THE ANTIGENIC VARIATION OF *SALMONELLA PULLORUM*

by

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INTRODUCTION

It has been established that antigenic variation occurs in Salmonella pullorum, and this fact has a marked influence on the routine testing for pullorum disease. From the literature cited, it is evident that this phenomenon of variation in S. pullorum presents a serious problem in pullorum disease control. One can readily appreciate that adequate control of pullorum disease is impossible while employing diagnostic methods and techniques that are subject to serious error. From the available experimental evidence it is apparent that maximum accuracy cannot be attained in testing for pullorum disease with a standard strain antigen alone. Limited reports indicate that a greater degree of accuracy can be obtained with the use of polyvalent antigens. However, nonspecific reactions occur when mixed regular- and variant-antigens are used, hence errors are introduced into this procedure.

This investigation was instigated in an attempt to determine what characteristics are associated with a variant strain of S. pullorum, and what influence the various selective media used in isolating Salmonella species may have upon the antigenic structure of this organism.

LITERATURE REVIEW

Following the initial recovery of a bacterium by cultural methods, the organism is subjected to a series of laboratory manipulations designed to insure a pure culture. In addition to furnishing pure cultures these isolation procedures lead to a "domestication" of the organism, or the bacterium becomes adapted to life on the artificial culture medium to which it is subjected (Parr and Robbins, 1942). In the process of adaptation a culture may undergo changes in form. These changes in form, termed "variations", may be manifested either in deviations from the normal colonial or cellular appearance, or in physiologic and antigenic properties. In some instances apparently one change may influence another. Thus, the conversion of a "smooth" form to the "rough" phase may be accompanied by changes in antigenicity (Dubos, 1945).

This paper is concerned mainly with antigenic variations in S. pullorum induced by environmental factors. It has been shown (Bushnell, 1949) that repeated subculturing of a "smooth" form of Escherichia coli mutabile on nutrient agar containing four percent agar-agar will result in the conversion of the "smooth" form to the "rough" phase. The resulting rough colonies will agglutinate nonspecifically when suspended in saline alone (Bushnell, 1949). It has been repeatedly shown

that growing an organism in the presence of its specific flagellar antibody brings about the inhibition of antigen production in the daughter cells, and in some cases ellicits the appearance of other flagellar constituents which the organism is capable of producing but otherwise were not exhibited (Dubos, 1945). Organisms appearing in a specific phase also have been induced to change to the group phase. These organisms may in turn be induced to revert to the specific phase by growing them in the presence of group serum (Bruner and Edwards, 1941; Bornstein, 1943). Edwards and Bruner (1942) transformed Salmonella salinatis, artificially, into a culture which could not be differentiated from Salmonella sandiego, by growing it in the presence of a serum containing agglutinins active against a specific flagellar antigen of S. salinatis. The resulting culture was identical in all respects with a species of S. sandiego isolated from a natural source. Dubos (1945) offered the opinion that such transformations of the Salmonella group give an insight into the many changes which are probably taking place in nature. This suggested to Dubos that "the immunological complexity of Salmonella is due to the fact that the members of this group are in an unstable state and are undergoing evolution at a rapid rate".

However, the reports on induced variation in species of the Salmonella genus deal, primarily, with those species bear-

ing flagellar antigens. In organisms, such as S. pullorum, where only a somatic antigen is concerned a search of the literature failed to reveal any reports on the successful artificial transformation of the regular to the variant form of the organism. Consequently, whether such a conversion can be artificially induced remains to be demonstrated. It may be pointed out here that much stress has been laid on artificial transformations with little emphasis placed on the role of natural environmental conditions. In considering Dubos' theory of instability and evolution in Salmonella, the writer is of the opinion that if evolutionary changes are occurring in S. pullorum, then the rate at which these changes occur bears a relationship to the nature of the environment.

Younie (1941) reported that during the hatching season of 1939 a few pullorum disease outbreaks occurred in chicks. The source of the infection could not be determined since the chicks were grown on pullorum free premises, and the eggs from which they hatched were obtained from pullorum negative flocks. Some of the flocks where these outbreaks occurred were retested and positive reactions were obtained for pullorum disease. Younie also observed that although a large number of flocks were affected there were few reactors in relation to the number of birds showing pullorum disease symptoms. With material obtained from these infected chicks

Younie infected three groups of normal chicks; one group at 24 hours of age, a second group at 72 hours, and a third group at 120 hours of age. At the end of the third week after inoculation there were no fatalities and at this time one-third of each group was posted for examination. All but one of the 24-hour group, less than one-third of the 72-hour group, and none of the 120-hour group showed lesions typical of "pullorum disease". Pooled serum from these chicks resulted in complete agglutination of antigens prepared from strains of S. pullorum isolated from chicks with both the septacemic and nonseptacemic type infection. However, two standard strain antigens were not agglutinated and there was only a trace of reaction with a third regular strain antigen as compared with complete reaction with homologous antigen. Inasmuch as the organisms isolated were similar to S. pullorum in every respect except for this antigenic difference, the organisms were designated by Younie as variants.

Younie (1941) carried out agglutinin absorption reactions which led him to believe the antigenic differences between regular and variant strains were of a quantitative rather than a qualitative nature. He suggested both types contained similar antigenic factors, but these factors were present in different amounts. He concluded that a standard strain would contain more of the regular factor and the variant strain more variant factor. Younie also found that when

absorbed with regular antigen all regular agglutinins would be removed from regular serum; however, absorption with variant antigen would only remove the variant agglutinins.

Bond (1942) found that serum from a fowl inoculated with variant organisms readily agglutinated standard antigen, and that variant antigens appeared to possess greater heterophilic tendencies. The experimental fowl received two doses of formalin killed variant cultures followed by three doses of living cultures. The sera of these birds gave high agglutination titers with variant antigens and low titers with standard antigens. Later work showed a pronounced specificity with variant sera for homologous antigens as compared with their reactions with standard antigens.

Byrne (1943) studied a number of the Younie variants by using absorbed rabbit anti-sera in agglutination reactions. He observed that variant strains were agglutinated by sera produced against regular antigens, and that the standard pullorum antigens were agglutinated by anti-variant sera.

Gwatkin (1946) infected a lot of chicks with regular and variant strains. Suspensions prepared from the lungs, heart, and liver of chicks infected with variant organisms were used to infect chicks of group II orally. In group I, 294 chicks were tested by the whole blood method with regular and variant antigens. In this test, 191 chicks were positive to the variant antigen and 20 were positive to the regular

antigen. Had this been a routine test and had the positive and questionable samples been grouped, 185 birds detected with the variant antigen would have passed as negative. A second group of chicks infected with variant organisms showed a preponderance of variant agglutinins six days after infection, but two months later, after injection of the same culture, the predominating agglutinins were of the regular type.

Durrell (1946) using (1) regular antigen made up with standard S. pullorum strains 4, 10, and 11, (2) variant antigen consisting of Younie's strains 2 and 96, (3) commercial antigen composed of a mixture of regular and variant organisms, and (4) commercial regular antigen, found that the variant antigen (2) missed 22 percent and the commercial regular antigen (4) 14 percent of the 100 percent pullorum infected birds as found by using the commercial (3) antigen. Durrell noted that 15 birds which reacted only to variant antigen in the initial test gave a positive test with the regular antigen when retested three to five weeks later. His findings in this respect were similar to those of Gwatkin (1945).

At the meeting of National Plans Commission on Standardization of Antigens and Procedure in Testing for Pullorum Disease (1949), it was reported that polyvalent antigens were being used by several of the states in testing for pullorum by the whole blood method. However, inasmuch as these antigens

tend to yield nonspecific reactions they are not entirely satisfactory. At this meeting, Dr. Pomeroy, in commenting on the incidence of variant pullorum in turkeys, estimated that approximately 20 percent of all pullorum isolates from turkeys in Minnesota are typed as the variant kind.

Edwards and Bruner (1946) sought an answer to the variational phenomenon of S. pullorum in terms of the "form" variation explanation advanced by Kauffmann (1940). Certain minor O antigens of the genus Salmonella overlap, that is, they occur in more than one of the major groups in which the bacilli are divided on the basis of their O antigens. Among these are antigens I and XII. Kauffmann (1940, 1941) observed that single colony isolations from certain types of Salmonella resulted in some colonies containing a large amount of factor I, while others contained a smaller concentration of this antigenic factor. On further transfers, however, each form gave rise to the other. Simularly, antigen XII was found to be even more variable. Kauffmann subdivided antigen XII into XII_1, XII_2, and XII_3 and found only the XII_2 to be variable. This type of variation he designated as "form variation".

Edwards and Bruner (1946) working with absorbed sera indicated the antigenic formula for S. pullorum as IX, XII_1, XII_2 and XII_3. They concluded that in normal cultures the XII_2 factor is variable and found that forms containing a

large or a negligible amount of this factor can be isolated from the same parent strain. These investigators believe cultures may become stabilized either as standard or variant strains. The standard strains contain a small amount of the XII_2 while the variant strains contain a relatively larger proportion of this factor.

Wright (1947) reported that strains of S. pullorum recently isolated were mixtures of regular and variant forms. By selection of colonies from such cultures, pure strains possessing the composite antigenic structure for both regular and variant S. pullorum were obtained. In view of his results Wright predicted that antigens prepared from strains possessing the complete antigenic structure will detect both regular and variant type infections.

Gwatkin and Bond (1947) followed a similar technique as Wright. In examining 100 cultures of S. pullorum by the slide agglutination method using regular, variant, and Proteus serum, these workers found 59 cultures to be regular and 41 cultures to be variants. Twenty one of the regular group showed a varying percentage of colonies which were agglutinated slightly by the Proteus serum. One of the variant strains had about two percent colonies that were slightly agglutinated by the regular serum. Tube antigens prepared from these colonies were not satisfactory, giving poor reactions with both regular and variant sera. The regular

serum employed by Gwatkin was prepared by absorbing S. paratyphi A. var. durazzo anti-serum with Proteus antigen. Variant sera were prepared by using variant cultures for the immunization of goats. The Proteus anti-serum prepared by immunizing a horse served as a duplicate variant serum. Gwatkin (1947) reported on the specificity of a Proteus strain for the XII_2 factor.

Wright (1947) reported unsuccessful attempts to increase the XII factor of S. pullorum by selection. Gwatkin (1948) reported that no change could be induced in the antigenicity of S. pullorum by passage of living cultures, in the presence of large quantities of killed heterologous form cultures, through mice, guinea pigs, and chickens. In his experiments Gwatkin seeded five tubes of beef infusion broth with regular S. pullorum strain 11. Five other tubes of the same medium were inoculated with a variant culture, strain 296. After incubation for 24 hours at 37° C. the growth was collected in saline, heated for one hour at 65° C. and then centrifuged. The sediment from the five tubes of strain 11 was suspended in 1 ml of a living 24 hour agar culture of S. pullorum, strain 296, which had a density of 2 by the McFarland nephelometer standards. This was injected intraperitoneally into a guinea pig. The killed sediment of strain 296 was similarly suspended in a live suspension of strain 11 and injected into another guinea pig. The first guinea pig

died from peritonitis on the first day after injection. The second was killed on the third day. The S. pullorum organisms recovered did not show any change in antigenic form. In the same paper Gwatkin reported that 20 serial subcultures of regular strains made on beef extract agar and on the same medium containing 1 ml of a heavy suspension of autoclaved variant organisms, strain 296, displayed no differences in growth characteristics or form. Growth of regular and variant cultures on beef extract agar containing 10 percent of variant serum and incubated at 37 degrees centigrade showed no changes which could lead to differentiation between the two forms. Gwatkin (1945) reported that 10 regular and 6 variant cultures of S. pullorum, isolated from chicks which had died from pullorum disease, were tested with a specific bacteriophage. His results indicated serological types could not be distinguished by bacteriophagy.

MATERIALS AND METHODS

Titrations of S. pullorum Cultures

Seventy cultures of S. pullorum were selected for study in determining which possessed regular tendencies and which were of the variant type. Of these, 42 were from the stock collection gathered at the Department of Bacteriology, Kansas

State College, over a period of years, and the remaining cultures were obtained through the courtesy of Dr. Van Roekel, University of Massachusetts; Alice B. Moran, University of Kentucky; Walter E. Burr, University of Connecticut; Dr. R. A. Packer, Iowa State College; J. E. Williams, University of Minnesota; and the United States Bureau of Animal Industry. Two of the 70 strains were isolated from humans; the remaining 68 were isolations from chickens and turkeys which had shown typical pullorum disease symptoms or had succumbed to this disease. Gram's stain reactions, microscopic examinations, and biochemical and serological tests were employed as a means of ascertaining the purity and identification of each culture. Only those which gave specified reactions for S. pullorum according to Bergey's Manual of Determinative Bacteriology, Sixth Edition, were used in this study.

Each of the 70 strains was transferred to Difco Dehydrated Stock Culture Agar[1] slants containing additional Bacto agar to make the total agar content of the medium 1.5 percent, and, therefore, assure sufficient solidity. The slants were then incubated for 48 hours at 37° C. At the end of this period antigens were prepared for each strain by washing off the growth with 0.3 percent phenolized saline and adjusting the washings to a McFarland nephelometer reading of 1.

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[1] Throughout the rest of this paper this medium will be referred to as modified stock culture agar.

Inasmuch as washing removes some of the antigenic components from S. pullorum (Bushnell, 1949), and since the aim was to arrive at quantitative estimates, the cells were not washed prior to making up the antigens. The resulting antigens were then tested with regular and Proteus anti-sera by the tube agglutination method. To determine quantitative estimates of the amount of XII_2 and XII_3 factor present in each culture, the amount of antigen was kept constant and the concentration of sera varied.

In the protocol (Table 1), a series of three rows of tubes for the titration of the antigens with each of the three sera was set up. The first tube of each row contained antigen and serum to give a dilution of 1:5, each of the tubes following contained 1.5 ml of antigen. The twofold dilution method was carried out in obtaining the above dilutions. This protocol represents one row of the series, that is, the titration of the antigen with only one serum.

Table 2 illustrates the method used in arriving at the antigenic types of the antigens.

A serum was prepared by inoculating a goat with a regular strain of S. pullorum, which produced an anti-serum of high titer against standard pullorum antigens. A variant serum, prepared by inoculating a horse with a Proteus strain obtained from Dr. Gwatkin, was supplied by Dr. L. E. Erwin, Department of Bacteriology, Kansas State College. In order

Table 1. Protocol for antigen titration.

	Tube									
	1	2	3	4	5	6	7	8	9	10
Dilution	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
Antigen	4.0 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml	1.5 ml
Serum	1.0 ml	1.5 ml	. . . . .							

Table 2. Method used in determining the antigenic types of the different antigens.

Culture no.[1]	Serum type	Dilution 1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280
3452	Regular absorbed	4+	4+	4+	4+	4+	4+	4+	-	-
	Proteus	-	-	-	-	-	-	-	-	-
Cal. P135	Regular absorbed	4+	4+	4+	4+	4+	4+	4+	-	-
	Proteus	4+	4+	4+	4+	4+	-	-	-	-
4803	Regular absorbed	-	-	-	-	-	-	-	-	-
	Proteus	4+	4+	4+	4+	4+	4+	4+	-	-
M 74	Regular absorbed	4+	4+	4+	-	-	-	-	-	-
	Proteus	4+	4+	4+	4+	4+	4+	4+	-	-
P 161	Regular absorbed	4+	4+	4+	-	-	-	-	-	-
	Proteus	4+	4+	4+	-	-	-	-	-	-

[1] The first two cultures were classed as regular strains of S. pullorum. The second two as variant, and the last as an intermediate culture.

to compare the titers obtained by using the above regular serum and an absorbed regular serum, a second regular serum was prepared by inoculating a goat with S. paratyphi A. var. durazzo. A series of nine doses, beginning with 2 ml of a saline suspension corresponding to a McFarland nephelometer reading of 1 and ending with 10 ml of a saline suspension adjusted to a McFarland nephelometer reading of 5, was administered to the goat subcutaneously at intervals one day apart. These inoculations were followed by three intravenous injections of 10 ml of a saline suspension of the organism, McFarland nephelometer 5, administered at weekly intervals. The goat was bled seven weeks after the first inoculation at which time the serum possessed a titer of 1:10, 240 against its homologous antigen.

S. paratyphi A. var. durazzo contains a relatively high amount of XII_3 factor and little XII_2. S. reading, on the other hand, contains little XII_3 and more of the XII_2 factor. Consequently, absorption of S. paratyphi A. var. durazzo anti-serum with S. reading antigen will remove the XII_2 agglutinins from the serum along with some of the XII_3. Thus, if the adsorption is not controlled and more antigen is added than is necessary for complete absorption of the XII_2 factor, the result will be a serum negative for both the XII_2 and XII_3 factors. By absorbing 50 ml of S. paratyphi A. var. durazzo anti-serum with 2.5 ml of S. reading antigen standard-

ized to a McFarland nephelometer reading of 100 in a water bath at 37° C., and then removing the cells by centrifugation a serum was obtained which gave titers of agglutination with regular S. pullorum antigens, but no reaction against Proteus. These tests were proof that the anti-sera were specific for the XII_3 factor, and that the XII_2 factor had been completely removed. The results of the antigen titrations with regular, regular absorbed, and proteus anti-sera are listed in Table 2.

Isolation of Colony Types from Various Media

The following experiment was an attempt to determine the relationship between colonies grown on various culture media, and the parent cultures which were being carried on the modified Difco Stock Culture Agar. A major portion of this experiment was concentrated on the study of six cultures typical of the entire group. These consisted of two regular strains which did not react to proteus anti-serum, two variants which gave negative results with regular absorbed serum, and two intermediate strains.

The Endo's, Eosin Methylene Blue, MacConkey's, Bismuth sulphite, and S. S. Agar media were prepared from Difco dehydrated products according to the accompanying directions. The nutrient agar medium was the ordinary laboratory preparation. The Stock Culture Agar was the modified Difco product used to carry the stock cultures.

Table 3. Highest dilution showing positive agglutination in the titration of S. pullorum antigen with regular, regular absorbed, and Proteus antisera.

Culture no.	Serum types		
	Regular	Regular absorbed	Proteus
	Regular group		
3933	1:2560	1:160	1:5
5096*	1:2560	1:320	1:20
4074	1:2560	1:120	0
50806	1:2560	1:160	1:10
Bl 23944	1:2560	1:640	1:20
3478	1:1280	1:640	1:5
5095*	1:1280	1:320	1:20
3245	1:1280	1:160	1:10
8732*	1:1280	1:320	1:20
3976-BS	1:1280	1:160	1:5
5094*	1:1280	1:320	1:20
3557	1:1280	1:160	1:20
M 82	1:1280	1:320	1:10
3239	1:1280	1:80	1:5
Iowa	1:1280	1:320	1:20
3794	1:1280	1:80	1:5
5098*	1:1280	1:320	1:20
N.120	1:1280	1:160	1:20
5102*	1:1280	1:320	1:40
854422	1:1280	1:640	1:20
5107*	1:1280	1:640	1:20
Jones#	1:1280	1:320	0

Table 3. (cont.).

Culture no.	Serum types: Regular	Regular absorbed	Proteus
5104*	1:1280	1:640	1:40
Crile	1:1280	1:160	1:20
11**	1:1280	1:320	1:20
Y17	1:1280	1:80	1:40
20**	1:1280	1:80	1:10
3976	1:1280	1:320	1:20
19**	1:1280	1:80	1:5
CI	1:1280	1:320	1:10
17**	1:1280	1:160	1:10
4760	1:1280	1:160	1:40
3568	1:1280	1:160	1:20
3299	1:1280	1:160	1:10
3920	1:1280	1:160	1:10
4905-3	1:1280	1:640	1:10
Cal. P135	1:1280	1:320	1:80
5078	1:1280	1:640	1:40
3199	1:640	1:160	1:10
W.W.E.S.	1:640	1:640	1:10
Turkey	1:640	1:160	1:5
3433	1:640	1:160	1:5
P21	1:640	1:160	0
3452#	1:640	1:320	0
3260	1:320	1:160	1:10
92510	1:320	1:320	1:10

Table 3. (cont.).

Culture no.	Serum types: Regular	Regular absorbed	Proteus
3481	1:320	1:160	1:5
4903-7	1:320	1:160	1:10
4903-6	1:320	1:320	1:5
C22-574	1:320	1:320	1:20
85777	1:80	1:160	1:10
3282	1:160	1:10	1:5
	Variant group		
79**	1:1280	1:5	1:320
C3070-1	1:2560	1:20	1:320
4002-3	1:640	1:10	1:320
4**	1:1280	1:5	1:160
A7080-3	1:1280	1:10	1:320
M74	1:1280	1:20	1:160
24628-34	1:1280	1:20	1:160
427**	1:1280	1:5	1:80
24628-33	1:160	1:20	1:160
419**	1:1280	1:5	1:160
C II#	1:80	0	1:130
4803#	1:5	0	1:320
3558	1:320	1:40	1:80
4016	1:160	1:20	1:40

Table 3. (concl.).

Culture no.	Serum types: Regular	Regular absorbed	Proteus
	Intermediate group		
P161#	1:640	1:20	1:20
B123T441	1:1280	1:20	1:20
85817#	1:1280	1:20	1:20
134	1:40	1:20	1:20

* Recent isolations

\# Cultures followed through in colony study

** Strains employed by the BAI in the preparation of S. pullorum antigens

Each culture was streaked on Petri plates containing the above media by the four flame technique, so that well isolated colonies would result, and incubated at 37 degrees centigrade for 48 hours. Incubation for this length of time produced heavier growth, facilitating the manipulation of single colonies. With the exception of a few minor deviations the procedure used for slide agglutination was essentially similar to that employed by Wright and Edwards (1948). A drop of regular absorbed serum diluted 1:3 was placed on a slide along side a drop of proteus anti-serum of the same dilution. A portion of growth from a single colony was emulsified in each of the two drops of sera and the slide tilted until agglutination occurred. Twenty-five colonies from each plate were tested by this method. Those colonies which agglutinated readily with regular absorbed serum were designated as regular, those giving a rapid reaction with Proteus anti-serum were typed as variant, and colonies which agglutinated equally well with each of the sera were classed as intermediates. Regular colonies produced complete agglutination with regular absorbed serum immediately after emulsification was complete. This was also true of variant colonies and intermediate colonies with their respective anti-sera. After emulsification of the colony with the anti-sera the mixture was observed for agglutination. The preparations were observed for about 5 minutes. Tables 4 and 5 list the colony types tested, the media they

were grown in, and remarks on the observations noted during the course of these studies.

Colonies which developed on nutrient agar were typical for S. pullorum as described by Bergey's Manual of Determinative Bacteriology (1948). Those on Difco's selective media illustrated the morphological characteristics of the Salmonelleae when grown on Difco products, Difco Manual (1948). However, the colonies which developed on the modified stock culture agar presented a macroscopic appearance vastly different from the typical growth on nutrient agar and the other media employed in this study. In this environment the colonies were much larger, ranging from 5 to 10 mm in diameter, more convex, pearly white, opaque, and markedly butyrous. Many of the colonies were concentrically ringed and had developed from one to three papillae. This growth was in opposition to the low convex, transluscent, two to three millimeter, typical S. pullorum colony grown on nutrient agar. The growth described above was so erratic, that it was deemed wise to check the colonies for purity in view of possible contamination. Growth on Kligler's Iron Agar and Bismuth Sulphite agar slants and in carbohydrate broths, together with Gram's stain reactions and microscopic examination produced evidence that the cultures were S. pullorum.

Table 4. Colony types of S. pullorum cultures growing on nutrient agar.*

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
4803	Nutrient agar	0	25	0	Slow, fine, incomplete agglutination in regular serum
C II		1	23	1	Very slow, fine, incomplete agglutination in regular serum
3452		25	0	0	Very slow, fine, incomplete agglutination in variant serum
Jones		25	0	0	Same as above
P 161		0	25	0	Slow, fine, incomplete agglutination in regular serum
85817		25	0	0	Slow, fine, incomplete agglutination in variant serum
3478		25	0	0	
49036		25	0	0	
Cal. P135		22	1	2	
C I		22	0	3	
Turkey		25	0	0	
4002-3		25	0	0	
3920		25	0	0	
N 120		25	0	0	

Table 4. (cont.).

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
3299		25	0	0	Slow, fine, incomplete agglutination in variant serum
4905-3		25	0	0	
4903-7		25	0	0	
85777		25	0	0	
4074		25	0	0	
3933		25	0	0	
3433		25	0	0	
3282		25	0	0	
3976		25	0	0	
3481		25	0	0	
854422		25	0	0	
W.W.E.S.		25	0	0	
4903-6		25	0	0	
3478		25	0	0	
Y17		25	0	0	
# 5078		25	0	0	

Table 4. (cont.).

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
3976	Nutrient agar	25	0	0	Slow, fine, incomplete agglutination in variant serum
3479		25	0	0	No agglutination in variant serum
50806		25	0	0	Slow,fine, incomplete agglutination in variant serum
5807		25	0	0	
Crile		25	0	0	
C22-574		25	0	0	
PL239441		0	21	4	Slow, fine, imcomplete agglutination in regular serum. Equal agglutination for intermediate
3558		0	25	0	Very slow, fine, incomplete agglutination in regular serum
24628#33		0	23	2	Reaction in regular serum rapid and complete; however, not as rapid as in variant serum
4016-3		0	25	0	Slow, fine, incomplete agglutination in regular serum
24628-34		0	0	25	Agglutination in both sera with equal rapidity

Table 4. (cont.).

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
4803	Stock culture agar	0	25	0	Colonies almost approach intermediate stage
C II		0	25	0	Slow, fine, incomplete agglutination in regular serum
3452		25	0	0	Slow, fine, incomplete agglutination in variant serum
Jones		25	0	0	Same as above
P161		0	25	0	Slow, fine, incomplete agglutination in regular serum
85817		25	0	0	Slow, fine, incomplete agglutination in variant serum
3479		25	0	0	
C22-574		25	0	0	
5078		25	0	0	
W.W.E.S.		25	0	0	
Crile		24	0	1	
3260		25	0	0	
50806		25	0	0	

Table 4. (concl.).

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
134	Stock culture agar	0	0	25	Complete agglutination in both sera with equal rapidity
4016-3		0	0	25	Slow, fine, incomplete agglutination in regular serum

* Those colonies which agglutinated rapidly with regular absorbed serum were designated as regular, those giving a more rapid reaction with Proteus anti-serum were classed as variant, and colonies agglutinating equally well in both sera were typed as intermediates.

Table 5. Colony types of S. pullorum cultures growing on various media.

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
3452	Nutrient agar	25	0	0	Slow, fine, incomplete agglutination
	Stock culture agar	25	0	0	Same as above
	Bismuth sulphite agar	25	0	0	Same as above
	Endo's agar	25	0	0	No agglutination in variant serum
	S.S. agar	25	0	0	Slow, fine, incomplete agglutination in variant serum
	Eosin methylene Blue agar	25	0	0	
	MacConkey's agar	25	0	0	
Jones	Nutrient agar	25	0	0	
	Stock culture agar	25	0	0	
	Bismuth sulphite agar	25	0	0	
	Endo's agar	25	0	0	
	Eosin methylene Blue agar	25	0	0	

Table 5. (cont.).

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
Jones	S.S. agar	25	0	0	No agglutination in variant serum
	MacConkey's agar	25	0	0	Slow, fine, incomplete agglutination in variant serum
4803	Nutrient agar	0	25	0	Colonies almost intermediate
	Stock culture agar	0	25	0	Same as above
	Bismuth sulphite agar	0	25	0	Same as above
	Endo's agar	0	25	0	Very slow, fine, incomplete agglutination in regular serum
	Eosin methylene Blue agar	0	25	0	Same as above
	S.S. agar	0	25	0	Same as above
	MacConkey's agar	0	25	0	Same as above
P161	Nutrient agar	0	25	0	Fine, slow, incomplete agglutination in regular serum
	Stock culture agar	0	25	0	Same as above

Table 5. (concl.).

Culture no.	Culture media	Colony types: Regular	Variant	Intermediate	Remarks
P161	Bismuth sulphite agar	0	21	4	Slow, fine, incomplete agglutination in regular serum
	Endo's agar	0	25	0	
	Eosin methylene Blue agar	0	25	0	
	S.S. agar	0	25	0	
	MacConkey's agar	0	25	0	
85817	Nutrient agar	25	0	0	Slow, fine, incomplete agglutination in variant serum
	Stock culture agar	25	0	0	
	Bismuth sulphite agar	25	0	0	
	Endo's agar	25	0	0	
	Eosin methylene Blue agar	25	0	0	
	S.S. agar	25	0	0	
	MacConkey's agar	25	0	0	

Colony Types from Successive Transfers of *S. pullorum* on Difco Stock Culture Agar Containing Four Percent Agar-Agar

In view of the results obtained by Bushnell (1949) concerning the conversion of "smooth" forms of *E. coli mutabile* to the "rough" phase, by growing this organism in an environment containing four percent agar-agar, similar work was carried out with *S. pullorum* to study the possibilities of antigenic changes being associated with induced dissociations.

Cultures 4803, CII, 3452, Jones, Pl61 and 85817 were streaked on Difco stock culture agar to which additional agar-agar had been added to result in a medium with a total agar-agar content of four percent. The plates were incubated at 37° C. for 48 hours. At the end of this period 25 well isolated colonies were picked and tested by the slide agglutination technique described previously. The growth from these plates was transferred to fresh plates of the same medium, incubated for 48 hours at 37° C., and at the end of the incubation period the same procedure followed. Colonies from the first, second, fifth, sixth, eighth and tenth transfer plates were picked and typed by the slide agglutination technique. The antigenic characteristics of all the colonies were essentially the same as the "smooth" types of the six parent strains reported in Table 4. However, there was an apparent slight retardation in the speed of agglutination when the daughter

colonies of the regular parent 3452 were tested with regular absorbed serum. The rate of agglutination was not influenced when these same colonies were tested with the Proteus anti-serum. On the other hand when daughter colonies of strain 4803 were tested with Proteus anti-serum the rate of reaction was enhanced. In regular absorbed serum no change was noted. Rough colonies gave negative agglutination when tested with saline and fowl sera negative for pullorum. Evidently the stability of the antigens remained uneffected in this phase.

On the first transfer to the high agar-agar content medium there was a distinct change in colony appearance. The colonies produced resembled the "rough" phase, however, there persisted a slight luster to the colonies. Subsequent transfers produced successive increases in colony roughness with the persistence of a slight luster. Colonies were not as large as those developing on the modified stock culture medium, ranged from 2 mm to 3 mm in diameter, and they bore a central papilla and were more compact and friable. No antigenic differences were observed between the periphery of a colony, and the central papilla. Rough growth from each transfer plate transferred to plain nutrient agar or modified stock culture agar reverted to the "smooth" form.

Gram's stain reactions of the organism in the rough phase revealed cells typical of normal S. pullorum when grown on nutrient agar, both in morphology and staining properties.

The Effect of Sulfamethazine on the Antigenic Structure of S. pullorum

Modified stock culture agar was tubed in 5 ml amounts and autoclaved for 15 minutes at 15 pounds pressure. At the end of this sterilization period the tubes were cooled to 50° C. and varying concentrations of Sulmet, a Lederle product consisting of a 12.5 percent solution of sodium sulfamethazine, were added to each tube. The tubes were then slanted and allowed to solidify. Six strains of S. pullorum were seeded on the slants and incubated for 48 hours at 37° C. Growth from the third serial subculture on this medium was transferred onto fresh slants of the stock culture media containing 0.1 ml of Sulmet solution. This process was continued until growth of S. pullorum occurred on slants containing 0.2 ml of the Sulmet solution per 5 ml of media.

Antigens were prepared from growth on the Sulmet containing medium by following the technique outlined on pages 11 and 12 and tested with both regular absorbed and Proteus anti-sera to determine the antigen type. Table 6 lists a comparison of the titers derived from cells grown on Modified Stock Culture Agar containing Sulmet and on the same medium free of the drug.

The six strains of S. pullorum streaked onto Petri plates containing 0.2 ml of Sulmet per 5 ml of stock culture medium produced daughter colonies which were of the mixed

type, since they all reacted to both the regular absorbed and Proteus anti-sera. Colonies from strain 4803 were intermediate in nature, while the remaining five strains produced daughter colonies which exhibited a marked increase in the rate at which they agglutinated with both sera; however, they did not quite approach the intermediate stage, and were either regular or variant depending on the nature of the parent strain.

Forty-eight hour cultures of each strain, after the first transfer on slants containing 0.05 ml of Sulmet, displayed a very marked increase in the rate of agglutination in both types of sera when tested by slide agglutination. Growth from strains 3452, Jones, P 161, 85817, and C II almost approached the intermediate stage. Strain 4803 was completely converted to an intermediate strain.

Growth resulting on slants containing Sulmet was smooth, moist, and glistening. The organisms had adsorbed pigment imparted by the drug and assumed a pinkish hue. Microscopic examination of the cells revealed Gram negative organisms ranging from plump cocco-bacilli to long slender "S" shaped forms. The number of involution forms was extremely marked. Transfer of these sulfa resistant strains to Modified Stock Culture Agar resulted in the conversion of the strains to their original form, both morphologically and antigenically.

Table 6. Effect of sulfamethazine on the antigenic structure of the organisms studied.

Culture no.	Titer of agglutination produced by cells grown in the absence of Sulmet		Titer of agglutination produced by cells grown in the presence of Sulmet	
	Serum type		Serum type	
	Regular absorbed	Proteus	Regular absorbed	Proteus
3452	1:320	1:0	1:640	1:80
Jones	1:320	1:0	1:640	1:40
4803	1:0	1:320	1:320	1:320
C II	1:0	1:160	1:5	1:160
P 161	1:20	1:20	1:20	1:320
58517	1:20	1:20	1:320	1:40

DISCUSSION

Each of the 70 strains of S. pullorum studied reacted with regular unabsorbed serum in a high titer. This was apparently due to the presence of factors IX and XII_1 in both the regular and variant strains of the organism. However, with regular absorbed serum where only the XII_3 agglutinin was present a different picture resulted. The results of the antigen titrations as listed in Table 3 show that 52 of the strains gave a higher titer with regular absorbed than with Proteus anti-serum. Of these 52 cultures, 4 did not react with Proteus anti-serum at all, 14 gave a higher titer with Proteus anti-serum (2 of these giving negative reactions with regular absorbed serum), and 4 of the strains gave equal titers with both types of sera. From these results, it seemed evident that stabilization in the pure regular and pure variant forms actually occurred. However, later this was shown to be in error, in that when stabilization does develop it is with the retention of both factors by the organism. In view of this it is desirable to have in mind a definition as to what constitutes a regular, variant, or intermediate strain of S. pullorum.

Thus, from the results listed in Table 3, a variant strain is one which will give a higher titer of agglutination when reacted with Proteus anti-serum than with regular absorbed serum; the converse would follow for regular strains; and an

intermediate strain is one giving equal titers of reaction with both sera. Bearing this in mind, the first 52 strains were classed as regular, the second group of 14 as variant, and the remaining 4 as intermediate, Table 3.

The results derived from strains 3452, Jones, C II and 4803, Table 3, indicate that stabilization in the pure regular and pure variant forms was possible. However, the experiments with colony-type isolations proved this to be erroneous. Strains 3452 and Jones, which gave negative reactions with Proteus anti-serum by tube agglutination (Table 3), when transferred to the various culture media gave rise to daughter colonies which agglutinated in both the regular absorbed and Proteus anti-sera, Tables 4 and 5. However, the reaction was much more rapid in the regular absorbed serum. Strains 4803 and C II produced daughter colonies which reacted to both sera, but which were predominantly variant in nature. This factor, which involves the speed of reaction, indicates the phenomenon is due to quantitative factors; that is, factors XII_2 and XII_3 are always present in a strain of S. pullorum, but one usually exceeds the other in concentration. If this be the case then it is possible to assume that the negative results recorded in Table 3 for the above strains were due to the reduction of the factors concerned to below the limits of detection by an agglutination study.

Of the 44 strains plated on nutrient agar, Table 4, only

three produced daughter colonies reacting with only one type serum. These three strains transferred to other media gave rise to colonies which reacted to both the regular absorbed and Proteus anti-sera. It is evident that the environment may have had an influence in determining just how much of each factor was retained by the organism. A study of Table 5 will reveal that strains 3452, Hones, 4803, C II, P 161, and 85817 when streaked on the various media developed some changes in form. In one instance daughter colonies of 3452 agglutinated with only regular absorbed serum, when these colonies were isolated from Endo's medium. Colonies from Jones agglutinated only with regular absorbed serum when the parent strain was streaked on S. S. agar. These same parent strains streaked out on the other media gave rise to colonies reacting with regular absorbed and the Proteus anti-serum. The above observations indicate that the original assumptions were correct, and that stabilization occurs with the complete antigenic structure inherent in a culture of S. pullorum.

Contrary to speculation, and to results reported by other workers, a single parent strain did not produce daughter colonies of both the regular and the variant form. Each of the six strains was plated on seven different media and 25 colonies were picked from each plate; thus, giving a total of 175 colonies of each strain studied by slide agglutination. Each of the colonies was agglutinated by both the regular

absorbed and Proteus anti-sera. With the exception of a few questionable instances in which variant strains gave rise to a few intermediate colonies, Tables 4 and 5, each of the colonies was regular or variant depending on the parent.

In agreement with Wright (1947), it is believed that strains possessing the complete antigenic structure for S. pullorum can be employed successfully for the detection of both the regular and variant forms of pullorum disease, however, before this can be done there are certain conditions to be taken under consideration. It may be noted from the data in Table 3 that regular strains gave titers ranging from 0 to 1:40, and in one instance 1:80 when tested with Proteus antiserum. Variant strains gave low titers when tested with regular absorbed serum, and intermediate strains gave very low titers with both these sera. This deficiency in titer strength for one, or both of the XII_3 and XII_2 factors probably accounts for the unfavorable results reported by Gwatkin and Bond (1947) who employed mixed culture antigens for the detection of regular and variant agglutinins. In view of these findings, the development of a satisfactory antigen may lie in locating a strain which will agglutinate equally well in both regular absorbed and Proteus anti-sera, with the ideal situation existing in a strain with a titer of at least 1:320 when reacted with both sera by tube agglutination. The location of such a strain possibly may be accomplished by chance,

or by growing numerous strains in an environment which will increase the XII_2 and XII_3 factors. With this latter end in view the six strains selected for study were grown in an environment containing sulfamethazine. The results obtained, Table 6, indicate that a definite change took place in the antigenic structure of the sulfa resistant strains. Previously it was stated that the growth took on a pinkish coloration imparted by the pigment of the drug. Evidently the adsorption phenomenon that took place changed the make up of the cell surface and thereby influenced the antigenic structure of the organism. Development of marked involution, or abnormal forms, occurred and this factor may offer unforeseen pitfalls in efforts to employ Sulfa resistant strains of S. pullorum as antigens. However, this preliminary work indicates such strains to be stable if maintained on the Sulmet medium described.

The Sulmet compound used in these experiments is sold commercially as a therapeutic agent for poultry diseases. It is reasonably logical to assume that if this compound influences antigenic changes in-vitro possibly it may produce alterations of a similar nature in-vivo if the blood level of the drug reached a sufficient concentration and persisted long enough. Hypothetically, then, a culture might assume variant tendencies while the drug level in the blood remained high, and revert to the regular form when the level became

lowered and new generations of organisms had not been in contact with sufficient doses of the drug.

For some time now the search for a more efficient S. pullorum antigen has been in progress. Referring back to Table 6, one can predict that possibilities exist in a strain such as 4803. A method employing such a modified strain in the production of an antigen would be economical, and would eliminate errors of nonspecificity caused by polyvalent antigens.

CONCLUSIONS

In the study of 70 strains of S. pullorum, it was found that the tendency is for antigenic stabilization to occur with the retention of both the XII_2 and XII_3 factors by the organism. Colony type studies revealed that parent strains give rise to daughter colonies that are complete in their antigenic make up and that all colonies are regular or variant depending on the parent. It was found that the culture medium may have an influence in determining the antigenic structure of S. pullorum.

Sulmet incorporated in the environment served to increase the antigenic factors of S. pullorum. In view of the results here reported it is suggested that strains possessing the complete antigenic structure for S. pullorum may be located or developed and employed successfully for the detection of both the regular and variant form of infection.

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