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A STUDY OF DIPHTHERIA BACILLI, WITH SPECIAL REFERENCE TO THEIR SEROLOGICAL CLASSIFICATION.

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ABSTRACT.

MUCH work has already been done in connection with the differentiation of true diphtheria bacilli from diphthomorphic organisms. Some observers have maintained that all disease producing strains could be distinguished from non-pathogenic types by their sugar reactions. Others have laid much stress on some particular forms of virulence test. More recently serological classification has been attempted in order to help to solve this problem.

With regard to sugar reactions Graham-Smith (1908) found that certain strains produced acid in media containing saccharose, while Hine (1913), on the other hand, maintained that no true diphtheria bacillus could ferment this sugar. Eagleton and Baxter (1922) have shown that diphtheria bacilli, virulent and non-virulent, ferment glucose but do not produce any change in saccharose. Jordan and his collaborators (1922) have found that virulent and non-virulent bacilli fermented glucose and maltose and produced no change in saccharose and that their action on dextrin was variable.

Work on the serological classification of these organisms has also shown some considerable variation. Havens (1920) isolated 206 strains of diphtheria bacilli. These represented cultures from acute cases, release cultures and cultures from healthy carriers. He found that these 206 strains could be divided into two serological groups, one group containing 169 strains, the other 37. There was no evidence of any cross agglutination. Durand (1920) isolated 255 strains of typical diptheria bacilli and after excluding those strains which would not form stable suspensions divided them into five groups, A. B. C. D. and E. containing 16, 8, 25, 61 and 40 strains respectively. Eighty-seven strains were found to be inagglutinable by these five sera but nevertheless could absorb agglutinins from such sera. His final figures for the five groups of bacilli are 18, 8, 31, 76 and 51. Durand and Guerin (1921) in a further paper showed that small isolated outbreaks were always due to the same type of bacillus. They also found that healthy carriers gave the same type of bacillus as was found in the cases arising from these sources of infection. Bell (1922) isolated 133 strains of *B. diphtheriae* indifferently collected from cases and carriers. He was able to agglutinate 80 per cent. of these strains by three monovalent sera. Group I contained 17 strains, Group II 8 strains, Group III 80 strains.

In view of the results which have just been described it has appeared important to investigate further a considerable number of strains as encountered in routine work, not only as regards the serological characteristics of the organisms but also in regard to their morphology, their biochemical reactions, and their virulence for guinea-pigs.

Technique.

Strains of diphtheria bacilli were collected from acute cases only. Strains which were isolated from cases without evidence of clinical diphtheria were discarded. Pure cultures having been obtained an 18-hour serum culture was used for studying the morphology and the morphological characteristics were noted according to the Wesbrook classification. Stock cultures were maintained on gelatin slants in the cool incubator at 20° C. The biochemical reaction of the various strains were tested in Hiss's serum water medium containing 1 per cent. of the various sugars—glucose, galactose, laevulose, maltose, dextrin and saccharose. Litmus was used as an indicator. The power of the organism to break down two alcohols, namely glycerol and mannitol, was also tested.

Many of the strains were tested for virulence by guinea-pig inoculation. In order to obtain a uniform amount of growth all strains were subcultured four times in a dextrose free broth containing 2 per cent. of Witt's peptone. The hydrogen ion concentration of this broth after sterilisation was *p*H 7·4. A fifth subculture was then made and incubated for 48 hours at 37° C. and 0·5 c.c. of the broth culture per 100 grams of weight of the guinea-pig was injected subcutaneously. A control animal was used on all occasions. This animal received the same amount of culture as was given to the test guinea-pig together with 500 units of antitoxin.

For the preparation of agglutinating sera half grown rabbits were used and the antigen was given intravenously in all cases. At first, the antigen employed was a formalised suspension of diphtheria bacilli which had been grown on horse serum slants. Some rabbits died from the toxic effects, but it was soon discovered that the response as measured by the agglutinins present in rabbits' serum was very poor and this method was abandoned. Living bacilli were then used as antigen. An 18-hour culture on a serum slant was washed off with 10 c.c. of salt solution and thoroughly emulsified. The requisite amount of suspension was incubated at 37° C. along with the necessary amount of antitoxin for one hour before being injected.

The following table serves to indicate in general the amounts of culture and antitoxin used:

Day	Amount of Serum slant	Antitoxin
1st	0·1	1000
6th	0·2	1000
11th	0·4	500
16th	0·6	500
21st	0·8	200

Sera with titres ranging between 1–1600 and 1–12,800 were obtained by this method.

The method of preparing the agglutination tests was the usual one of doubled dilution. The tests were set up in tubes placed in racks as devised by Dreyer and incubated for two hours at 37° C. A control tube containing salt solution and bacillary emulsion was used in connection with each test. All bacterial suspensions were standardised by the method of Kirwan and Brown (1915). Occasionally considerable difficulty was encountered owing to strains showing persistent spontaneous agglutination.

DISCUSSION OF RESULTS.

1. *The Biochemical Reaction.* The sugar reaction of 89 strains was tested by using Hiss's serum water medium as a basis. The reactions were noted after three days' incubation and again after ten days' incubation. Within the three-day incubation period all strains were found to produce acid in glucose, galactose, laevulose, maltose and dextrin. On glycerol the reaction varied, sometimes acid was produced and at other times no change was observed. On saccharose and mannite uniformly negative results were obtained. The production of clot in the media was usually well established within the three days' period on glucose, galactose, laevulose, and maltose. The action on dextrin and glycerol was variable. At the end of ten days' incubation very few strains had failed to produce acid and clot on glucose, galactose, laevulose, and maltose. On dextrin in which all strains had already produced acid, 34 strains failed to produce clot. On mannite and saccharose there was again no change noted.

2. *Virulence Test.* By the method already described 52 strains were tested for virulence as soon after isolation as possible. Forty-six strains were found to kill the guinea-pigs within four days. In nine instances the guinea-pigs died within 24 hours after being inoculated. Thirty strains killed in two days, six strains killed in three days, and only one guinea-pig died on the fourth day. All control animals survived.

The six non-virulent strains were isolated from ordinary cases of diphtheria. In two instances it was found that the strain isolated from the nose was non-virulent while the strain isolated from the throat was virulent.

3. *Serological Classification.* Eighty-nine strains were collected from eighty clinical cases of diphtheria, in nine of the cases the organism being isolated from both the throat and nose. It was found, however, as the investigation proceeded that the strains isolated from the throat and nose of the same patient belonged without exception to the same serological group. Accordingly 80 strains of diphtheria bacilli were grouped as follows: Type I, 68 strains; Type II, one strain; Type III, six strains; Type IV, one strain; Type V, two strains; Type VI, one strain; Type VII, one strain.

Type I serum had a titre of 1 in 1600. Strains belonging to other types were not agglutinated in a greater dilution than 1 in 100.

Type II serum with a titre of 1 in 1600 only agglutinated the homologous strain to titre. It agglutinated Type I strains to 1 in 100 dilution and the other types in dilution of 1 in 50 to 1 in 200 only.

Type III serum agglutinated seven strains to full titre (1 in 1600). It agglutinated Type I strains to a dilution of 1 in 400, but strains belonging to other types were only agglutinated up to 1 in 50 dilution or not at all.

Type IV serum had a titre of 1 in 6400 and only agglutinated the homologous strain to full titre. It agglutinated Type I strains to 1 in 100 dilution of the serum, but strains belonging to other types were unaffected in this dilution.

Type V serum had a titre of 1 in 6400 and again only agglutinated the homologous strain to titre. Type I strains were agglutinated to 1 in 800. Strains belonging to other types were not agglutinated above 1 in 100.

Type VI serum only agglutinated the homologous strain to titre, 1 in 1600. Strains of Types II and III were unaffected by a 1 in 100 dilution. Strains belonging to Types IV and V were agglutinated to 1 in 100. Strains belonging to Type I were agglutinated to 1 in 200.

Type VII serum, with a titre of 1 in 3200, only agglutinated one strain. No other strain was agglutinated to titre. Strains of Types II, III and IV were not agglutinated by 1 in 100 dilution. Type V strains were agglutinated by 1 in 100, Type I strains by 1 in 400 dilution.

4. *Serological Groups in Relation to Morphology.* It was thought at first that strains of diphtheria bacilli having the same morphological characteristics might also correspond in their serological reactions. All strains were classified according to the Wesbrook classification, but no relationship between the morphological classification and the serological classification could be established.

5. *Serological Groups in Relation to Biochemical Reactions.* As the biochemical reactions were very similar for all strains no grouping of the strains on this basis was possible.

6. *Serological Groups in Relation to Virulence Tests.* Of 52 strains tested for virulence 38 belonged to Group I. All but six of these were found to be virulent. The remaining 14 strains included all strains belonging to the other six groups. These were all virulent. It is of interest to note that although one batch of antitoxin was used throughout in the protection of control animals none of these control animals died.

7. *Serological Groups in Relation to Cases.* It has already been noted that strains isolated from the nose and throat of the same patient always belong to the same serological group. It has further been found that when two or more cases of diphtheria occurred in the same family the bacilli isolated from these cases also belonged to the same serological group.

The following table shows the mortality rates for the various groups:

	No. of cases	No. of deaths	No. of cases in which paralysis occurred
Cases from which Type I strains were isolated	68	4	2
,, ,, ,, II ,, ,,	1	0	0
,, ,, ,, III ,, ,,	6	1	0
,, ,, ,, IV ,, ,,	1	0	1
,, ,, ,, V ,, ,,	2	2	0
,, ,, ,, VI ,, ,,	1	0	0
,, ,, ,, VII ,, ,,	1	1	0

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As the number of cases harbouring bacilli other than Type I is small no conclusions can be drawn as to one type being more virulent than another.

It has already been demonstrated by Havens (1920) who classified his strains into two groups that when one minimal lethal dose of toxin of a Group I strain together with five units of standard antitoxin (made from the toxin of a Group I bacillus) was injected into a guinea-pig, the animal survived. When the same dose of toxin of a Group II strain was given with the antitoxin the guinea-pig died. In the case of Group II toxin 20 units of the standard antitoxin were required to protect against 1 unit of toxin. These findings of Havens have not been confirmed. On the contrary, Hartley (1922) has found that the antitoxin made from the toxin of one serological type afforded equal protection against the toxins of other types.

SUMMARY.

1. The fermentation of glucose and non-fermentation of saccharose was a constant finding with 80 strains of *B. diphtheriae*.

2. From 80 clinical cases of diphtheria seven serological types of bacilli have been isolated.

3. The morphological characteristics and the sugar reactions do not indicate the serological grouping of the strains.

4. Fifty-two strains were tested for virulence and 46 were found to be virulent. In two instances the strain isolated from the nose was avirulent, while the strain isolated from the throat was virulent.

5. Virulent and avirulent bacilli are not differentiated by serological tests.

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