A SURVEY OF THE INCIDENCE OF SEROLOGICAL VARIANTS OF SALMONELLA PULLORUM IN MICHIGAN

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BY

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INTRODUCTION

When discussing pullorum disease eradication with hatcherymen, the diagnostician is often asked why repeated blood-testing of breeder flocks has failed to bring about reduction or elimination of the disease in chicks. Sometimes one can find management practices which are at fault. In other cases, however, one is at a loss to explain this failure to his own satisfaction; much less to the satisfaction of the hatcheryman. The reports of various Canadian workers concerning the presence of so-called "variant" strains of Salmonella pullorum have offered another possible explanation. It was felt that a survey to determine the incidence of "variant" strains of S. pullorum among cultures isolated from fowl presented for diagnosis at the Poultry Clinic might aid in the evaluation of the importance of this aspect of the Michigan pullorum disease control plan.

REVIEW OF LITERATURE

Younie (10) states that in 1939, a few outbreaks of pullorum disease occured in chicks hatched from breeders which were negative to pullorum disease tube tests. In 1940, an increase in the number of such outbreaks was noticed. A retest of the breeders of one breed revealed only 11 reactors. Losses continued to the end of the hatching season. Neither the post-mortem appearance of affected chicks nor the cultural characteristics of the organisms isolated from them differed from the usual findings in pullorum disease. Certain serological differences were seen. Slide agglutination tests showed differences in rapidity and type of agglutination. In tube agglutination tests, antiserum of the pullorum variant did not react with Eastern Conference strains 19 and 20 and gave only a 1+ reaction in 1/50 dilution with strain 17 while three variant strains gave complete agglutination in a 1/800 dilution.

Younie reported further study in 1942 (11). A flock of 94 pullets was tested for pullorum disease in the fall. Three percent were found to be reactors. A retest made six weeks later showed no new reactors. On July 5th of the following year, the flock was tested with antigens made from the three conference strains, five variant strains, with three commercial antigens, and with one commercial whole-blood antigen. Serum from three birds reacted with the con-

ference strains' antigens and commercial antigens while 64 sera reacted with the variant strains' antigens. Cultures of S. pullorum were obtained from 65% of 90 birds autopsied. Additional history supplied after the study was begun indicated that the variant infection may have started in the spring preceding the July test. A flock of baby chicks purchased at that time suffered a 40% mortality from pullorum disease caused by a variant strain.

Attempts to verify Younie's reports and to find an explanation for them have been made. Glover and Connell (3) used 47 cultures of S. pullorum obtained from consignments sent to the diagnostic laboratory. Agglutinin absorption tests showed that the strains fell into at least two groups--"normal" and "variant". Low titer sera from experimentally infected birds agglutinated the homologous antigen but did not agglutinate the heterologous type. "Consequently," they state, "infected birds carrying a low serum titer are in many instances not detected by the standard agglutination test." Byrne (1) used ten strains of S. pullorum and their respective rabbit antisera in his studies. He found cross agglutination between variant and standard strains but he points out that the titers of these sera were higher than might be expected to occur in fowl naturally infected with the chronic form of pullorum disease. Agglutinin absorption studies showed a distinct division into standard and atypical or variant strains.

Wright (9) reported that, in over 7000 blood samples tested by the tube agglutination method with antigen of both conference strains and variant strains, 33.8% were positive with variant antigen but only 1.4% were positive with conference strain antigen. Mixing conference and variant strains in "aliquot" proportion yielded an antigen intermediate in results between its two components.

Gwatkin and Bond (8) infected 338 chicks with five variant strains of S. pullorum. Of 294 which were tested for agglutinins at five to six weeks of age, 191 were positive with variant antigen but only 20 with regular type antigen. Therefore, about 39% of the reactors would have been missed by the regular antigen if positive and suspicious reactions were grouped. A second test was made between 80 and 102 days of age. Regular (or conference strains) antigens failed to detect 68.8% of the birds whose sera were either positive or suspicious with variant antigen. In 315 tests, only two (0.25%) known negative birds reacted with variant antigen.

This study was continued by Gwatkin (5). At five and a half months of age, 49.2% of the chickens positive with variant antigen were negative with regular antigen. At this time, the fowl were divided into two flocks; one positive and one negative. The negative group (110 birds) was tested at seven and a half months; ten reactors to variant antigen were found. Five of these birds did not react to regular antigen; however, S. pullorum was recovered

from seven. At nine months, two reacted with variant antigen and one of these with the regular antigen. At ten months no reactors were found with either antigen in either the plate or tube tests. At one year of age, one hen reacted with variant antigen but was negative to the regular antigen; S. pullorum was not recovered. Three settings of eggs from each of the positive and negative flocks were made. The mortalities for each of the settings from the negative group were 12%, 12%, and 10%; S. pullorum was not recovered from any of these. The percent mortalities in chicks hatched from the variant positive group were 84, 42, and 93. The pullorum organism was recovered from 100%, 80% and 91%, respectively. Gwatkin states, [sic] "It appears that variant infection was controlled by the same methods employed in dealing with regular type infection with the very important difference that a variant antigen was employed."

Gwatkin reported another study of serological reactions of variant S. pullorum infections in 1946 (7). Sixty birds which had been infected by mouth as chicks with variant strains of S. pullorum were killed or died between the 6th and 17th months after infection and bacteriological examinations were made on these birds. All were positive in tube agglutination tests with variant antigens. Eight of the 60 had the same serum titer with both antigens; the remainder had higher titers with variant than with regular antigens. Thirty-two were negative with regular antigen in

dilutions as low as 1/10. The organism was recovered from 25 of these. Fourteen of the 32 reacted only in a 1/20 dilution with variant antigen and the organism was recovered from nine of these. Seventeen other birds of the 60 reacted with regular antigen only in a 1/10 or a 1/20 dilution and the organism was recovered from 15. With advancing age, fewer birds were missed by the regular antigen but during the age when they would normally have been tested, many would have been missed by regular antigen only.

Some other observations which Gwatkin made during the course of his studies are reported (7). Parenteral infection with variant type strains did not give clear cut results and serum had to be collected early to detect differences in agglutinins. Absorption experiments showed that regular agglutinins were more easily removed by either regular or variant strains than were variant agglutinins. In some cases, however, massive treatment with either regular or variant type organisms removed both types of agglutinins. That an antigenic difference between variant and regular types of S. pullorum exists was shown by cross agglutination tests with a strain of Proteus sp. isolated from the ovary of a hen whose serum agglutinated variant antigen. Eighteen variant strains were agglutinated in fairly high titer (1/160 to 1/640) in Proteus antiserum. Thirty regular strains were agglutinated only in a 1/10 or a 1/20 dilution.

Gwatkin (6) tested 746 birds from variant infected flocks and 645 from regular type infected flocks using both tube and plate tests and with variant, regular, and mixed antigens. Whole blood antigens gave an agreement of 99.4% between variant and mixed antigens and 96.8% between regular and mixed antigens. In the first tube test comparison, positive and suspicious reactions were grouped. Mixed antigen detected 98.6% of reacting birds detected by variant antigen and 93.4% of regular antigen reactors. Regular antigen detected only 85.7% of the variant reactors. In the second tube test comparison, agreement between variant and mixed antigens and regular and mixed antigens was 97.3% and 92.8% respectively, when tested at 1/50 dilution. When tested at 1/25 serum dilution, the agreement was 94.9% and 90.2%, respectively.

Finally, a detailed antigenic analysis has been made by Edwards and Bruner (2). The authors state that the antigenic formula of S. pullorum is IX, XII_1, [XII_2,] XII_3. In normal cultures, the XII_2 factor is variable, and forms containing a large amount or a negligible amount of XII_2 can be isolated from the same normal strain. It is possible for cultures to become fairly well stabilized in either form, thus giving rise to the so-called "standard" (St.) strains and "variant" (X) strains. The standard strains contain only a small amount of XII_2 but the variant strains contain a large amount.

The reactions in S. paratyphi A var. durazzo serum (II, XII_1, XII_3) and S. reading serum (IV, XII_1, XII_2) were particularly revealing. The Proteus serum of Gwatkin gave results comparable to those obtained with S. reading serum. The Proteus culture used to prepare the serum contains all or a portion of XII_2. It was evident that S. pullorum St. had a very weak XII_2 content whereas S. pullorum X had a very strong XII_2 component.

When S. Typhi 0901 (IX, XII_1, XII_2, XII_3) or S. pullorum X serum was absorbed by S. typhi T 2 (IX, XII_1, XII_3) a strong residue of agglutinins remained for S. pullorum X, S. typhi 0901 and S. reading. This residue represented the antigen XII_2. When the same sera were absorbed with S. pullorum (standard), a much weaker residue of agglutinins was left for S. pullorum X, S. typhi 0901 and S. reading. This indicated that while the XII_2 component was not sufficiently developed in S. pullorum St. to cause evident agglutination, a sufficient amount was present to absorb most of the XII_2 agglutinins. This conclusion was supported by absorption of S. pullorum St. serum with S. typhi T 2. A residue of XII_2 agglutinin was left in the serum which agglutinated S. pullorum X, S. typhi 0901 and S. reading. Apparently, while S. pullorum St. did not contain sufficient XII_2 antigen to be agglutinated, it did contain enough to produce a low titer of XII_2 agglutinins.

When durazzo serum was absorbed by S. reading it still agglutinated S. pullorum X in a low dilution, indicating that the variant strains contained a small amount of XII_3 antigen. S. reading serum absorbed with durazzo no longer agglutinated S. pullorum St. indicating that the standard strain did not contain sufficient XII_2 antigen to cause it to agglutinate in XII_2 serum.

The $XII_2^{\pm}$ and $XII_2{+}{+}$ forms were easily distinguished in the slide agglutination tests using S. reading serum absorbed by durazzo (XII_2) and durazzo serum absorbed with S. reading (XII_3). The ++ forms agglutinated rapidly in XII_2 serum and very slowly or not at all in XII_3 serum. In the $\pm$ forms, the behavior was just the reverse.

An attempt to differentiate the two forms of S. pullorum by using a phage (4) isolated from organs of chicks naturally infected with a regular strain of S. pullorum showed no significant difference in activity for any of six regular or five variant strains tested.

A few statements concerning the relative incidence of variant infection occur in the literature. The outbreaks of pullorum disease in chicks which Younie reports (10,11) were primarily of the variant type. Glover and Connell (3) typed 161 strains of S. pullorum isolated from post-mortem material and found 110 variant strains, 49 normal (or standard or regular) and 2 which agglutinated with both sera equally well. Reference has already been made to Wright's (9) series of agglutination tests.

MATERIALS AND METHODS

Two hundred and two cultures of Salmonella pullorum representing 199 consignments were isolated from chickens and turkeys of various ages presented over a period extending from November 1, 1945 to September 1, 1946. In many instances, the consignments were shipped to the laboratory. Often it was not possible to learn the name of the hatchery from which the chicks were purchased. Hence, it is not known how many different hatcheries are represented nor how many of the strains collected originated from the same hatchery's stock.

Isolations were made by streaking material from birds suspected of having pullorum disease on Difco "SS." agar plates. After 24 hours incubation, colonies having the gross appearance of S. pullorum were subcultured and identified. Usually after identification, one culture, chosen at random from each consignment, was placed in stock. Stock cultures were maintained by stab seeding semi-solid agar containing 0.5% beef extract and 0.5% proteose-peptone. These were incubated 48 hours, sealed with paraffin, and stored in a 36° to 40° F. refrigerator. The stock cultures were transferred at 4 to 6 week intervals.

An antigen was prepared from each strain in the stock culture collection by seeding about 5 cc. of broth with a loopful of the stock culture. The broth tubes were

incubated for 8 hours at 37° C. on a mechanical horizontal shaker. This procedure gave rather uniformly good growth. Each broth culture was then used to seed agar slants contained in 6 or 8 ounce screw-cap prescription bottles. The slants were incubated in a vertical position for 40 hours. After incubation, the surplus broth inoculum was aspirated and discarded. The bacterial growth was washed from each slant using about 5 cc. of physiological saline per slant. This yielded a very dense suspension of live organisms.

Gwatkin (7) reported that antiserum prepared with a strain of Proteus obtained from the ovary of a hen agglutinated variant strains of S. pullorum in high dilution but did not agglutinate standard strains beyond 1/20. He kindly supplied a transplant of this Proteus strain. An antigen was prepared from it by boiling the growth washed off a 24 hour agar slant culture. A rabbit was hyperimmunized by 4 intravenous injections of 0.5 ml. each at 4 day intervals. Another rabbit was similarly immunized with Salmonella paratyphi A variety durazzo. A third rabbit was immunized with S. pullorum, Eastern Conference strain 19. Four days after the last injection of antigen, each rabbit was bled by cardiac puncture and the serum was harvested.

The 202 recently isolated strains of S. pullorum and 6 strains maintained in the laboratory for several years were typed by placing approximately 0.02 ml. of each of the three sera on a glass plate and mixing with each serum a

similar quantity of antigen. If agglutination was not immediate, the plate was gently rotated. The intensity of the reaction was not classified except in those strains which were positive to Proteus antiserum. With the pullorum antiserum used, all strains, except number 800, gave a definite positive reaction. Strain number 800 was classified as 2+ or partial. The paratyphi A antiserum rarely gave more than 2+ reaction with any of the pullorum strains.

Since most of the typing of pullorum strains reported prior to the time of this study had been done with the tube agglutination test, it was desired to check the results of the serum plate test with those of the tube test. Accordingly, antigens prepared from 54 strains were each diluted to a McFarland nephelometer turbidity of 1 and combined with Proteus antiserum and with S. paratyphi A variety durazzo antiserum in serum dilutions of 1/20, 1/80, 1/160 and 1/320. These were incubated 20 hours at 50° C. in a water bath and 4 hours at room temperature.

The results of the serum-plate typing of 156 strains are recorded in Table 1 A. The results of the typing of the other 52 cultures, examined by both the serum-plate test and the tube test, are recorded in Table 1 B. The results of all of the serum-plate tests are summarized in Table 2. Of the 31 strains (15% of the total) which agglutinated with Proteus antiserum, 29 agglutinated quickly and distinctly. The cell aggregates were, however, smaller

Table 1 A.

Serum Plate Agglutination Results

Consignment Number	Antiserum		
	Proteus	Para A	Fullorum
46 - 591	-	+	+
595	-	+	+
606	-	+	+
607	-	+	+
627	-	±	+
628	-	+	+
629	-	±	+
631	-	+	+
636	2+	±	3+
637	-	+	+
638	-	+	+
640	-	+	+
644	-	+	+
646	-	+	+
650	-	+	+
653	-	+	+
657	-	+	+
658	1+	-	3+
664	-	+	+
681	-	+	+
686	-	+	+
688	2+	±	+
690	-	+	+
691	-	+	+
693	-	+	+
694	-	+	+
695	-	+	+
698	-	+	+
709	-	+	+
711	-	+	+
712	-	+	+
713	-	+	+
714	-	+	+
715	-	+	+
716	-	+	+
719	-	+	+
721	-	+	+
722	-	+	+
725	-	+	+
728	-	+	+
732	-	+	+

Table 1 A. (continued)

Consignment Number	Antiserum		
	Proteus	Para A	Pullorum
46 - 736	-	+	+
738	-	+	+
739	-	+	+
741	-	+	+
742	-	+	+
743	2+	±	3+
745	-	+	+
746	-	+	+
748	-	+	+
753	2+	-	4+
754	-	+	+
756	-	+	+
757	-	+	+
759	-	+	+
760	-	+	+
761	-	+	+
765	-	+	+
766	2+	±	4+
768	-	+	+
769	-	+	+
770	-	+	+
771	-	+	+
772	-	+	+
778	-	+	+
781	-	+	+
782	-	+	+
783	-	+	+
784	-	+	+
786	-	+	+
788	-	+	+
810	1+	±	3+
811	-	+	+
815	-	+	+
824	2+	-	4+
865	-	+	+
876	2+	±	3+
880	-	+	+
884	-	+	+
885	-	+	+
886	-	+	+
887	-	+	+
888	-	+	+
892	-	+	+

Table 1 A. (continued)

Consignment Number	Antiserum		
	Proteus	Para A	Pullorum
46 - 893	-	+	+
894	-	+	+
895	-	+	+
897	-	+	+
898	-	+	+
899	-	+	+
900	-	+	+
902	-	+	+
904	-	+	+
906	-	+	+
907	-	+	+
908	-	+	+
909	-	+	+
910	-	+	+
912	-	+	+
913	-	+	+
917	-	+	+
920	-	+	+
922	-	+	+
930	-	+	+
931	-	+	+
933	-	+	+
934	-	+	+
937	-	+	+
938	-	+	+
939	-	+	+
949	-	+	+
953	-	+	+
958	-	+	+
961	-	+	+
962	2+	-	3+
965	-	+	+
967	-	+	+
968	-	+	+
971	-	+	+
973	-	+	+
988	-	+	+
995	-	+	+
996	-	+	+
1005	2+	-	3+
1006	-	+	+
1007	-	+	+

Table 1 A. (continued)

Consignment Number	Antiserum		
	Proteus	Para A	Fullorum
46 - 1040	-	+	+
1042	-	+	+
1069	-	+	+
1071	-	+	+
1079	-	+	+
A - 4	2+	+	4+
46 - 15	-	+	+
37	-	+	+
48	-	+	+
50	-	+	+
64	-	+	+
70	2+	+	4+
79	-	+	+
87	-	+	+
103	2+	-	4+
105	-	+	+
106	-	+	+
117	2+	-	3+
122	-	+	+
164	-	+	+
165	-	+	+
170	-	+	+
188	-	+	+
196	-	+	+
P - 179	-	+	+
P - 19	-	+	+
M - 17	-	+	+
M - 19	-	+	+
M - 20	-	+	+
Variant 201	2+	-	3+
Proteus	4+	-	+

Table 1 B.

Consignment Number	Serum Plate Antiserum			Tube Agglutination Test								
				Proteus				Para A var. Durazzo				
	Proteus	Para A	Pullorum	1/20	1/80	1/160	1/320	1/20	1/80	1/160	1/320	Control
46 - 727	2+	2+	3+	3+	2+	1+	-	3+	2+	1+	±	-
794	2+	-	3+	3+	4+	-	-	2+	3+	-	-	-
796	-	+	+	-	-	-	-	2+	4+	2+	2+	-
800	2+	-	2+	3+	3+	±	-	3+	4+	±	±	-
801	-	+	+	-	-	-	-	3+	4+	3+	2+	-
802	2+	±	4+	4+	4+	1+	-	2+	4+	2+	1+	-
803	-	+	+	-	-	-	-	2+	4+	1+	±	-
805	-	+	+	±	-	-	-	4+	4+	2+	1+	-
809	-	+	+	-	-	-	-	3+	4+	2+	2+	-
817	-	+	+	-	-	-	-	4+	2+	2+	1+	-
820	-	+	+	-	-	-	-	3+	2+	2+	±	-
821	-	+	+	-	-	-	-	3+	3+	2+	1+	-
831	2+	-	4+	2+	2+	±	-	3+	2+	±	±	-
832	-	+	+	-	-	-	-	4+	2+	2+	±	-
833	-	+	+	-	-	-	-	4+	2+	2+	±	-
834	-	+	+	-	-	-	-	3+	2+	1+	±	-
837	-	+	+	-	-	-	-	4+	3+	3+	2+	-
840	-	+	+	-	-	-	-	3+	1+	1+	1+	No record
845	2+	1+	3+	2+	1+	1+	±	3+	2+	1+	±	-
846	-	+	+	-	-	-	-	4+	2+	1+	±	-
847	2+	1+	3+	2+	2+	1+	-	3+	2+	1+	±	-
850	2+	±	3+	2+	1+	±	±	2+	2+	1+	±	-
851	-	+	+	-	-	-	-	4+	3+	3+	±	-
852	-	+	+	-	-	-	-	4+	2+	1+	±	-
854	2+	±	4+	-	-	-	-	4+	2+	1+	±	-
855	-	+	+	-	-	-	-	4+	2+	1+	±	-
856	-	+	+	-	-	-	-	3+	3+	3+	3+	-

Table 1 B. (continued)

Consignment Number	Serum Plate Antiserum			Tube Agglutination Test								
				Proteus				Para A var. Durazzo				
	Proteus	Para A	Pullorum	1/20	1/80	1/160	1/320	1/20	1/80	1/160	1/320	Control
46 - 858	-	+	+	-	-	-	-	3+	3+	2+	2+	-
860	-	+	+	-	-	-	-	4+	3+	2+	±	-
946	2+	2+	3+	2+	±	-	-	4+	2+	1+	-	-
1010	-	+	+	-	-	-	-	3+	2+	2+	2+	-
1012	-	+	+	-	-	-	-	3+	2+	2+	2+	-
1021	-	+	+	-	-	-	-	3+	1+	+	-	-
1044	-	+	+	-	-	-	-	2+	1+	1+	±	-
1047	2+	-	3+	2+	1+	±	-	3+	1+	±	-	-
1050	-	+	+	-	-	-	-	2+	1+	1+	±	-
1052	-	+	+	-	-	-	-	3+	2+	2+	-	-
1053	-	+	+	-	-	-	-	3+	2+	1+	±	-
1065	-	+	+	-	-	-	-	4+	4+	4+	4+	-
1067	-	+	+	-	-	-	-	3+	2+	2+	1+	-
1080	-	+	+	-	-	-	-	4+	2+	2+	±	-
A - 1	2+	±	4+	2+	1+	-	-	4+	2+	1+	±	-
A - 2	-	+	+	-	-	-	-	4+	2+	1+	±	-
A - 5	2+	1+	4+	3+	2+	1+	±	4+	2+	2+	±	-
A - 8	2+	1+	3+	3+	2+	-	-	3+	2+	1+	-	-
H - 1	-	+	+	-	-	-	-	4+	2+	3+	3+	-
46 - 9	-	+	+	-	-	-	-	4+	2+	2+	1+	-
17	2+	±	3+	4+	1+	-	-	4+	3+	1+	±	-
27	-	+	+	-	-	-	-	4+	4+	2+	1+	-
29	-	+	+	-	-	-	-	4+	3+	3+	2+	-
33	-	+	+	-	-	-	-	4+	4+	4+	4+	-
34	-	+	+	-	-	-	-	4+	2+	1+	±	-

than those which formed with pullorum antiserum; hence, they were classified as only 2+ reactions. Two strains, although positive, were considerably slower in agglutinating and the reactions were classified as 1+.

The tube agglutination tests and the serum-plate agglutination tests were in good agreement. Positive reactions did not occur in any of the tube agglutination test dilutions of Proteus antiserum which were not also positive to the serum-plate test. Only one strain, number 854, agglutinated in the serum-plate test but did not agglutinate in the tube test. No explanation for this is offered.

Table 2.

Summary of serum-plate typing of 208 strains of S. pullorum.

Proteus antiserum reaction	Number of Strains	S.paratyphi A var. Durazzo anti-serum reaction	Number of Strains
+	31	+	6
		±	14
		-	11
±	0		
-	177	+	175
		±	2
		-	0

DISCUSSION

Edwards (2) has shown that the so-called variant strain of S. pullorum is a form in which the production of large amounts of XII_2 antigen has become stabilized. The "standard" strains of S. pullorum contain small amounts of the XII_2 factor. He found that recently isolated strains, considered to be the "normal" form, gave rise to two forms of colonies; those having much and those having little XII_2 factor. However, these forms when transferred twice on agar again gave rise to both forms. In other words, they did not stabilize in either form. Inasmuch as the antigens tested represented the growth of masses of colonies, it is felt that only those strains which originally were stabilized in the variant or XII_2 ++ form reacted with Proteus antiserum. Variant forms probably did exist in some of the other strains but were not detected because the strains were not stabilized in that form.

The strains which had been maintained in stock longest were examined for the presence of rough colonies and were found to be quite smooth and entirely suitable for this survey.

SUMMARY

Two hundred and two strains of S. pullorum isolated from consignments presented for diagnosis at the Poultry Clinic and six laboratory strains were classified as variant or standard type according to their reaction with Proteus and with S. paratyphi A var. Durazzo antisera. The serum-plate test was used. Thirty-one strains (15%) of the variant type were found. The results of the serum-plate test were in good agreement with the results of the tube test. In one case ("A" strains) where the flock history was known, and, despite repeated testing of breeders, pullorum disease persisted, it was found that 4 of the 5 strains examined were of the variant type.

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