fish Each Sample	No. Positive Samples	Serotype
Bullhead catfish (*Ameiuris* sp.)	5	1	1	*S. enteritidis* (1)
" " "	1	5	0	
" " "	1	10	1	*S. enteritidis* (1)
Channel catfish (*Ictalurus* sp.)	3	2	3	*S. blockley* (2), *S. thompson* (1), *S. bredeney* (1)
" " "	1	3	1	*S. tennessee* (1), *S. anatum* (1)
" " "	4	5	4	*S. typhi-murium* (2), *S. blockley* (1), *S. montevideo* (1), *S. thompson* (1), *S. oranienburg* (1), *S. enteritidis* (1)
" " "	3	10	3	*S. muenchen* (1), *S. thompson* (1), *S. enteritidis* (2)
Carp sucker (*Catostomus* sp.)	7	1	5	*S. enteritidis* (1), *S. anatum* (1), *S. blockley* (1), *S. bredeney* (1), *S. newport* (1)
" " "	1	3	1	*S. enteritidis* (1), *S. typhi-murium* (1)
" " "	2	4	2	*S. enteritidis* (1), *S. anatum* (1), *S. tennessee* (1)
Drum (*Carpiodes* sp.)	2	4	2	*S. blockley* (2), *S. anatum* (1)
Total	30		23	

Survey of Sewage: Thirty-three "sewer swabs" were cultured from the two sewage treatment plants over a twelve day period preceding the collection of fish from the rivers receiving the respective effluent from the Manhattan and Junction City sewage plants (Table 3). All 33 (100 percent) "sewer swabs" were positive for salmonellae. Seventeen serotypes were isolated with only two serotypes common to both towns. Three swabs were lost at the Manhattan location.

Eight serotypes were recovered from the "sewer swabs" at Manhattan, Kansas. All eight of these serotypes were also recovered from the fish cultured from the Kansas River (Table 4). In addition to those serotypes which were common to both fish and "sewer swabs", three others were isolated, one of which, S. enteritidis (isolated 8 times), was the most common serotype isolated from the fish taken from the Kansas River. Since this serotype was not isolated from the "sewer swabs", it can be construed to indicate some other source of heavy salmonella contamination of the Kansas River, or that this serotype may have been very common in the sewer at some time earlier than the "sewer swabs" would have been able to detect.

A total of 20 different serotypes were isolated from the combined sewage and fish surveys of both locations (Table 4). Seventeen different serotypes were isolated from all fish cultures and the same number, seventeen, was also isolated from the combined "sewer swabs" of the combined locations. Only 6 serotypes were not common to both fish and sewer, and none of these 6 types were recovered more than once each.

No effort was made to quantitate salmonella from the river water during this study, but the results of the cultures of the alimentary tracts

Table 3

Sewer Swab Isolations

(Location)

Date	Manhattan, Kansas			Junction City, Kansas		
	Raw Sewage	Treated Sewage	River at Outfall	Raw Sewage	Treated Sewage	River at Outfall
7/2-4	S. muenchen S. typhi-murium	S. anatum S. bredeney	S. anatum	S. enteritidis	S. panama	S. cubana
7/4-6	S. monte-video	S. bredeney	S. montevideo	S. enteritidis	S. infantis S. enteritidis	S. infantis S. enteritidis
7/6-8	S. tenn-essee	S. thompson	S. anatum S. newport	S. enteritidis	S. derby S. blockley	S. infantis S. enteritidis
7/8-10	*	S. anatum	S. montevideo	S. anatum S. enteritidis	S. give	S. blockley
7/10-12	S. monte-video	S. thompson	*	S. anatum	S. blockley	S. senftenberg
7/12-14	S. monte-video	S. anatum	*	S. montevideo	S. meleagridis	S. senftenberg

*Sewer Swabs Lost - No Culture

Table 4

Frequency of Isolations of Salmonellae Serotypes from Sewer Swabs and from the Alimentary Tracts of Fish from Manhattan and Junction City, Kansas

Serotype	Manhattan, Kansas No. Isolations from Sewer	Manhattan, Kansas No. Isolations from Fish	Junction City, Kansas No. Isolations from Sewer	Junction City, Kansas No. Isolations from Fish	Total Times Isolated
S. anatum	5	4	2	-	11
S. enteritidis	-	8	7	4	19
S. blockley	-	6	3	1	10
S. montevideo	5	1	-	-	6
S. thompson	2	3	-	-	5
S. bredeney	2	2	-	-	4
S. typhi-murium	1	3	-	-	4
S. tennessee	1	2	-	-	3
S. muenchen	1	1	-	-	2
S. newport	1	1	-	-	2
S. oranienburg	-	1	-	-	1
S. infantis	-	-	3	2	5
S. senftenberg	-	-	2	1	3
S. cubana	-	-	1	1	2
S. panama	-	-	1	1	2
S. berta	-	-	-	1	1
S. lomita	-	-	-	1	1
S. derby	-	-	1	-	1
S. give	-	-	1	-	1
S. meleagridis	-	-	1	-	1

of the fish and the "sewer swabs" indicate a substantial pollution problem exists in the rivers concerned.

Commercial Fish Feed: Twenty-seven samples of commercial fish feed from 7 manufacturers in 5 widely separated states and 15 samples from the Kansas State University Mill were all negative for salmonellae. Each sample was cultured in duplicate.

Ingredients used in the Kansas State Mill fish feed were cultured individually and two of these ingredients found to be contaminated with salmonellae. One of 2 samples of meat scrap was positive for S. binza and S. montevideo. Only 1 fish meal* sample was cultured and from it S. derby, S. bredeney, S. kaapstad, and S. livingstone were isolated. Additional samples of meat scrap were available and 12 of 12 samples yielded S. montevideo. The other ingredients which were either vegetable, mineral or a highly refined product such as vitamins were negative for salmonellae on a single culture for each ingredient.

There are two possible reasons for the failure to isolate salmonellae from the finished feed. First, there is a dilution of the contaminated ingredients which comprised 10 percent by weight of the feed. Second, and most important, is the possibility that any or most salmonellae were killed by the temperature of the pelleting process used in the manufacture of feeds (28).

Experimental Control Fish: The alimentary tract of 20 of the fish to be used in this experiment were cultured for salmonellae individually.

*Not to be confused with "fish feed".
Fish meal is a high protein fishery product which contains many growth factors and is commonly incorporated into animal feeds.

No salmonellae were recovered. These fish had been fed pellets made at the Kansas State University Mill.

Quantitative Survival: The decrease in the numbers of salmonellae in the intestine of fasting channel catfish is precipitous (Table 6), but the ability of the salmonellae to survive for at least 29 days is well demonstrated. Salmonellae also survived in the stomach of these fish in even higher numbers than were recovered from the intestine, though this may be a direct result of the salmonellae being inoculated into the stomach at the start of the experiment. Counts on the stomachs were not done at the beginning of the experiment because at that time the author felt the survival time would be very short. The salmonella count in the water was followed throughout the study, and in the author's opinion, the salmonellae in the water did not have any appreciable effect on the counts of either the stomach or the intestine of the fish. On the contrary, the shedding of salmonellae by the fish probably maintained the salmonellae level in the water. The erratic rise in the salmonella count of the water on 7/8 is confusing; it might have been the result of collecting a water sample just after a fish had eliminated a large number of salmonellae. With this one exception, the slope of the water MPN of salmonellae was gradual and predictable. On the last day of the experiment, 4 MPN's were done on water from four different locations within the tank. No significant difference was noted (Table 5).

Qualitative Survival: The results of all the individual cultures of the intestine and stomach of the fasting channel catfish is shown in Table 7. The reason for not isolating salmonellae from the intestine of one of fifteen fish on 6/30 and again on 7/4 is difficult to explain in any meaningful way.

Table 5

The Most Probable Numbers (MPN) of Salmonellae in Each Pool of Intestine, Stomach, and Water

Date	WATER		INTESTINE		STOMACH	
	MPN of Each Sample	Log of MPN	MPN of Each Pool	Log of MPN's	MPN of Each Pool	Log of MPN's
6/26	1600+	3.20	1,720,000 5,420,000 918,000 16,090,000	6.91*	Not done	
6/30	17	1.23	3,480 1,090,000 16,090,000	6.76*	Not done	
7/4	6	0.78	17,200 920 9,180	3.96*	Not done	
7/8	542	2.73	2,210	3.34	24,000	4.38
7/12	4	0.60	920	2.96	24,000	4.38
7/16			940	2.97	24,000	4.38
7/24	2 1 1 1	0.10*	30	1.48	920	2.96

*Log of average of MPN's where more than one pool was used

Table 6

Survival of Salmonellae in the Stomach and Intestine of Artificially Inoculated Catfish and the Corresponding Salmonellae Concentration in the Water of the Experimental Tank

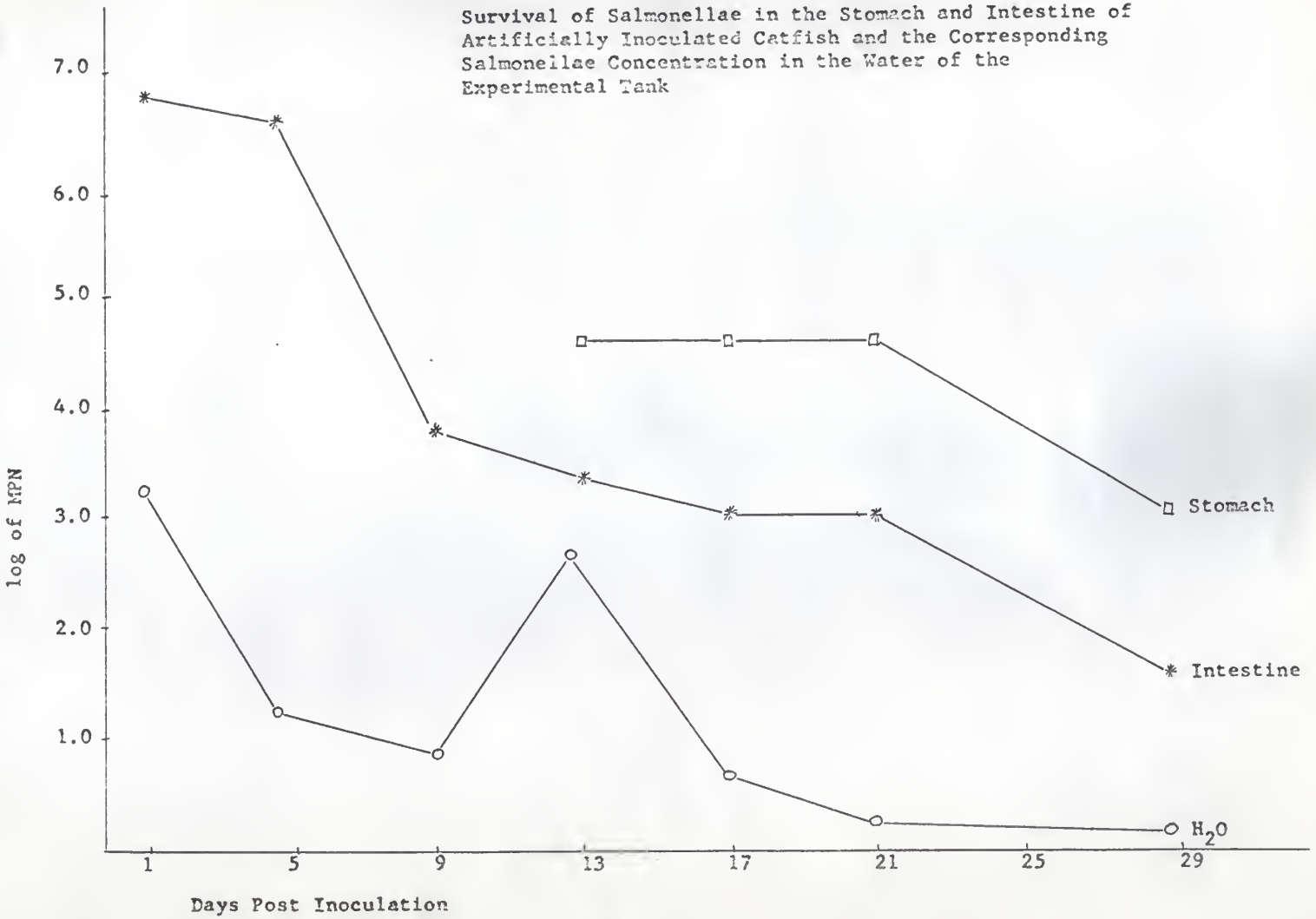
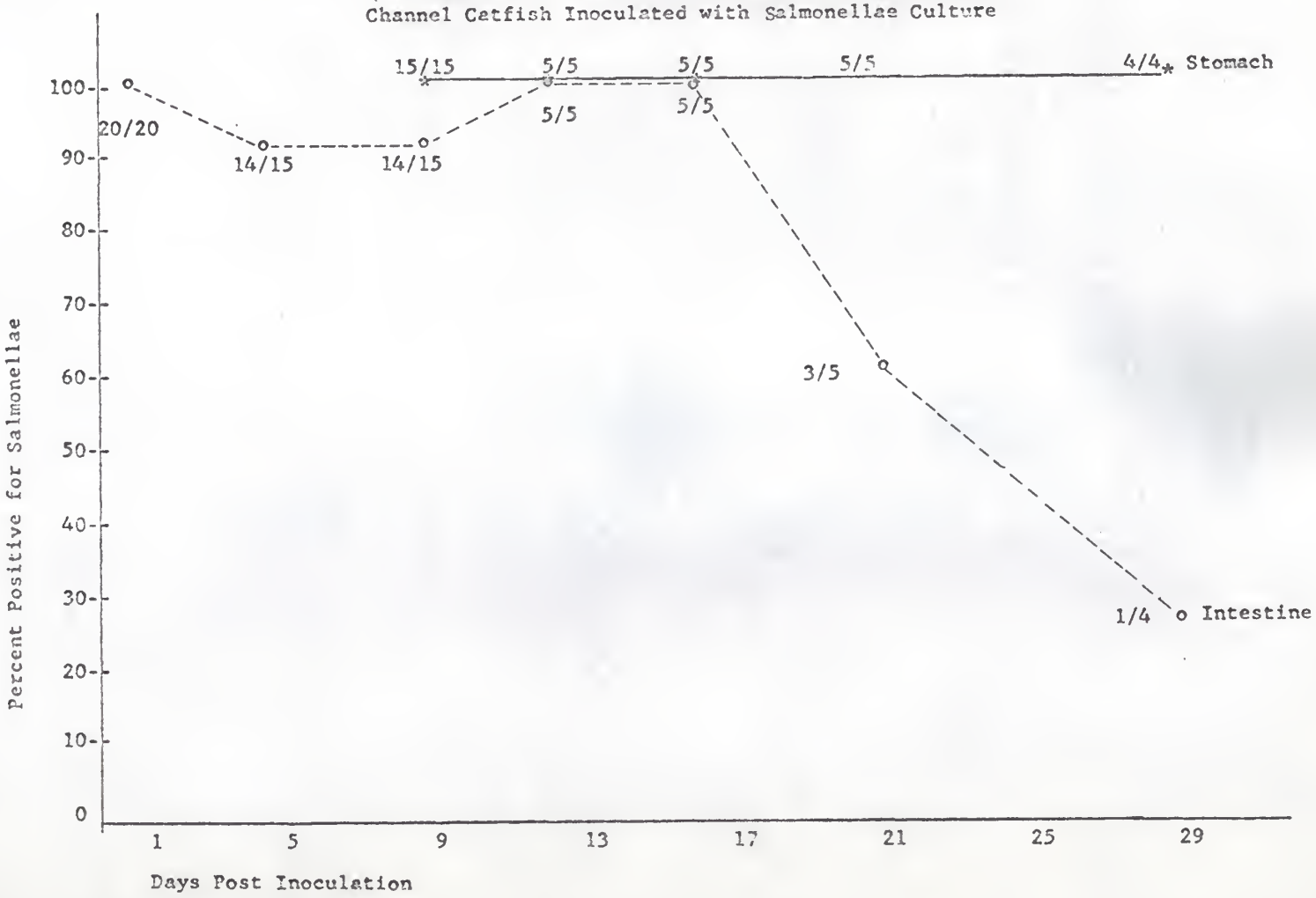


Table 7

Qualitative Cultures of the Stomach and Intestine of Channel Catfish Inoculated with Salmonellae Culture



The most logical explanation would be that they were in fact positive but slipped by undetected. It is noteworthy that despite the decrease in the number of positive individual intestine cultures, every fish was still harbouring salmonellae in their alimentary tracts as shown by the stomach cultures.

DISCUSSION

Moore's technique of using "sewer swabs" to trace typhoid carriers through the tributary branches of a sewer system has often been applied to survey work. Certainly the merit of this technique was demonstrated with salmonellae isolations from 100 percent of the "swabs" cultured in this study. The intended purpose of the "sewer swabs" was only to demonstrate a source of salmonellae for the rivers from which fish were to be taken. Thus, only one or two colonies were picked for each positive "swab". Despite the relatively short span of time (12 days) over which cultures were taken and the limited number of colonies which were serotyped, a few interesting observations were possible. First, of 17 total serotypes isolated only 2 were common to both towns. These towns were not greatly different in size and were only 20 miles apart. There was a considerable amount of commuter traffic from each town to the other with Kansas State University in Manhattan and Fort Riley near Junction City. The serotype isolated from 7 of 18 "sewer swabs" (S. enteritidis) at Junction City was not isolated from any "sewer swabs" at Manhattan although 8 isolations of this serotype were made from fish at Manhattan. Four isolations of S. enteritidis were made from fish taken at Junction City which compares with the 8 isolations from Manhattan because twice as many cultures of fish were done at Manhattan as

at Junction City (30 and 16 cultures respectively). This observation must mean one of several things: there was another heavy source of salmonellae for those fish below the Manhattan sewer, that S. enteritidis had been an abundant type in the Manhattan sewer at some time prior to the "sewer swab" survey, or that the fish, S. enteritidis, or both were carried downstream to Manhattan.

A point for speculation would be the manner in which salmonellae might have gotten into the stomach and intestine of the river fish. There are three possible routes which come to mind. The first is by the consumption of water, which fish are not supposed to do since they have no system specifically for the purpose of eliminating excess or waste water from their bodies. Secondly, surface contamination of their food whether it be insects or smaller fish might account for some salmonella intake. Thirdly, the species of fish which were cultured were practically all scavengers, some of which do not feed on other fish or live creatures. The best example of this is the carp sucker which is a bottom feeder. It is most likely that organic particles from the sewage which contain salmonellae are eaten as a predominant part of the diet of these fish.

Other points of interest which were observed concern the gut and stomach of the channel catfish inoculated and held in the laboratory. Upon opening the abdomen of these fish it was observed that several had intussusception of the intestine, the cause of which was not determined. When it was found that salmonellae were surviving in the stomach of the channel catfish beyond what the author expected, a rough check of the pH of the fish stomach revealed a neutral condition. This was determined by opening

the stomach with scissors to expose the mucosa, and placing a drop of Brom Thymol Blue directly on the mucosa. The green color which developed indicated a neutral condition.

The numbers of salmonellae present in both the stomach and intestine on the 29th day of the experiment were still well above the level of sensitivity of performing the quantitation by the MPN technique. It is also of interest that from cultures of the last four fish, which were sacrificed on the 29th day, all three of the original serotypes inoculated into the fish S. typhimurium, S. thompson, and S. muenchen were recovered.

Contrary to the survival time of E. coli in various fishes of from one to 14 days (10,13,15) salmonellae can survive for more than 29 days in both the stomach and intestine of the channel catfish. The numbers and kind of bacteria, the route of inoculation, the species of fish, and the physical conditions of the experiment were different from those of Gualin (15), Glantz (13), and Geldreich (10), and may have been responsible for some of the difference in the survival time. However, it was observed that the salmonellae are hardy organisms in this environment and in fact did survive for a longer period of time than anticipated.

There are no results to show there was any multiplication of salmonellae in the alimentary tract of the channel catfish under the conditions of this experiment. However, it is not concluded that under favorable conditions salmonellae cannot multiply in the gut of these fish.

The isolation of many of the same salmonella serotypes from sewage and from the alimentary tracts of fish taken from the river polluted by that sewage is indicative of a public health problem which is not fully

appreciated by either health officials or the public. It demonstrates just how inadequate primary sewage treatment is, and that a dangerous source of infection to the public exists, not just in the form of salmonellae but in many other pathogens eliminated in human excreta. Thus, inadequate sewage treatment provides a ready source of infection not only to man but to wild and domestic animals and birds which drink from the river and in turn may serve as human food directly or which may serve as carriers of pathogens to animals which do serve as human food. The ability of fish to pick up salmonellae and to harbor them within their gut also give those pathogens an effective means of transportation to waters where no direct pollution exists. Man may infect himself as a result of catching and cleaning such fish.

The fact that salmonellae may survive in the intestine of fish for considerable periods of time should be taken into account in producing fish products for human consumption. As previously mentioned in this paper, outbreaks of human salmonellosis have been traced to fish and fish products (20,24,25), but little consideration has been given to the possibility that the fish may have been contaminated prior to being caught and not necessarily as the result of handling during its processing. In the case of oysters, outbreaks have been traced to the oyster bed as well as to the processing operation. The knowledge of oyster contamination is due to the fact that oysters are frequently eaten raw, thus causing more outbreaks than fish. Oysters, by the nature of their method of feeding and their habitat are most likely to pick up pathogens. With the greater pollution pressure of a growing population on our fresh water supplies which in turn pollute our coastal salt water as well, contaminated fish products may some day be frequently incriminated as the source of human infection.

Finding an inexpensive source of protein for the undernourished people of the world has caused an increased interest in commercial fish culture in the United States. This industry must be cognizant of the dangers which might develop as a result of using feed contaminated with salmonella. Such feed may be in the form of a commercial product, scraps, garbage, or offal which some individuals may find to be a cheap source of food for growing fish.

Cold water species of fish such as trout would probably create much less problem than warm water fish such as the catfish. The warmer water temperatures would allow for more multiplication of pathogenic organisms in the water and possibly even in the fish's alimentary tract as well. The catfish which is a scavenger to begin with, is very hardy, grows large rapidly and inhabits warm water, all of which make it a desirable species for commercial culture. These same traits make it more susceptible to becoming a public health problem.

The relationship between fish and bacteria of public health importance is probably coincidental. There is no evidence to suggest these bacteria are true parasites of fish, and there are no reports of diseases of fish being caused by these bacteria. It was very surprising to find so many fish taken from polluted water to be harboring salmonellae. There is one distinct difference between fish and turtles in their relationship to salmonellae: the turtle seems to carry the salmonella indefinitely and multiplication must take place in the turtle without any evidence of disease, while as yet there is little evidence which would indicate the multiplication of salmonellae in the gut of fish.

More study into the relationship of salmonellae and fish is needed to determine the effect of type of feed, water temperature and species of fish on the survival and multiplication of salmonellae in the gut of fish. The present level of knowledge only serves to demonstrate a possibility of health significance and future investigations may well prove the importance of contamination of fish.

The cyclic phenomenon of salmonellosis is probably the most dramatic of all the modern diseases of man and animals. This is primarily due to the extremely wide host range, the development of carrier states, and modern mass production as it relates to multiple exposure. Both man and lower animals constitute a large reservoir for salmonellae. Wastes of both man and animal are emptied untreated or inadequately treated into our streams and rivers which in turn serves as a water supply for both domestic and wild animals that may further spread the disease which eventually will come back to man in his food.

An example of mass production which aids in the spread of salmonellosis is the massing together of large numbers of animals or fowl that are fed a rich diet which contains animal protein made from offal and scraps of infected animals. This animal protein, due to recontamination of the finished product, still contains salmonellae and thus reinfects even larger numbers of animals. These animals in turn are consumed by man who in his turn may become infected. This disease may be transmitted from man to man indirectly through food contamination or may pass through multiple cycles in animals before returning to man.

No control measures will be effective until all sources of salmonellosis are attacked simultaneously with the goal of eliminating this disease from both man and animal.

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OCCURRENCE AND SURVIVAL OF SALMONELLAE IN THE ALIMENTARY TRACT OF SOME FRESH WATER FISHES

by

WILLIAM T. MARTIN

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Department of Bacteriology

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A variety of fish collected as far as 1 1/2 miles below the sewage treatment plants of two towns on a river in Kansas were cultured for salmonellae. Fish weighing over approximately five pounds were cultured individually, while alimentary tracts of smaller fish were pooled depending on size and species with from two to ten fish per pool. Of the individually cultured fish, seven of fourteen (50 percent) yielded salmonellae. The pooled specimens were 90.6 percent positive for salmonellae (twenty-nine of thirty-two pools). Seventeen different serotypes were recovered from the fish.

Thirty-three sewer swabs, each exposed for 48 hours, were cultured over a 12 day period preceding the collection of the fish. All 33 of these sewer cultures were positive, yielding seventeen serotypes of salmonellae. Eight of eleven and six of ten of the serotypes were common to the respective sewer and fish cultures at both locations.

Channel catfish were inoculated with a mixture of three serotypes of salmonellae and kept in tanks without feed. Salmonellae were isolated from the gut of the catfish in decreasing numbers over a twenty-nine day test period. All fish were culturally positive twenty-nine days after inoculation and all three of the serotypes introduced into the fish were recovered at this time.