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THE VIRULENCE TESTING OF THE DIPHTHERIA BACILLUS AND ITS PRACTICAL APPLICATION.

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PRECISE methods for the diagnosis of diphtheria involving a virulence test are only slowly being adopted by the general public health administration of this country. This is largely due to the trouble and expense entailed in the proper investigation of bacteriological material. The intradermic test on account of its simplicity and relative cheapness offers, in our opinion, a method which could be applied in any properly equipped public health laboratory.

The purpose of the present paper is to describe in detail the technique of the intradermic test as we have applied it, and to give numerical results which may be of help in giving precision to certain commonly held views on the epidemiology of the disease.

Technique. We have followed the technique of Eagleton and Baxter which is as follows:

The swab when received is smeared over the surface of a Loeffler slope, which is then incubated over-night. The following morning a general smear is made from the surface of the slope, and if this proves positive on examination, it is plated out on Loeffler's medium in Petri dishes. One part of each plate should be thickly implanted.

Loeffler slopes which appear negative at 24 hours should be re-incubated, as occasionally they become positive at 48 hours. We have no evidence that longer incubation is of any value.

It is sometimes convenient to damp the swab with sterile broth and plate directly from it.

The colony of *C. diphtheriae* is picked from the plate preferably on the 3rd or 4th day of incubation. The colony is then very characteristic, being a circular disc with raised centre, creamy in colour, and with a matt surface. If the bacilli are at all numerous in the implant, the colonies can generally be picked off at an earlier stage, *e.g.* 48 hours. A difficulty occasionally met with is due to liquefaction of the Loeffler by one of the organisms present. It is then necessary to resort to plain nutrient agar plates, or plates of Douglas's tellurite medium.

The original swab should be kept in a dark place and retained until isolation has been successful, as it may be advisable to replate directly from the original swab if the primary plate is overcrowded.

At least two colonies are selected, and sown on Loeffler slopes. Smears are made from the over-night growth of these and stained by Neisser's method.

If pure, the colonies are emulsified in saline, and standardised, by comparison with an opacity tube, to contain roughly 500 million organisms per cubic centimetre. Immediately before injection into animals the standardised suspensions are transferred to clean deposit glasses. No special precautions to ensure sterility are necessary between emulsification and injection.

Two guinea-pigs are required for each series of tests. They should be from 250 to 350 grm. in weight, and preferably of light colour. No guinea-pig which has previously been injected with horse serum should be used. One of the guinea-pigs serves as the control animal and is injected intraperitoneally with 500 units of diphtheria antitoxin the night before the test. If the antitoxin is given intravenously or by the intracardiac route it may be administered immediately before the test. In practice this will be found inconvenient except under exceptional conditions, *e.g.* when the control guinea-pig prepared overnight has died before the test.

The guinea-pigs are shaved or depilated over an area sufficient for the injections. We always use the following depilator, the formula of which we owe to our colleague, Mr. C. G. Pope.

Barium sulphide	35 grm.
Flour	35 grm.
Talc (powdered)	35 grm.
White Castile soap	5 grm.

The dry powder will keep several months. To prepare for use, add water to the powder, and mix to a thin creamy consistency. The paste should be used the same day as it is made.

The animal is prepared with clippers or scissors, and the cream is applied to the clipped area with a brush and left on for two minutes. It is then thoroughly washed off with warm water, and the hairs removed with a swab of cotton wool. A perfectly depilated area should result. If the cream is left on the skin of the animal too long or not washed off properly afterwards, it causes irritation of the skin.

Both the unprotected and control guinea-pigs are injected intracutaneously with 0·2 c.c. of each of the standardised suspensions. Each injection should raise a small circumscribed swelling upon which the hair follicles are visible as small pits. The technique of the injection is similar to that of the Schick and Dick tests. An all-glass 1 c.c. syringe should be used, with the plunger fitting so closely that no leaking-back along the barrel is possible. If less than 0·15 c.c. of the suspension is delivered into the skin the injection should be repeated.

We employ the following method of cleaning the syringes after the injection of each culture:

Three small pots are filled respectively with 20 per cent. phenol, methylated spirits and saline. Two syringes are used. While one is in use the other is washed out by an assistant three times successively with the phenol, methylated

spirits and saline. After washing, each syringe is filled once with the suspension under test and then emptied before drawing up the 0·4 c.c. or more that is required for the injections.

Not less than 4 hours (preferably 6 hours) after the injections a "following dose" of 125 units of diphtheria antitoxin is given intraperitoneally to the unprotected (or test) guinea-pig which might otherwise die from diphtheria toxaemia before proper readings are made. Usually both control and test animals survive, and may be used for other purposes afterwards. It is important that the animals should be kept in a warm place, and care taken with their feeding.

Reading of Reactions. Readings should be made daily from the 1st to the 3rd day after injection. Under favourable conditions the reactions may be easily readable at the end of 24 hours, although as a rule the best readings are made on the 2nd or 3rd day. The lesion produced by a virulent culture consists of a small erythematous patch about 15 mm. in diameter which advances to superficial necrosis within 48 to 72 hours. A day or two later the necrotic patch becomes a dry scab which may eventually be shed from the surface leaving a small scar. Avirulent organisms produce little or no reaction.

On the control (protected) guinea-pig there is practically no reaction or, at the most, a raised red papule which fades away rapidly on the 2nd or 3rd day, and is easily distinguished by the practised eye from the true reaction. Occasionally the control animal may fail to become protected, though the titre of the antitoxin is carefully controlled; we have not been able to find any explanation for the occurrence, and have thought it is possibly due to accidental injection of the serum into one of the hollow viscera of the peritoneal cavity.

Very occasionally staphylococci or streptococci from the nose or throat produce reactions on both the control and the test animal. The lesion is smaller in area and of a more purulent nature than the true diphtheritic lesion with which it can rarely be confused. Only once have we met with a reaction due to infection with an organism of the gas gangrene group.

On no occasion have we met with an organism resembling the diphtheria bacillus (other than true toxigenic *C. diphtheriae*) which gave consistent and characteristic reactions. Certain diphtheroids (proved on investigation not to be *C. diphtheriae*) which gave misleading results by the subcutaneous method were quite clearly avirulent by the intradermic test.

In the event of typical diphtheritic reactions developing on the control animal the test should be repeated, but if urgently required, a provisional report may be given, since even without the control guinea-pig typical diphtheritic reactions are rarely read erroneously.

Recently, whenever we have had eight or ten suspensions for injection, we have found it advisable to use two unprotected animals to guard against vitiation of the tests through the death of one of them.

A known virulent strain of *C. diphtheriae* should always be included in the

series of cultures for injection as a control. We always employ the American strain "Park Williams 8," since suspensions of this organism tend to produce slightly smaller reactions than most, though it is fully virulent. If this gives no reaction the whole test is repeated. An avirulent control culture may also advantageously be included.

A dozen injections (*i.e.* two colonies from six cultures) may easily be put up on each guinea-pig. Frequently we have tested as many as 25 and occasionally 30 cultures on one guinea-pig with success, though with large numbers there is a risk of overcrowding causing fusion of the lesions, and of the guinea-pig dying of toxaemia.

Without the "following dose" of 125 units of antitoxin, the intradermic injection of very few, *e.g.* 4 or 6 virulent cultures, may lead to the death of the guinea-pig.

Unsatisfactory readings are comparatively rare. In one hundred consecutive pairs of guinea-pigs entirely satisfactory readings were obtained in 79 pairs. The control guinea-pigs showed undue reactions in four instances, the control virulent culture (Park Williams 8) gave poor reactions in 10 cases, the protected guinea-pig died in one case, and the test guinea-pig in six cases. Only in these six cases where the unprotected guinea-pig died (6 per cent. of tests) was it impossible to return a provisional report on the virulence of the cultures pending the completion of a properly controlled test. We now use two guinea-pigs unprotected except for the "following dose" of antitoxin, to ensure rapid reporting of results of the tests.

Owing to the economy of the test and the ease with which it can be performed, repetition of the tests is advisable whenever there is the least uncertainty as to the readings. If two colonies of each culture are put up on two unprotected animals the tests rarely call for repetition.

It would perhaps be well at this point to make clear our use of the words "virulent" and "avirulent."

We apply the word "virulent" only to those organisms morphologically resembling the diphtheria bacillus which ferment glucose but not sucrose, and which produce a toxin causing characteristic lesions in guinea-pigs. The toxic effects must of course be neutralisable with diphtheria antitoxin. In practice, we do not now consider it necessary to check the sugar reactions except in the case of cultures of special interest. (*Vide* Okell and Baxter, 1924.)

By "avirulent" bacilli we mean organisms morphologically indistinguishable from virulent bacilli, fermenters of glucose but not of sucrose, and not virulent in the above restricted sense. As a matter of fact, we have never met with an organism likely to be confused with the diphtheria bacillus, which in properly controlled tests gave consistent and characteristic intradermic reactions in guinea-pigs. Reactions resembling somewhat the true reaction have been obtained with staphylococci, streptococci and in one case with *B. welchii*, but these cultures gave reactions both in the test animal and in the protected control.

Analysis of Results.

In the period under review 2368 swabs were received, of which 1388 (59 per cent.) proved positive for the presence of diphtheria-like bacilli on examination of the over-night growth on Loeffler slopes. Of these 1388 cultures, the bacilli were isolated in apparently pure culture in 1286 (92·7 per cent.). These figures include cultures from cases of diphtheria and "carriers," and, as one would expect, more difficulty was met with in isolating the bacilli from "carriers" than from cases. In a consecutive series of 234 positive swabs from cases clinically diagnosed as diphtheria, and examined under particularly favourable conditions, the "suspicious" bacilli were obtained in pure cultures in 231 (98·7 per cent.), and of these 229 (99·1 per cent.) were virulent (Caiger and O'Brien, 1924).

In a series of 758 positive cultures from carriers, "suspicious" bacilli were isolated in 715 instances (94 per cent.). Of these 715 cultures 50 per cent. were virulent *C. diphtheriae*, 39·7 per cent. avirulent *C. diphtheriae*, 3·6 per cent. bacilli of the Hofmann group, 0·3 per cent. bacilli of the Xerosis group, and 6·4 per cent. unclassified diphtheroids. "Carriers" were therefore found virulent in 50 per cent. of cases. This is a higher percentage than that found by Eagleton and Baxter, and by Forbes, and is possibly explained by the fact that so many of our cultures came from persons who had had some association with outbreaks of diphtheria. In this connection it is noteworthy that contacts rarely harbour avirulent bacilli (Graham-Smith, 1908).

Avirulent Cultures and Cultures of Intermediate Virulence.

Large numbers of cultures found avirulent by the ordinary test have been re-tested with tenfold strength suspensions, *i.e.* 5000 million organisms per c.c., and without the "following dose." In no instance was a culture which had been definitely read as avirulent on the ordinary test found to be virulent when thus tested. One of the outstanding results of this series of tests was that we could find practically no evidence of the existence of cultures of intermediate virulence. From time to time cultures were found which gave poor or doubtful readings, but on carefully repeating the tests, it was nearly always found that they would either give a standard virulent reaction, or be entirely avirulent. One culture only was met with which gave an uncertain reading with a 500 million suspension, but gave definitely virulent reactions with a 5000 million suspension. This culture was examined very carefully, and plated many times; many colonies were tested and always showed the same type of intermediate virulence. A similar result was obtained on the subcutaneous test. There was some local swelling, but no general symptoms were produced when a suspension from the growth from two Loeffler slopes was injected subcutaneously. On injecting 10 c.c. of an eight-day unfiltered broth culture, death resulted with the typical post-mortem appearances of diphtheria toxaemia. With some difficulty a filtrate was prepared from this strain which was proved to contain very weak specific diphtheria toxin. This culture came from the throat of a carrier.

It has frequently been asserted that cultures of diphtheria vary greatly in virulence. We suggest that these differences generally depend on accidental experimental factors, and that under suitable conditions the large majority of strains can be shown to be of equivalent virulence. There seems to be at the moment no experimental evidence that the variations in the virulence of the infection in the clinical sense can be correlated with variations in virulence in the restricted laboratory sense.

The Co-existence of Virulent and Avirulent Organisms in the same Culture.

From two out of 626 "positive" cultures from cases of diphtheria, both virulent and avirulent diphtheria bacilli were isolated, once from the throat and once from the nose. From three cultures out of 758 from "carriers," etc., both virulent and avirulent diphtheria bacilli were isolated, twice from the throat and once from the nose.

In four instances a virulent diphtheria bacillus was found to have been replaced by avirulent *C. diphtheriae* in the same nose or throat when a swab was taken some days or weeks later. No instance was met with of an avirulent *C. diphtheriae* being later replaced by a virulent in the same patient.

Out of 234 positive swabs from cases carefully diagnosed by an experienced physician as clinical diphtheria, avirulent *C. diphtheriae* alone were isolated in two cases (0·9 per cent). The explanation suggested is that the patient was harbouring both virulent and avirulent bacilli, and the avirulent colonies happened to be selected on the plate. When this was discovered it was unfortunately too late in both cases to re-examine the patient and make a search for the virulent organisms that were presumably the cause of the disease.

One Antitoxin Protects against all Strains of C. diphtheriae.

In our examination of 819 virulent strains isolated from 1388 positive cultures, we have never met with a virulent organism (morphologically resembling *C. diphtheriae*, fermenting glucose and failing to ferment saccharose) against which antitoxin prepared with the Park Williams 8 strain failed to protect. Thus the usual view that there is but one diphtheria toxin has been amply confirmed.

Value of Nose and Throat Cultures from the same Patient.

In many cases nose and throat cultures were examined, but isolation was only proceeded with from the swab showing most diphtheria bacilli. The following table gives records of a small series where a thorough effort was made to isolate the organisms from both throat and nose:

	Throat and nose virulent	Throat virulent nose negative	Nose virulent throat negative
Cases and convalescents	13	20	4
Carriers, contacts and ordinary population	12	4	2
Total	25	24	6

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The figures are small, but show the value of taking swabs from both throat and nose. It need hardly be emphasised that in the examination of a suspected carrier, cultures should be taken from the nose as well as the throat, and indeed from any discharge as in otitis media, etc.

Cultures from Ear.

Out of 39 cultures from ears, mostly from post-diphtherial conditions or where a diphtherial element was suspected, true virulent *C. diphtheriae* were isolated from 14.

Agreement of Intradermic with Subcutaneous Test.

The general experience of previous workers rendered it unnecessary to carry out a large series of intradermic and subcutaneous tests in parallel, and the cultures which we submitted to the subcutaneous test were usually chosen because of their special interest and importance.

In our investigations the subcutaneous test has always been done by injecting the entire over-night growth of a Loeffler slope emulsified in saline. It has been applied to 59 cultures of the present series—33 virulent, and 26 avirulent. In every case there was complete agreement in the final results of the two methods.

Impure Cultures.

The testing of impure (primary) cultures from swabs has been studied by Havens and Powell (1922), Force and Beattie (1922), Bull and McKee (1923) and Kelly and Potter (1923). Such primary cultures have been given the name "whole" or "field" cultures by American writers. We have examined the reactions of some 300 cultures before and after purification.

In the case of patients suffering from diphtheria or of profuse "carriers" where upwards of a third of the organisms present in the smear resemble *C. diphtheriae*, the results from pure cultures confirmed in a large percentage of cases those obtained by using impure cultures. Non-specific reactions are, however, more common. The results from impure cultures where less than one-third of the organisms resemble *C. diphtheriae*, have been found unreliable, though a definite positive reaction can be accepted with caution as evidence of virulence. A few contaminating organisms in the test suspension give no difficulty whatever in properly controlled tests. Primary cultures obtained from cases of diphtheria usually contain a preponderating number of *C. diphtheriae* and are nearly always virulent. Only exceptional cultures from carriers show numerous diphtheria bacilli. The application of the primary culture test is therefore strictly limited, since, if the culture comes from a properly diagnosed case of diphtheria its virulence can be predicted, while if it comes from a "carrier" the bacilli are rarely present in such quantities as to render a primary culture test reliable. Table II shows the agreement between primary and final virulence tests in tests on 311 cultures.

Table II.

Showing agreement of Impure and Pure Culture Tests when approximately one-third or more of the organisms present in the Impure Culture were C. diphtheriae.

Agreements		Discrepancies	
Impure culture confirmed by pure culture	Impure culture virulent and pure culture mixed—virulent and avirulent	Impure culture avirulent, and pure culture virulent	Impure culture virulent and pure culture avirulent
290	2	18	1
93·9 %		6·1 %	

The testing of primary cultures to some extent guards against the misinterpretation of one rare occurrence, namely the presence of a culture containing a mixture of avirulent and virulent bacilli. Thus in one undoubted case of diphtheria, where the primary culture had proved virulent, two purified colonies were avirulent—an obviously disconcerting result. On replating from the original swab and testing many colonies virulent and avirulent colonies were separated.

One advantage of tests on impure cultures is that a provisional report can often be made within two or three days from the receipt of the swab.

In conclusion it may be stated that tests on primary cultures which have given virulent results have, with one exception in our series, always been confirmed by tests on the pure culture. On the other hand, it has been frequently found that the impure cultures (especially when less than one-third of the organisms in the smear resemble the diphtheria bacillus) give "avirulent" or "doubtful" readings, while after purification they prove quite definitely virulent.

Discussion.

Recent work on the Schick Test and active immunisation against diphtheria has emphasised the great importance of the virulence criterion for the true diphtheria bacillus in all clinical and epidemiological problems. It is not too much to say that no bacillus should be considered as a source of diphtherial infection until its virulence has been proved. It is true, of course, that the bacilli which can be isolated from what appear to be undoubted lesions of diphtheria are almost invariably virulent, but in the case of patients with less typical lesions or of suspected "carriers," the "suspicious" bacilli which may be present are not infrequently avirulent.

The relationship, if there is any, between avirulent and virulent diphtheria bacilli is still an unsolved problem, but there was every indication throughout this investigation, as indeed throughout almost all investigations on the subject, that the avirulent bacillus is of no public health significance.

Without resort to the virulence test great confusion would arise in assessing the value of the modern methods of diphtheria prophylaxis. The occurrence of avirulent diphtheria bacilli in Schick positive individuals is by no means

uncommon, and should such patients develop a sore throat, a "safety diagnosis" of diphtheria may be made. We have now met with a considerable number of cases where such a "safety diagnosis" of diphtheria had been made in which the bacilli were avirulent. In all these instances the clinician in charge abandoned without misgivings the diagnosis which in the first place was made on bacteriological rather than clinical grounds.

Again, cases occur when a "safety diagnosis" of diphtheria is made in Schick negative individuals who harbour virulent bacilli but who otherwise have little that is abnormal about them. It does not seem yet to be sufficiently realised that the "carriers" properly so-called, with virulent bacilli in their throats, are always Schick negative and are immune to the disease. This is not only indicated from first principles but has been abundantly confirmed in all recent investigations. The Schick positive individual, or, more correctly, the individual with little or no antitoxin in his blood, if he harbours virulent diphtheria bacilli in his throat, has either got diphtheria or is in imminent danger of getting it. He does not appear to be able to "carry" virulent diphtheria bacilli in his throat for more than a day or two without succumbing to their attack.

It is not yet clear that all Schick positive individuals are unable to carry virulent diphtheria bacilli in the nose without symptoms of faucial diphtheria, but it is certainly true in the majority of cases. The subject of nasal diphtheria and its diagnosis is in urgent need of investigation on modern bacteriological and immunological lines, and it is possible that on fuller knowledge it would be found that Schick positive individuals cannot carry virulent *C. diphtheriae* without either suffering from or incubating diphtheria.

Our experience of the applications of virulence testing to diagnosis in fever hospital administration is recorded in the report of Caiger and O'Brien (1924).

The combination of simple microscopic diagnosis of the diphtheria bacillus with the necessity of giving antitoxin to every patient in case of doubt has led to much confusion in handling the administrative problems of diphtheria, and, though it is still important not to withhold antitoxin in doubtful cases, it is equally important concurrently to collect more precise data than has been the custom in the past.

We have pointed out on another occasion (Okell, Eagleton and O'Brien, 1923) the danger involved in managing epidemics in institutions in the usual way by isolating together all those patients showing positive swabs without reference to the virulence of the culture or the state of immunity of the patient. Under such an arrangement there is a considerable risk of susceptible carriers of avirulent bacilli being placed in close contact with non-susceptible carriers of virulent bacilli. The danger to the susceptible individual of such an arrangement is obvious.

The value of virulence testing in School administration has been investigated by Graham Forbes with whom we co-operated in the enquiry. Forbes

calculated that in the case of 33 avirulent "carriers" an average of five months of school life would have been lost in two years, had the result of the virulence test not been known.

In our own experience we have met with many similar cases where it was possible to release from isolation or restriction individuals who had been wrongly thought to be dangerous carriers. In some of these cases hardship to the individuals and the expense to the administrative authority may be great. Under such conditions the omission of a virulence test may be lamentable, and one is forced to the conviction that every important public health body which has to deal with problems of diphtheria should be either equipped with facilities for performing such tests, or at least have access to laboratories where such tests can be done.

The intradermic test is both economical and reliable. From our experience of its practical application we are convinced that it is eminently suitable as a routine method, whenever numbers of cultures are to be tested for virulence.

Summary.

1. In the intradermic test for virulence of "morphological diphtheria bacilli," 0·2 c.c. containing 100 million organisms is injected. The control guinea-pig receives 500 units of antitoxin the night prior to the test. The test animal receives 125 units six hours after the injection of the cultures. A known virulent organism is always injected as a control; 12 to 20 cultures can be tested on one guinea-pig.

2. Of 2368 swabs received, 1388 (59 per cent.) showed morphological *C. diphtheriae*. From these 1286 pure cultures were isolated (92·7 per cent.). In 234 positive swabs from cases of clinical diphtheria, in a series examined under very favourable conditions, morphological *C. diphtheriae* were obtained in 231 (98·7 per cent.) and of these 229 (99·1 per cent.) were virulent. In 758 positive cultures from carriers morphological *C. diphtheriae* were isolated in 715 (94 per cent.); 50 per cent. were virulent *C. diphtheriae*, 39 per cent. avirulent, 3·6 per cent. of Hofmann group, 0·3 per cent. Xerosis and 6·4 per cent. unclassified diphtheroids.

3. Only one culture of intermediate virulence was found, *i.e.* uncertain reactions occurred with a dose of 100 million organisms while clear reactions occurred with 1000 million.

4. Out of 626 positive cultures from cases of diphtheria, both virulent and avirulent bacilli were isolated in two instances, once from the throat and once from the nose.

5. A monovalent antitoxin (Park Williams 8) neutralised all of 819 virulent strains.

6. Amongst 39 cultures obtained from the ear in post-diphtherial conditions, virulent *C. diphtheriae* were isolated fourteen times.

7. Impure primary ("field") cultures gave reliable results (94 per cent.) when more than one-third of the organisms seen in the smear made from the

overnight culture were morphologically *C. diphtheriae*. If fewer *C. diphtheriae* were present in the smear the primary culture gave uncertain results.

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