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Study of the influence of certain environmental conditions on the duration of viability of Salmonella pullorum

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STUDY OF THE INFLUENCE OF CERTAIN ENVIRONMENTAL CONDITIONS ON THE DURATION OF VIABILITY OF SALMONELLA PULLORUM

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STUDY OF THE INFLUENCE OF CERTAIN ENVIRONMENTAL CONDITIONS ON THE DURATION OF VIABILITY OF *SALMONELLA PULLORUM*

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INTRODUCTION

An increase in the knowledge of the bacterium, S.(almonella) pullorum is of industrial as well as scientific value because of the great economic and financial importance attached to the organism as it affects the poultry industry. Since the first identification of S. pullorum as the causative organism of the disease which results in such a high percentage of mortality among baby chicks and losses in production from adult birds, investigators, spurred on by the questions and demands of poultry raisers, have sought for sufficiently complete knowledge to permit of eradication or control of the scourge. Published articles and references regarding the characteristics of the organism and the menace of pullorum disease (the most recent name for the disease caused by infection with S. pullorum) have multiplied. The bibliography of Hooker (7) listing some three hundred forty-nine references in 1927 and a recent monograph by Rettger and Plastridge (14) in which they have collected and summarized one hundred and ninety-four articles are evidence of the interest in and the importance of pullorum disease. Eighty-seven of the abstracted articles have been published since 1927. The monograph of Rettger and Plastridge contains a description of the organism and its etiological significance to pullorum disease. The characteristics of the bacterium by which it is identified form a part of the description.

In spite of the work along the line of prevention and control, unaccountable outbreaks of the disease still occur and poultrymen

are asking that an explanation be found. If it could be proved that the organism lived and retained its virulence for some time in material about a poultry plant, the appearance of the organism and consequent infection of fowl might be explained.

This investigation was undertaken with the immediate object of determining one or both of two things; first, the length of time the organism lived in a few materials and second, that the organisms lived for at least an extended period in some materials. This information would serve as a foundation for further study on the virulence or non-virulence of the bacterium under the certain experimental conditions and might explain the rôle of materials in the spread and perpetuation of infection.

Since the importance of the organism is closely associated with the disease of poultry and the poultry industry, that point of view influenced the selection of material and method of attack. Reference is made in many instances in this report to the controlled or artificial conditions adopted for this study. For obvious reasons field conditions cannot be duplicated in the laboratory. Poultry raisers have urgently sought an explanation of certain situations in the hope of helping themselves and preventing losses which in the last analysis are financial. Much aid has already been given the poultrymen but in spite of their greatest care and precautions in sanitary practice, instances of loss through pullorum disease exist. It is those losses which investigators are seeking to prevent by acquiring more complete knowledge.

The innumerable factors and substances which might influence the duration of viability of S. pullorum made it necessary to choose certain materials which could be subjectively studied within the period of time and scope of the investigation. Soil, water, chicken feces and dry cloth were selected as of widely different character and at the same time materials which would inevitably become contaminated and with which birds would continually be in contact.

LITERATURE

A study of research literature and periodicals in animal diseases and allied problems yielded very meagre information concerning definite experiments on the viability of S. pullorum over a period of time. The few instances found were not in very close agreement and are more evidence that further study is necessary regarding the viability period of S. pullorum.

The earlier references show that some believed feces, water and food could contain the living organism. Rettger, Kirkpatrick and Stoneburn (13) stated "the disease may spread through the medium of infected food and water" and "normal chicks may acquire it by picking up infected droppings." Doyle (4) stated that infection of healthy birds might occur from ground infection. Mathews (11) has written that two experiments involving the feeding of chicks with feces from reacting hens gave negative results and concluded that feces did not contain viable and virulent S. pullorum. Apparently his view was in direct opposition to that of other workers. Kerr (8) gathered some data both as to the presence of S. pullorum in feces and the length of time it survived. Feces were collected from twenty-four cases of pullorum disease and examined at intervals for the presence of the viable organism. In two cases, after thirty days and thirty-two days S. pullorum had died. In the majority of cases it was alive for a period between fifty and ninety days and the maximum period for one sample was one hundred one days. The feces were emulsified with sterile physiological saline, stored

in test tubes and kept moist by the addition of a small amount of glycerine. Allen and Jacob (1), as a preliminary to their experiments regarding disinfection of soil with sodium acid sulphate, determined the length of time S. pullorum remained alive in previously sterilized soil. They recovered the organism from red clay loam and rich black clay loam fourteen months after inoculation but not from sand after the tenth month. Beaudette (2) inoculated sterile dry soil of known pH with S. pullorum. He found the bacterium had died in all soils with a pH less than 7.0 at eight days, but was alive at that pH (7.0) for the sixty-four days during which he carried on the investigation. The addition of 0.3 cc. of sterile distilled water for each gram of soil increased the ability of the organism to remain alive. By the addition of the sterile distilled water he was able to recover it from soil of pH 5.2 to 7.0 for three days and for more than eight days from soils of pH between 6.2 and 7.0. No description of the type of soil was given in the brief report. The use of sterilized soil in these experiments eliminated the influence which the presence of other organisms might have been expected to exert. The results of Gwatkin (6) "indicate that B. pullorum has marked powers of resistance against putrefactive changes." His success in recovering the organism from partially decomposed carcasses and that of Mallmann (9) and Kerr (8) substantiate the same opinion. Investigators at the Ontario Veterinary College (12) report some observations on the effect of dessication. After seven days storage at incubator temperature growth was retarded and at room temperature the same effect was produced after eighteen days storage. Rettger, Kirkpatrick and Stoneburn (11), Brunett (3) and

Edwards and Hull (5), in experimenting to determine the possibility of infecting birds, found infection following exposure to infected litter and birds. They did not state that subsequent infection was contracted from contaminated soil.

EXPERIMENTAL

Culture and Materials

The strain of the organism selected for the investigation was isolated a few weeks before the first material was inoculated. It seemed advisable that a recently isolated strain from a normally infected bird be used in order that conditions approximate those occurring naturally. Changes in the organism resulting from continued cultivation on artificial media would also be eliminated. A flock had been tested by the routine procedure and a number of reactors were bought by the laboratory for an experimental project. One of these died and at the necropsy S. pullorum was isolated from the peritoneal fluid, pericardial fluid and ovary. A culture from a pathological ovule was chosen and its identity carefully rechecked. The isolated culture fulfilled all of the requirements for the identification of S. pullorum:- a small Gram negative bacillus occurring singly, typical dew drop colonies on agar plate, fermentation of dextrose with production of acid and gas, failure to attack lactose, maltose, dulcite or sucrose, and proper serological reactions. The organism was used in the preparation of an agglutinogen by inoculating the whole surface of an agar slant and washing off the growth after 24 hours incubation at 37°C. with carbolized saline. This heavy suspension was diluted with physiological saline containing 0.25% phenol to a turbidity approximately equal to tube 0.75 of the McFarland nephelometer standard. The antigen was tested against serum known to contain specific agglutinins for S. pullorum and serum not containing specific agglutinins. Macroscopic agglutination tests were incubated at 37°C. for 24 hours and an additional

20 to 24 hours at room temperature after which they were read and the results noted.

After isolation and identification the organism was cultured throughout the experiment on meat extract agar slants which were stored at room temperature. Sub-cultures were prepared on fresh medium once a month.

In the different experiments a number of different media, some solid and some liquid were used. The basic solid medium was an agar medium made by the formula and technique recommended for standard practice from the "Report of the Referee and Associate Referee on Standard Methods of Diagnosis of pullorum Disease in Barnyard Fowl." This is quoted by Rettger and Plastridge (14).

Water	1000 cc.
Difco Beef Extract	0.3%
Difco Bacto Peptone	1.0%
Dry granular (Difco) agar	1.5%

Reaction -- pH 6.8 - 7.2

The Referee's Report gives specific and detailed instructions concerning the method of combining the ingredients and adjusting the reaction. Nutrient broth was prepared according to the above formula except that the agar was omitted. Synthetic media for soil organisms and differential media for the identification of organisms of the colon group were tried but did not afford any assistance toward the isolation of S. pullorum. These media did not inhibit other forms or give S. pullorum colonies a distinctive appearance. The carbohydrate media for biochemical reactions were made with the meat extract broth as a base and sufficient sterile 10% solution of the given carbohydrate was added to make a final concentration of 1% of the carbohydrate. Dunham

type fermentation tubes were used to detect gas formation and bromthymol blue was the indicator added to show acid production.

The possibility that contaminating and rapidly growing organisms present in the soil, fecal material or drinking fountain water might crowd out or obscure the colonies of S. pullorum was an obstacle to be removed. The work of Kerr (8), Stafseth and Mallmann (15) and Mallmann, Thorp and Semmes (10) suggested that the triphenyl methane dye, brilliant green, had the desired bacteriostatic effect and that it had been given sufficient study by them to establish its suitability. This dye has the advantage of inhibiting the growth of the Gram positive and coliform groups of organisms without interfering with the development of S. pullorum. Kerr had found by careful comparison through the inoculation of media with and without the dye that fewer S. pullorum colonies developed on the medium containing the brilliant green. He did not consider the inhibitory effect toward the organism sufficient to nullify its value. Both Kerr and Mallmann, Thorp and Semmes examined different concentrations recommending a final concentration of 1 to 50,000 or 1 to 100,000 as best suited for routine work. These concentrations did not exert too powerful bacteriostatic action toward S. pullorum. The keeping quality of solutions and the relative quality of different brands were examined and recommendations made. Mallmann, Thorp and Semmes added the dye to broth and to agar; Kerr used it in peptone broth and agar in isolating S. pullorum from feces.

For the work of this investigation the concentration of brilliant green in both broth and agar was 1 to 50,000 with controls always in-

oculated to insure against inhibiting S. pullorum and falsely interpreting results as negative. The dye was added directly to tubes of sterile broth. For agar plates, it was placed in the Petri dish before the melted agar was poured and mixing was accomplished by tilting and rotating the dish.

As the serological characteristics of each culture were considered an essential part of the identification, it was necessary to make an antigen from each culture each time that it was recovered from the material examined. A previously described uniform method was adopted.

The materials used to furnish the environment for the S. pullorum were course cotton cloth of the type used in some cases for feed sacks, tap water and water discarded from the drinking fountain in the poultry pen, soil samples of two different pH values and chicken feces. The cloth was stored at room temperature, the water at various temperatures according to the data to be secured, the soil and feces at a temperature above freezing but below room temperature (fluctuating between 5 and 15° C.).

Technique

The scope of this investigation was to gather data as to the length of time S. pullorum remained viable in the selected material under experimental conditions. The soil samples were a composite of soil actually taken from poultry runs and not sterilized. The fresh feces were collected from the pen and were inoculated as soon as collected. The water, as alread stated, was part tap water and part taken from the drinking fountain in the poultry house. It was not sterilized or treated in any way except that coarse particles of shavings were filtered from the drinking fountain water. The cloth was from a feed sack, washed and sterilized. With the exception of the cloth, a water suspension of S. pullorum standardized in turbidity to equal tube 0.5 of the McFarland nephelometer was used for inoculation. It was diluted immediately and plated in order to learn the approximate number of viable organisms. These varied in the different suspensions from 518 million to 878 million per cc. Table I shows the results of the plating and the material inoculated.

Table I. Number of Viable Organisms in Suspensions Used for Inoculation of Material.

Suspension	Dilution plate counted	Number of colonies	Geometric average per cc.	Material inoculated
I	1 million	480	592 million	Water
	10 "	73		
II	10 million	47	518 million	Water
	10 "	57		
III	1 million	472	626 million	Water and soil
	10 "	83		
IV	10 million	94	878 million	Water and soil
	10 "	82		
V	10 million	68	791 million	Soil and feces
	10 "	92		
VI	10 million	59	647 million	Feces
	10 "	71		
VII	1 million	428	535 million	Feces
	10 "	67		

Dry Cloth

The question was raised of the possible danger of feed sacks serving as a vehicle of infection when the sacks were refilled and delivered to another plant without being disinfected. In response some preliminary investigational work was undertaken to determine the length of time the organism would remain viable in dry stored cloth. A laboratory strain of S. pullorum, received at this laboratory from the University of New Hampshire and designated P-11, was used. This culture has been grown on nutrient agar slants since the summer of 1929. The cloth was saturated with a broth culture of the test organism and then dried in the incubator at 37° C. before storing. A sample was examined each week for viability. Cloth was prepared four times but in no case was the quantity sufficient to outlast the organism. The results of the preliminary work are summarized in Table II.

Table II. Results of Preliminary Inoculation of Cloth with S. pullorum

Trial	No. samples prepared	No. sterile	No. contaminated and S. pullorum not recovered	No. S. pullorum recovered	Maximum period viable
I	16	1	3	12	75 days
II	13	3	6	4	100 days
III	19	2	1	16	175 days
IV	20	7	4	9	249 days

With the experience of the preliminary experiment, 150 samples were prepared with an additional 50 uninoculated to serve as controls. A feed sack of unbleached muslin was washed clean, dried and sterilized in the autoclave. It was cut into rectangles approximately 8 x 10 centimeters. These were slashed most of the way across to leave four strips 2 x 10 centimeters attached at one end. One hundred cc. quantities of broth were sterilized in wide mouthed flasks in the autoclave for one half hour at 15 pounds extra pressure. Later the pieces of cloth were introduced into the flasks of broth and the whole sterilized again. These were incubated to determine sterility and then were inoculated with a 20 hour agar slant culture of S. pullorum. The inoculated broth containing the cloth was then incubated for twenty-four hours. The cloth was then removed aseptically to sterile Petri dishes and each group of strips was spread flat in a separate dish. These were placed in the incubator at 37° C. to dry. The controls were handled in an identical manner except that the inoculation of the broth was omitted. After 48 hours, all of the cloth appeared to be dry and the separation

of the strips and storage was completed. Care was taken as far as possible to prevent contamination and each strip was placed separately in a test tube 18 x 150 mm. previously plugged with cotton and sterilized. The tubes were then stored in the dark at room temperature. The broth from each flask in which cloth had been treated was streaked on an agar slant and a pure culture of S. pullorum developed from each inoculated flask. No growth developed on the slants from the control flasks.

The general plan for the examination of the cloth briefly was as follows. Examination for the presence of viable S. pullorum was made at intervals of four weeks using three tubes of inoculated cloth and one control at each test. Had the organism not been recovered from all three inoculated tubes, it had been planned to shorten the interval between examinations assuming that the organism had begun to die out. Examination was made by adding 20 cc. of sterile broth medium to each of three inoculated tubes and one control and incubating the tubes at 37°C. A loopful of the broth was streaked on the agar plate after 48 hours of incubation or less if turbidity indicated growth in a shorter time. From the resulting growth, colonies were fished to agar slants for culture and identification as S. pullorum. When growth in the broth was not macroscopically visible after 48 hours incubation, plates were streaked at intervals until growth was obtained or the incubation had been continued for one week. If no growth resulted from a week's incubation, the flask was considered sterile and discarded. In the few cases where contaminating organisms appeared in the flask, repeated

platings using both brilliant green and plain agar plates were made to procure a pure culture for identification. In all cases the pure culture was identified by colony type on agar plate, Gram stain, carbohydrate reactions and serological reactions. The methods have already been described. S. pullorum was found viable and has been recovered from all inoculated cloth tested up to the time this report was written. A maximum period of 314 days elapsed after the original inoculation of the material.

Water

For the determination of the length of time the bacterium was able to live in water, natural conditions of temperature variation could not be duplicated and instead extreme conditions were substituted. The temperatures selected were (1) frozen and not thawed until the time of examination, (2) frozen and thawed once every 24 hours, and (3) room temperature at the laboratory, approximately 20-24° C. with an occasional greater fluctuation. Growth was obtained from a 20 hour culture spread over the entire surface of agar in Kolle flasks. After 24 hours incubation at 37° C., the growth was washed off with a small quantity of sterile physiological saline. This heavy suspension was centrifuged and the supernatant saline removed. Sterile distilled water was added to replace the saline and this suspension was further diluted with the type of water chosen for the investigation to a turbidity equal to tube 0.5 of the McFarland nephelometer. The final suspension was tubed in 2 cc. quantities in sterilized tightly corked tubes and place at the designated storage temperature. Tap water was used as the dilu-

ent for the water to be frozen without thawing and that to be held at room temperature. Water taken from the henhouse was used to dilute the suspension which was subjected to daily freezing and thawing. The turbidity of the latter made it necessary to determine the number of times the heavy suspension should be diluted with distilled water in order to approximate the number of organisms in the other suspensions. The calculated amount of turbid diluent was then added to the suspension of organisms. The distilled water suspension was used for plating to determine the number of viable S. pullorum organisms.

Some preliminary work had also been done to obtain a rough idea as to the length of time the organisms remained viable in ice. The strain used for the preliminary work was the same as that used for the preliminary cloth examination and the suspension was made with saline instead of water. Samples were to remain frozen during the time of the investigation. No great value was attached to the results because at two times the power was shut off the electric refrigerator and the material accidentally thawed. S. pullorum was recovered from a sample of the saline suspension 171 days after it was prepared and first frozen.

Sufficient quantity of tap water suspension for one hundred and fifty 2 cc. vials was prepared and tubed in order that fifty trials in triplicate might be made in the course of the investigation. Fifty tubes of uninoculated tap water were prepared as controls. The tubes were placed in the freezing compartment of the electric refrigerator and to guard against previous accidents care-

ful watch was kept of the refrigerator and the tubes removed to another machine when it was necessary to defrost. To determine viability, three inoculated vials and one control were removed from the refrigerator and permitted to thaw at room temperature. In the early part of the experiment, the thawed suspension was shaken; a one cc. portion placed in a Petri dish; melted and partially cooled agar was added. The other portion was added to a tube of broth which served as an enrichment medium in case colonies failed to develop on the plate. After the earlier tests, the plating of the suspension was omitted and the entire contents of the vial added to the tube of broth since that method seemed to be the most certain to produce results. A loopful of the broth was streaked on an agar plate at 24 hour intervals until growth developed or until the tube had been incubated for a week. If S. pullorum had not been isolated in this period the culture was discarded. Brilliant green plates were used if the nutrient agar plates showed sufficient contaminating organisms to make fishing of S. pullorum colonies difficult or the danger of S. pullorum being overgrown appeared as a possibility. S. pullorum-like colonies were fished and a pure culture obtained and identified by the methods already described in connection with the isolation from dry cloth.

The organism was readily recovered 238 days after it was first frozen which was the last examination made before the writing of this report. The results are presented in Table III. No trials before the twelfth are tabulated because in each case S. pullorum

was isolated from all three of the inoculated tubes and no irregularities occurred.

Table III. Results of Freezing Water Suspension.

No. of examination	Age of suspension	No. of vials examined	No. of Vials Yielding viable S. pullorum
12	84 days	3	3
13	91 "	3	0
14	94 "	6	6
15	98 "	3	3
16	105 "	3	3
17	112 "	3	3
18	119 "	3	2
19	126 "	3	3
20	130 "	3	1
21	136 "	6	6
22	141 "	3	3
23	148 "	3	1
24	155 "	6	0
25	160 "	6	6
26	168 "	3	3
27	175 "	3	1
28	182 "	3	1
29	189 "	6	2
30	192 "	6	0
31	194 "	6	5
32	195 "	6	3
33	196 "	3	2
34	197 "	3	3
35	200 "	3	1
36	203 "	3	3
37	210 "	3	3
38	217 "	3	2
39	224 "	3	3
40	231 "	3	3
41	238 "	3	3

The second variation in the temperature factor was daily freezing and thawing. The test organism was suspended in water taken from poultry drinking fountains. Contaminating organisms necessitated a change in the technique of examination for viable S. pullorum cells. Brilliant green was quite regularly employed as a means of inhibiting the contaminants. The suspension was tubed in 2 cc. quantities. The tray

containing the 2 cc. tubes was taken from the freezing compartment of the refrigerator each day and allowed to remain at room temperature until all of the vials had become free of ice. The control tubes did not prove of any value and their consideration is omitted. Each week three tubes were tested by adding the contents to nutrient broth containing brilliant green. At first S. pullorum was not recovered from all three tubes and plain broth was substituted for subsequent tests. Plates were made from this broth on both brilliant green and plain agar. In all cases practically the same amount of growth appeared on both sets of plates. These results further verify the work of Kerr (8) in his studies on the effect of brilliant green on the quantitative development of pullorum colonies.

The period that S. pullorum survived the extreme treatment of freezing and thawing was so short it was possible to repeat the trial a second and third time. The data from the three trials are tabulated in Table IV. The first time the culture apparently had died by the eighteenth day though the organism was recovered on the sixteenth day. In the second lot of material, the organism remained alive somewhat longer. No growth of S. pullorum was obtained after twenty-nine days of treatment. The divergence between the results of these two trials was so great that the experiment was repeated a third time. The results of the third experiment compared favorably with the second. S. pullorum was recovered from three samples examined at twenty-six days but not from any at twenty-seven, thirty or thirty-six days. At thirty-six days all of the remaining vials were tested and no viable pullorum was found. The results of the three trials are summarized in Table V.

Table IV. Results of Freezing and Thawing Suspension of S. mullorum

	No. of examination	Age and No. times thawed	No. vials examined	No. vials yielding viable S. mullorum
Trial I	1	3 days	3	3
	2	7 "	3	3
	3	14 "	3	2
	4	16 "	6	2
	5	18 "	6	0
	6	25 "	14	0
Trial II	1	10 days	3	3
	2	17 "	3	1
	3	22 "	6	1
	4	29 "	6	1
	5	31 "	6	0
	6	37 "	21	0
Trial III	1	7 days	3	3
	2	13 "	3	3
	3	20 "	3	2
	4	22 "	3	0
	5	24 "	6	2
	6	26 "	6	3
	7	27 "	6	0
	8	30 "	6	0
	9	36 "	12	0

Table V. Summary of Freezing and Thawing Results.

Trial	Maximum age and No. times thawed	No. vials examined till dead	No. vials yielding viable S. mullorum
I	16	15	10
II	29	18	6
III	26	24	13

The third temperature influence investigated was room temperature. A suspension of S. mullorum in tap water was prepared to study the length of time the organism could survive at room temperature, 20-24°C.

The method of preparation was identical with that used in the case of the frozen material. The tubes were stored in a case in the laboratory not protected from the light but never reached by the sun. Three tubes were examined each week. The contents of the tube were added to a tube of nutrient brilliant green broth and plates were streaked from this tube after it had been incubated. When necessary for isolation, more than one plate was streaked from a single tube of broth. At the time the investigation was terminated, S. pullorum was recovered from a sample of the suspension which had been stored at room temperature for a maximum period of 140 days. Table VI shows the intervals and the results of the work.

Table VI. Results of Storing Water Suspension of S. pullorum at Room Temperature

No. of examination	Age of suspension	No. of vials examined	No. of vials yielding viable S. pullorum
1	3 days	3	3
2	7 "	3	3
3	14 "	3	2
4	21 "	3	1
5	28 "	6	6
6	35 "	3	3
7	42 "	3	0
8	49 "	6	6
9	56 "	3	3
10	63 "	3	3
11	70 "	3	3
12	77 "	3	3
13	84 "	3	3
14	91 "	3	3
15	98 "	3	3
16	105 "	3	3
17	112 "	3	2
18	119 "	3	3
19	126 "	3	3
20	133 "	3	3
21	140 "		

Soil.

The length of time that S. pullorum remains viable in soil may be of even greater importance than the length of time that it is able to persist in water. The fact that sterile soil has been found to be toxic for plants led to the belief that unsterilized soil would be preferable for inoculation with S. pullorum. It was feared that the presence of other organisms might interfere with the isolation of S. pullorum and a number of differential media were tried. The best results were obtained by using brilliant green in the broth and in the agar plates. By this method little difficulty was experienced in inhibiting the other types of organisms sufficiently to make the fishing of S. pullorum colonies readily accomplished. It has been shown by Beaudette (2) that the pH of soil and moisture were important factors in maintaining viable S. pullorum in soil. The influence of temperature was eliminated for experimental reasons although its importance must be recognized. In order that as much practical value as possible might result from the data collected, the soil to be inoculated was taken from poultry yards in the state of Massachusetts. The final samples used were composites of poultry yard soils procured from different parts of the state. Soil from seven different points scattered throughout the state was collected and the pH value determined. One sample from the Cape Cod section was of a pH notably less than that from other sections. As it was impossible to obtain more samples from this section and as a relatively small portion of the state was concerned, the sample was disregarded. The Eastern part of the state, as judged by the samples received, had the less

acid soil and the Western part the more acid, the range being 5.6-5.8 and 4.7-5.2 respectively. A composite sample from two eastern towns and a composite sample from three towns from the middle of the state westward to the Connecticut valley were finally used for inoculation. A suspension similar to that used for the water experiments was used to inoculate the air dried soil. The amount of the suspension was approximately equal to two thirds of the water holding capacity of the soil. The soil was kept in flower pots which were stored in the attic where the temperature was quite low (5 - 15°C.) but did not at any time reach freezing. The samples were stirred and the water lost by evaporation made up every two or three days with sterile distilled water. Before a portion was removed for examination for viable S. pullorum, the water loss was made up and the equivalent of 10 grams of dry soil removed. The sample was shaken by hand for ten minutes in a dilution bottle containing 90 cc. sterile distilled water. One cc. of the suspension was added to a tube of nutrient broth and a tube of broth with brilliant green. After 24 hours of incubation these were streaked on both plain and brilliant green agar plates. If no pullorum-like colonies were found on the first plates, they were streaked on plates again after 48 hours. Broth tubes were incubated and tested up to one week before they were discarded as negative for S. pullorum. Table VII shows that there was variation between the results of the three lots of inoculated material at the different times and the maximum period elapsing between inoculation and the last recovery. For the soil of higher pH it was 11, 15 and 28 days in the three lots; for the lower pH, 4, 6 and 5 days.

Table VII. Results of Inoculation of Soil with S. pullorum

	No. of examination	Age of material	S. pullorum recovered Higher pH	Lower pH
Trial I	1	2 days	+	+
	2	4 "	+	+
	3	6 "	+	-
	4	8 "	+	-
	5	11 "	+	-
	6	15 "	-	-
Trial II	1	3 days	+	+
	2	4 "	+	+
	3	5 "	+	+
	4	6 "	+	+
	5	8 "	+	-
	6	11 "	+	-
	7	15 "	+	-
	8	18 "	-	-
Trial III	1	5 days	+	+
	2	6 "	+	-
	3	7 "	+	-
	4	14 "	+	-
	5	22 "	+	-
	6	28 "	+	-
	7	31 "	-	-
	8	35 "	-	-

The results of the three trials are summarized in Table VIII.

Table VIII. Summary of Soil Inoculation Results.

Trial	No. days tested	Maximum age found viable	
		Higher pH	Lower pH
I	13	11 days	4 days
II	18	15 "	6 "
III	35	28 "	5 "

Feces.

The problem of inoculating fecal material was rather different from any of the other experimental materials because natural infection must of necessity be entirely different from any artificial inoculation. A water suspension like that used for the other inoculations was prepared and mixed with freshly collected feces from a pen of negative birds. One hundred sixty cc. of the suspension were added to three hundred twenty grams of feces. The material was stirred slightly to thoroughly mix the inoculum. It was stored in wide mouthed glass jars protected by gauze at attic temperature. The added moisture made the mixture rather too thin and when the second supply was prepared a suspension only half as dilute was used. The examination was made by weighing a 15 gram sample (12.5 grams in the second and third trials) and shaking it with water in a dilution bottle similarly to the way the soil was handled. Fecal material and one cc. of the water suspension was added to plain and brilliant green broth. The best results were obtained from brilliant green broth tube to which the water suspension was added. The steps following the incubation of the broth tubes were identical with those used in examining the soil. Control plates of brilliant green agar were used and were inoculated with a pure culture of the experimental strain and added to fecal material to guard against too great inhibition.

The organism was recovered from the first preparation twenty-two days after the inoculation; from the second, twenty-four days

days after inoculation; and from the third, twenty-one days after inoculation. The data is presented in Table IX.

Table IX. Results of Inoculation of Feces with S. pullorum

	No. of examination	Age of material	S. pullorum recovered
Trial I	1	7 days	+
	2	14 "	+
	3	22 "	+
	4	28 "	-
	5	31 "	-
Trial II	1	14 days	+
	2	21 "	+
	3	24 "	+
	4	28 "	-
	5	31 "	-
Trial III	1	14 days	+
	2	21 "	+
	3	23 "	-
	4	25 "	-
	5	28 "	-

DISCUSSION

When this investigation was undertaken, it was recognized that a multiplicity of factors such as sunlight, variable temperature, variable moisture etc. could not be reproduced in the laboratory. Extreme and exaggerated conditions were set up in order that there might be compensation for any protection which artificial conditions might afford against destructive factors naturally present. Considering the ability of S. pullorum to survive under some of the treatment to which it was subjected, it seems likely that it could survive also from one season to the next in the poultry yard or in some parts of the poultry house. In some cases it also is quite possible that the organism might survive from one generation of poultry to the next.

Cloth was the material from which the organism was recovered after the longest period of investigation. It was the first material inoculated and no weakening had been observed or sterile samples found throughout the allotted time. If delayed growth in the preliminary investigation were evidence of the weakening or dying of the organism, it appears that the freshly isolated strain was more potent than the strain which had been cultivated for some time on artificial media. In this investigation, growth was always visible in the broth within 48 hours of incubation. The final test was made 314 days after the cloth became dry and 317 days after it was inoculated. Practically no contamination occurred and in cases where it did appear, a pure culture of S. pullorum was readily obtained by plating the broth.

Water seemed to furnish a relatively favorable environment for S. pullorum. From the time the water was inoculated and frozen to the end of the investigation period, 238 days later, the organism was still being recovered without difficulty. Little trouble was caused by contamination and the isolation was made by using brilliant green media. No attempt was made to determine the rate of decrease in the number of viable organisms because the study was not planned to be quantitative. In the earlier tests when part of the suspension was plated direct, a diminution in numbers was noted. Because of unsatisfactory plating results and the necessity of relying more and more on the broth tubes for growth, the plating was discontinued after the twelfth week. Failure to recover S. pullorum from all three vials at the twenty-fifth week led to an increase in the number of vials used for each examination. This was followed by a shortening of the time interval. After four examinations to conserve the material, the number of vials examined was reduced to three. The results were somewhat irregular for part of the period, but S. pullorum was isolated with regularity for the last six weeks of the investigation. At this time the suspension was being added to plain broth tubes and after growth had developed it was streaked on brilliant green agar. The pure culture was isolated from this medium and identified by the reactions previously mentioned. The results show the strong resistance of S. pullorum to such conditions.

The freezing and thawing treatment gave rather unexpected results for it is generally conceded that freezing and thawing is especially destructive to bacterial cells. Other forms which were

present in the unsterile water also survived for a longer period than the S. pullorum. None of these were examined for identification and they may have been largely spore-formers which would naturally be expected to survive longer under such conditions. They were readily inhibited by brilliant green in the medium.

In the tap water suspension of S. pullorum at room temperature, the viable organisms outlasted the time allowed for the investigation. The contamination was more than expected from the type of water used but inhibitory media made possible the isolation of S. pullorum for 140 days. Brilliant green broth streaked on brilliant green agar was the most satisfactory method. The failure to isolate S. pullorum from some of the vials in some of the earlier examinations might be explained by the change in the technique from direct plating to enrichment in brilliant green broth. It may be that the contaminating organisms without the inhibitory effect of the brilliant green were sufficient to crowd out the S. pullorum.

The results of the experiments with water suggest that S. pullorum could live for a long time in that medium since it survived considerable freezing and thawing as well as storage in continuously frozen or unfrozen water.

From the results of this investigation, the danger of infection being continued from one season to the next through the agency of soil is not very great. The longest period that it survived was only twenty-eight days and the period for the more acid soils was much shorter. Before any positive statement can be made much more

data would have to be collected. All the work seemed to indicate a relation between the length of time that S. pullorum remained viable and the acidity of the soil. No mention was made of the pH of the soil in the work reported by Allen and Jacob (1) although the soil was described as "rich black loam","red clay loam" and "sand." Nothing was said of the water content which Beaudette (2) found to be a factor. These facts probably may be rightly taken as an explanation of discrepancies among the findings reported by different investigators. The variability of the complex factors in soil seems to indicate that many difficulties stand in the way of establishing a definite limit as to the length of time that S. pullorum may live in soil.

The results of the fecal examination did not compare very well with the work of Kerr (5). Whether this is due to artificial inoculation of the feces or to some other factor cannot be determined without further experimentation. The period during which S. pullorum was recovered was short enough to indicate little danger from feces as a means of spreading infection.

Purely as a matter of interest and entirely outside of the original scope of this problem, a little work was undertaken to determine the pathogenicity of the organism recovered from the experimental material. Only one recovered culture was examined, that isolated from cloth after 286 days storage. This was the culture which had been under experiment for the longest period. Baby chicks were chosen as the susceptible animal. Ten, day old chicks were bought from a reliable source known by history to be pullorum disease free

and healthy Rhode Island Red stock. The culture which two days previously had been established as S. pullorum was inoculated into meat extract broth. The 24 hour old broth culture was used as the inoculum for five of the chicks which were confined in a cage and separated from other birds. The other five chicks were used as controls. The chicks were not fed previous to the administration of the infective broth. The treated group were given two drops of the broth culture administered orally from a pipette. The control group were treated identically with sterile broth. After a half hour grit and water were placed before the chicks, and later baby chick mash was fed. Care was taken at all times that no infection was carried from the treated birds to the controls or to either group from an outside source.

All the chicks seemed to thrive and develop normally and made good growth. No symptoms of disease were observed at any time. Eleven days after the infective dose was given, the chicks were killed and necropsies performed. No lesions were found in four of the control group; in the fifth slight congestion of the liver was observed but cultures were negative. As routine procedure, cultures were taken from the liver, lungs and heart blood and any lesions observed. All cultures from the control group were negative for S. pullorum and in most cases sterile though on one or two plates a few colonies developed probably from air contamination. The chicks of the control group were numbered from 1 - 5 inclusive; the treated group, from 6 - 10 inclusive. The results of the necropsies are presented in Table X.

Table X. Results of Necropsy of Inoculated and Control Chicks.

Chick No.	Organs cultured	Lesions observed	S. pullorum isolated
1	Liver, lungs, heart blood	No lesions	Negative
2	Liver, lungs, heart blood	No lesions	Negative
3	Liver, lungs, heart blood	No lesions	Negative
4	Liver, lungs, heart blood	No lesions	Negative
5	Liver, lungs, heart blood	Slight congestion in liver	Negative
6	Liver, lungs, spleen, heart blood, nodule on lung	Congestion of liver, nodule on lung	Spleen, liver
7	Liver, lungs, heart blood, heart nodule	Nodules on heart	Heart nodule, lung
8	Liver, lungs, heart blood	Small areas of leucocytic infiltration in liver	Liver
9	Liver, lungs, heart blood	No lesions	Negative
10	Liver, lungs, heart blood	No lesions	Negative

Cultures were identified by the methods used for all of the cultures of the previous work of this investigation. Each proved to be a short Gram negative rod occurring singly; colonies on agar plates after 24 hours incubation were small, discrete, dew drop colonies; dextrose was attacked with acid and gas produced; dulcite, maltose, lactose and sucrose were not attacked. A suspension of the organism in phenolated physiological saline as an antigen was agglutinated by serum containing agglutinins for S. pullorum but not agglutinated by serum not containing specific agglutinins.

S. pullorum was recovered from three of the five chicks inoculated with the broth culture. From this limited amount of work it appears that S. pullorum retained its virulence after it had been dried in cloth for 286 days.

SUMMARY

S. pullorum remained viable outside the animal body in such substances as dry cloth, water, soil and feces when the material was maintained under specific conditions produced at the laboratory for the periods given below:

1. In dry cloth previously saturated with nutrient broth and free from other organisms, *S. pullorum* was viable after 314 days when the experiment terminated.

2. In tap water, *S. pullorum* was viable after it had been frozen without thawing for 238 days after which examination was discontinued.

3. In water containing other micro-organisms, frozen and thawed daily, *S. pullorum* was viable for periods varying from 16 - 29 days and after it had been frozen and thawed from 16 - 29 times.

4. In tap water stored at room temperature, *S. pullorum* was found to be viable after 140 days after which examination was discontinued.

5. In artificially inoculated soil of a pH between 4.7 and 5.2, *S. pullorum* was found to be viable for periods varying from 4 - 6 days but in no case was it viable after seven days.

6. In artificially inoculated soil of a pH between 5.6 and 5.8, *S. pullorum* was found to be viable for periods varying from 11 - 28 days but in no case was it viable after 31 days.

7. The pH of soil had an effect on the length of time *S. pullorum* was viable in the soil.

8. In feces artificially inoculated, *S. pullorum* was viable for a maximum period of 24 days after inoculation in one case but was not recovered 25 days or more after inoculation in any case.

Apparently *S. pullorum* recovered from cloth after it had been dried for 286 days had retained its pathogenicity because it was subsequently recovered from tissues of chicks to which it had been fed

eleven days before.

S. pullorum remained viable in experimental material under artificial conditions long enough to be a factor in the transmission of pullorum disease, provided the organism retained its virulence during the time that it remained viable.

REFERENCES

(1) Allen, P. W. and Jacob, M. 1930.
Sodium Acid Sulphate as a disinfectant against Salmonella pullorum in poultry-yard soils.
Tenn. Agr. Expt. Sta. Bul. 143. pp. 3-14

(2) Bemudette, F. R. 1928.
Report of the poultry pathologist.
N. J. Stas. Report

(3) Brunett, E. L. 1930.
Transmission of Bacterium pullorum infection among mature chickens.
Jour. Amer. Vet. Med. Assoc. 76 (n.s. 29) pp 667-669

(4) Doyle, T. M. 1925.
Bacillary white diarrhoea of chicks.
Jour. Compar. Path. and Ther. 38, pp. 266-282

(5) Edwards, P. R. and Hull, F. E. 1929.
The transmission of bacillary white diarrhea among hens.
Jour. Amer. Vet. Med. Assoc. 75 (n.s. 28) pp. 333-336

(6) Gwatkin, R. 1926.
Some experiments on the disinfection of eggs and incubators.
Ontario Vet. Col. Rpt. 1925. pp. 58-61

(7) Hooker, W. A. 1927.
Bibliography of Bacillary white diarrhea infection of the fowl.
U. S. Dept. Agr. Off. Expt. Sta. pp. 2-29

(8) Kerr, W. R. 1930.
Selective media for the cultivation of Bacillus pullorum and Bacillus sanguinarium.
Jour. Compar. Path. and Ther. 43 pp. 77-85

(9) Mallmann, W. L. 1929.
Salmonella pullorum in the intestinal contents of baby chicks.
Jour. of Infec. Dis. 44 pp. 16-20

(10) Mallmann, W. L., Thorp, F. Jr. and Semmes, M. 1928.
A medium for the isolation of S. pullorum and other members of the paratyphoid group from avian tissues.
Jour. Amer. Vet. Med. Assoc. 73 (n.s. 26) pp. 825-838

(11) Mathews, F. P. 1929.
Factors influencing the control of bacillary white diarrhoea.
Jour. Amer. Vet. Med. Assoc. 71 (n.s. 24) pp. 585-589

(12) Ontario Veterinary College. 1928.
The visibility of B. pullorum in the tissues of dead chicks.
Ontario Vet. Col. Rpt. 1927. pp. 32-33.

(13) Rettger, L. F., Kirkpatrick, W. F. and Stoneburn, F. H. 1912
Bacillary white diarrhea of young chicks. (Third Report)
Conn. (Storrs) Agr. Expt. Sta. Bul. 74 pp. 153-185

(14) Rettger, L. F. and Plastridge, W. N. 1932.
Pullorum disease of domestic fowl. A monograph.
Conn. (Storrs) Agr. Expt. Sta. Bul. 178. pp. 109-192.

(15) Stafseth, H. J. and Mallmann, W. L. 1928.
Selective media.
Poultry Sci. 8 pp. 19-22.

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