ZONE REACTIONS IN THE AGGLUTINATION TEST FOR PULLORUM DISEASE IN TURKEYS

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ZONE REACTIONS IN THE AGGLUTINATION TEST FOR PULLORUM DISEASE IN TURKEYS

by

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It is characteristic of Science and Progress that they continually open new fields to our vision.

Pasteur

THESIS

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INTRODUCTION

Pullorum disease in turkeys was first recorded by Hewitt in 1928. Since then a great deal of work has accumulated on means of proper diagnosis of diseased and carrier birds. The official tests prescribed by the National Turkey Improvement Plan (1946) include: (1) Standard tube agglutination test (U. S. Live Stock Sanitary Association Meeting, 1932), (2) The rapid serum test (Runnells, Coon, Farley and Thorp, 1927), and (3) The rapid whole-blood test. In the case of the standard tube agglutination test, it is recommended that either of the two serum dilutions 1:25 or 1:50 may be employed.

Corporon (1945), while studying pullorum disease in turkeys found that in standard tube agglutination tests some birds which gave the maximum agglutination reaction in 1:100, 1:200, 1:400 and even in 1:800 serum dilutions showed only slight reactions in 1:25 and 1:50 serum dilutions.

This phenomenon, referred to in the literature as zone reaction, prozones, prezones, zones of inhibition and pro-agglutinoid zones is not uncommon in antigen-antibody reactions. Thus Coventry (1930), while studying the trypanocidal effects of immune serum in rats infected with Trypanosoma lewisi, found that 1.1 ml of the immune serum had no trypanocidal effect, 1.5 ml was markedly trypanocidal, 1.9 ml had no such effect, 213 ml was only slightly trypanocidal and 2.7 ml was markedly trypanocidal. She also found that these zone phenomena were independent of the inactivation of the serum. Taliafferro and Johnson (1926) observed similar zones of inhibition in mice infected with T. equinum.

Felton and Bailey (1926) observed zone reactions in their studies on the protective action of pneumococcus anti-serum in mice infected with

pneumococci. They found higher doses of the antiserum less effective as compared to certain smaller doses in some mice. They attributed this absence of protection when higher doses of antiserum were used to a substance different from the protective antibody, but which paralyzed the defence mechanisms of the mice.

Lorgan and Nigg (1928) observed that in some positive Wassermann reactions for syphilis in leprous individuals, there was more complement fixation in tubes containing smaller amounts of patients serum than in the ones containing the maximum amounts. They found the incidence of such reactions to be as high as 50% of the positive Wassermann reactions.

Priestley (1931) observed zones of inhibition in standard tube agglutination tests in sera from animals infected with the Brucella group of organisms. He, also, found that, provided a standard technique was adhered to, the zone generally occurred in the same position. Seddon (1915) made a similar observation. White (1931) has drawn attention to the occurrence of prozone inhibition phenomenon in tests performed with freshly drawn sera of typhus sufferers and convalescents. Shibley (1929) has made an extensive study on zones produced artifically by heating immune sera to various temperatures for different periods of time.

The present study was undertaken to gain more information on the subject of zone reactions as they occur in standard tube agglutination tests for pullorum disease in turkeys.

Materials and Methods

Bacterial strains: The organisms used in this study were:

1. Salmonella pullorum strain.....4
2. Salmonella pullorum strain.....10
3. Salmonella pullorum strain.....11

All antigens discussed in this report were prepared from the organisms listed above and are referred to by their numbers hereafter.

All organisms were plated on tryptose agar (Difco), and incubated for 24 hours at 37° C. Single, smooth colonies as indicated by reflected light were picked up and all antigens were prepared from cultures originating from such colonies. The morphological, cultural and biochemical reactions of all the three strains correspond to those listed in Bergey's Manual (1948).

Preparation of standard antigens: Kolle flasks containing tryptose agar (Difco) were seeded with about 2 ml of 24 hours tryptose broth (Difco) cultures. Simultaneously a loopful of the same broth culture was streaked on a tryptose agar plate, and after 24 hours incubation at 37° C., the plates were examined by reflected light for the presence of any rough or dissociated forms. Kolle flasks corresponding to plates showing only smooth colonies were selected for antigen preparation.

The Kolle flasks, after 24-48 hours of incubation, were washed with phenolized (0.5%) physiological sodium chloride (0.85%) solution (referred to hereafter as 0.5% carbol-saline solution) to form thick suspensions. These suspensions were filtered through sterilized cotton pads, and the filtrates stored in the refrigerator as stock antigens.

To prepare standard antigens, each stock antigen, just before use, was diluted with enough 0.5% carbol-saline solution to correspond in turbidity to tube three in McFarland's nephelometer (McFarland: 1907).

To prepare polyvalent standard antigen, equal quantities of each of the stock antigens 4, 10 and 11 were mixed in a test tube and the mixture then diluted with 0.5% carbol-saline solution to match in turbidity of tube three in McFarland's (1907) nepholometer.

Sera used: - Three turkeys (previously tested and found negative to the agglutination test for pullorum disease) were inoculated intravenously with 0.5 ml of 24 hours tryptose broth culture, as follows:

Turkey 395.....S. pullorum strain..... 4

Turkey 615.....S. pullorum strain.....10

Turkey 11921.....S. pullorum strain.....10

The turkeys were bled at various intervals of time, and the sera were separated by centrifugation of the coagulated blood. All sera were stored in the refrigerator without added preservatives. The time of the post-infection bleeding was indicated by labelling the serum containers as follows: 11-day serum or 16-day serum, etc.

Methods: The standard macroscopic tube agglutination test was used. All serum-antigen mixtures were incubated in a water bath at 56° C. for 18 hours unless otherwise stated. In view of the considerable importance attributed to convection currents in affecting an increase in the speed of clumping in agglutination tests(Topley and Platts: 1918, Fleming 1928) the tubes were immersed in the water bath up to three-fourths of the depth of the contained fluid.

The degrees of agglutination have been indicated on the following scale: -

+++ --- Complete agglutination - supernatant fluid clear

++ --- Markedly granular with flocculation

+ --- Granular without flocculation

± -- Doubtful

- Negative

Experiments and Results

I. Influence of temperature of incubation on agglutination tests

Shibley (1929) observed a serum (pneumococcus Type I, horse) in which an inhibition zone appeared only when the incubation was at 56° C. but disappeared when incubation was at 37° C. Heidelberger and Kendall (1929), while studying precipitation reaction, observed flocculations at icebox temperatures in mixtures which showed no precipitation at room temperature. Spencer (1930) on the other hand, found that the zone was destroyed if the serum-suspension mixtures were incubated at 56° C. Priestley's (1931) results indicated a definite narrowing of the zone when the incubation temperature was 56° C.

The following experiments were planned to study the comparative influence of the incubation temperatures of 37° C. and 56° C. on agglutination tests in pullorum positive sera.

Agglutination tests in duplicate were performed with the 11, 16 and 21-day sera of turkeys 615 and 11921. One set of tubes of each serum sample was incubated at 37° C. and the other at 56° C.

Table I shows the results of these experiments.

Table I

Influence of temperature of incubation

Antigen used -Standard polyvalent

Turkey Number	Time of post-infection bleeding in days	Incubation Temperature	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Controls
615	11	37° C	+++	+++	+++	+++	+++	++	+-	-	-	-	-
615	11	56° C	+	+++	+++	+++	++	++	+	-	-	-	-
615	16	37° C	++	+++	+++	++	++	+	+	-	-	-	-
615	16	56° C	++	++	+++	+++	+++	++	+	+-	-	-	-
615	21	37° C	+++	+++	++	+	+-	+-	-	-	-	-	-
615	21	56° C	+-	+	++	++	+	+-	-	-	-	-	-
11921	11	37° C	+++	+++	+++	+++	+	+-	+-	-	-	-	-
11921	11	56° C	++	+++	+++	+++	++	++	+	+-	-	-	-
11921	16	37° C	++	+++	+++	+++	++	++	+	+-	-	-	-
11921	16	56° C.	++	++	+++	+++	+++	++	+	+-	-	-	-
11921	21	37° C	+++	+++	++	++	++	+	+-	-	-	-	-
11921	21	56° C	+-	+	++	++	++	++	+-	-	-	-	-

It will be seen from this table that there were no zones of inhibition in 11 and 21-day sera when the incubation temperature was 37° C. but definite though weak zones appeared in 1:10 serum dilution of the 11-day serum and well marked zones appeared in 1:10 and 1:20 serum dilutions of 21-day sera when tests were incubated at 56° C. In the case of 15-day sera, the already existing weak z ones in 1:10 serum dilutions widened to 1:20 serum dilutions at 56° C. incubation.

This observation is very significant since in any pullorum eradication program in turkeys, where a single dilution method is employed and the tests are incubated at 56° C. some positive birds are liable to be missed and will be potential sources of infection for the healthy stock.

II. Effect of dilution of the antigen

Heur (1922) and da Costa Cruz (1929) showed that zone reactions in agglutination tests could always be demonstrated when very light bacterial suspensions were titrated in constant amount, against increasing dilutions of antiserum. Seddon (1915) found that by reducing the density of the bacterial suspension, the dilutions in which zone occurred were raised. Priestley (1931) by halving the density of the bacterial suspension raised the zone to the next serum dilution. Spencer (1930) made a similar observation. Corpron (1945) did not find much difference in the zone reactions by changing the density of the antigen. In the following experiments the results obtained by the use of standard antigens and the standard antigens diluted 1:1 with 0.5% carbol-saline solution are shown in Table II.

Table II

Influence of dilution of the antigen on zones in the serum

Time of post-infection bleeding in days	Antigen used	1/10	1/20	1/40	1/80	1/160	1/320	1/540	1/1280	1/2560	1/5120	Control
5	S	-	+	+++	+++	++	+/-	-	-	-	-	-
5	D	-	-	+++	+++	+	+	+/-	-	-	-	-
11	S	+/-	+	++	+++	+++	++	+/-	-	-	-	-
11	D	-	+	++	+++	++	+	+	-	-	-	-
16	S	-	+	+++	++	+	-	-	-	-	-	-
16	D	-	-	++	+++	+/-	-	-	-	-	-	-
21	S	-	+	++	+/-	-	-	-	-	-	-	-
21	D	-	-	+++	++	+/-	-	-	-	-	-	-
5	Stand. Poly.	-	++	+++	+++	+++	++	+/-	-	-	-	-
5	Diluted Stand poly.	-	+	+++	++	++	+	+/-	-	-	-	-

S= Standard antigen 10

D= Diluted antigen 10

By studying the data in Table II it will be observed that by reducing the density of the antigens by one half the zones of inhibition became more pronounced and in most cases these are raised to 1:20 and then to 1:40 serum dilutions. It thus seems that the position of the zone of inhibition in the agglutination test depends on the ratio between the amount of serum and the number of organisms. From this it will be seen possible that further dilutions of the antigen might extend the position of the zones to 1:80 serum dilutions or even higher.

These observations have important bearings on the central program of pullorum disease. The use of one serum dilution of 1:25 or of two serum dilutions of 1:25 and 1:50 with a light bacterial suspension as recommended by the National Turkey Improvement Plan (1946) and the subsequent incubation of the serum-suspension mixtures at 56° C. might fail to detect some diseased and carrier birds.

In view of the above observations, a better and more definite way to detect the diseased and carrier birds would probably be either to replace the use of two serum dilutions of 1:25 and 1:50 as recommended by the National Turkey Improvement Plan (1946) by four serum dilutions to include 1:100 and 1:200 serum dilutions or if only two serum dilutions must be used, a serum dilution of 1:25 combined with 1:100 or 1:200 serum dilutions to cross over the area of occurrence of zone.

III. Effect of heated serum

Detre (1927) stated that the zone was removed and agglutination occurred when zone sera were inactivated by heat at 53° C. Spencer (1930)

showed that if the serum was inactivated at 56° C. for periods varying from 10 to 60 minutes, the zone widened in proportion to the length of time of inactivation. In some of the sera used by him, heat treatment introduced new zones of inhibition. Priestley (1931) and Corpron (1945) also made similar observations. Corpron (1945) also noticed a fall in the end-titres of the heated sera.

Dreyer and Jem. Blake (1906) studied the production of "Zone effects" with sera heated at 70-75° C. They noticed intermediate zones of inhibition in such sera and attributed them to the production by heat of a substance which impeded agglutination. Shibley (1929) also investigated the production of zones by heat treatment of immune sera. His experiments showed three states of immune serum induced by heat, (a) The unheated serum gave agglutination in all concentrations to the limit of the titer; (b) After heating at about 60 to 70° C. a zone of inhibition occurred in the lowest serum dilution, but agglutination took place in the higher dilutions to the limit of the titer; (c) After further heating at about 76° C. there was no zone, but the titer was lowered, and with serum heated to 78° C. no agglutination appeared.

The following experiments were conducted to determine the effect of heat treatment on 11-day sera of turkeys 615 and 11921. Sera used in this study were heated at 56° C. and 65° C. for ½ hour, the ones which were heated at 65° C. were diluted 1:5 with 0.85% sodium chloride solution before heat treatment to avoid their coagulation. Table III shows the results of this experiment.

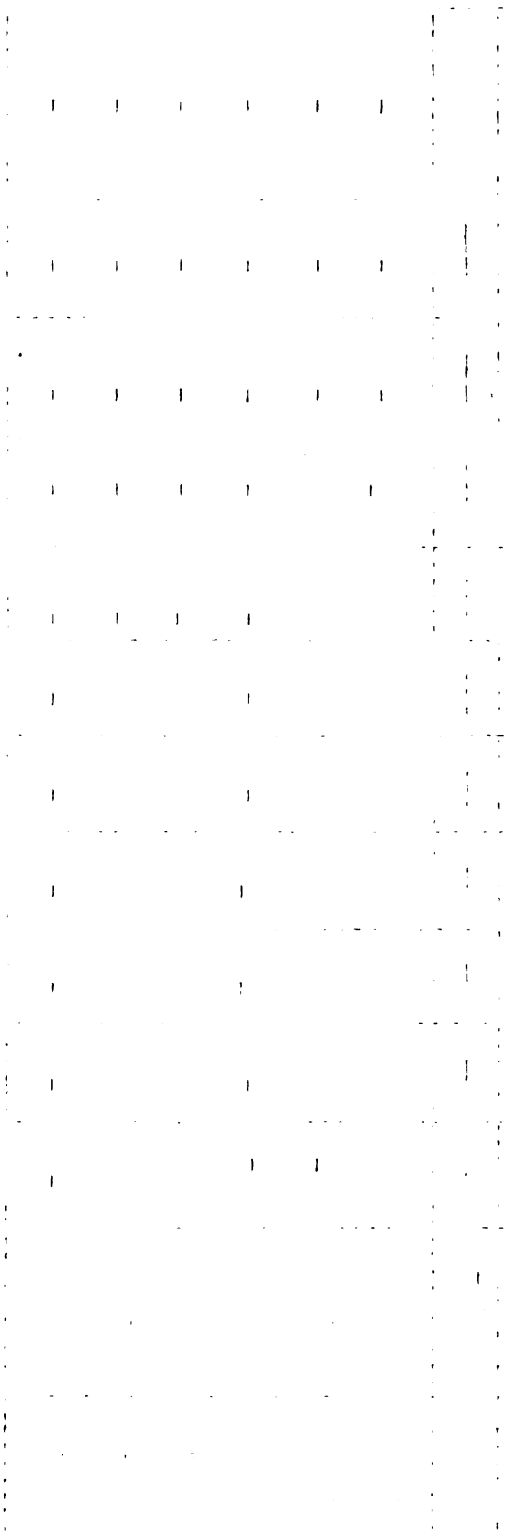
It will be seen from the results that heating the sera at 56° C. for ½ hour did not cause any material change in the position of the

Table III

Zone reactions as affected by heating of sera

Antigen used - Standard polyvalent

Turkey	Treatment	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
615	Unheated	++	+++	+++	+++	+++	++	++	±	-	-	-
615	56°	-	++	+++	+++	+++	+++	++	±	-	-	-
615	65°	-	±	±	±	-	-	-	-	-	-	-
11921	Unheated	++	+++	+++	++	++	++	±	-	-	-	-
11921	56°	++	+++	++	++	+	+	-	-	-	-	-
11921	65°	-	-	-	-	-	-	-	-	-	-	-



zone reaction but when the sera were heated at 65° C. no agglutination appeared. Apparently all the agglutinins had been destroyed at that temperature.

IV. The shift of zone in the serum of Turkey 395

The agglutination reactions of turkey 395 are shown in Table IV. It will be seen from that table that the position of the zone shifts from 1:320 serum dilution in the 16-day serum to 1:80 serum dilution in the 20-day serum, to 1:20 in the 25-day serum and 1:10 and 1:20 serum dilutions in the 30-day serum. This shift in the position of the zone coincides with the fall in the end-titers of the sera, which shifts from 1:640 in the 16-day serum to 1:320 in the 20-day serum and 1:80 in the 25 and 30-day sera. A similar reaction was observed by Spencer (1930) who noted in his studies that in the later samples of the serum from the same patient, the zone of inhibition had shifted from the intermediate position to the tubes of maximum serum concentration.

This observation is in line with that obtained in experiment II (Influence of dilutions of antigen; Table II). There is apparently a correlation between the proportion of serum and antigen and the occurrence of zone. It was noticed in experiment II that if the antigen was diluted, the zone shifted to include a correspondingly higher dilution of the serum. In this experiment when the amount of antibodies is reduced the zone shifts to higher concentrations of the serum.

V. Agglutinin absorption in zone areas

The following experiment was planned to see what happens to the agglutinins present in the tubes exhibiting zone reactions. For such

a study, it was considered necessary to produce wider and definite zones and so a lighter bacterial suspension (Standard antigen 10 diluted 1:1 with 0.85% sodium chloride solution) was employed as an antigen. Eleven, sixteen and twenty-one-day sera of turkey 615 were titrated against the diluted antigen and the tubes incubated at 56° C. for 18 hours. Table V shows that there were definite zones present in all the three sera(reactions A, B and C). All the tubes showing reactions A, B and C were centrifuged for one-half hour at 3,000 r.p.m. and the supernatant fluid from each tube was again titrated against the diluted antigen. The results obtained are detailed in Table V (reactions A', B' and C'), and show that the supernatant fluids from A, B and C contain fairly high concentration of agglutinins in the zones of inhibition, as indicated by ++ agglutination reactions. This indicates that in the original agglutination tests (A, B and C), few, if any, antibodies combined with the antigen.

The absence of agglutination in the presence of antibodies reveals the presence of an inhibitory factor. The exact nature of such a factor has been the subject of much speculation and debate.

Two hypotheses have been put forward to explain the appearance of zone phenomenon in agglutination reactions. Eisenberg and Volk (1902) explained the phenomenon on the lines of Ehrlich's conception of the agglutinin as consisting of a "haptophore" and a "zymophore" group, the haptophore group bringing about the specific union of the agglutinin with the bacteria and zymophore group causing agglutination. They further assume that by heating or aging, some of the agglutinin is so modified

Table IV

Shifting of the zone reactions in serum of turkey #395

Time of post-infection bleeding in days	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	1/2560	1/5120	Control
16	+++	+++	+++	+++	+++	+	++	±	-	-	-
20	+++	+++	+++	-	++	+	-	-	-	-	-
25	+++	+	++	+	±	-	-	-	-	-	-
30	-	+	+++	+	±	-	-	-	-	-	-

Table V

Antigen used -Standard antigen 10 diluted 1:1 with 0.85% Sodium chloride solution -
Serum Turkey 615

Time of post-infection bleeding in days	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1280	Control	Serum antigen-dilutions
11	-	+	++	+++	++	+	+	-	-	A
11	++	++	+ -	-	-	-	-	-	-	A '
16	-	+	++	++	+ -	-	-	-	-	B
16	++	++	+	-	-	-	-	-	-	B '
21	-	++	++	+	-	-	-	-	-	C
21	++	+	+	-	-	-	-	-	-	C '

A, B, C = reaction in primary serum-antigen mixture titrations.

A', B', C' = reactions resulting from mixing supernatant fluids from tubes A, B, and C, following centrifugation, with fresh antigen.

(agglutinoid) that the clumping component is destroyed without, however, affecting the binding portion. To explain the inhibition of reaction in lower serum dilutions it is assumed that the agglutinoid in these concentrations has a greater affinity for the bacteria and is, therefore bound to them to the exclusion of effective agglutination.

The second hypothesis was put forward by Zinsser (1923) and has been widely accepted. It explains the inhibition of clumping in a zone of stronger serum concentration, due to the presence of a non-specific protein colloid which coats the bacterial bodies in the antigen and thus prevents their agglomeration.

Recently Wiener (1944) and Race (1944) have shown the presence of both "incomplete" and complete antibodies in the Anti-Rh serum of the individuals showing zone reactions in lower serum dilutions. Incomplete antibodies, according to Wiener (1945) are univalent and are unable to serve as a link between red cells that effectively combine with the receptors on the red cells thus preventing agglutination by bivalent or complete antibodies - a trend back to Ehrlich's hypothesis.

SUMMARY

Weak, if any, zone reactions were produced when the serum-antigen mixtures were incubated at 37° C. but definite and wide zones were noticed when the mixtures were incubated at 56° C.

Dilution of the antigen raised the zone to as high as 1:40 serum dilution.

It is suggested that in any pullorum eradication program, either of four serum dilutions of 1:25, 1:50, 1:100 and 1:200 should be employed or if only two dilutions must be used, serum dilutions of 1:25 and 1:100 or 1:200 should be preferred.

Heating the sera at 56° C. for ½ hour did not produce any change in the position of the zone reactions, but heating the sera to 65° C. destroyed all agglutinins in them.

The shift of zones in one turkey correlated to shifts in its end titer.

Supernatant fluids from tubes showing zone reactions revealed the presence of high titre of agglutinins when titrated with fresh antigens.

Literature on the subject is reviewed.

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