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BACILLARY WHITE DIARRHŒA OF CHICKS (B.W.D.).

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THE main problem we are now investigating with relation to B.W.D. is whether systematic testing by the agglutination method of all fowls in a breeding flock and the disposal of the positive reactors according to the methods developed during the past few years, particularly in U.S.A., will in England ultimately result in the entire prevention of the disease among chicks bred from the " clean " birds. All the indications are favourable hitherto, but such an investigation must extend over several years. A number of questions arise in carrying out this work which have been the subject of our investigation during the past three years. While this work was in progress the paper by Doyle (5) appeared covering some of the ground. It will be seen that we confirm many of his findings, though not all.

(*a*) THE " CARRIER " FOWL.

It has been known for some years that the " carrier " hen is the chief source of spreading B.W.D. Rettger and Harvey (1) first showed that eggs laid by " carrier " hens may be infected with *B. pullorum* and that, if such eggs are fertilised and incubated, the developing chick may die before reaching maturity, or, if hatched out, may be infected with B.W.D. and spread the disease to healthy chicks in the same brood. We (2) have shown that litter, etc., infected by diseased chicks may retain infection for several weeks.

AT WHAT AGE CAN INFECTION WITH B. PULLORUM PER OS TAKE PLACE ?— We (3) showed that infection of young chicks up to six days old is easily brought about by feeding *B. pullorum* culture. We have experimented with older birds. Two laying hens, each one year old (Nos. 13 and 14) and six chicks aged six weeks were placed in a small pen and received 50 c.c. of *B. pullorum* 24 hours old broth culture mixed with their food daily for one week. The chicks were bred from stock known to be negative reactors to many agglutination tests and the hens had been repeatedly tested and were negative on all occasions. Agglutination tests were now carried out periodically for a period of seven months. One chick died nine days after the cessation of the feeding and a second on the 14th day. Six months later the remaining fowls were killed and *post-mortem* examinations were carried out. Table I is a summary of the experiment and shows that fowls up to one year old may be infected by feeding very large amounts of *B. pullorum*, and chicks six weeks old may die from such

treatment. During the past year a further experiment was carried out. Three laying hens, each one year old and three pullets about six months old were tested by the agglutination method and gave negative reactions. A 100 c.c. *B. pullorum* broth culture was fed twice daily for one week. One hen died nine days after the last feed : *B. pullorum* was recovered from the heart blood and liver. Agglutination tests were carried out at intervals on the remaining five fowls for the next nine months, when all were killed and careful *post-mortem* examination made. Table II is a summary of the findings. It is very improbable that under natural conditions, any fowl or chick would be subjected to the chance of as heavy a dosage as occurred here.

Artificial Infection of the Male Bird.—Many workers have recorded that the cockerel, if a " carrier " as shown by being positive to the agglutination test, may be a source of spread of B.W.D. Doyle (4) records two experiments in which attempts were made to transmit B.W.D. from an infected cockerel to clean hens and from infected hens to clean hens via a clean cockerel.

We planned two experiments in which cockerels, infected artificially, one by an intratesticular and one by an intravenous injection of *B. pullorum*, were mated with normal non-reacting hens. In one experiment a cockerel, known to be a negative agglutination reactor over a long period received 0·1 c.c. of *B. pullorum* broth culture into the substance of the right testis. He was mated with five normal, non-reacting hens and agglutination tests were carried out on 11 occasions during the next three months, after which time the birds were killed and *post-mortem* examinations made.

The second experiment was identical except that the cockerel was injected intravenously instead of intratesticularly. Between the third and fourth weekly agglutination tests the cock in each experiment showed an agglutination titre of 1/160, which was maintained throughout the experiment. All 10 hens in the two experiments showed an indefinite agglutination reaction at 1/20. All 12 birds were killed and a *post-mortem* examination made. No *B. pullorum* was found. It would appear that intratesticular and intravenous injection of living *B. pullorum* can cause non-reacting cocks to become agglutinators, but that hens mated with them remain normal.

Mating a Normal Cockerel with Reacting Hens.—Twelve hens, positive on several occasions to the agglutination test, were penned with a non-reacting cockerel. The contact experiment lasted for nine months. Agglutination tests with the cockerel's serum were carried out periodically during that period and on no occasion was a positive reaction recorded. *Post-mortem* examination failed to show any infection with *B. pullorum*. It would appear that agglutinating

hens may be in contact with a cock for long periods without its becoming infected.

Do "Carrier" Hens Infect Normal Hens?—Six normal hens, proved non-reactors by repeated agglutination tests, were penned with six proved "carriers." A normal non-reacting cockerel was kept with them. During the nine months of contact 22 agglutination tests were carried out on each fowl. The negative reacting hens and the cockerel never reacted, while the "carriers" continued to react; the titres of their serum causing reaction varied from 1 in 40 to 1 in 320. Apparently, hens with persistent positive agglutination reactions may be in contact with normal hens and a cock for many months without infecting the healthy birds.

Infection Via the Cloaca.—Two hens, tested on several occasions and proved negative to agglutination tests, received 5·0 c.c. *B. pullorum* broth culture into the cloaca. The injection was made with great care so that the bulk of the injected culture was retained for several minutes. A clean cockerel and four clean hens were placed in contact with the injected birds. Agglutination tests were carried out periodically with serum from all birds for nine months. Both of the injected hens developed an agglutination titre of 1/160, maintained during the experiment, and at autopsy one bird only yielded *B. pullorum* from the ovary. The cock and the five hens in contact showed practically no agglutination at 1/20 and at autopsy no sign of infection was found; cultures proved negative.

Can Day-Old Chicks Contract B.W.D. by Contact with "Carriers"?—Doyle (5) carried out experiments from which he concluded that "carrier" hens liberate *B. pullorum* in the fæces and that the organism may be picked up by and infect day-old chicks. We have been unable to confirm that day-old chicks in contact with "carrier" hens develop B.W.D. Twenty chicks were hatched from eggs produced by non-agglutinator hens bred to a clean cock. The chicks were divided into two lots of 10. One lot was placed under a "broody" hen and kept in a small pen with 10 heavily infected natural agglutinator hens. These hens were specially selected for the experiment; in addition to their being natural agglutinators, they had received several injections of living *B. pullorum* culture. The other lot was kept in contact with 10 "clean" hens. In each group contact lasted for two months. No deaths occurred and when the chicks were five months old agglutination tests were carried out; no reactions were noted.

(*b*) Agglutination Test.

Variation in the Agglutination Titre of "Carrier" Hens.—We believe that the agglutination titre of the serum of "carrier"

hens may vary considerably over a set period of time and that in some cases agglutination, even in low dilutations, fails to be demonstrated for a short period though it reappears later. Knight (6) states that the test should be made when hens are in lay and cocks ready for mating. Doyle (4) notes that marked fluctuations have been observed in the agglutination titre of carriers when tested at frequent intervals over a long period and gives this as an explanation of those cases where birds pass a first test and react later without having been exposed to infection.

We collected eight " natural agglutinating " hens and kept them in the same pen for eight months, during which time agglutination tests were carried out periodically. The dilutions of serum used were 1 in 40 up to 1 in 320. Table III shows the " end points " of the various samples of serum on different dates and the results of *post-mortem* examinations. The same antigen for the agglutination tests used throughout the experiment was stored during the whole period in an ice box. We believe that the variations shown are really variations in the titre and outside the error of experiment. (The only reading we would feel inclined to question is that of 25/2/25, which is unduly high.) On this occasion it was not possible to carry out the tests under the usual laboratory conditions and we suspected that the " end point " adopted on this occasion may have been somewhat high. We therefore conclude that a considerable variation in the titre shown by an individual hen may occur from time to time. Hen 20 is a good example of a fowl whose serum had a variable agglutination titre, sometimes showing negative reactions and from whose ovary *B. pullorum* was isolated.

Agglutination by Egg Albumin.—Beaudette (7) stated that the albumin of eggs laid by hens giving a positive serum agglutination will also give a positive agglutination if the albumin of their eggs is used in place of serum. We (3) carried out many experiments and have already recorded some of our findings. More recent work confirms our previous results. Agglutination by egg albumin is non-specific. A precipitation can be produced if it is mixed with tap water, saline solution made with tap water, saline solution made with copper distilled water, etc., and albumin from any egg produced the reaction. Doyle (1) has investigated the method and found it valueless.

Treatment of Antigen for Use in Agglutination Tests.—We have inquired into the different methods of preparing *B. pullorum* antigen for use in the carrying out of agglutination tests because of the variety of methods described. Two strains of *B. pullorum* were grown on agar for about 40 hours. The cultures were washed off in sterile normal saline solution and diluted in the same solution till a

suspension of about 1,000 million per c.c. was got. Samples were treated as follows :—

(*a*) Heated at 60° C. for one hour ; (*b*) Heated at 60° C. for one hour and 0·5 per cent. phenol added ; (*c*) 0·5 per cent. phenol added, then heated at 60° C. for one hour ; (*d*), (*e*) and (*f*) Unheated and 0·2 0·3 and 0·5 per cent. formalin respectively added, then incubated at 37° C. for 24 hours ; (*g*) A mixed sample of all treated lots. Agglutination tests were carried out, using immune hen serum with the various treated samples of *B. pullorum* suspension as antigen. Dilutions of serum ranged from 1 in 20 to 1 in 1280. No appreciable difference in the end points of the various tests was recorded. We conclude that the feature of importance in the tests is not the kind of antigen used, but the choice and dilution of the hens' blood which is to be called positive. We think in general practice it would be well to keep a " standard " agglutinating serum in a laboratory doing tests and to use the standard as a check on current tests.

(*c*) B. Pullorum from Eggs.

Different workers give different results as to the percentage of eggs, from which *B. pullorum* can be isolated, laid by carrier hens. Doyle (5) concludes from his experiments that infected eggs are laid at very irregular intervals and that the percentage, at least in some cases, is much higher than is generally supposed. We have carried out experiments on the isolation of *B. pullorum* from eggs laid by (*a*) natural " carrier " hens and (*b*) hens artificially infected with *B. pullorum*. Our technique is to incubate the eggs at 37° C. for seven days. On removal from the incubator the surface of the eggs is wiped with hot lysol followed by methylated spirit and then flamed. All or part of the yolk is removed with sterile precautions and placed in a large tube of broth, which is now incubated for 24 hours. Agar slopes are then sown with the contents of the broth tubes and incubation is carried out for 24 to 48 hours. Examination of any growths on the slopes is made and any gram negative organisms found are submitted to sugar tests and agglutination tests using a hyper-immune *B. pullorum* serum.

(*a*) Eggs from Naturally Infected Hens.—Thirty-eight eggs laid by five hens were examined. From two eggs only were cultures of *B. pullorum* recovered.

(*b*) Eggs from Artificially Infected Hens.—Forty-eight eggs laid by 10 hens, used in the production of *B. pullorum* anti-serum and which had received many injections of living culture, were examined. From two eggs cultures of *B. pullorum* were isolated.

Thus, from natural and artificially infected " carrier " hens about

four per cent. of the eggs laid over a period yielded cultures of *B. pullorum*. We found that contaminating organisms grow from about four per cent. of the eggs tested.

We have also shown that *B. pullorum*, injected into eggs laid by clean hens, can be recovered after incubation for a week. 0·1 c.c. of varying dilutions of culture (1-1,000 to 1-1,000,000) was injected, and cultures were recovered from all eggs except those injected with a dilution of 1 in 1,000,000. Three eggs were used for each dilution.

We (8) have already recorded that of 273 unhatched eggs, of which 127 were unfertile and 146 contained dead chicks, cultures of *B. pullorum* were grown from 55. These eggs were produced by hens of which 11 per cent. reacted positively to the agglutination test.

Passage of B. Pullorum Through Egg Shells.—We have carried out experiments to test whether, in the isolation of *B. pullorum* from eggs laid by carrier hens, the organisms may have penetrated through the shell from some external sources. Two litres of broth culture of *B. pullorum* was prepared. Eighteen eggs from non-reacting hens, the surface of which were cleansed and dried, were soaked in the culture for periods varying from five to 60 minutes. They were then incubated for seven days and, using our ordinary technique, attempts were made to isolate *B. pullorum* from the yolks. The results were negative. A second experiment in which 12 eggs were used and immersed in culture for 30 minutes, likewise gave negative results when yolks and albumin were cultured.

(*d*) B. Pullorum—Gas Production.

Rice (9) states that a strain of *B. pullorum* may develop or lose gas producing power during cultivation.

Strains of *B. pullorum* isolated from chicks in our laboratory are subjected to the various laboratory tests including their ability to ferment carbohydrates with gas formation. Before being used for any experimental work they are again subjected to the various tests. We have noted that strains, aerogenic when freshly isolated may, after several subcultures, become anaerogenic. Seven cultures were aerogenic when freshly isolated: when tested four months later, all had lost the power of producing gas through producing acid in glucose. It would appear there there is no necessity to describe two types of *B. pullorum* (A and B).

Absorption of Agglutinins.—We first tested emulsions of *B. pullorum* and *B. gallinarum* with their respective agglutinating sera and also carried out cross agglutination tests, and found that the end points of the sera were identical; we then carried out absorption tests to see, if by such means, the two organisms can be differentiated. A heavy emulsion of *B. pullorum*, grown on agar and washed off in

normal saline, was made and killed by heat. To it was added some hyper-immune *B. pullorum* serum. Agglutination was complete after about one hour at 55° C. The mixture was centrifuged and the clear supernatant fluid was used in agglutination tests, in which the antigens were several strains of *B. pullorum* and one of *B. gallinarum*. Agglutination took place at the same dilution of supernatant fluid irrespective of whether the antigen was *B. pullorum* or *B. gallinarum*. A further experiment was carried out using a heavy emulsion of *B. gallinarum* in place of *B. pullorum*. The results were similar. Therefore we can conclude that, in our hands, as far as agglutination is concerned *B. pullorum* and *B. gallinarum* are identical.

(*e*) Use of Hyper-Immune Serum.

We (2) have already recorded that hyper-immune *B. pullorum* serum gave protection when injected into chicks infected artificially with *B. pullorum*. Further laboratory experiments show that such serum injected into day-old chicks will protect them against culture given per os or injected subcutaneously 24 hours later. Table IV is an example of many experiments giving similar results.

Some field trials have been carried out. In one of the most interesting of these, 800 chicks in three hatches were injected with 1·0 c.c. of serum immediately on removal from the incubator, i.e. from six to 48 hours after hatching. Rather to our disappointment a number of further deaths from B.W.D. occurred amongst the injected chicks. By this time the examination of the unhatched eggs had been carried out and we found that of 273 eggs left in the incubators when hatching was over, about 25 per cent. contained dead chicks from which *B. pullorum* was isolated. We then bled the hens on another farm which supplied the eggs. Of these hens 45 per cent. gave clear positive agglutination reactions. It was evident that the eggs for hatching were heavily infected with the bacillus and that many of the infected eggs failed to hatch, while others just hatched giving rise to heavily infected chickens. The incubator, therefore, during the first few hours after hatching was an " incubator " in the bacteriological sense and became a hotbed of concentrated infection for all the hatched chicks. These chicks were, therefore, heavily infected before the serum was given. It thus became clear why the serum-injected chicks showed only a somewhat lower death rate than the injected controls.

We conclude that serum will be of service only in hatches with a light infection. Where a very few chicks are noted with B.W.D. within a day or two of removal from the incubator, the serum given to all the brood will probably give a high degree of protection. It is difficult to arrange such an experiment on the laboratory scale,

it is also difficult to find a poultry farm with the exact conditions required for this observation. We are at present trying to arrange for such a test.

Summary.

(1) Fowls up to one year old have been infected by feeding large volumes of *B. pullorum* broth culture. Deaths may occur soon after infection or hens may live for months when *B. pullorum* may be isolated from the ovary.

(2) Non-reacting cockerels may be made to react to the agglutination test by intravenous or intratesticular injections of *B. pullorum*. Such cockerels do not appear to transmit infection to hens mated with them.

(3) Natural and artificially infected hens do not appear to transmit infection to normal hens in the same pens or to chicks bred from clean stock when placed in contact.

(4) A clean cockerel mated with infected and clean hens does not appear to cause infection of the clean stock.

(5) A considerable variation in the agglutination titre of fowls' serum may occur from time to time, and there is evidence that a strongly positive hen whose ovary contains *B. pullorum* may at times show a negative agglutination reaction.

(6) Various methods of making antigens for use in agglutination tests have been the subject of experiment.

(7) *B. pullorum* was recovered from about four per cent. of the eggs laid by natural and artificial " carrier " hens.

(8) Attempts to cause *B. pullorum* to pass through the shells of eggs were unsuccessful.

(9) Gas-producing strains of *B. pullorum* may lose this quality in the laboratory.

(10) In our hands, as far as agglutination is concerned, *B. pullorum* and *B. gallinarum* are identical.

(11) Hyperimmune serum may be useful in hatches of chicks in which a light infection of B.W.D. is present.

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TABLE I.

Feeding Experiments.

Fowl.	*Agglutination Tests.*	*P.M. Findings.*
13	Complete 1/40 ..	Positive Ovary.
14	Complete 1/80 4 weeks after feeding and maintained.	Positive Ovary.
Chick 1	Died 9 days after feeding.	*B. pullorum* isolated heart blood.
2	Died 13 days after feeding.	*B. pullorum* isolated heart blood.
3 Cock ..	Negative	—
4 ,, ..	,, ...	—
5	,, ...	Negative Ovary.
6	,, ...	Positive Ovary.

TABLE II.

Fowl.	*Agglutination Tests.*	*P.M. Findings.*
Hen 1	Nil	Negative.
,, 2	Rose to 1 in 160 ..	B.W.D. Ovaries.*
Pullet 1	Nil	Negative.
,, 2	Rose to 1 in 160 ..	B.W.D. Ovaries.*
,, 3	Nil	Negative.

* *B. pullorum* was isolated in pure culture from the ovaries of Hen 2 and Pullet 2.

TABLE IV.

B. Pullorum Hyperimmune Serum.
Chick Protection.

Chicks two days old given 0·5 c.c. serum subcutaneously. Twenty-four hours later were injected with culture.

Dilution of Culture.	*Result.*		
1 in 1,000	+4	+6	+8
1 in 10,000	+6	+6	L
1 in 100,000	L	L	L
1 in 1,000,000	L	L	L
Control Chicks (No Serum).			
1 in 1,000	+6		
1 in 1,000,000	+5		
Control Chicks (Normal Fowl Serum).			
1 in 1,000	+4		
1 in 1,000,000	+7		

+4, etc. = Died in 4 days, etc. L = Lived.

TABLE III.

Variation in Agglutination Titres.

	1925.	*Dates of Tests and End Points.*																			
Hen No.	20/1	11/2	19/2	25/2	5/3	10/3	18/3	25/3	1/4	8/4	16/4	22/4	29/4	5/5	12/5	27/5	4/6	27/6	24/7	25/8	17/9	28/9
44 ..	80	80	160	320	80	40	160	160	160	—	40	40	—	40	160	—	—	—	—	80	—	80
115 ..	80	—	40	320	160	160	160	160	160	80	80	40	40	160	160	—	40	—	40	80	40	80
125 ..	80	160	160	320	160	160	160	160	160	160	—	—	—	—	—	—	—	—	160	160	160	160
26 ..	80	—	N.D.	320	160	160	160	160	160	80	160	160	80	160	160	—	160	160	160	160	80	160
18 ..	80	160	N.D.	320	—	40	160	—	Sitting							40	—	—	—	—	—	—
77 ..	80	—	40	320	40	80	80	80	160	Died.												
785 ..	640	160	160	320	160	160	160	160	Died.	*B. pullorum* isolated from ovary.												
20 ..	—	—	40	—	—	—	—	—	—	80	40	—	—	40	—	—	—	—	—	—	40	

P.M. on Hens.	*Ovary.*	*B. Pullorum Isolated (Ovary).*
44	+	+
115	+	—
125	+	—
26	+	+
18	+	—
77	+	Not done
785	+	+
20	+	—

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