THE RECOGNITION OF TOXICOGENIC BACTERIAL STRAINS IN VITRO

BY

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The detection of toxicogenicity *in vitro* has not in the past proved a practical proposition for routine purposes, and this paper is concerned with a new technique which, it is believed, makes this possible. In diagnostic bacteriology most pathogens are recognized by their cultural and biochemical properties, and are finally characterized by serological study of their antigenic structure. In those groups of organisms which produce disease through the agency of exotoxins the surface antigenic pattern is of little diagnostic use, and final recognition is effected by specific serological neutralization of the toxin in a susceptible experimental animal. Clearly a rapid test is required to bring exotoxin-producing organisms into line with those which can be identified serologically. Technical difficulties have, however, prevented the use of *in vitro* toxicogenicity for diagnostic purposes. The lecithinase action of the alpha toxin of *Clostridium welchii* is an exception to the rule that toxins rarely produce convenient effects *in vitro*. Flocculation of diphtheria toxin with its homologous antitoxin was first demonstrated by Nicolle, Césari, and Debains in 1920 and is widely used for titration of antitoxin (Ramon, 1922), but the procedure in its original form is too complex for routine identification of toxicogenic strains.

Petrie and Steabben (1943) introduced a technique for the identification of the pathogenic clostridia of gas-gangrene in plate cultures. Crude specific antitoxic serum was incorporated in an agar-base medium designed to yield abundant toxin production. After prolonged anaerobic incubation the plates were left at room temperature, and under these conditions a ring of opacity developed around the colonies of a toxicogenic strain in the presence of the homologous antiserum. The zone of opacity was shown by the plate microscope to consist of Liesegang rings, and was accepted as a specific reaction.

The same workers attempted to employ this technique for the recognition of toxicogenic strains of *Corynebacterium diphtheriae*, but the results were unsatisfactory. Repetition of their experiments suggested to me three main reasons for the failure. In the first place, isolated colonies produce only minute amounts of toxin even after prolonged incubation. Secondly, as flocculation occurs only within a narrow zone, and as both toxin and antitoxin excess may inhibit the reaction, it is necessary to adjust the antitoxin content of the plate most carefully; strain variations in toxicogenicity make it very difficult to gauge the antitoxin concentration so as to be within the optimum range. Thirdly—and this is perhaps the most serious difficulty—certain aerobic and anaerobic organisms may produce a zone of opacity around their colonies when grown in serum-agar media. Clearly this non-specific reaction may lead to erroneous interpretation, especially as a number of strains of *C. diphtheriae* produce concentric rings irrespective of whether they are toxicogenic or not. The methods described below were devised to overcome these difficulties and to place in the hands of laboratory workers a technique which it is hoped will find a useful routine application.

The New Technique

In working out this technique three principles have had to be considered: to obtain maximal and rapid toxin

production; to produce an antitoxin gradient in the medium so as to give a reaction with varying toxin concentrations; and to provide non-specific serum constituents in sufficient quantity to make the flocculation reaction easily visible. The nutritional and gaseous requirements for rapid toxin production in most toxicogenic organisms are well known. In the case of *C. diphtheriae* a modified Pope's medium was found suitable, whereas for staphylococci a slight modification of Walbum's medium proved satisfactory. Inoculation in the form of a wide streak will yield *pari passu* with increased bulk of growth far more toxin than a single colony. The minimum length of incubation can be determined only by experiment: it is the shortest time which will reveal all virulent strains as toxicogenic.

The required antitoxin gradient can be achieved by the simple method of placing on the surface of the medium a strip of filter paper moistened with commercial antitoxin in suitable dilution. The concentration of antitoxin at any point on the plate is determined by the laws of diffusion

FIG. 1.—Completed test with corynebacteria. Strain 1=positive control; strain 2=non-toxicogenic; strain 3=toxicogenic.

governing this complex system, and varies from a maximum (corresponding to the concentration of the antitoxin used) to a minimum in the part of the plate most remote from the strip. In order to provide non-specific serum constituents in sufficient quantity to make flocculation reaction easily visible it has been found better to incorporate 20% normal horse serum in the medium rather than to rely on the constituents of the crude antitoxic serum, which diffuse at a different rate from the immune globulins.

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 (Fig. 1).

The principles outlined above have been adapted for the examination of *C. diphtheriae* and staphylococci, and the special procedures for these organisms are detailed below; the technique, however, should be capable of adaptation for any toxicogenic organism—aerobic or anaerobic. Furthermore, it is possible that substances diffusing from bacterial cultures, but not commonly regarded as exotoxins, can also be used for purposes of recognition if suitable antisera are employed.

Method for Examination of *C. diphtheriae*

Materials.—The medium is prepared by dissolving 4 g. of protose-peptone ("difco"), 0.6 g. of maltose, and 0.14 ml. of lactic acid (B.P.) in distilled water. The volume is made up to 100 ml. and the pH adjusted to 7.8. A 3% solution of agar in 1% sodium chloride is cleared by filtration through paper pulp and its reaction is adjusted to pH 7.8. It is further clarified by treatment with charcoal, and again filtered through pulp. Equal parts of the peptone and agar solutions are mixed together and distributed in 10-ml. quantities, which are sterilized by steaming for 30 minutes on three successive days. The antitoxin used is refined diphtheria antitoxin globulins (Burroughs Wellcome and Co.) diluted with physiological saline to a concentration of 1,000 units per ml. The filter-paper strips are prepared from sheets of good quality filter paper (Postlip 633 was used). This is cut into strips measuring 6 by 1.5 cm. and these are placed in a plugged test-tube and sterilized in the hot-air oven.

Procedure.—Plates are prepared by melting 10 ml. of the medium, adding 2 ml. of sterile normal horse serum, and pouring into 3½-in. (8.75-cm.) Petri dishes. A sterile filter-paper strip is now dipped in diluted antitoxin, and after excess antitoxin has drained off it is laid across the surface of the freshly poured plate and allowed to sink into the medium while it is still fluid. The plate is then dried thoroughly in the incubator and should be used the same day. The organisms to be tested are inoculated by a loop in the form of a wide streak right across the plate. Three or four such streaks are readily accommodated on a single plate, and each set should include a known toxicogenic strain as a positive control. The plate is incubated and examined after 24 and 48 hours.

Results

A positive reaction is denoted by the appearance of fine white lines forming an arrow-head within 1/2 in. (1.25 cm.) of the filter paper and pointing towards it. It makes its appear-

FIG. 2.—Non-specific opacities. The strain on the left shows opacity and arrow-head formation (toxicogenic); the centre strain shows opacity only (non-toxicogenic); the strain on the right shows arrow-head only (positive control).

ance after about 22 hours' incubation in some strains, and in its earliest stages has to be sought with a hand lens. It is best seen by reflected light against a dark background. After 48 hours the arrow-head is easily visible to the unaided eye by transmitted light. A completed test is shown in Fig. 1.

If the plates are left at room temperature the density of the lines gradually increases, but secondary lines may develop which possibly represent the interaction of bacterial substances diffusing out from the growth with antibodies corresponding to them present in the serum. The presence of such antibodies may be assumed from the fact that the material used for the immunization of animals contains numerous substances in addition to the toxin itself. These secondary lines may appear with both toxicogenic and non-toxicogenic strains, and with the latter might lead to difficulty in interpretation. If, however, the final readings are made directly after 48 hours' incubation no confusion arises from this cause, as several days at room temperature are required to bring out the secondary lines. Even then they are very soft, and their detection usually requires the aid of a lens.

The plate shown in Fig. 2 demonstrates that the opacity produced in serum media by certain strains of corynebacteria is independent of toxin production. The strain on the left shows opacity and the arrow-head denoting toxin production; the strain in the middle shows the opacity but no toxin-antitoxin line. The right-hand strain (the positive control) shows the arrow-head only. On standing at room temperature these opacities lose homogeneity and become distributed in the form of concentric denser and clearer areas resembling Liesegang rings. Clearly the development of rings cannot be accepted as proof that a toxin-antitoxin reaction is taking place.

Results of Tests on 20 Strains of *Corynebacteria*

Strain No.	Origin and Type of Organism	Animal Virulence Test	In vitro Test	
			24 hrs.	48 hrs.
1	W.I. No. 77	Virulent	+	+
2	Diphtheroid, St. Geo. Hosp.	Avirulent	—	—
3	W.I. No. 90	Virulent	+	+
4	L.C.C. 11680, gravis	"	+	+
5	L.C.C. 11674, gravis	"	+	+
6	St. Geo. stock, gravis	"	—	+
7	L.C.C. 12224, mitis	Avirulent	—	—
8	L.C.C. 12151, mitis	"	—	—
9	L.C.C. 12494, gravis	Virulent	+	+
10	L.C.C. 12511, gravis	"	—	+
11	L.C.C. 12594, mitis	Avirulent	—	—
12	L.C.C. 12595, mitis	"	—	—
13	L.C.C. 12597, mitis	"	—	—
14	N.C.T.C. 3985, gravis	Virulent	—	+
15	N.C.T.C. 3986, gravis	"	+	+
16	N.C.T.C. 3988, intermedius	"	—	+
17	N.C.T.C. 3989, mitis	"	—	+
18	L.C.C. 12825, mitis	Avirulent	—	—
19	L.C.C. 12826, gravis	Virulent	+	+
20	L.C.C. 12828, mitis	"	+	+

W.I. = Wellcome Institute.

L.C.C. = London County Council, Southern Group Laboratories.

N.C.T.C. = National Collection of Type Cultures.

The accompanying Table shows the results obtained with this test on 20 strains of corynebacteria, all but one of which (No. 2) gave typical morphological and cultural characters of *C. diphtheriae*. Of the 13 virulent strains eight showed a positive reaction in 24 hours, while with the remaining five the reaction became positive only after 48 hours' incubation. None of the seven avirulent strains showed a positive reaction after 48 hours.

Method for Examination of Staphylococci

With the staphylococci ability to form a toxin is clearly not the sole factor determining virulence, since these organisms invade the body. Nevertheless a simple procedure for the detection of toxicogenicity might well prove to be of value for both clinical and experimental purposes. That staphylococcal toxin-antitoxin reaction can be recognized in an agar plate was shown by an accidental observation made in 1944 on a routine plate (Fig. 3). A method similar to the one described for the diphtheria bacilli was devised and tried in a small number of cases.

Materials.—The medium used consists of heart-infusion broth (difco) to which 0.2% potassium acid phosphate (KH_2PO_4) and

0.03% magnesium sulphate (MgSO_4) are added. Both salts are used crystalline. The pH is adjusted to 6.8, and the broth, which is somewhat yellow, is clarified with charcoal. Agar is now added to a concentration of 1.5% and the medium filtered and clarified in the usual way. It is distributed in 10-ml. quanti-

FIG. 3.—Routine plate showing staphylococcal toxin-antitoxin reaction. Plate inoculated with patient's strain of *Staph. aureus*. Upper cups contain penicillin solution (1 and 5 units per ml.); lower cups contain pus from patient's abscess—the left shows presence of penicillin and a ring believed to be a toxin-antitoxin reaction.

ties in tubes and sterilized in the autoclave. For antitoxin refined staphylococcal antitoxin globulins (Burroughs Wellcome and Co.) are employed, diluted so as to contain 1,000 units per ml.

Procedure.—This is precisely as in the former method, except that normal horse serum is not incorporated in the medium, as its addition was not found to improve the reaction. Readings are taken with a lens after 48 hours' aerobic incubation. The effect of incubation in an atmosphere of carbon dioxide was not tried.

Results

The lines which form with toxicogenic strains are multiple and are fainter than those formed with *C. diphtheriae*, so that their presence has to be sought with the aid of a hand lens. A typical plate is shown in Fig. 4. The test has been applied

FIG. 4.—Completed test with staphylococci. All strains show multiple line formation.

to 32 strains of staphylococci. Of these nine were obtained from the fingers or were obviously contaminants on plates, and only one—an *aureus* strain obtained from the hand of a laboratory worker—gave a positive result in 48 hours. Ten strains, being obtained in pure culture from typical staphylococcal lesions, were assumed to be pathogenic, and nine of them gave positive results in 48 hours. The remaining 13 strains were obtained in the course of routine work from various sites and three of them failed to give a positive reaction.

Discussion

The application of these methods has not yet reached a stage at which detailed discussion of the results would be profitable, and the object of this paper is to present the new *in vitro* technique and to indicate directions along which development is possible.

Clear-cut results have been obtained with a small series of *C. diphtheriae* strains, and they show that there is a high correlation between *in-vitro* toxicogenicity as detected by this test and the so-called "virulence" as detected by the usual guinea-pig test. It is a generally accepted fact that pathogenicity is not a good basis for bacteriological classification, as virulence is not a permanent intrinsic property of a given species. It is, however, precisely this point which is of paramount interest to the medical bacteriologist: to him a non-virulent strain is not a "true" representative of the species, and is indeed the worst nuisance, since its unimportant character is revealed only after lengthy investigations, involving animal experiments. Animal tests have numerous disadvantages in a routine laboratory, and it is hoped that the *in-vitro* test may well prove of practical value on account of its great simplicity and the relatively short time needed to obtain the result.

Once an organism has been assigned to the *Corynebacterium* group on morphological grounds and grown in pure culture the test can be applied without investigating the biochemical properties of the strain and without classification as regards its type, as the specificity of the reaction with diphtheria antitoxin is axiomatic. Theoretically not every toxicogenic strain need be virulent. If this differentiation really does exist between the *in-vitro* and the animal test a larger series of parallel tests should reveal this. As, however, every virulent strain is by definition toxicogenic, from the utilitarian point of view there is no source of error in the test. Clearly, further work is needed to establish whether or not this test can be relied upon to replace the accepted virulence tests in practice.

The results with the staphylococci are less well defined, but here again there is high correlation between a positive test and clinical pathogenicity. In addition to the possible use of the test in clinical bacteriology the procedure may prove to be of value in investigating the diffusible products of staphylococci. The multiplicity of lines developing with the positive strains suggests the presence of several interacting substances and clearly requires further study.

Without substantial modification the same technique can be used for the identification of the anaerobes by means of their specific toxins. The technique should also be capable of wide application to other groups of organisms by investigating diffusible antigenic substances produced by the culture but not usually regarded as exotoxins.

Summary

The principles of a new technique for the *in-vitro* detection of bacterial toxicogenicity are outlined.

Tests utilizing these principles and adapted for examination of *C. diphtheriae* and staphylococci are described.

Results with a small number of strains show a high correlation with the results of animal virulence tests in the case of *C. diphtheriae* and with clinical pathogenicity in the case of staphylococci.

The possibilities of wider application of the technique for both clinical and experimental purposes are discussed.

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SPERANSKY'S METHOD OF SPINAL PUMPING IN RHEUMATOID ARTHRITIS

A REVIEW OF FOUR CASES

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The recent interest shown in Speransky's method of treating rheumatic cases by means of "spinal pumping" make it worth while to report four cases of rheumatoid arthritis which were treated in this way and followed up for a year. Speransky (1935) gave reasons for his belief that the nervous system played an important part in acute and chronic disease. Ponomareff and Tchechkoff (1927) and Jowelew (1930) studied the passage of antibodies through the blood-stream barrier of the spinal cord in rabies and attempted by spinal pumping to break it down.

The operation, which consists in withdrawing and replacing 20 ml. of spinal fluid slowly twenty times, takes about an hour. The spinal fluid in each of our cases was sterile, and laboratory tests showed no abnormality. The only reactions were vomiting in one case and a transient rise of temperature in another. Spinal pumping is not an easy procedure to carry out in cases of active rheumatoid arthritis, for the patients find it difficult and painful to get into a suitable position for lumbar puncture.

Speransky claimed good results with this treatment in malaria and typhus, and also in the rheumatic diseases. He treated 100 cases of "polyarthritis" by spinal pumping, giving intensive salicylate therapy before and after, and found that of 52 patients with their first attack of rheumatic fever 70% appeared to show complete recovery. Of 15 cases of chronic arthritis, 4 were reported recovered, 9 improved, and 2 unchanged.

Later the Gillman brothers (1946) issued an account of their trials of spinal pumping, and interest was reawakened in the subject. They reported good results in 36 cases of subacute rheumatism following rheumatic fever and in 22 cases of chronic arthritis which had failed to respond to gold and physiotherapy. In 12 of these chronic cases there was great improvement, with a return of the sedimentation rate to normal in two weeks; in the remaining 10 the condition was unchanged. After the spinal pumping they noted severe sweating, a rise in skin temperature, and visible dilatation of the peripheral skin capillaries which lasted for 48 hours. Some unpleasant side-effects were encountered, such as severe headache and vomiting, and, in one

case with hypertension, death due to multiple cerebral haemorrhages followed the third pumping. After publication of that report it was decided to try spinal pumping in the following cases of rheumatoid arthritis which had failed to respond to more rational methods.

Case 1

A hospital sister aged 54 had had arthritis for a year. It had spread rapidly to her hands, wrists, feet, knees, and elbows in the last six months. She was admitted on June 18, 1946, with clinical rheumatoid arthritis—afebrile. Radiographs revealed generalized decalcification with loss of joint space. A blood examination showed: red cells, 3,850,000; Hb, 70%; white cells, 5,500; differential count normal; W.R. negative; uric acid, 2.6 mg. per 100 ml.; sedimentation rate, 25 mm. in one hour (Westergren), which rose to 40 mm. in the next five weeks. Treatment with "myocrysin," salicylates, 1,000,000 units of penicillin, complete bed-rest, and plasters brought no improvement, and as she was rapidly becoming crippled it was decided to carry out spinal pumping. This was performed on Aug. 4 after a preliminary course of 10 g. of salicylate in 24 hours. The C.S.F. pressure at the onset was 180 mm., and at the end 70 mm. Salicylate, 10 g., was given during the next 24 hours. Five hours after pumping the patient complained of feeling "burning hot, especially in the forearms and feet," though there was no rise in general or skin temperature and no demonstrable capillary dilatation. This lasted for 12 hours. The joints then improved, and in a month the sedimentation rate had dropped to normal and the patient was up, walking, and free from pain. She proceeded to a convalescent home.

In February, 1947, the B.S.R. was 5 mm. In September, 1947, it was 8 mm., and she was doing light work, typing and playing the organ with some difficulty, and having to take three or four "veganin" tablets a week for pain in her wrists and ankles. There was a moderate degree of deformity and crippling, though it was no worse than in August, 1946. She had had no treatment since October, 1946. Radiographs showed no change from August, 1946.

Case 2

A Polish housewife aged 54 had suffered from intermittent arthritis in the knees, wrist, and hands for 20 years. It had been much worse in the last six years, since she had come to England. In spite of physiotherapy and gold injections at the B.R.C.S. Clinic, Peto Place, her condition had got worse. When admitted on Aug. 18, 1947, she was crippled with rheumatoid arthritis and had been unable to walk for two months. The

FIG. 1.—The blood sedimentation curve in Case 2.

B.S.R. was 110 mm. in one hour (Westergren); blood uric acid, 2.5 mg. per 100 ml.; Hb, 83%; white cells, 7,200.

Spinal pumping was carried out on Sept. 6. Salicylate was not given before or after. The C.S.F. pressure was 180 mm. After 10 withdrawals the pressure dropped to 35 mm. and the pumping was stopped. A reaction similar to that of Case 1 came on six hours afterwards, with a feeling of heat in forearms and legs lasting 12 hours. She had a general fever of 100° F. (37.8° C.), but no demonstrable capillary dilatation. During the next week the joint condition improved, and four weeks later she was discharged. Fig. 1 shows the course of