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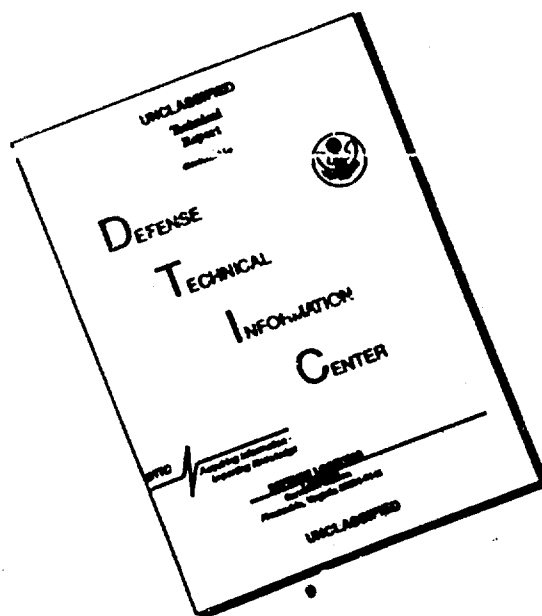
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Voenno-Meditsinski Zhurnal, 1961, vol. 10, pp. 65-68.

The Use of the Indirect Hemagglutination Reaction for Detection of
Botulin Toxin

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(Entered January 1960)

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The preparation of a quick method for a laboratory diagnostic of the types A and B botulin toxins appears to be an actual problem. The isolation of Bac. botulinus from foodstuffs, or from substances of a patient or cadaver, cannot be regarded as a determinant in the diagnostics of botulism. The only authentication can be a detection of the toxin and determination of its type. P.A. CHERTKOVA, S.M. HASLEDYCHEVA, P.D. DOMATCHENKO and other authors tried to use for this purpose a ring-precipitation reaction. However, the method was not sufficiently developed and it failed to find the acceptance. For these reasons also the reaction of the complement fixation did not become prevalent. S.M. MINERVIN et al. proposed a method of determination of the phagocytic index in order to detect botulin toxin and this test is about to be approved.

The purpose of the current work is to discuss BOIDEN'S method of the indirect hemagglutination reaction, which we modified for the purpose of determination of the types A and E botulin toxins in some foodstuffs. T. RYTSAL used this principle for the first time in 1956 in order to determine botulin toxin type A.

The data and the investigative methods, Botulin toxins types

W. M. T. S. R.

A and B were obtained by the following methods. We poured into a bottle of 3 liter capacity 2.5 liters of HOTTINGER'S broth that contained 200 mg% of nitrogen amine. Then, we inserted a cellophane sac with 200 ml of the phosphate buffer (pH 6.8) in a physiological solution of table salt. We covered the broth and the phosphate buffer with a layer of vaseline oil. The entire arrangement was sterilized in an autoclave. Having checked the sterility, we inoculated the cellophane sacs with 1 ml each of a 2-day culture of *Bac. botulinus* type A (strain 98), or with the type B (strain 255). This we cultivated for 7 days at 37° temperature. After this period, we pumped the culture liquid from the cellophane sacs, separating it from microbial bodies and we centrifuged it at 8,000 revolutions per minute for 20 minutes. Then, the obtained botulin toxin was titrated on white mice weighing 10 to 12 gm. We used for LD a minimal amount of the toxin, which, after intraabdominal administration in a volume of 0.5 ml, caused death of mice within 96 hours. One LD part of botulin toxin type A was equal to 0.000015 ml and that of the type B toxin was equal to 0.0003 ml.

The serums obtained from the KHARKOV Scientific Research Institute of the Armed Forces were purified by the "diaferm-3" method of the Academy of Medical Sciences and we used them as antitoxin serums.

For nonspecific antigen we used the culture liquid of *Bac. histolyticus* No.5, *Vibrion septique* No.59, *Bac. oedematiens* No. 277, *Bac. perfringens* type A, *Bac. sporogenes*, also diphtherial and tetanic anatoxins. We cultivated *Bac. botulinus* and causative agents of the gaseous gangrene group according to the indicated

method of KIGG-TAROTSTSI. We used for nonspecific antiserum diphtherial antitoxin from TOMSK Scientific Research Institute of the Armed Forces.

Sheep corpuscles. We took sheep blood into a sterile vessel, defibrinated and stored it in a refrigerator at +4° temperature. On the day of experiment we washed erythrocytes five times with a physiological solution of table salt (pH 7.2) and we prepared in it a 3% suspension.

Tannic acid. We prepared 0.1% solution of tannic acid in a physiological solution of table salt (pH 7.0) every time prior to experiment.

Solution of normal rabbit serum. We took rabbit blood from the heart the day before the experiment. On the day of experiment we inactivated the serum in a water bath at 56° temperature within 30 minutes. In order to avoid nonspecific agglutinins, we used adsorption by sheep corpuscles. We added 0.5 volume of a 3% suspension of sheep corpuscles to one volume of a whole serum of a rabbit. Then, we left this at room temperature (17 to 25°) for 10 minutes and later centrifuged, pumped and diluted with a physiological solution of table salt (pH 7.0) at 1:100.

Research material. Prior to carrying out the experiment, and in order to avoid a nonspecific agglutination, we adsorbed the material by means of sheep corpuscles (two drops of precipitated erythrocytes per 1 ml of investigated liquid) at room temperature within 10 minutes. The suspension was centrifuged; as the liquid above sedimentation was separated, we made from it necessary dilutions in a solution of a normal rabbit's serum.

Processing of erythrocytes with tannic acid. We added one volume of a tannic acid solution to one volume of a 3% suspension of erythrocytes. Then, we stirred the contents of the test tubes and we heated them for 10 minutes in a water bath at 37° (erythrocytes should settle down almost fully on the bottom of a test tube following a proper selection of the concentration of tannic acid). After heating, erythrocytes were washed once with a physiological solution of table salt (pH 7.2) and resuspended in a physiological solution of table salt (pH 7.0). The resuspension of erythrocytes should provide a stable homogeneous suspension. The presence of minute conglomerates and their rapid settling on the bottom of a test tube indicate that a larger amount of tannic acid was used. Similarly tanned erythrocytes are not suitable for carrying out an experiment, because they produce a spontaneous agglutination in the course of a basic experiment. If, however, after centrifuging, loose sediment of erythrocytes is on the bottom of a test tube, using them will lead to a sharp decrease of the sensitivity of reaction. Hence, for using a new consignment of tannic acid we recommend that its optimum concentration be determined in advance. One should consider as an optimum dose such, with which the hemagglutination titer is the highest in the basic experiment with botulin toxin and with a specific serum.

Sensitization of sheep corpuscles. To one volume of erythrocytes we added 0.5 volume of whole antitoxin serum type A, or one volume of antitoxin serum type B and a fourfold amount (according to the serum) of physiological solution of table salt (pH 6.4). We mixed this carefully and then we kept it in water bath

Table 1
Comparative Evaluation of the Sensitivity of the Indirect Hemagglutination Reaction with
Biological Test on White Mice after Determination of Botulin Toxin in a Herring

Type of botulin toxin	Dilution of the extract from herring	Antibotulin serum		Nonsensitized erythrocytes	Antibotulin serum		Nonsensitized erythrocytes	Biological test on white mice
		Type A	Type B		Type A	Type B		
		Preparation of the extract						
Type A	1:5	++++	-	-	++++	-	-	17* 19 19 24
	1:10	++++	-	-	++++	-	-	24 24 24 24
	1:20	++++	-	-	++++	-	-	24 24 24 24
	1:40	++++	-	-	++++	-	-	24 48 48 48
	1:80	++++	-	-	++++	-	-	72 72 96 96
	1:160	++++	-	-	++++	-	-	a** a a a
	1:320	++++	-	-	-	-	-	a a a a
Type B	1:5	+++	-	-	-	-	-	a a a a
	1:10	-	-	-	-	-	-	a a a a
	1:20	-	-	-	-	-	-	a a a a
	1:40	-	-	-	-	-	-	a a a a
	1:80	-	-	-	-	-	-	a a a a
	1:160	-	-	-	-	-	-	a a a a
	1:320	-	-	-	-	-	-	a a a a
Solution of normal rabbit's serum	1:640	-	-	-	-	-	-	a a a a
	1:1280	-	-	-	-	-	-	a a a a
	1:5	++++	++++	-	++++	++++	-	17 18 22 23
	1:10	++++	++++	-	++++	++++	-	17 17 17 23
	1:20	++++	++++	-	++++	++++	-	24 24 24 48
	1:40	++++	++++	-	++++	++++	-	72 72 96 96
	1:80	++++	++++	-	++++	++	-	96 96 96 96
Solution of normal rabbit's serum	1:160	++++	++++	-	-	-	-	a a a a
	1:320	++++	++++	-	-	-	-	a a a a
	1:640	++++	+++	-	-	-	-	a a a a
	1:1280	+++	-	-	-	-	-	a a a a
	-	-	-	-	-	-	-	a a a a
	-	-	-	-	-	-	-	a a a a
	-	-	-	-	-	-	-	a a a a

a) - Death of mice after administration of the extract is indicated by figures denoting hour
a**) - Mouse remained alive for 5 days.

at 37° for 15 minutes. Subsequently, we centrifuged this, washed twice with a physiological solution of table salt (pH 7.0) and resuspended in a physiological solution of a normal serum of rabbits in a volume equal to the volume of the tanned erythrocytes.

Arrangement of the basic experiment. We poured into test tubes, with inner diameter of 8 to 10 mm, 0.5 ml each of dilution of the examined liquid, then we added 0.1 ml each of the suspension of sensitized erythrocytes; we stirred this and placed under thermostatic control at 37° until the passing time of the control reaction. Usually, the recognition of reaction was possible within 1.5 hours.

Every investigation was accompanied by the following control:

- 1) 0.5 ml of the minimum diluted investigated material + 0.1 ml of nonsensitized sheep corpuscles;
- 2) 0.5 ml of solution of normal serum of a rabbit + 0.1 ml of nonsensitized sheep erythrocytes;
- 3) 0.5 ml of normal serum of a rabbit + 0.1 ml of nonsensitized sheep erythrocytes.

We used antidiphtherial antitoxic serum for the specificity test as a control during the examination of various foodstuffs, as well as in testing soil and water.

Biological test on white mice. Since we worked having previous knowledge of the types of botulin toxins, we did not effect the neutralisation reaction. We made biological tests in the following way. We administered to white mice (weighing 10 to 12 gm), intra-abdominally, various solutions of the extract of investigated material in a volume of 1 ml each.

Before we passed to the basic investigations, we checked the specificity method with the culture liquid of Bac. hystoliticus No.5,

Vibrio septique No.59, Bac. oedematiens No.277, Bac. perfringens No.28 type A, Bac. sporogenes, also of diphtherial and tetanic anatoxins.

The examination revealed that the indirect reaction of hemagglutination is strictly specific and it gives positive results only with a combination of antigen with a specific antiserum. Moreover, while working with antibotulin serums types A and B (series 133 and 154), we noted a reciprocal agglutination between the types A and B. Following a specific exhaustion of these serums by botulin toxins, we did not observe a reciprocal reaction and, at the same time, the hemagglutination titer of serums remained as before.

Determination of botulin toxin in artificially infected salted herring. A brief medical-chemical analysis of the herring: a slight smell of ammonium; the litmus reaction slightly alkaline; the ammonia reaction positive; the hydrogen sulfide reaction slightly positive and the 9.9% content of table salt.

We cut the herring into pieces weighing 20 gm each and we administered to each piece 0.5 ml of botulin toxin type A, or 1 ml of type B. We ground this in a porcelain mortar and added for each experiment 10 ml of solution of normal rabbit's serum and left the mixture at room temperature for 1.5 hours. Subsequently, we transferred the paste-like mass on a double-layer gauze-napkin and suspended it in a 5-ml centrifugal test tube for a 15-minute centrifuging at 4,000 revolutions per minute. We pumped the liquid from the test tube and centrifuged it again for 10 minutes. Then, we prepared necessary dilutions from the liquid found above the sediments. In order to avoid a spontaneous aggluti-

nation of erythrocytes, we heated the tissue extract in water bath at 56° for 2 to 3 minutes until its coagulation set in, and then we centrifuged for 5 minutes at 4,000 revolutions per minute. The liquid obtained above the sediment was perfectly clear. To prevent a nonspecific agglutination, one can use 0.1 N solution of hydrochloric acid, which is necessary to add to the extract by drops during constant mixing until the coagulation sets in. A further preparation is carried out analogically to the first method after heating.

We used for biological tests the extract from a herring without any preparation. As is obvious from the results of one investigation (see table 1), the detection of botulin toxin in a herring is possible with the aid of the indirect hemagglutination reaction. The method of the indirect hemagglutination reaction is more sensitive than the biological test on white mice. While heating the extract that is prepared from a herring, a higher hemagglutination titer can be obtained than by the acid method.

Determination of botulin toxin in artificially infected sausage.

We used for this purpose two kinds of sausages: "sectioned", and "muscovite", i.e. boiled and thoroughly smoked.

Weighed 5 gm portions of each kind of sausage were ground in a porcelain mortar. We added to each test tube 0.5 ml of botulin toxin type A, or 1 ml of type B, mixed it and left it at room temperature for 1 hour. Subsequently, we added to each 10 ml of solution of a normal rabbit's serum and kept this 30 minutes longer under the same conditions. Then, we centrifuged it, pumped off the liquid

above the sediment and, after adsorption by sheep corpuscles, we prepared from this suitable dilutions, using the solution of normal rabbit's serum for indirect hemoglutination reaction, and the physiological solution for biological test.

The results of the examination of sausage as to the presence of botulin toxin indicated that the method of determination of botulin toxin with the aid of the indirect hemoglutination reaction is also more sensitive than the biological test on white mice.

The examination of canned green peas and flounder canned in tomato juice also revealed pertinently to the presence of botulin toxin that the test of indirect hemoglutination reaction is effective in determination of botulin toxins in the cans. The method of the indirect hemoglutination reaction is specific and more sensitive than the biological test on white mice.

Hence, the test of the indirect hemoglutination reaction is highly specific. Due to the reciprocal agglutination of types A and B toxins, it is necessary to carry out a specific exhaustion of the antibotulin serums. The indirect hemoglutination reaction permits to determine and to differentiate a type of botulin toxin (A from B) in foodstuffs with salted herring, in sausage and in canned goods (green peas and flounder in tomato juice). This reaction is more sensitive than biological tests on white mice; depending on material, the investigation time is reduced by 3 to 5 hours. The test of the indirect hemoglutination reaction can be used as a quick method for checking foodstuffs that are suspected of containing botulin toxins of A and B types.

Russian word "aprobirovat"

Russian-English Dictionary, Prof. V.K. MÜLLER, 1949, page 20:
aprobatsiya = approbation, approval; aprobirovat = to
approve, approve.

Russian-English Technical and Chemical Dictionary, LUDMILLA
IGNATIEV CALLAHAN, 1947, page 19: aprobatsiya = approbation,
approval; aprobirovat = approve, approve.

Russian-English Physics Dictionary, IRVING EMIN, 1963, page 19:
aprobatsiya = approbation, approval, confirmation;
aprobirovannyi = approved, tested;
aprobirovat = approve, approve, test.