SEROLOGICAL DIFFERENTIATION OF POPULATIONS OF SOCKEYE SALMON, *ONCORHYNCHUS NERKA*

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ABSTRACT

As part of a study applying serological methods to the distinction of salmon races, the normal serums of human beings, cattle, horses, sheep, pigs, goats, brown bullheads, and other salmon were tested for agglutinative activity with the erythrocytes of sockeye salmon. Only horse serum and pig serum reacted strongly with the salmon cells. With some of the pig serums tested, consistent differences were found in the strength of reaction of the cells of individual sockeye salmon. The frequencies of individuals displaying the various strengths of reaction were found to differ significantly between areas of origin of the salmon tested. When techniques of population genetics were used, evidence for the possible genetic control of the observed differences was obtained.

INTRODUCTION

This is a report on the results of a collaborative investigation[1] of the differentiation of sockeye salmon (*Oncorhynchus nerka* Walbaum) populations by serological methods. The aim of the work was to estimate the potential value of agglutinins found in the nonimmune serums of animals (so-called " natural antibodies ") for detecting racial variations among salmon erythrocyte antigens. Such agglutinins have been used successfully in research on the erythrocyte antigens of man, cattle, sheep, chickens, and other warm-blooded animals. (Cf. Cushing and Campbell, 1957, for a general account of these reactions). They have also been used to demonstrate individual variations in the erythrocyte antigens of skipjack, *Katsuwonis pelamis* Linnaeus (Cushing, 1956) and brown bullheads, *Ictalurus n. nebulosus* Le Sueur (Cushing and Durall, 1957), and other fishes (Suyehiro, 1949).

Variations in erythrocyte antigens provide favorable material for racial analyses, since whenever studied their genetic determination has been found to be simple and direct. Also their specificity has not been found to be subject to environmental influences. Evidence for the genetic determination of such variations in fish has recently been provided by Hildeman (1956), and the potential utilization of these antigens in fishery research has been discussed by Cushing and Sprague (1953), and by Ridgway (1957).

MATERIALS AND METHODS

Samples of whole salmon blood were obtained by field crews from the major salmon-producing rivers of North America and were either shipped immediately by commercial air transport or brought directly to the laboratory. These samples were collected by bleeding salmon from the tail into open-mouth screw-cap bottles without preservative or anticoagulant. The largest collections were made in the rivers of Bristol Bay and at various localities in the Columbia River System. The clotted bloods (approximately 5 to 50 ml.) were transported in bottles in insulated picnic jugs containing ice. They were usually received from 1 to 3 days after collection and were kept refrigerated at 5 to 10 °C. until used. Under these conditions most of the samples remained useful for a week or two.

Suspensions (1.5 per cent) of fresh cells were prepared on the day of use by washing 1.5 ml.

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aliquots of whole blood in three 20 ml. changes of Alsever's solution. All washings were carried out in a refrigerated centrifuge, and the cell suspensions were kept refrigerated except when in immediate use. Individiual tests were performed by placing two drops of suspension with two drops of suitable serum dilution in 10 x 70 mm. test tubes. After 15 minutes incubation at room temperature, the tubes were centrifuged for 1 minute. The sedimented cells were resuspended by lightly tapping the tube and the degree of agglutination was scored in the conventional manner (4 indicating very strong, 3 strong, 2 moderate, 1 light and ± barely detectable agglutination with 0 indicating no agglutination).

The influence of variables such as the speed of centrifuging, age of cells, temperature, etc., were considered and controlled. Efforts were made to control the objectivity and accuracy of the method by testing the same sample on different days and making the readings without knowledge of the sample sources. Readings were also made on the same material by independent observers. Such controls substantiated the comparisons of different tests, provided a basis for evaluating the limits of experimental error in these tests, and suggested refinements in technique for control of such error.

EXPERIMENTAL RESULTS AND DISCUSSION

Several serums from humans,[2] cattle, horses, sheep, pigs, goats and fish, all preserved by freezing, were titrated against sockeye cells. Included were a number of cross matches (approximately 700) of cells and serums (1 in 4) between sockeye samples from various localities. All of the latter gave negative results, an observation that is in contrast to those that have been made on the brown bullhead, the white croaker (*Genyonemus lineatus* (Ayres)) and skipjack (Cushing and Durall, 1957). Some brown bullhead serums (6 out of 16) were found to react strongly with sockeye cells at 1 in 4 dilution. However, these serums did not differentiate among sockeye from different areas. There also appeared to be no relation between the positive reactions and the blood group of the brown bullhead involved.

It is of interest that bovine serum which proved so useful in detecting individual differences in chickens and in skipjack reacted very poorly with sockeye cells.

Pig and horse serums were found to agglutinate sockeye cells more strongly than the other serums examined. As titrations of several single pig serums with the cells of different fish showed differences in the strength of reactions with these cells, attention was concentrated on this observation. While the optimum temperature of agglutination for this serum was approximately four degrees, it was practical to work only at room temperatures which consistently remained at 22° to 24°C.

A standardized procedure was adopted for the comparison of readings of different sets of samples. This procedure consisted of testing the cells of each fish against four dilutions (1 : 16, 1:32, 1:64 and 1:128) of heat-inactivated (56°C. for 30 minutes) serum from a single pig designated W.P.4. The scores of the readings at the latter three dilutions were added to give a single total score. Table 1 presents an example of the protocol just described. Figure 1 presents a summary of scores from many such protocols, which combines the samples into three groups. The first of these consists of samples taken from the rivers of Bristol Bay, the second of samples taken from rivers of the eastern Pacific coast from Kodiak Island to the Fraser River, the third of samples taken in the Columbia River System. An analysis of variance was performed (Table 2), which shows that the three groups differ significantly. This analysis was performed by the method given in Snedecor, 1957, page 283, which takes into account the differences in variances. This is the first time that it has been possible to demonstrate a statistically significant serological difference between populations of individuals within a single species of fish.

It should be borne in mind that the salmon in each of these areas are homing toward a variety of separate and distinct spawning grounds. In spite of this it seems reasonable that genetic exchanges may be more frequent within the Columbia and Bristol Bay groups than between them, and that this would plausibly account for the differences observed.

Consideration of all the individual scores of the above groups suggests that these form a trimodal distribution (Figure 1). Some statistical justification for the validity of this suggestion was obtained by applying a test for bitangentiality (Haldane, 1952).

Additional evidence of antigenic heterogeneity was obtained from absorption experiments. Samples of the serums of several pigs were absorbed with strong and weakly reactive cells. In general, two capacities for antibody removal were observed. Several absorptions indicated that weaker reacting cells (Bristol Bay) were less

2. Human typing serums kindly supplied by Hyland Laboratories, Los Angeles, California.

TABLE 1. Comparison of the Strength of Agglutination of Bristol Bay and Columbia River Sockeye Salmon Blood Samples by Serum WP4.

Serum Dilutions	Areas																					
	Kvichak (Bristol Bay)												Columbia River									
	Samples												Samples									
	1	2	3	4	5	6	7	8	9	10	11	12	12	13	14	15	16	17	18	19	20	21
1 : 16	2	3	3	3	3	3	4	3	4	3	4	4	4	4	4	4	4	4	4	4	4	3
1 : 32	2	2	2	2	2	1	3	1	2	2	2	2	3	3	3	3	3	3	3	3	3	3
1 : 64	±	±	±	±	±	±	1	±	1	±	1	1	1	2	3	1	2	2	2	2	2	1
1 : 128	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	±	±	±	±	0	0
Score	2.5	2.5	2.5	2.5	2.5	1.5	4	1.5	3	2.5	3	3	4	6	7	4	5.5	5.5	5.5	5.5	5	4

4 indicates very strong, 3 strong, 2 moderate, 1 light and ± barely detectable agglutination. The scores are obtained by adding the readings of the highest three dilutions.

TABLE 2. Frequencies of Agglutination Scores of Sockeye Salmon

Scores	Areas		
	Bristol Bay No. of Fish	Gulf No. of Fish	Columbia No. of Fish
0	1	0	0
0.5	3	0	0
1.0	6	0	0
1.5	2	1	0
2.0	9	2	0
2.5	14	6	3
3.0	14	23	4
3.5	3	4	1
4.0	4	11	7
4.5	1	3	1
5.0	0	7	4
5.5	0	1	7
6.0	0	0	11
6.5	0	0	1
7.0	0	0	3
7.5	0	0	0
8.5	0	0	1

Analysis of Variance

Source of Variation	Degrees of Freedom	Mean Square	F
Between means	2	61.3638	61.32**
Error	96	1.0007	

** Highly significant

effective in removing antibodies than stronger reacting cells (Columbia River).

One exceptional serum was discovered. This serum appeared to have antibodies that were relatively more amenable to fractionation. Absorption of this serum with the cells of a Bristol Bay fish left considerable antibody for the cells of a Columbia River fish while absorption with the cells of the Columbia River fish removed all the antibody for both types. The small amount of this serum that was available prevented a systematic expansion of this observation. However, the occurrence of this serum shows that a study of the serums of other pigs may prove to be profitable.

The discovery that the erythrocytes of salmon from different geographic areas vary significantly with respect to their reactions with antibodies in pig serum raises the question of the nature of the mechanism controlling this variation. While the answer to this question awaits further study, several sources of evidence indicate that a genetic mechanism probably exists. First, the erythrocyte antigens of mammals and birds have been found to be genetically determined wherever investigated. Second, Hildeman (1956) has shown that certain erythrocyte antigens of goldfish have hereditary determinants. Third, different species of salmon (Ridgway and Klontz, 1957), as well as other species of fish (Suyehiro, 1949; Cushing and Sprague, 1953), show characteristic species specificities with respect to their erythrocyte antigens. This suggests that the antigenic specificities involved are genetically determined.

An analysis of the frequency distributions of the antigenic variations in sockeye salmon populations was made with the Hardy-Weinberg formula (Srb and Owen, 1953) in an attempt to obtain further information concerning the possible genetic background for these variations. The Hardy-Weinberg formula relates the frequencies of allelic genotypes to the frequencies of allelic genes in a randomly mating population, in terms of the binomial expansion. It was through the use of this formula that Bernstein determined the exact method of inheritance of the human ABO groups (see Race and Sanger, 1954) and it is an indispensable tool in the study of the population genetics of blood groups in man and animals.

Our interpretation of the data presented is based upon the assumption that the observed variations in strength of agglutination, expressed in terms of high, intermediate and low scores,

TABLE 3. Application of the Hardy-Weinberg Formula

Populations	Total Number of Fish N	Categories						"Gene" Frequencies	
		aa		aA		AA			
		Observed	Expected (p^2N)	Observed	Expected ($2pqN$)	Observed	Expected (q^2N)	p	q
Bristol Bay	57	49	49	8	7	0	<1	0.43	0.07
"Gulf"	58	32	29	18	24	8	1	0.71	0.29
Columbia R. Mainstream	43	7	3	9	17	27	23	0.27	0.73
Okanogan	43	5	5	18	19	20	19	0.33	0.67
Lk. Wenatchee	64	4	2	16	19	44	42	0.19	0.81
Composite "Population"	265	97	65	69	133	99	67	0.496	0.504

$$p = \frac{2(aa) + (aA)}{2N} \qquad q = \frac{2(AA) + (aA)}{2N}$$

The frequencies of weak (aa), intermediate (aA), and strong (AA) reactors observed in the various populations of sockeye salmon are compared with those expected on the basis of the Hardy-Weinberg Law.

are due to two primary causes.

The first is that the active agglutinin in serum WP4 has a lower affinity for a qualitatively different but related antigen on the surface of the lower scoring cells than for the antigen on the higher scoring cells. Since our absorption results indicate that these antigens must be serologically related, because they react with the same agglutinin, we assume that they are determined by allelic genes. Differences in the strength of agglutination brought about by the reaction of one type of antibody with different antigens determined by allelic genes are well documented in the literature on blood groups (Race and Sanger, 1954).

The second primary cause of variation must be postulated to account for the intermediate reactions. This is assumed to be a dosage effect causing a stronger reaction with cells of individuals homozygous for a particular gene than those of individuals heterozygous for this gene. Dosage effects exhibited by blood grouping reagents are also well documented in the literature; in fact, the scoring system used by Race, Sanger and Lehane (1953) in their study of the dosage effect in the human Duffy blood groups serves as a model for our scoring system.

The apparent trimodality of the distribution of agglutination scores (Figure 1) served as a basis for assigning individual fish to one of three categories, rated as strong, intermediate and weak reactors. These categories, as explained above, may be postulated to correspond to three genotypes determined by a single gene and its allelic alternates. Strong reactors are considered as homozygous for this gene (symbolized AA), intermediate reactors as heterozygous (Aa) and weak reactors as lacking this gene (aa). The numbers of fish occurring in each category are compared with those expected on the basis of the Hardy-Weinberg formula in Table 3.

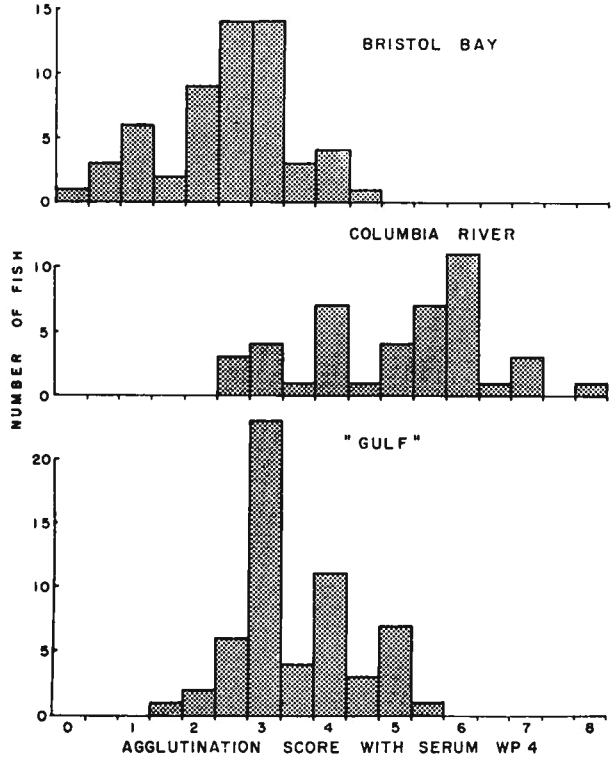

FIGURE 1. Histograms showing the agglutination strengths of sockeye salmon erythrocyte samples from three populations.

Two groups of fish drawn from spawning populations were also studied. These were taken

from the Okanogan River and the Little Wenatchee River which are the major spawning areas for Columbia River sockeye salmon. Since these two populations were examined at a later date and under somewhat different conditions, it is necessary to assign the individual fish to the three categories on the basis of the modes present in the population, rather than according to standard score values. For this reason, the present analysis must only be considered as a guide toward further studies under more refined conditions.

It is of interest that the Mendelian populations taken from spawning grounds gave data that appear to fit the Hardy-Weinberg equation quite well, while two out of three populations that were known to be mixtures of breeding stocks did not, nor did a composite of all populations (Table 3). In the two cases where the number of fish was sufficient in all categories for the application of the Chi Square test these conclusions appear to be statistically sound. (Okanogan River $\chi^2 = 0.106$ $d.f. = 2$, "composite population" $\chi^2 = 61.9$ $d.f. = 2$).

Although the close agreement of the actual Bristol Bay data with the Hardy-Weinberg Law may possibly be due to chance alone, the results may tentatively be ascribed to the several populations in this system being genetically homogeneous with respect to the relative frequencies of "A" and "a". While the reasons for and implications of this homogeneity remain to be determined, three not mutually exclusive possiblities suggest themselves. The first is that the various "parent-stream" stocks, while genetically isolated, have not diverged with respect to the frequencies of these genes as they obtained in a common ancestral stock. The second is that the various "parent-stream" stocks are not sufficiently isolated to allow for divergence of the frequencies of the two alleles among them. The third is that identical selection pressure in the separate populations may impose identical points of genetic equilibrium.

The failure of the Columbia River and Pacific Coast samples to conform to the Hardy-Weinberg formula can be explained by the fact that each represents a mixture of populations differing with respect to the frequencies of A and a. This explanation receives support from consideration of the good fit of samples obtained on the spawning grounds of the upper Columbia after the Wenatchee and Okanogan runs had separated from each other. In these cases, where fairly large samples were taken from known Mendelian populations, the distribution of scores does conform to the Hardy-Weinberg law. In view of these various considerations there seems to be good reason to postulate that the variations in erythrocyte antigens which we are comparing are genetically controlled by a simple allelic system of genes, the frequencies of which may vary in racial stocks of different geographic origins.

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SUMMARY

1. In over 700 cross matches between the cells and serums of samples from several localities, no evidence was obtained for the existence of natural isoagglutinins in sockeye salmon.
2. The normal serums of several humans, cattle, horses, sheep, pigs, goats and brown bullheads were tested for agglutinative activity with the erythrocytes of sockeye salmon. Only horse serum and pig serum reacted strongly with the salmon cells.
3. Consistent differences were found in the strength of reaction of some pig serums with the cells of individual sockeye salmon.

   The frequencies of the various strengths of reaction, which were expressed as scores, were found to differ significantly between areas of origin of the salmon tested.
4. A hypothesis to explain the possible genetic determination of these differences was presented and tested with the Hardy-Weinberg law.

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ADDENDUM

Since the preparation of this manuscript we have tested somewhat larger samples from Bristol Bay and the Columbia River, taken in the 1957 season, and have repeated the distinction with considerable precision. In 1956, 86 per cent of Bristol Bay sockeye salmon tested had scores less than 3.5 while 83 per cent of those from the Columbia River had scores greater than 3.5. In 1957, 92 per cent of the Bristol Bay fish tested had scores less than 3.5 while 79 per cent of those from the Columbia River had scores greater than 3.5. This year to year stability provides further evidence for the genetic control of the characters determined by this method.