The *in vitro* Test for Virulence of *Corynebacterium diphtheriae*

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THE testing of *C. diphtheriae* for virulence has been carried out, until very recently, exclusively on experimental animals, though the need for a reliable *in vitro* test has long been recognized. Using the principle of toxin-antitoxin flocculation devised by Nicolle, *et al.*,[2] for titrating the L_f unit of toxin, Elek [1] has worked out an *in vitro* method which seems entirely reliable when certain conditions are strictly adhered to.

The test, as devised by Elek, consists of the preparation of an agar base which must be clear and contain 0.3 gm. maltose and 0.07 ml. lactic acid per 100 ml. To this melted, cooled base is added 20 per cent normal horse serum, and the mixture is poured into a Petri dish (100 x 10 mm). A sterile filter paper strip, 6 x 1.5 cm., is dipped into diphtheria antitoxin containing 1,000 u/ml. drained, and placed in the center of the dish and allowed to sink into the warm agar. After solidification, the plates are dried in the incubator and inoculated on the same day. Four organisms may be tested on each plate; a known virulent strain should be included.

In describing the test and the nature of the reaction Elek says [1]:

"If the organism to be tested is inoculated in the form of a wide line at right angles to the filter paper the toxin produced by the organism will diffuse out in descending concentrations, just as the antitoxin diffuses from the filter strip. In these circumstances the points of optimum proportions for toxin-antitoxin reaction fall on a continuous line, and the flocculation which occurs along this line provides striking graphic representation of the law of optimal ratios. On each side of the inoculum a white line develops at an angle, so that a toxicogenic strain becomes virtually marked with an arrow-head."

While endeavoring to confirm Elek's work by running a long series of virulent and avirulent cultures in comparison with tests in animals, sources of error were noted and are here described.

Preliminary Experiments with Materials

A. *Preparation of Agar Base*

The first lot of medium was prepared according to the original technique. It was soon found that a clearer agar solution was obtained by omitting both filtration through paper pulp and clarification with charcoal. Difco granulated agar was used and the base was prepared by dissolving and titrating all ingredients together. The base was tubed and divided into two parts. One half was sterilized in flowing steam for 30 minutes on 3 successive days as recommended by Elek. The other half was autoclaved at 15 lbs. for 15 minutes.

Preliminary experiments were made to determine the efficacy of autoclaving. Eight different virulent strains were used, and no significant difference could be noted between the two methods of sterilization.

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Preliminary experiments to determine the optimum pH indicated that the reaction took place in agar most readily between pH 7.6 and 8.2. More acidic or basic media inhibited the reaction of some strains.

While working with the base medium, several media commonly used in many laboratories were tried. Difco Heart Infusion Agar and Proteose No. 3 Agar were found to be unsatisfactory even with the addition of maltose and lactic acid in the recommended amounts.

As a result of the preliminary experiments preparation of the basal medium was modified for use in our work as follows:

Proteose peptone—Difco	20 gm.
Maltose Difco	3 gm.
Lactic acid C. P.	0.7 ml.
Agar, granulated Difco	15 gm.
Sodium chloride C. P.	5 gm.
Distilled water	1,000 ml.

Dissolve the ingredients in a boiling water bath and adjust to pH 7.8. Dispense in desired amounts by measuring fairly accurately. Autoclave at 15 lbs. for 15 minutes. Store in icebox.

B. *Antitoxin*

Different concentrations of antitoxin for the filter paper strips were tried. It was found that a dilution containing 500 u/ml. (units of diphtheria antitoxin per ml.) was as satisfactory as the 1,000 u/ml. originally recommended.

C. *Petri Dishes*

For use with the regular-sized Petri dish (100 x 10 mm.) filter paper strips were cut to measure 6 x 1.5 cm. and were sterilized by hot air. Agar base was dispensed in 10 ml. amounts, to which were added 2 ml. of serum. One filter strip was placed in the center of each dish, which could accommodate 4 tests.

When running large numbers of cultures it was found convenient to use Petri dishes measuring 150 x 15 mm. Filter paper strips were cut to measure 10 x 1.5 cm. For these dishes, agar base was tubed in 25 ml. amounts to which 5 ml. of serum were added. Two filter paper strips were placed in each dish, one on each side. This made it possible to make 14 tests per dish.

D. *Serum*

As horse serum is difficult to obtain in some laboratories it was thought advisable to compare sera from other animals and to substitute a more readily available serum if it proved to be satisfactory. Sera from horse, sheep, rabbit, and human beings were compared using 36 strains of *C. diphtheriae*. Rabbit serum was found to be as effective as horse serum. Sheep serum was also satisfactory, but human serum was definitely inferior. As rabbit serum was easily available, it was used throughout this study. Citrated and oxalated plasma were found to give no reactions whatsoever.

PERFORMANCE OF THE TEST

The present technique for preparing the plates is as follows:

Melt the agar base and cool to 50° C. Add 20 per cent sterile serum, mix, and pour into a sterile dish. While the agar is fluid, place the filter strips, which have previously been saturated with diphtheria antitoxin, (500 u/ml.) in the desired position and press into the agar. Allow the plate to harden with the cover partially removed to give a dry surface, and dry further in the incubator for several hours before streaking. For this study plates were inoculated the same day they were prepared. Inoculations are made with a loop approximately 3mm. in diameter. A loopful of broth culture (24–72 hours old) is streaked in a continuous line at right angles to the paper strip across it, and at least ½ to ¾ inch on both sides of it. Inoculum should be large enough to spread easily but not large enough to spread out of bounds.

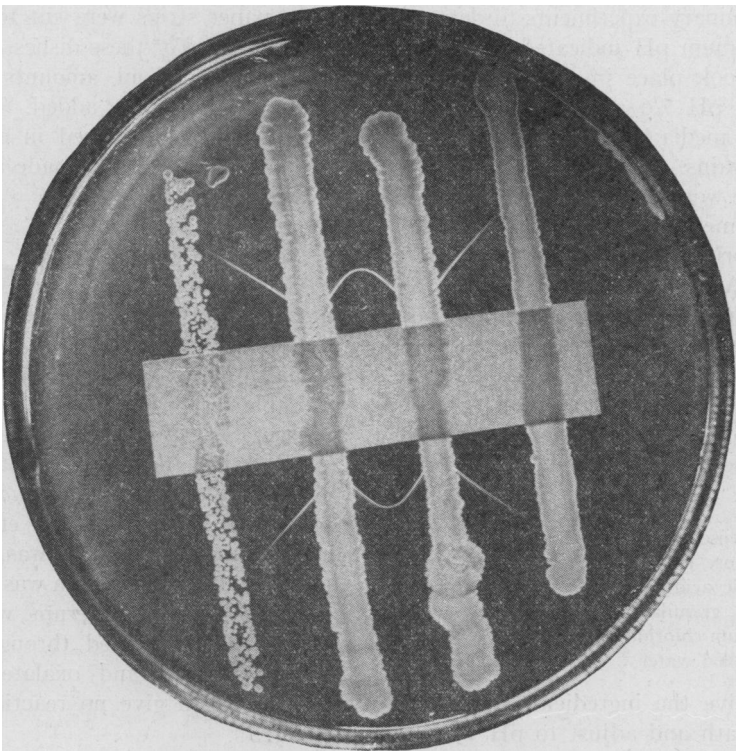

FIGURE 1—The *in vitro* virulence test in a 100 x 15 mm. plate observed after 72 hours at 37° C. For method of preparation see text. The two central cultures are virulent and each forms a "4s" reaction (four well marked oblique lines of precipitation). The two outer cultures are avirulent. The tips of the lines of precipitation between the two central streaks of growth have merged, forming arcs.

The surface of the agar should not be broken. Preliminary tests indicate that growth on slants may be used successfully and may even prove superior to broth inoculum for the *minimus*[3] cultures since these grow lightly in broth. The inoculated plates are incubated at 37° C.

Readings are made at 24, 48, and 72 hours. At 24 hours the lines of reaction between toxin and antitoxin may be very faint, and it is often necessary to search for them with a hand lens. A strong source of illumination is needed, and an ordinary gooseneck lamp with a 100 w. bulb, shaded, has been found to be satisfactory. Readings are made by viewing the unopened plate from the bottom while moving it from side to side. A little experience is needed to recognize early reactions, but strong reactions may be seen plainly without the lens and in any good light.

Extending outward from the growth of each virulent organism there appear two pairs of fine white lines of flocculation, one pair of lines on each side of the filter strip. The two lines of each pair project from each of the sides of the line of inoculation at angles of approximately 45° to the line of inoculation and away from the filter paper. Each pair of lines suggests the "arrow-head" which Elek mentions. The lines arise 6–8 mm. away from the filter paper. Early reactions are seen close to the growth of the organism and the lines increase in length

and intensity as the incubation time increases. These reactions are shown in Figure 1.

All four lines do not appear simultaneously, nor do they show the same intensity. It is therefore convenient, in reading the results of tests, to record the number of lines appearing, with a statement of intensities. For example, a reading of 1w,2s would indicate 1 weak and 2 strong flocculation lines, with the fourth line not appearing at the time the reading was made.

Strong reactions are those which may be seen without a hand lens. Weak reactions are seen only with difficulty without magnification.

A few avirulent organisms produced very indistinct lines and several produced an excessive number of lines at various abnormal angles to the line of inoculation. These developed later than 72 hours, usually only after 5 days at 37° C. These reactions, because of their diffuseness, or abnormal numbers, or late development, cause no confusion after their true nature was recognized.

RELATION OF *in vitro* VIRULENCE TEST TO TYPE OF *C. diphtheriae*

At the time this study was undertaken, large numbers of field cultures were being received from the Georgia State Board of Health. These were streaked on cystine-tellurite agar [4] for isolation. Pure cultures, morphologically positive or doubtful, were typed according to McLeod's [5] method of classification.

Virulence tests were run on rabbits with 48 or 72 hour broth cultures, and the same broth cultures were used to determine virulence by the *in vitro* method.

One hundred and forty-one cultures thus tested showed complete agreement between the rabbit and the *in vitro* virulence tests. A review of the strains tested showed that they fell into the following types:

Type	*Virulent*	*Avirulent*
Gravis	31	1
Gravis-like	13	0
Mitis	18	2
Mitis-like	38	7
Indeterminate	2	1
Diphtheroids	–	28
Totals	102	39

As no minimus strains had been isolated while this study was in progress, 24 virulent minimus strains, isolated earlier in the year, were taken from stock. At the same time a number of avirulent strains of various other types were selected for testing.

The *in vitro* reactions produced by the virulent minimus strains were generally of lesser intensity and were slower in appearance than those of mitis or gravis strains. Tests with minimus strains were also adversely affected by various factors which had little or no influence on the reactions of other strains.

For example, the first 24 minimus strains tested were included along with a series of 38 other virulent and avirulent strains. This series of test plates was inoculated from 72 hour broth cultures. The minimus reactions were weak or negative in 24 hours, but all of these strains gave strong readings in 48 hours. However, in another test in which these same 62 cultures were streaked on the test plates from solid medium, variable and unsatisfactory results were obtained.

RELATION OF SERUM TO *in vitro* VIRULENCE TEST

In seeking for an explanation of the unsatisfactory results it was noted that the rabbit serum used in preparing the agar for the tests showed considerable hemolysis. There were thus two new factors in this experiment: inoculation from slants of solid medium (Pai slants) and hemolysis in the rabbit serum. To determine which factor, if either, was

TABLE 1

Effect of Hemoglobin (or Hemolysis) on in vitro Virulence Test

Culture Number	Type of Culture	Clear Medium						Slight Hemolysis						Marked Hemolysis
		24 hrs.		48 hrs.		72 hrs.		24		48		72		72
		S	B	S	B	S	B	S	B	S	B	S	B	S and B
A-1409a	Mitis.	..	3w	3s	4s	4s	4s	..	..	1w	..	1w	..	..
A-366a	Min.	2w	..	3s, 1w	2w	4s	4s	..	..	..	..	..	..	..
A-389	Min.	1w	..	4s	4s	4s	4s	..	..	..	..	..	..	..
A-391	Min.	..	..	4s	4w	4s	4s	..	..	..	..	..	..	..
A-418	Min.	4w	..	4s	4s	4s	4s	..	..	..	..	..	..	..
A-480	Min.	3w	..	4s	4s	4s	4s	..	..	..	..	..	..	..
A-492	Min.	4w	1w	4s	4s	4s	4s	..	..	..	..	..	..	..

S = Slant inoculum
B = Broth inoculum

Figures = Number of lines of flocculation
w = Weak reactions
s = Strong reactions

responsible for the poor results, 7 strains (6 minimus and 1 mitis), each in both 48 hour broth cultures and on Pai slants, were used as inoculum. Rabbit serum was collected carefully so as to avoid hemolysis, and was separated from the clot as soon as possible. Hemolysed erythrocytes were prepared by washing 2 ml. of defibrinated rabbit blood with saline, then lysing 1.5 ml. of packed cells with 13.5 ml. of distilled water. Three tubes each containing 25 ml. of agar base were melted and cooled to 50° C. To each was added the following:

Tube	*Clear Serum*	*Distilled Water*	*Red Cell Solution*	*Resulting Medium*
1	5 ml.	1 ml.	0	Clear
2	5 ml.	0.7 ml.	0.3 ml.	Slightly hemolysis
3	5 ml.	0	1 ml.	Marked hemolysis

Test plates, with paper strips and antitoxin, were prepared from these 3 tubes in the usual manner. Slant and broth cultures of each organism were streaked side by side on each of the three types of media. Results are as shown in Table 1.

In Table 1 it is clear that inoculations may be made from slants even more successfully in the case of minimus cultures than from broth. It is also clear that some substance was released from the erythrocytes when lysed that markedly inhibited the reactions, particularly those of the minimus strains.

To determine the extent to which the method of preparation of the serum affected the reaction two rabbits were bled and equal portions of their blood were pooled in three large tubes. The three tubes were treated as shown below.

The results obtained with these sera in the *in vitro* test are shown in Table 2. The results obtained with both types of inoculum were essentially the same.

Tube 1	(A) Centrifuged immediately and serum removed	Only slightest tinge of hemolysis
Tube 2	Placed in icebox overnight. Next day;	
	(B) Centrifuged, 5 ml. serum removed.	Same degree of hemolysis as (A)
	(C) Clot and remaining serum stirred. Centrifuged and serum removed.	Slightly more hemolysis than (B)
Tube 3	Placed in icebox for 48 hours.	
	(D) Centrifuged and serum removed.	Same degree of hemolysis as (A)
	(E) Clot and remaining serum mixed. Centrifuged and serum removed.	More hemolysis than (C) but still very slight

TABLE 2

Effect of Method of Preparing Serum on the in vitro Virulence Test

Culture Number	Type of Culture	Tube 1 (A) 24 hrs.	Tube 1 (A) 48 hrs.	Tube 2 (B) 24	Tube 2 (B) 48	Tube 2 (C) 24	Tube 2 (C) 48	Tube 3 (D) 24	Tube 3 (D) 48	Tube 3 (E) 24	Tube 3 (E) 48
A-391	Min.	2w	4s	..	4s	..	2w	..	..	..	..
A-366a	Min.	4w	4s	2w	4s	..	4w	..	..	..	..
A-389	Min.	4w	4s	..	4s	..	3w	..	..	..	..
A-1307a	Mitis-like	3s, 1w	4s	4s	4s	2s, 2w	4s	4s	4s	4s	4s

This experiment indicated that prolonged contact of serum with the clot materially affects the value of the medium for virulence tests with minimus strains.

This reduction in reactivity seemed out of proportion to the amount of hemoglobin released by the red cells. As potassium is also released in comparatively large amounts during hemolysis, graded concentrations of KCl up to 2 mg. per cent were added to tubes of basal medium. When used for *in vitro* virulence tests, the presence of the KCl did not cause any inhibition of the reactions. As small amounts of iron are known to inhibit toxin production, it is suggested that iron may be responsible for the unsatisfactory results in media containing much free hemoglobin.

The following method for collection of serum has been found to give consistently large yields of serum free of hemolysis:

Withdraw, aseptically, 50 ml. of blood from the heart of a rabbit; with as little force as possible place the blood in a sterile Petri dish 150 x 15 mm. in size. Allow this to stand for several hours then remove the exuded serum with a pipette. Do not break or in other ways disturb the clot. Centrifuge the serum twice to make sure that all erythrocytes have been removed.

The usual method of placing the blood in a test tube and loosening the clot before centrifugation has been found to be unreliable as some lysis occurs when the clot is disturbed in any way.

PRACTICAL APPLICATION OF THE *in vitro* VIRULENCE TEST

Using the previously noted modifications of the original test, and using, in the cases of minimus cultures, serum previously tested for reactivity with minimus strains, a total of 290 cultures were tested for virulence by the *in vitro* and *in vivo* methods during this study. Some of these tests were made during the experiments referred to above. There was complete agreement between the two methods. The data are shown in Table 3.

TABLE 3

Comparison of in vitro with in vivo Virulence Tests

Type Strains	Virulent Cultures: in vitro	Virulent Cultures: Rabbit	Avirulent Cultures: in vitro	Avirulent Cultures: Rabbit
Gravis	31	31	4	4
Gravis-like	15	15	4	4
Mitis	30	30	10	10
Mitis-like	62	62	45	45
Minimus	24	24	6	6
Indeterminate	3	3	9	9
Saccharose positive	2	2	..	..
Diphtheroids	..	..	49	49
	167	167	123	123

DISCUSSION

Assuming an effective agar base is used, and such obvious factors as pH, type of inoculum, and amount of anti-

toxin are properly adjusted, it seems evident that the most important other factor in assuring correct results by the *in vitro* virulence test for *C. diphtheriae* is the collection and preparation of the serum used in the agar base. Since many lots of serum are not satisfactory even though they show only very slight hemolysis, it is important to test each different lot for reactivity especially *with minimus strains*. Most specimens of serum will be satisfactory for the majority of other strains of *C. diphtheriae*. This is shown by the fact that in the first long series of tests, when no minimus strains were encountered, complete agreement was obtained with the animal test although no special precautions were observed in the collection of the serum.

SUMMARY

The study of the *in vitro* virulence test described in this paper has led to the following observations concerning it:

1. In preparing the medium it was found satisfactory to:
   a. Use purified Difco agar in a simplified formula which omits clarification.
   b. Sterilize by autoclave.
   c. Use diphtheria antitoxin in concentrations of 500 u/ml.
2. The optimum pH range for the reaction was found to lie between pH 7.6 and 8.2.
3. Difco Heart Infusion Agar and Proteose No. 3 Agar with maltose and lactic acid added are unsatisfactory basal media.
4. Rabbit and sheep sera were shown to be as satisfactory as horse serum. Human serum seemed to be less satisfactory than the others.
5. The reaction of minimus strains is inhibited by using serum faintly colored by lysis of erythrocytes. Other strains are less affected by this factor.
6. Some unknown substance or condition, which interferes with the *in vitro* virulence test, appears in the serum when it remains in contact with the clot for one day or longer. This may cause a high percentage of falsely negative reactions, particularly in testing minimus strains.
7. In a series of tests, including 167 virulent and 123 avirulent strains of all types, complete agreement was obtained between the *in vivo* and *in vitro* tests when the technique described in this paper was followed.
8. A positive reaction in the *in vitro* virulence test seems to be an entirely reliable indication of virulence of *C. diphtheriae*.
9. A negative reaction in the *in vitro* virulence test may be given by some virulent strains unless conditions favorable to a positive reaction by those strains be provided. In the present state of our knowledge, conditions favorable for a positive reaction by all virulent strains cannot be stated with certainty. Therefore, any negative reaction to the *in vitro* virulence test should be verified by animal inoculation.
10. The test deserves further study.

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