THE PRACTICAL VALUE OF THE GUINEA-PIG TEST FOR THE VIRULENCE OF DIPHTHERIA BACILLI

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Owing to the frequency of diphtheria and the comparative ease of making bacteriologic examinations in this disease, the laboratory has been freely used as an aid in its diagnosis and management, so that the bacteriology may be considered one of the best developed subjects in the domain of bacteriology.

The morphologic, biologic and tinctorial qualities of diphtheria bacilli are so distinctive that with comparatively little experience they are readily detected in properly made cultures, and if diphtheria bacilli were to be found on mucous membranes only in diphtheria and disappeared in a reasonable length of time after the clinical evidences of infection had disappeared, nothing would be simpler or more satisfactory than the bacteriologic diagnosis of the majority of these infections.

From a practical standpoint, however, the bacteriologic diagnosis of diphtheria is greatly complicated by reason of the fact that many persons harbor diphtheria-like bacilli in the mucous membranes, particularly of the nose and throat, that are apparently harmless and compatible with normal health. In the presence of inflammatory changes in these parts, or following direct exposures to a known case of diphtheria, the presence of these "carrier" bacilli greatly complicates the situation when it is necessary to determine whether or not a condition of infection with diphtheria bacilli may be present.

Likewise, following an attack of diphtheria, the bacilli may persist for long periods on the mucous membranes even though the patient is in all respects restored to health; most physicians have encountered these cases in private and hospital practice when prolonged, expen-

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sive and tiresome quarantine was necessary because of the possible danger of transmitting virulent bacilli to others, until repeated cultures showed the patient to be, temporarily at least, free of diphtheria bacilli.

We now know that these "carrier" cases, both the convalescent and the person who has never had clinical diphtheria, may carry either virulent and disease-producing bacilli or nonvirulent bacilli; in not a few instances both virulent and nonvirulent forms are present on the same mucous membrane. In practice it would be simplicity itself to prolong the quarantine of all convalescents until repeated cultures show the absence of diphtheria bacilli, and likewise to quarantine all healthy "carriers" who have been in contact with a person with clinical diphtheria. These procedures, however, may bring great hardship to a person or family by reason of prolonged isolation and detention; the attending physician may regard lengthy quarantine as a reflection on his ability and a reversal of his opinion regarding the condition of his patient, and he may in this manner suffer in professional reputation.

It is readily understood, therefore, that some practical and reasonably sure means of differentiating virulent from nonvirulent diphtheria bacilli is highly desirable. The test should be one that is easily applied; that is sufficiently delicate to detect the potential harmfulness of bacilli with low grade virulence and it should give the result as quickly as possible.

Before any test, however delicate and accurate it may be, can be accepted as sufficiently safe in practical work to regard a "carrier" as free of danger to himself and others on its evidence, several important questions must be answered with reasonable positiveness: 1. Can bacilli that are without disease-producing powers according to our tests resume virulence and induce disease? 2. May not bacilli of low pathogenicity produce an infection in a second individual of low resistance while causing no apparent harm to the carrier? 3. May not virulent bacilli gradually lose virulence and later resume full pathogenic powers under conditions over which we have no control?

1. Without detailing the large amount of research work by many investigators which these questions have rendered necessary, we may state that the great weight of experimental evidence is to the effect that diphtheria bacilli which have proved nonvirulent with every test, remain so indefinitely despite prolonged efforts to give them even feeble pathogenic powers. A few bacteriologists claim to have successfully accomplished this feat, but their work has not been generally confirmed.

2. It is entirely different, however, with bacilli of low pathogenicity; with these, passage through animals or from throat to throat among men, may readily enough restore a high degree of pathogenicity and for this reason any test for the virulence of diphtheria bacilli must be

one sufficiently delicate to detect the weak pathogenic powers of such cultures. It is reasonable to suppose, and clinical experience supports the contention, that a bacillus of low pathogenicity and apparently harmless to its host, may be capable of harm in a second individual.

3. The third question is more difficult to answer. Many observers have noted that diphtheria bacilli retain their virulence until they disappear, regardless of the length of their stay. We have found this to be true in not a few instances, as with patients fully recovered from diphtheria who continue to harbor virulent bacilli for prolonged periods of time. These instances are readily understood when it is remembered that diphtheria bacilli may retain their virulence for years under the more unfavorable conditions of artificial culture mediums. But in our experience we have also reason to believe that after a time, the minimum being not less than two weeks, a proportion of bacilli prove non-virulent when tested by animal inoculation. Whether or not they regain virulence when passed from throat to throat is the important question, difficult to answer because the disease may be spread in such a varied and confusing manner, but in a considerable experience with these cases we have no direct evidence that this occurs. Most physicians depend on two or three consecutive negative cultures before discharging a patient as cured, but when one stops to consider the fallacies of this method and the goodly proportion of those who escape still carrying their bacilli, as shown by a more extended series of cultures, it is a question whether or not a good virulence test is not to be preferred.

Further than this, we have reason to believe that in some instances the prolonged number of positive cultures following diphtheria are due to the presence of harmless diphtheria-like bacilli of "carriers" that had no connection with the attack of clinical diphtheria. They were present before the attack of diphtheria and are likely to remain for an indefinite time afterward. At first they may be overgrown by the disease-producing bacilli or overlooked in cultures, but as the latter disappear the nonpathogens become prominent again and yield a formidable line of positive cultures.

It is clearly apparent, therefore, that some means of differentiating between pathogenic and nonpathogenic diphtheria bacilli must be available if the diagnosis and control of diphtheria is to be based on bacteriologic examination. Differentiation according to morphology is not usually possible or sufficiently accurate, although it is well known that the granular or beaded and barred types are more likely to be virulent than the solid types; likewise, biologic characters do not serve in this capacity; a rich creamy white growth is more frequently found with nonvirulent, solid types, while the granular, virulent bacilli grow less luxuriantly, but these characteristics are not definite. Acid production

with various sugars is likewise irregular and indefinite, although the granular, virulent types generally produce acids with dextrose and dextrin, while the nonvirulent Hoffman's bacillus produces no acid at all. Between these extremes cultures of various degrees are encountered, and indeed the variations in virulence among diphtheria bacilli have practically no parallel among other pathogenic bacteria.

The animal inoculation test therefore remains the only means now known for determining the virulence of a culture of diphtheria bacilli. To be reliable and satisfactory as a practical measure, an animal inoculation test must fulfill certain fundamental requirements:

1. It must be specific and sufficiently delicate to detect the pathogenicity of bacilli of low virulence on the basis that such bacilli may be capable of harm when transferred to the mucous membranes of persons less susceptible than the carrier.

2. The test must be simple and easily interpreted; also economical and possible of completion in as short a time as consistent with accuracy.

Aside from these requisites certain technical factors, which need not be detailed in this communication, must be considered in order to obtain the best results.

Experimental work has shown that the tissues of the guinea-pig are best adapted for conducting virulence tests, in addition to the general adaptability of this animal for such laboratory purposes.

While there is no parallel between the mechanism of human infections and animal inoculation tests, yet experience has shown that a micro-organism found nonvirulent in a carefully conducted animal inoculation test may be regarded as harmless to human beings, although the final and decisive test is actual transference of these bacilli from throat to throat; and since diphtheria is so frequently spread in such a varied and confusing manner, absolute statements are not justifiable. As pointed out in this paper, our experience has taught us that a properly conducted virulence test may be regarded as reliable and a safe method, and much to be preferred in general, to two, or even three, consecutive negative cultures, in deciding the question of quarantine of a person harboring diphtheria bacilli over a long period of time.

In the Philadelphia Hospital for Contagious Diseases virulence tests have been conducted routinely for the past six years on cases showing positive cultures after two or three weeks' detention in the hospital, and in all cases showing diphtheria or diphtheria-like bacilli but with the diagnosis still more or less in doubt. In this class of cases are included moist or dirty noses, discharging ears or wounds, and inflamed throats showing no exudate.

Reports on the technic and results of these tests have been made by Weston and Kolmer¹ and by Kolmer.²

During the past two years Kolmer and Moshage³ have made a study of various methods for determining the virulence of diphtheria bacilli, with the result that from the standpoints of delicacy and comparatively brief time required for a virulence test the subcutaneous injection into 250 to 300 gm. pigs of 4 c.c. of twenty-four-hour Loeffler culture removed with 10 c.c. of normal salt solution, yielded the best results (Table 1).

TABLE 1.—A SUMMARY OF RESULTS REGARDING THE VIRULENCE OF DIPHThERIA BACILLI AS DETERMINED BY VARIOUS METHODS

Total Examined	Culture Medium	Hours Incu-bation	Route of Injection	Per Cent. Positive Results	Culture Medium	Hours Incu-bation	Route of Injection	Per Cent. Positive Results
37	Pl. Dext. broth	72	Subcut.	86.5	Pl. Dext. broth	72	Intracut...	64.9
23	Pl. Dext. broth	72	Subcut.	65.1	Loeffler.....	24	Subcut. ...	69.6
8	Serum Dext. broth	72	Subcut.	62.5	Loeffler.....	24	Subcut. ...	62.5
27	Pl. Dext. broth	72	Subcut.	74.0	Pl. Dext. broth	24	Intraperit.	63.0
8	Serum Dext. broth	72	Subcut.	75.0	Serum Dext. broth	24	Intraperit.	75.0
27	Pl. Dext. broth	72	Subcut.	63.0	Pl. Dext. broth	72	Intraperit.	55.5
9	Pl. Dext. broth	72	Subcut.	45.5	Pl. Dext. broth	9 days	Subcut. ...	66.6
8	Pl. Dext. broth	72	Subcut.	50.0	Serum Dext. broth	72	Subcut. ...	75.0

The subcutaneous injection of seventy-two-hour serum dextrose broth cultures in doses corresponding to the weights of the animals yielded good results but required more time for the conduct of the test; likewise the intraperitoneal injection of twenty-four-hour serum dextrose broth cultures yielded good results and had the merit of showing the result in less time than the subcutaneous method. The intracutaneous injection of 0.1 c.c. of seventy-two-hour plain dextrose broth cultures was found inferior to the subcutaneous injection of larger doses, as 1.3 c.c. for a 260 gm. pig; furthermore, the results of the intracutaneous injection were more difficult to read, especially with bacilli of a low degree of virulence. For further details and results we refer the reader to the paper mentioned.

During the past four years 1,054 virulence tests have been conducted, and the object of this paper is to report on the results observed and their practical value in the management of diphtheria.

1. Weston, P. G., and Kolmer, J. A.: Jour. Infect. Dis., 1911, viii, 295.
2. Kolmer, J. A.: Jour. Infect. Dis., 1912, xi, 56.
3. Kolmer, J. A., and Moshage, E. L.: Jour. Infect. Dis. (To be published).

TECHNIC

The technic employed in conducting the tests herein reported may be briefly described as follows:

1. Isolation of the bacilli by the "streak" method on plates of Loeffler's blood serum medium.

2. The inoculation of tubes of plain 0.2 per cent. dextrose broth with a reaction of +0.8 with several different colonies so as to make more certain that the cultures will contain virulent bacilli if the plates contain colonies of virulent and nonvirulent bacilli.

3. Cultivation of the broth cultures at 35 to 37 C. for seventy-two hours (tubes in a slanting position so that the bacilli may receive the maximum of oxygen, which favors toxin production).

4. The examination of the broth culture for purity, followed by the subcutaneous injection in the median abdominal line of a 250 to 300 gm. pig of a dose of unfiltered culture corresponding to 0.5 per cent. of the weight of the animal expressed in cubic centimeters. The total amount injected is brought up to 4 c.c. by the addition of sterile normal salt solution in order to equalize pressure and absorption.

5. The animal is observed for at least four days for evidences of local inflammatory edema and general toxemia. If death occurs the animal is necropsied for the typical changes of diphtheritic infection, namely, local inflammatory edema, congestion of the suprarenal glands, pulmonary changes, etc. Cultures are prepared from the site of injection and the internal organs.

6. Frequently and especially when in doubt of a result and with positive or presumably virulent cultures, a second pig is injected with the same amount of culture, plus 1 c.c. of a 500-unit antitoxin serum.

7. Local edema and evidences of toxemia are regarded as indicating virulence of a culture, even though the animal does not succumb in the usual period of four days.

As will be noted, unfiltered cultures are used entirely because the soluble toxins secured by filtration are but one element of the pathogenicity of a culture, and in virulence tests it is desirable to include a test of all possible pathogenic agents.

Types of Bacilli.—In classifying the cultures submitted to the virulence test the types were recorded after the classification of Westbrook, Wilson and McDaniel. For the sake of brevity all the types found in the cultures are not recorded. For example, in a given culture of granular bacilli Types A, B and C may be found, although if one predominates, the culture is recorded according to that type. The cultures for the virulence test were recorded as follows:

- A. Granular bacilli, A, B, C and D—mostly C.
- B. Barred bacilli, A₁, B₁, C₁ and D₁—mostly B₁.
- C. Solid bacilli:

Long solid—A₂, B₂ and C₂—mostly C₂.

Short solid—D₂ and E₂—mostly D₂.

RESULTS

The results of 1,054 tests conducted after the method outlined with granular, barred and solid bacilli from the throat, nose, ear and other mucous membranes, are shown in Tables 2, 3, 4 and 5.

TABLE 2.—RESULTS OF VIRULENCE TESTS WITH 551 THROAT CULTURES OF DIPHTHERIA BACILLI

Types	Total	Results		Per Cent. Positive
		+	—	
Granular (mostly C).....	202	141	61	69.8
Barred (mostly B ₁).....	17	10	7	58.8
Long solid (mostly C ₂).....	311	144	167	46.3
Short solid (mostly D ₂).....	21	21	20	4.7

TABLE 3.—RESULTS OF VIRULENCE TESTS WITH 261 NOSE CULTURES OF DIPHTHERIA BACILLI

Types	Total	Results		Per Cent. Positive
		+	—	
Granular (mostly C).....	102	74	28	72.5
Barred (mostly B ₁).....	7	5	2	71.4
Long solid (mostly C ₂).....	129	52	79	40.3
Short solid (mostly D ₂).....	23	0	23	0

TABLE 4.—RESULTS OF VIRULENCE TESTS WITH 217 EAR CULTURES OF DIPHTHERIA BACILLI

Types	Total	Results		Per Cent. Positive
		+	—	
Granular (mostly C).....	105	70	35	66.6
Barred (mostly B ₁).....	6	5	1	83.1
Long solid (mostly C ₂).....	73	31	42	42.4
Short solid (mostly D ₂).....	83	1	82	3.0

TABLE 5.—RESULTS OF VIRULENCE TESTS WITH 25 MISCELLANEOUS CULTURES OF DIPHTHERIA BACILLI

Sources	Types	Total	Results		Per Cent. Positive
			+	—	
Penis.....	Long and short solids.....	6	0	6	0
Vagina.....	Long solids.....	6	0	6	0
Eye (normal).....	Long solids.....	4	0	4	0
Gallbladder.....	Long solids.....	1	0	1	0
Skin.....	Long and short solids.....	8	0	8	0

Table 6 is a summary of the virulence of the different types of bacilli from the different sources.

TABLE 6.—COMPARATIVE VIRULENCE OF THE DIFFERENT TYPES OF BACILLI FROM DIFFERENT SOURCES

Type of Bacilli	Percentage of Positive Results of Cultures			
	From Throat	From Nose	From Ear	From Various Other Sources
Granular (mostly O).....	69.8	72.5	66.6	None tested.
Barred (mostly B ₁).....	58.8	71.4	88.2	None tested.
Long solid (mostly C ₂).....	46.3	40.3	42.4	0
Short solid (mostly D ₂).....	4.7	0	3.0	0

An examination of these tables shows the following:

1. The granular and barred types of bacilli give the highest percentage of positive tests. In clinical diphtheria they are almost invariably virulent for guinea-pigs. No matter how long they persist in the throat after recovery, they should be regarded as dangerous until proved otherwise. Of all types they are most likely to retain their virulence.

2. The value of animal inoculation tests is especially appreciable when dealing with solid types of bacilli. The longer types are more likely to retain virulence than the shorter varieties. Both, however, may be descendents of the "carrier" bacillus rather than of the bacillus producing the clinical evidences of the disease.

3. The short solid types of bacilli are especially common in the nose in the absence of clinical diphtheria. In a small percentage of cases these bacilli may be truly pathogenic but the majority of cultures are found without virulence when tested by animal inoculation.

4. The long solid types of bacilli, so frequently found in otitis media, are in the majority of cases nonvirulent.

5. Animal inoculation tests have a special field of usefulness in testing the solid types for virulence when they persist in the tissues over a long period of time or when found after contact with "carrier" cases.

6. Cultures of the short solid D₂ types have proved nonvirulent with such regularity that whenever we are sure that the type is true we do not hesitate to report negatively on the culture and release quarantine. Although this method has been in practice for several years and concerned the discharge of hundreds of cases, no return cases can be attributed to the presence of these bacilli in the throats or noses of their hosts.

The majority of the cultures tested by us were from patients who had been in the hospital for fifteen to twenty-two days, and after the disappearance of clinical evidences of the disease. A few cultures were tested in the first week of the disease, especially when the clinical evidences of diphtheria were slight or atypical.

The results of the virulence tests according to the day of disease are shown in Tables 7 and 8.

Table 7 is a summary of the positive results observed with granular or beaded bacilli from the throat, nose and ear; Table 8 is a similar summary of the positive results with the long, solid C_2 types of bacilli. The numbers of cultures tested of barred bacilli were too few to prepare a table, and the short solid B_2 types of bacilli were uniformly without virulence in these tests.

TABLE 7.—A SUMMARY OF POSITIVE VIRULENCE TESTS WITH GRANULAR DIPHTHERIA BACILLI AT VARYING INTERVALS AFTER INFECTION

Week of Disease	Percentage of Positive Virulence Tests		
	Throat	Nose	Ear
1	100	70	50
2	70	100	68.7
3	67.5	80.6	80.0
4	73.9	75.0	60.0
5	83.3	75.0	66.6
Over 5	91.0	86.6	100.0

TABLE 8.—A SUMMARY OF POSITIVE VIRULENCE TESTS WITH SOLID DIPHTHERIA BACILLI AT VARYING INTERVALS AFTER INFECTION

Week of Disease	Percentage of Positive Virulence Tests		
	Throat	Nose	Ear
1	None tested	40.0	50.0
2	68.2	14.3	14.3
3	41.7 ^a	41.3	37.5
4	51.1	42.8	30.0
5	57.1	28.5	None tested
Over 5	47.7	40.0	0

These tables are not composed of the results observed of testing the cultures of a group of patients at regular intervals, but were compiled from the 1,054 tests.

As shown in these tables, a percentage of cultures retain their virulence for indefinite periods after the disappearance of clinical evidences of infection. In other words, virulent bacilli are likely to retain their virulence for prolonged periods when tested at intervals, so that negative virulence tests are not to be expected on the basis of duration of

disease alone; in a given case the tests are likely to yield persistently positive results as long as that particular culture of diphtheria bacilli is secured in culture. As previously stated, however, in our experience a small percentage of cultures presenting the same morphology were observed to lose virulence as tested at varying intervals; it is probable, however, that the majority of negative virulence tests following previous positive results are due to the fact that the virulent bacilli have gradually disappeared or their colonies were overlooked in the cultures.

PRACTICAL VALUE OF VIRULENCE TESTS

In the handling of diphtheria, second only in importance to the prompt, vigorous and specific treatment of the disease itself is the determination of the time when the individual ceases to possess the power of infecting others. This applies to "carriers" as well as to convalescents from clinical diphtheria.

As already stated, virulence tests may serve a useful function in differentiating between virulent and nonvirulent diphtheria bacilli when the micro-organisms persist over long periods of time on the mucous membranes or in persons who carry these bacilli, either after or without contact with a clinical case of diphtheria.

For the proper management of diphtheria according to bacteriologic examinations, an effort must be made to differentiate between the harmful and harmless bacilli. For instance, a "carrier" may be quarantined forever if guided by the fact that the presence of a diphtheria-like bacillus means diphtheria. The medical attendant must be convinced of the value of bacteriologic examinations and of the earnest efforts of the bacteriologist to work with him both in the interests of his patient and for the public welfare. The practitioner must be taught not to be afraid to report all of his cases and to culture faithfully. If, however, bacteriologic examinations serve to give unnecessary embarrassment to the practitioner by quarantining "carrier" cases, or by maintaining a quarantine unnecessarily long after convalescence as a result of conscientious efforts on the part of the latter to do his duty in taking cultures, he is not likely to give his best cooperation, and without this no real good is possible.

In the Philadelphia Hospital for Contagious Diseases in all instances in which the virulence test is negative the patient is considered noninfectious and fit for release from quarantine, regardless of what the cultures may show. These cases have been followed closely and the number of "return" cases has become practically negligible. On the other hand, we have on several occasions observed that convalescent children harboring virulent bacilli as determined by animal inoculation test, but subsequently released from quarantine on the basis of two or

three negative cultures, have been responsible for the infection of others.

The results have shown quite definitely that the virulence test is the safest and nearest perfect method that has been devised, and regardless of all things else, in the face of negative pig test, the patient is discharged; in the face of a positive test he is detained.

If the physician will take cultures carefully, conscientiously and freely, especially several cultures, before excluding the diagnosis of diphtheria in a given case, and if three consecutive negative cultures are required instead of two before quarantine is lifted, combined with the efforts of the bacteriologist to differentiate between virulent and nonvirulent bacilli, with the judicious use of the animal inoculation test in suitable cases, we believe that the bacteriologic diagnosis and management of diphtheria will be efficient and satisfactory to all.

CONCLUSIONS

1. Animal inoculation tests have been found of great value in determining the virulence of diphtheria bacilli.

2. Virulence tests are the only practical and safe means of determining the possible harmfulness of diphtheria bacilli.

3. Virulence tests have been found useful in the management of diphtheria bacillus "carriers," especially from the standpoint of duration of quarantine. This applies to healthy "carriers" discovered as the result of cultures, and to convalescents and persons showing atypical or slight clinical evidences of infection of the throat, nose, eye, wounds, etc., with cultures showing the presence of diphtheria bacilli.

4. Virulence tests in order to be practical and reliable must be sufficiently delicate to detect low degrees of virulence and this constitutes the first and important essential; it is desirable to conduct the test as economically and as speedily as consistent with specificity and delicacy.

5. Subcutaneous injection of 250 to 300 gm. guinea-pigs with cultures was found to yield better results than intracutaneous and intraperitoneal injection. The subcutaneous injection of 4 c.c. of a good twenty-four-hour culture of diphtheria bacilli on a tube of Loeffler's medium washed off with 10 c.c. of normal salt solution, was found as delicate and specific in its results as any method, besides being a test requiring a short space of time for its conduct.

A second method yielding excellent results, but requiring more time, consists in cultivating the bacilli in serum dextrose broth for seventy-two hours and injecting 250 to 300 gm. pigs subcutaneously with 1.3 to 1.5 c.c. of unfiltered culture.

6. Local edema and even slight evidences of toxemia of the test animal with absence of those signs in the antitoxin control, are regarded

as indicating virulence even though the animal does not succumb in the four-day period of observation.

7. Granular types of diphtheria bacilli were found virulent in about 70 per cent. of cultures from the throat, nose and ear; barred bacilli were found of equal virulence with the granular types. Long solid bacilli were found virulent in about 42 per cent. of cultures; cultures of short solid bacilli were found uniformly to possess no virulence.

8. Virulence tests were found of most value in determining the potential harmfulness of the solid varieties of diphtheria bacilli because these types are especially likely to be found in carriers.