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The Immunological Response of a Freshwater Fish to Bacteria of Marine Origin

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The Immunological Response of a Freshwater Fish to Bacteria of Marine Origin

by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIFE

Robert S. Pekarek was born in Berwyn, Illinois, May 1, 1940.

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The author began study in the Department of Microbiology, Stritch School of Medicine of Loyola University in September, 1962.

I

PURPOSE

The aims of this study are to show the immunological response of a freshwater fish, Carassius auratus, to a bacterium of marine origin, Milford 17, and to see if there are any serological relationships between the Milford 17 strain and other marine bacteria with similar physiological and morphological characteristics.

II

HISTORY

Natural immunity can be defined as an animal's resistance to the harmful agents of its environment. However the term immunity is more commonly restricted to the resistance of an animal to various microorganisms and their toxins (Raffel, 1961).

For the most part, investigations and practical applications of the immune response, the ability of an animal to produce antibodies, has been limited to man and a variety of other warm blooded animals. Volumes of experimental and applied data have been published to this effect, and several theories have been postulated on the mechanisms involved. The literature contains relatively limited experimental data and reports on the immune response of cold-blooded vertebrates and invertebrates. Yet these animals are also subject to parasitism and, therefore, must possess some type of defense mechanism in order to survive.

A variety of diseases have been described in cold-blooded animals, especially in fish, amphibians, and reptiles. In many cases the etiological agent was isolated and characterized (Bisset, 1947). Early investigators on the diseases of these animals believed that the sole defense mechanism in cold-blooded

vertebrates was phagocytosis. Kanthack and Hardy (1892) and Mesnil (1895) demonstrated and gave full accounts of phagocytosis in frogs. However, in both investigations, the microorganism studied was Bacillus anthracis, which is primarily a pathogen of herbivorous mammals and man.

Further investigations on cold-blooded vertebrates revealed that phagocytosis was by no means the only internal defense mechanism of these animals. Widal and Sicard (1897) found that frogs could produce agglutinins at 21° and 37°C when injected with a suspension of live typhoid bacilli, but no agglutinins could be demonstrated in frogs kept at 12°C. Following investigators, using a variety of antigens, also demonstrated agglutinin production in various cold-blooded vertebrates, and in almost every case their attention was drawn to the important role temperature played in the production of agglutinins.

Nybelin (1935), using Vibrio anguillarum and Pseudomonas fluorescens as antigens with eels, perch, and crappi, found that he could get high agglutinin titers at 18° and 21°C but very low or no titers at temperatures below 10°C. Pliszka (1939) obtained similar results, using Pseudomonas fluorescens with carp. In both of these investigations the antigen was injected intraperitoneally, but the bleeding techniques were only vaguely described.

Allen and Mc Daniel (1937) investigated the immune response in frogs, Rana pipiens and Rana catesbeiana, using a

20% suspension of humen red blood cells as antigen. Five tenths cc of antigen was injected every third day, for a total of four injections. The frogs were divided into two groups, one group being kept at a temperature range of 22° to 27°C and the other between 8° and 10°C. The frogs were later sacrificed, and the blood was collected from the heart. Using complement fixation tests, they found that the frogs kept at the higher temperatures gave positive results up to a serum dilution of 1:3, while the ones kept at the lower temperatures gave negative results. It may be noted that in all of the above and following experiments, the investigators did use controls.

In another experiment, Smith (1940), using adult carp, Cyprinus carpio, and semi-weekly intraperitoneal injections with a vaccine prepared from Bacterium salmonicida, obtained agglutinins at both 20° and 10°C. He did agree with earlier investigators that the higher the temperature the better is the antibody production in cold-blooded animals. However he concluded that antibody production takes place at 10°C if enough antigen is injected over a long enough period of time. The author stated that most of the fish died after a few weeks because of "confinement". Therefore the test sera were obtained from post-mortem blood samples.

Cushing (1942) also investigated the effect of temperature on antibody production in fish. He used two groups of goldfish, Carassius auratus, one group at 15°C and the other at 28°C.

Each fish in both of the groups received a 5cc intraabdominal injection of a suspension of sea urchin sperm. The fish were bled every four days for one month, and the sera tested for agglutinins. From the results of the agglutination tests Cushing found that antibodies were produced at both temperatures. However he concluded that a more rapid production and higher titers could be obtained at the higher temperature.

Extensive experimentation on the immune response of cold-blooded vertebrates and the effect of temperature was carried out by Bisset in the late 1940's. He reported that fish are often infected by what appear to be saprophytic water bacteria. When parasitized fish were placed in a tank at 20°C the infection would become progressively worse, and some of the fish would die. However the survivors would clear themselves completely of the infection. Another group of parasitized fish were placed in a tank at 10°C, and it was observed that these fish could not rid themselves of the infection. Yet the infection would not become worse, and a state of symptomless parasitism existed (Bisset, 1947a). It was also observed that the incidence of infections of fish decreased in the summer as the temperature of the water increased, and, conversely, increased in the fall as the temperature of the water decreased (Bisset, 1948).

This condition of symptomless parasitism was also observed in adult and larval frogs at 8° and 20°C. These amphibia, like fish, could rid themselves of the infection at the higher

temperature. As observed by earlier investigators, the production of agglutinins was not apparent at 8°C in frogs, whereas, at 20°C, they produced agglutinins to a high titer (Bisset, 1947b).

According to Bisset (1948) evidence indicates that the immunological response of frogs and fish is almost identical. He used frogs in many of his experiments, since they were easier to maintain and handle. In another experiment the frogs were divided into four groups and innoculated with a killed suspension of Pseudomonas fluorescens on six consecutive days. The first group was immunized at 20°C and kept at this temperature, while the second group was immunized at 20°C, but transferred to 8°C after one week. The third group was immunized at 8°C and kept at 8°C, while the fourth group was immunized at 8°C and transferred to 20°C after one week. Two weeks following the innoculations the frogs were sacrificed, and their sera tested for agglutinins. The first group at 20°C had a titer of 1:50+, while the second group showed no titer. The third group also showed no titer, while the fourth group showed titers of 1:10 to 1:25 (Bisset, 1948). Bisset concluded that a balance exists between the invading organism and the host at a low temperature. However when the temperature is raised, the defensive power of the host increases as does the offensive power of the invading organism. Unless the virulence of the bacterium is great enough to destroy the host, the bacterium will be completely eliminated. It is possible for cold-blooded animals to acquire immunity at a low

temperature, although it will not be manifested until the temperature is raised sufficiently. Similarly an animal immunized at a high temperature will not exhibit any antibody titer at a low temperature. Therefore two distinct mechanisms seem to be in operation. First there is the aquisition of the antigen or potential for antibody production, and secondly, the actual production and appearance of antibodies in the blood. Of these two, the production appears to be affected by changes in the temperature (Bisset, 1948).

About this same time, another type of immunological response was demonstrated in fish by Dreyer (1948). By injecting .2cc of horse serum or egg albumen intraperitoneally, followed by a similar dose 15 days later, an anaphalactic reaction was observed. This reaction was characterized by agitated swimming and excessive fanning of the anterior portion of the dorsal fin. This was followed by a complete folding of the dorsal fin along the body and extrussion of the gill clefs. The fish would then sink to the bottom of the tank, appear sluggish, and would refuse food. This reaction would last from 4 to 12 hours. The same reaction could be induced with .1cc of a histamine phosphate solution, and it could be modified by the injection of epinephrine.

Duff (1942) was the first to try a practical approach to immunization of fish against furunculosis caused by Bacterium salmonicida. However he felt that the injection of antigens, especially in a hatchery, would be too time consuming, so he

experimented with an oral vaccine. The vaccine was prepared from Bacterium salmonicida and mixed into the food. The fish used were cutthroat trout, Salmo clackii, and a few were sacrificed periodically to test their sera for agglutinins. When vaccinated and unvaccinated fish were challenged with virulent organisms, there was only a 25% mortality in the vaccinated fish, while the unvaccinated fish had a 75% mortality.

Recently, Epshtein (1960), stated that carp do not produce specific antibodies. He used horse serum and sheep red blood cells as antigens, the former being injected in doses of .005 to .01cc per gram of carp ten times with three day intervals, and the latter in doses of .1cc daily for five weeks. Blood samples were taken from the caudal vein from 4 to 30 days after the innoculations. Precipitation and complement fixation tests gave negative results. However no temperatures were given.

Uhr (1962) found that after a single injection of ΦX 174 bacteriophage, with a plaque titer of 10^8, into chickens, frogs, and goldfish, all produced the same level of rapidly sedimenting neutralizing, gamma globulin antibodies. These globulins were studied by ultra-centrifugation methods, and their sedimentation coefficients were determined. When he raised the temperature of the fish to 32°C, he found molecules with the sedimentation coefficients of 19 S and 7 S, which correspond to the figures found for the gamma globulins of mammals.

It can be concluded from the above presentation that

cold-blooded vertebrates have the ability to produce antibodies against a variety of antigens, and that this response is influenced by the temperature of the environment. Yet there are still many unanswered questions, which require further investigation.

In the past, most immunological studies have dealt with man and other warm blooded animals, with the main objective being the suppression of disease. Laboratory rabbits, guinea pigs, mice, and rats have been primarily used in the studies of pathogenicity, identification, and serology of microorganisms. However these studies have usually been confined to the study of terrestial warm blooded hosts to microorganisms that are not of marine origin. But today there is a new field of microbiology blossoming out, marine microbiology. Here the environment for the host and the microorganism are quite different from the terrestial forms. It seems only logical, that if a marine bacterium is pathogenic for certain fish, to study this phenomenon in the normal environment of the fish. It has been shown that many of the bacteria isolated from the sea are psychrophilic and halophilic and that the immunological response of cold-blooded animals is influenced by the temperature, as well as the optimal growth and virulence of a marine microorganism. In the following study the immunological response of a freshwater fish to bacteria of marine origin will be shown. Also, the use of fish as laboratory animals for the serological study of marine bacteria will be demonstrated.

III

IMMUNIZATION OF A FRESHWATER FISH TO A BACTERIUM OF MARINE ORIGIN

Introduction

It has been shown that fish produce antibodies, and that the production of these antibodies is influenced by the temperature. In this study the aims are to build up an antibody titer in freshwater fish, using a bacterium of marine origin as the antigen, to observe how long a fish will retain a titer without the influence of changing temperatures, and to see if an anamnestic response takes place.

Materials and Methods

The fish used were common pool goldfish, Carassius auratus, 10 to 12 inches in length. They were obtained from the Auburndale Goldfish Company, Chicago, Illinois. The fish were maintained in a large galvanized metal tank, which could hold approximately 94 gallons of water. Since high concentrations of zinc and lead ions are fatal to fish, by interfering with their respiration (Afflick, 1952), the tank was painted with three coats of a lead free epoxy pool paint.

Figures I and II illustrate the installation of the tank and the equipment used to aide in the maintainance of the fish.

The tank was fitted with a bottom drain and an overflow drain. The overflow drain allowed fresh water to enter the tank as needed, which was an aide in maintaining the temperature and cleanliness of the water. A centrifugal pump, Cole-Palmer Model 7106, was used to circulate the water in the tank through a filter containing glass wool and charcoal. This filter was suspended over the tank from wall brackets, and it served two purposes. It allowed the water in the tank to be cleaned of debris, and it enabled sufficient aeration of the tank's water. The pump circulated 6 liters of water per minute.

Being a relatively large volume of water, the temperature of the water was affected very little by slight temperature changes in the room. The temperature of the water was taken daily, and it was found that over the period of the investigation, the temperature of the water ranged between 20° and 22°C, except for one instance, which will be discussed in the Results.

After the addition of fresh tap water to the tank, the water was tested for chlorides. Little chlorine was found in the water, which could be easily neutralized. Incidentally, the fresh tap water was also put through a filter of glass wool and charcoal before entering the tank.

The fish were originally tagged by putting aluminum animal tags on their dorsal fins. However this caused an infection in the fins, which will be described in the Results. The tagging was abandoned since the individual fish could be

identified by its natural markings, which was easy when working with a limited number of fish.

The fish were fed daily, contrary to the instructions given by the Auburndale Goldfish Company, which instructed to feed the fish only twice a week. However since the fish were being bled weekly, they were fed daily.

The antigen used in this investigation was a bacterium of marine origin, Milford 17, isolated by the United States Fisheries Service, Milford, Connecticut, and obtained from Dr. E. Leifson, Stritch School of Medicine, Loyola University, Chicago, Illinois. The physiological and morphological characteristics of this organism are given in Table I. The organism was grown on agar slants, and the medium consisted full strength artificial sea water, 1.5% agar, .2% Casitone, .1% yeast extract, and .05% Tris Buffer, adjusted to pH 7.5. After two days of growth the slants were washed with .85% saline to harvest the cells. Flagella stains were made in order to insure the presence of the flagallar H antigen, and the stain technique employed was that of Leifson (1951). The antigen was a suspension of live organisms in .85% saline, and it was adjusted to an optical density of .600 on the Klett-Summerson colorimeter. The antigen used in the agglutination tests was adjusted to an optical density of .200. The antigen for testing of O antibodies was prepared by boiling a suspension of organisms for one hour, thus inactivating the H antigen (Smith and Conant, 1960).

Before injection of the antigen, preliminary experimentations on the handling and the bleeding of the fish were conducted. Most of the earlier investigators sacrificed their cold-blooded test animals in order to obtain the blood and the sera for testing. In this investigation it was desirable to collect the blood from the fish without killing it. Taking into consideration the anatomy of the fish and the size of the fish being used in this investigation, it appeared that the best method to obtain the blood would be by heart puncture. One fish was sacrificed and disected to find the exact location of the heart. It was found that if one person would hold the fish inverted in a pan of water, blood could be obtained with a 5cc syringe and a 22 gauge needle. The needle was inserted just anterior to the pectoral fins on the ventral side and angled dorsally and posteriorly to the heart. In the first attempt the blood clotted in the needle. However after the syringe and the needle were rinsed with a 1 to 2% sodium citrate solution to prevent clotting, the blood could be obtained easily without any apparent injury to the fish. Approximately 1 to 2cc of blood could be obtained from each fish, and the serum was separated by centrifugation.

A preliminary bleeding was done on each fish, and the serum was tested for agglutinins against the Milford 17 O and H antigens. Macroscopic serial tube dilution agglutination tests were conducted with the serum dilutions given in Table II.

Agglutination tests for the H antigen were incubated at 37°C and read after four hours, while those for the O antigen were incubated at 52°C for eighteen hours. Later in the investigation, agglutination tests were also conducted at 20°C with interesting results.

Seven fish were injected intraperitoneally with .5cc of the antigen every five days until the titer reached a peak and leveled off. Two fish were used as controls and received injections of sterile saline in the same manner as the antigen. All the fish were bled on the day prior to the next injection and then weekly after the titer leveled off in the manner described, and their sera were tested for agglutinins. At first both individual and pooled sera were tested to check the titers, but since the titers of the individual fish were almost identical, only the pooled sera were tested. The reason for this was to conserve the sera for further experiments, which will be discussed in Chapter IV. After the titer levels started to decrease, a booster shot of .5cc of the antigen was given to see if an anamnestic response could be obtained in the test fish.

Results

It was mentioned that the use of aluminum animal tags caused an inflamation of the dorsal fin. Cultures were made from the infected area on nutrient agar plates, and isolated white mucoid colonies developed. Colonies were picked and innoculated

into nutrient broth for further tests. Gram stains and flagella stains from both the plates and the broth revealed a gram negative polar monotrichous flagellate rod. Further diagnostic tests characterized the organism as a species of the genus Aeromonas (Galarneault, personal communication).

It was found that the Milford 17 organism, upon injection, did not have any apparent pathogenic effect on the fish. However this organism has been shown to be pathogenic for rabbits (Ortiz, personal communication). One of the test fish and one of the controls did die during the course of the experiment, but this was the result of them jumping out of the tank.

The agglutinaion tests on the preliminary bleeding, before injection of the antigen, showed that the fish did not have any agglutinins for the Milford 17 organism (Table III). Table IV shows the titers of the individual test fish, the control fish, and the pooled sera of the test fish after each bleeding up to the time the titers leveled off. It can be seen that their immunological responses to the Milford 17 O and H antigens were practically identical. All of the test fish reached a maximum titer of 1:256 against the O antigen and a maximum titer against the H antigen of 1:128. The pooled sera also reached the same maximum titers (Table IV and Figure III). In every case the control gave a negative response to the Milford 17 antigens.

It can be seen from Figure III that the fish attained a maximum titer approximately 34 days after the injections began,

and that a further injection did not raise the titer any higher. From Figure III a one tube dilution drop in the titers of both the O and H antibodies were observed, followed by a rise to the original maximum titers. However at this time of this bleeding the temperature of the water was found to be 15°C. The reason for this drop in temperature was due to a cold wave of sub-zero Fahrenheit temperatures which dropped the room's temperature considerably, since the room had no means of being heated except by the heat coming from the adjacent rooms. The rise to the original maximum titers came after the temperature of the water in the tank was raised to 21°C.

Further it was found that agglutination tests conducted for both the O and H antigens at 20°C gave higher antibody titers, 1:512 for the O antiserum and 1:256 for the H antiserum. The reason for running agglutination tests at this temperature was to try to set up a condition that would be more natural to the antigen-antibody reaction that would occur in a fish in its natural environment. These reactions will be discussed in Chapter IV.

Finally the titers were observed to start dropping about 70 days after the injections began. After 124 days the O titer was 1:64 and the H titer was 1:32, and at this time a booster shot of .5cc of antigen was administered. It can be seen from Figure III that an anamnestic response was obtained in the fish, and the titers began to climb.

FIGURE I

FRONT VIEW OF TANK

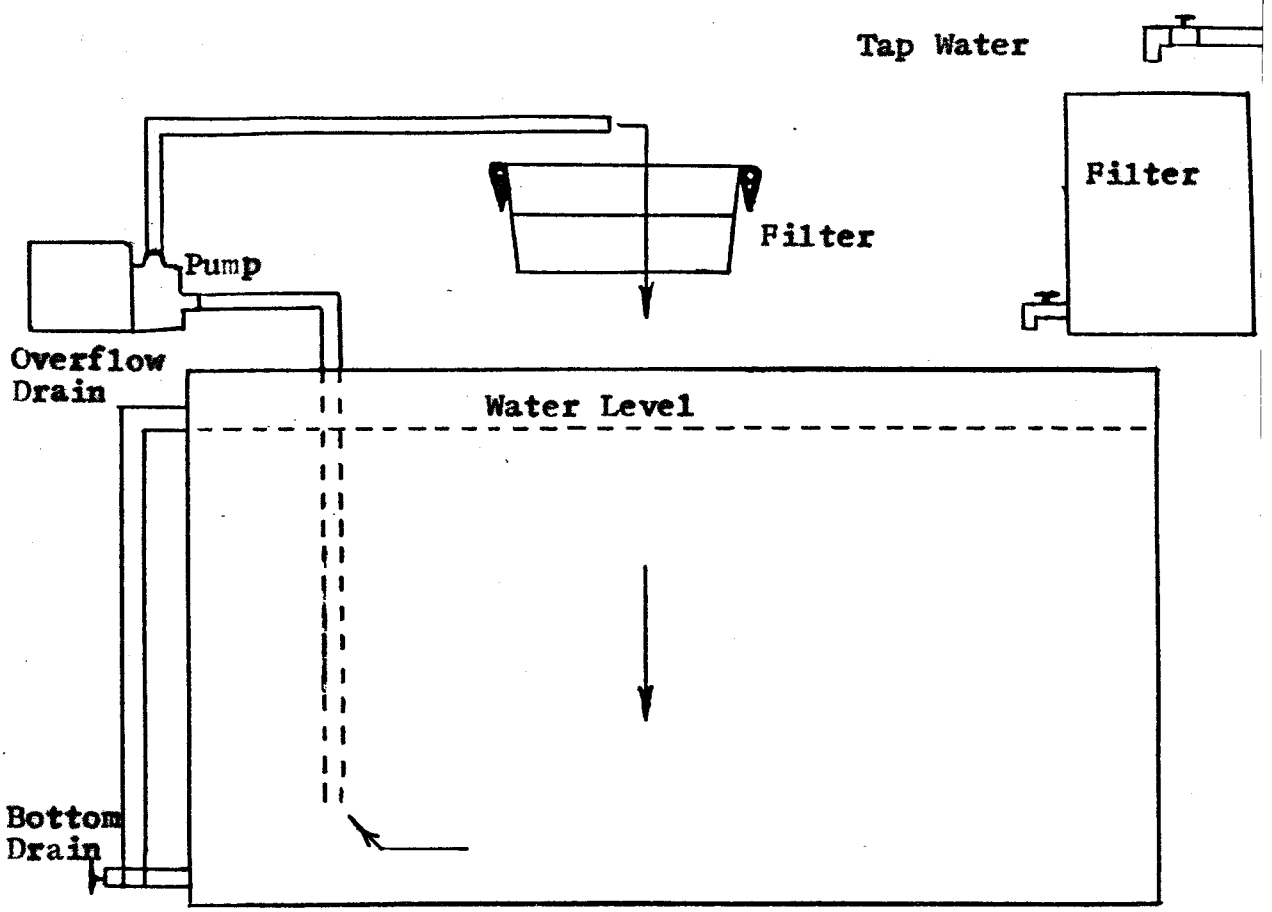


FIGURE II

SIDE VIEW OF TANK

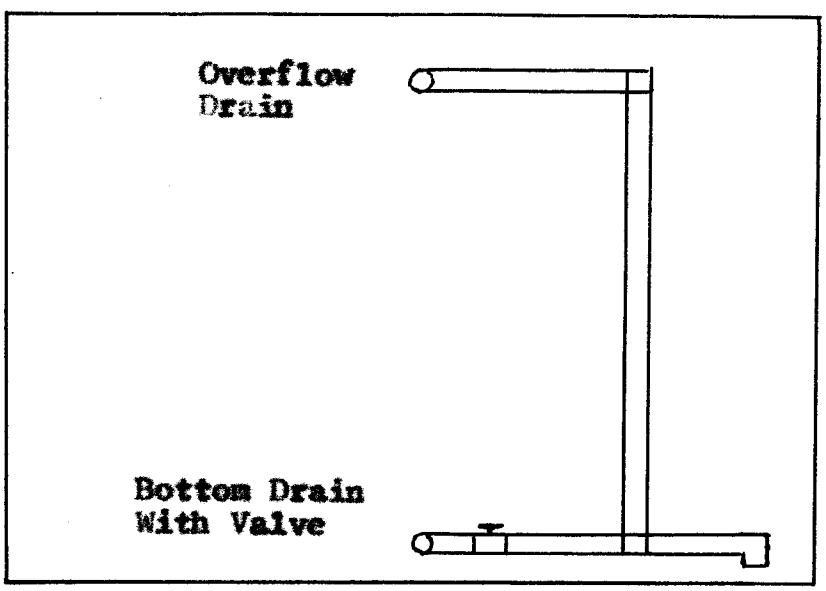


TABLE I

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF MILFORD 17

Growth at 37°C	+
Nitrate	+
Indol	+
Catalase	+/-
Glucose	F*
Sucrose	F
Lactose	-
Xylose	-
Maltose	F
D-mannitol	F
Growth on slant	No speading
Gram stain	-
Flagellation	
Slant	Mixed
Broth	Polar monotrichous

*F = Fermentaion without gas

TABLE II

SERIAL TUBE AGGLUTINATION TEST EMPLOYED

Tube	Saline	Unknown Antiserum	Dilution	Antigen	Total Volume	Final Dilution of serum
1.	.3ml	.3ml	1:2	.3ml	.6ml	1:4
2.	.3ml	.3ml	1:4	.3ml	.6ml	1:8
3.	.3ml	.3ml	1:8	.3ml	.6ml	1:16
4.	.3ml	.3ml	1:16	.3ml	.6ml	1:32
5.	.3ml	.3ml	1:32	.3ml	.6ml	1:64
6.	.3ml	.3ml	1:64	.3ml	.6ml	1:128
7.	.3ml	.3ml	1:128	.3ml	.6ml	1:256
8.	.3ml	.3ml	1:256	.3ml	.6ml	1:512
9.	.3ml	.3ml	1:512	.3ml	.6ml	1:1024
10.	.3ml	--	--	.3ml	.6ml	--

TABLE III

RESULTS OF AGGLUTINATION TESTS ON

FISH SERA BEFORE INJECTION OF ANTIGEN

Fish	O Agglutinins at 52°C	H Agglutinins at 37°C
1.	-	-
2.	-	-
3.	-	-
4.	-	-
5.	-	-
6.	-	-
7.	-	-
Control	-	-
Pooled	-	-

TABLE IV

RESULTS OF AGGLUTINATION TESTS OF INDIVIDUAL AND POOLED SERA UP TO MAXIMUM TITER LEVELS

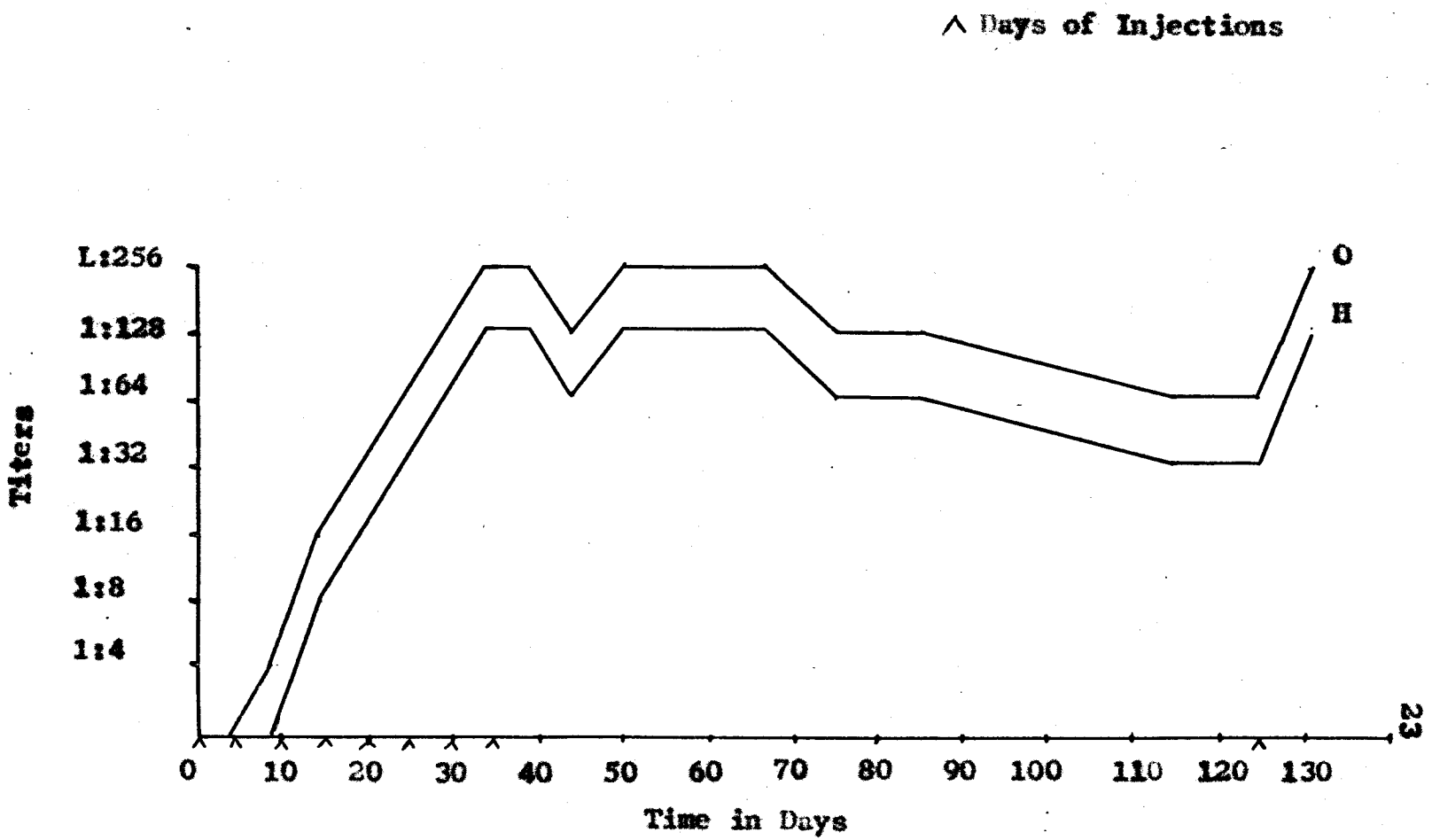
Total Amount of Antigen Injected	.5cc		1.0cc		1.5cc		2.0cc	
Bleeding	1		2		3		4	
	O	H	O	H	O	H	O	H
Fish								
1.	-	-	1:4	-	1:16	1:8	1:32	1:16
2.	-	-	-	-	1:8	1:4	1:32	1:8
3.	-	-	1:4	-	1:16	1:8	1:32	1:16
4.	-	-	1:4	-	1:16	1:8	1:32	1:16
5.	-	-	1:4	-	1:16	1:8	1:64	1:16
6.	-	-	-	-	1:8	1:4	1:32	1:16
7.	-	-	Died					
Control	-	-	-	-	-	-	-	-
Pooled	-	-	1:4	-	1:16	1:8	1:32	1:16

TABLE IV (con't)

Total Amount of Antigen Injected	2.5cc		3.0cc		3.5cc		4.0cc	
Bleeding	5		6		7		8	
	O	H	O	H	O	H	O	H
Fish								
1.	1:64	1:32	1:128	1:64	1:256	1:128	1:256	1:128
2.	1:32	1:32	1:128	1:64	1:256	1:128	1:256	1:128
3.	1:64	1:32	1:128	1:64	1:256	1:128	1:256	1:128
4.	1:64	1:32	1:128	1:64	1:256	1:128	1:256	1:128
5.	1:128	1:32	1:256	1:128	1:256	1:128	1:256	1:128
6.	1:64	1:32	1:128	1:64	1:256	1:128	1:256	1:128
Control	-	-	-	-	-	-	-	-
Pooled	1:64	1:32	1:128	1:64	1:256	1:128	1:256	1:128

FIGURE III

THE RESULTS OF THE TITERS OF POOLED ANTISERA FOR O AND H AGGLUTININS, PLOTTED AGAINST TIME



Discussion and Conclusions

It has been shown that the fish in this investigation produced antibodies for both the O and H antigens of the Milford 17 organism. This tends to substantiate the work of earlier investigators that cold-blooded animals have the ability to produce agglutinins. As mentioned, the earlier investigators demonstrated the influence of temperature on the production of antibodies in cold-blooded animals, showing that the titers in these animals would fluctuate with changes in their environmental temperatures. However in this study it was the aim to immunize the fish and observe their titers over a period of time without the effects of changing temperature.

From the results shown in Figure III, it was observed that the titers in the fish for the O and H antigens reached their maximum levels within 34 days. A further injection of antigen at this time did not raise the titers any higher. The fish maintained these titer levels for approximately 33 days at temperatures of 20° to 22°C before a decline started. However in one instance a one tube dilution drop in both titers was observed on the 44th day after the innoculations began, followed by a rise on the 51st day to the original levels. It was found that the temperature of the water in the tank at the time of the bleeding on the 44th day was 15°C. The water was immediately adjusted to 21°C, and by the 51st day the titers were back up as described.

This reaction shows the affect that temperature has on the antibody titers of cold-blooded animals.

It can further be seen that after the titers began to decline an anamnestic response was produced when the fish were given a booster shot of the Milford 17 antigen. Thus it is concluded from this study that when fish are held at a relatively constant temperature, their antibody response is similar to that observed in warm blooded animals. That is, they can build up an antibody titer to an antigen, maintain it for a period of time, and produce an anamnestic response to a booster shot. Thus the mechanisms involved seem to be either the same or similar. The only apparent difference is in the effect of temperature, which, in cold-blooded animals, would seem to affect their metabolic rates. It seems very probable, that if the metabolism of an organism is decreased, its ability to produce gamma globulins or antibodies would also be decreased. However further phylogenetic, qualitative, and quantitative studies would have to be employed to substantiate this.

IV

SEROLOGICAL STUDIES ON MORPHOLOGICALLY AND PHYSIOLOGICALLY RELATED ORGANISMS TO *MILFORD 17*, USING FISH AS LABORATORY ANIMALS

Introduction

Marine microbiology, a new field of microbiology, has blossomed out in the past few years. Many microorganisms have been isolated, studied, and classified, and in some instances, certain organisms have been found to be pathogenic for both man and marine animals (Leifson, 1964a, and 1964b; and Sakazaki, 1963). These organisms live and survive under conditions that are quite different than those found on land. Many marine bacteria are psychrophilic and halophilic. Since certain of these bacteria have been associated with disease in certain marine animals (Tubiash, personal communication), it only seems logical to study such organisms under their natural conditions. Thus it is the aim in this part of the investigation to conduct serological studies on a group of marine bacteria that share similar morphological and physiological characteristics with the *Milford 17* strain to see if there are any antigentic relationships between these organisms, using fish as laboratory animals.

Materials and Methods

The organisms used in this investigation are of marine origin, and they have similar morphological and physiological characteristics to the Milford 17 strain, which are described in Table V. They were obtained from Dr. E. Leifson and Dr. T. Galarneault, Stritch School of Medicine, Loyola University, Chicago, Illinois.

The test organisms were grown and harvested in the same manner described in Chapter III for Milford 17, and their optical densities were adjusted to .200 for the agglutination tests. The agglutination tests were also performed in the same manner as described in Chapter III with the employment of tests at 20°C for both the O and H antibodies. As a control, the serum of the control fish was tested against each one of the organisms, with negative results.

Results

It can be seen from Table VI that the Milford 17 antiserum gives low titers for the O antigens of XP45, YBC17, and YBC18, whereas negative results for the H antigens were observed at 52° and 37°C respectively. However at 20°C a titer of 1:128 against the O antigen of XP45 and titers of 1:64 against the O antigens of YBC17, YBC18, YSC3, YC4, and YM2 were found, while titers against the H antigens of 1:64 and 1:32 were found for

XP45 and YC4 respectively.

While conducting agglutination tests at 20°C for the H antigen, an interesting phenomenom was observed in certain tubes. Clots formed in the tubes containing low dilutions of the antiserum, thus indicating that the serum must have also contained some plasma. This was noticed in the tubes containing Milford 17, XP3, XR18, and YB7 antigens.

TABLE V

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS OF ORGANISMS TESTED AGAINST THE MILFORD 17 ANTISERUM, PRODUCED IN FRESHWATER FISH

Organism*	Lateral Flagella**	Growth at 37°C	Gelatin	NO_3	Indol	Catalase
Milford 17	+	+	+	+	-	+/-
XA3	-	-	+	+	-	+/-
XE1	+	+++	+	+	-	++
XF3	-	-	+	+	-	+++
XP45	+	+++	+	+	$+_w$	+++
XP46	+	+++	+	+	$+_w$	+++
XR18	-	-	+	+	$+_w$	+/-
YB7	-	+++	+	+	-	+
YB10	-	-	+	+	-	+
YBC17	-	+++	+	+	$+_w$	++
YBC18	+	+++	+	+	+	+++
YSC3	+	+++	+	+	$+_w$	+++
YC4	+	+++	+	+	-	++++
YM2	-	+++	+	+	$+_w$	+++

* All organisms are gram negative motile rods.

** Flagellation was determined from slant cultures only.

TABLE V (con't)

Organism	Glucose	Sucrose	Lactose	Xylose	Maltose	D-Mannitol
Milford 17	+	+	-	-	+	+
XA3	+	+	-	-	+	+
XE1	+	+	-	-	+	+
XF3	+	+	-	-	+	+
XP45	+	+	-	-	+	+
XP46	+	+	-	-	+	+
XR18	+	+	-	-	+	+
YB7	+	+	+/-	-	+	+
YB10	+	+	-	-	+	+
YBC17	+	+	-	-	+	+
YBC18	+	+	-	-	+	+
YSC3	+	+	-	-	+	+
YC4	+	+	-	-	+	+
YM2	+	+	-	-	+	+

TABLE VI

RESULTS OF AGGLUTINATION TESTS

WITH MILFORD 17 FISH ANTISERUM

Organism	Titers of Antiserum			
	O at 52°C	H at 37°C	O* at 20°C	H* at 20°C
Milford 17	1:256	1:128	1:512	1:256
XA3	-	-	-	-
XE1	-	-	-	-
XF3	-	-	-	-
XP45	1:8	-	1:128	1:64
XP46	-	-	-	-
XR18	-	-	-	-
YB7	-	-	-	-
YB10	-	-	-	-
YBC17	1:4	-	1:64	-
YBC18	1:16	-	1:64	-
YSC3	-	-	1:64	-
YC4	-	-	1:64	1:32
YM2	-	-	1:64	-

O* incubated 20 hours.

H* incubated 4 hours.

Discussion and Conclusions

It can be seen from Table V that these organisms have similar morphological and physiological characteristics to the Milford 17 organism. Thus it was the aim in this study to see if these organisms might share antigenetic properites with the Milford 17 strain, using the Milford 17 antiserum produced in fish. Low titers were found against the O antigens of XP45, YBC17, and YBC18 when incubated at 52°C, while no apparent titers were observed against the H antigens incubated at 37°C. However when the tubes were incubated at 20°C, fairly high titers were observed against the O antigens of XP45, YBC17, YBC18, YSC3, YC4, and YM2. Apparently these bacteria share O antigens with the Milford 17 strain. The reason for the presence of higher titers at 20°C is not exactly clear. The immune response of fish and perhaps other cold-blooded animals seems to be optimal at this temperature. Thus an antigen-antibody reaction with such organisms in the laboratory would give better results if the conditions of the tests were set as close as possible to the conditions that such reactions would take place in nature.

The formation of clots in some of the tubes apparently is due to the presence of plasma in the serum, since an anti-coagulant was used when the fish were bled. However clots were not produced in all of the tubes, and no clot was present in the tube containing only the antiserum. Furthermore the clots were

produced only in the tubes that were incubated at 20°C. It is possible that the clotting is due to coagulase or a coagulase-like substance produced by the test organisms. Further investigation on this observation is now being done.

From this investigation it can be seen that fish can be used for immunological and serological studies. Marine microbiology is a relatively new field in microbiology, which offers a broad spectrum of future investigations. The use of aquatic cold-blooded animals in the laboratory may provide more natural conditions for the study of marine microorganisms.

V

SUMMARY

A study was conducted on the immunological response of a freshwater fish, Carassius auratus, to a bacterium of marine origin, Milford 17, and it was found that antibody production was similar to that of warm blooded animals, when the temperature of the fish was constant. An anamnestic response was also observed in the fish. Serological studies were made with bacteria of marine origin which share morphological and physiological properties with the Milford 17 strain, showing that certain of these organisms must have antigens in common with the Milford 17 strain. In this part of the investigation, fish were used as laboratory animals for the production of Milford 17 antiserum.

VI

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APPROVAL SHEET

The thesis submitted by Robert S. Pekarek has been read and approved by three members of the Department of Microbiology.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 26, 1964
Date

Signature of Advisor