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SENSITIVITY AND SPECIFICITY OF THE COMBINED ACCELERATED METHOD OF DETECTING ANTHRAX SPORES IN THE SOIL

/Following is the translation of an article by A. Tomov and T. Todorov, Higher Military-Medical Institute and the Veterinary Institute of Infectious and Parasitic Diseases (Sofia), published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology) No. 10, 1966, pages 62-66. It was submitted on 22 February 1966. Translation performed by Sp/7 Charles T. Ostertag, Jr./

The study of the sensitivity of the fluorescent antibody method for the detection of anthrax bacilli in objects of the external environment, which are rich in saprophytic microflora, is difficult due to the non-specificity of the reactions. For this purpose in 1963-1964 we used the combined method, which includes the application of fluorescent antibodies and a capsule-forming medium. On the basis of these inventigations we proposed a new selective capsule-forming medium (Tomov et al, 1965) which guarantees the obtaining of specific results.

In previous investigations (Tomov and Todorov, 1965) we presented the results of checking the sensitivity and specificity of the combined method (selective capsule-forming medium and fluorescent antibodies) of detecting anthrax spores in tap water and river water. In the present communication we present the results of studying the sensitivity and specificity of the combined method of detecting anthrax spores in the soil.

The selective capsule-forming medium used by us has the following composition: 50 ml peptone agar (10% agar, 2.5% peptone, 0.5% sodium chloride), 100 ml beef serum, 50 ml of chicken egg protein, 25 ml of 0.4% hemin in an 100 H. solution of sodium hydroxide, 25 ml of 100 H. solution of acetic acid; the pH of the medium was established at a level of 7.2. The procedure for preparing the medium was described in a previous communication (Tomov, et al, 1965).

The fluorescent antibodies were obtained from anti-anthrax precipitating serum, which was prepared by means of immunization with capsule-forming anthrax bacilli at the production department of the Veterinary Institute of Sera and Vaccines (Sofia). The whole globulin fraction of the serum was isolated by means of salting out with ammonium sulfate, and tagging was done with the help of fluorescein isocyanate "Fluka" with a dye to protein ratio of 3:100 (in milligrams). The preparation was purified of unbound dye by filtration through a column with Sephadex g-25. The conjugates were saturated additionally with bacillary bodies of the vaccine anthrax strain 34F, which does not form capsules. A suspension, obtained by means of washing off of agar cultures (18-20 Roux flasks), was

heated to 80°, centrifuged washed with physiological solution (ph 7.4), and then 50 ml of conjugate was added to the precipitate. The mixture was maintained for 2 hours at room temperature and again centrifuged. The resulting conjugate was poured into 1 ml ampoules and subjected to lyophilic drying. During the work the contents of the ampoules were diluted in 1 ml of distilled water.

Contamination of the soil samples was performed with a suspension, containing 200 million spores in 1 ml, obtained from the capsule-forming anthrax strain Dimitrovo. From this main suspension we prepared the appropriate dilutions, with which we contaminated the samples of garden soil (10 grams). Subsequently, the samples were investigated by 2 methods: (1) into a flask, containing the contaminated soil, we added 20 ml of physiological solution, then after a thorough shaking we inserted 3-4 ml of trichloroethylene and again shook it for 5-10 minutes in order to destroy the vegetative forms. Then 0.1 ml of the mixture was inoculated (with the help of a spatula) on the surface of the selective capsule-forming medium in a Petri dish; (2) the other samples of contaminated soil were placed in a cylinder (500 ml capacity) and after the concentration of anthrax spores were investigated by the "Olstabmethode" (Conradi, 1913), which in essence is the flotation method. Inoculation on the selective medium was carried out by means of imprints of an oily tampon with a subsequent scattering with a spatula. All the tests were accompanied by control samples (we investigated non-contaminated soil, treated in the same manner).

The seedings were cultivated for 15-18 hours at 37° in an atmosphere of CO_2. Then the growth from the surface of the medium on each Petri dish was carefully washed off with a glass spatula into 2 ml of physiological solution. Two preparations from the resulting suspension were prepared for microscopic investigation. One of these was fixed in methanol for 15 minutes, and the other - for one minute in a solution of formalin-gentian violet (40% formaldehyde and 15% gentian violet). The latter preparation was thoroughly washed with water and dried. Both preparations were then treated with fluorescent antibodies in a wet chamber at 37° for 30 minutes. After this the preparations were again washed in flowing tap water. After drying a little they were placed under a cover slide and studied in a ML-2 luminescence microscope. Photography was done on "Ultrarapid" film with an exposure time of 10-30 seconds.

The anthrax bacilli were clearly distinguished in the preparations. A richly luminous capsule with a dark center, corresponding to the bacillary body, was apparent. The size of the capsule exceeded the cross section of the microbial body by several times. Most often the anthrax bacilli were disposed singly or in the form of short chains. In preparations made from samples of heavily contaminated soil numerous encapsulated luminous bacilli were seen in each visual field, and in the preparations made from samples, infected with a small amount of spores, the anthrax bacilli were encountered rarely (Figures 1 and 2).

The preparations containing anthrax bacilli differed sharply from those prepared from control samples of uncontaminated soil. In the latter case the strongly luminous bacilli were absent. Saprophytic microorganisms, disposed singly or in chains did not fluoresce or had a hardly noticeable shine. If the saprophytes were grouped together the fluorescence increased to a certain degree, however it never reached the intensity observed in the tests with encapsulated bacilli (Figure 3).

In view of the sharpness of the differences between the "positive" and "negative" preparations, faultless results were obtained in spite of the fact that the tests were set up exclusively with coded preparations.

In preparations, fixed with formalin-gentian violet, even sharply expressed groupings of saprophytes did not fluoresce, since the anthrax bacilli were characterized by an intense fluorescence. The results of a comparative study of the method of direct seeding and the method of Conradi treatment are presented in Tables 1 and 2.

The results obtained testify to the expressed sensitivity of the combination method. Thus, during the investigation by the simple method with direct seeding (see Table 1) the anthrax spores were detected at an intensity of contamination equal to 100-200 spores in 1 gram of soil. The occasional detection of bacilli is possible with a lower content of spores. Based on the data of Petrova and Larina (1962), with the help of the usual methods the anthrax bacilli, isolated from animals, are not revealed when soil is contaminated in quantities no greater than 800 spores per 1 gram. Under the conditions of our tests treatment by the Conradi method did not have any advantages over the method of direct seeding; on the contrary, even worse results were obtained (see Table 2). It is possible that this was conditioned by the great dilution of the spores when 500 ml of water for flotation was added to the cylinders; it is also possible that not all the spores could be transferred to the selective medium, using the oily tampon. The negative result, obtained in all the control samples with uncontaminated soil, supports the high specificity of the method.

In our opinion, the specificity of the proposed method of detecting anthrax spores in the soil is conditioned mainly by the peculiarities of the selective capsule forming medium. The latter promotes the formation of a rich capsule in anthrax bacilli, while at the same time it inhibits the growth of many saprophytes, and those which grow just the same do not form capsules (Tomov et al., 1965). The suppressing action of the medium on saprophytic flora also has significance on the sensitivity of the method, since conditions are created here for the more favorable growth and capsule formation of anthrax bacilli, in connection with a weakening of the antagonistic effect of saprophytic microorganisms.

The fluorescent antibodies also exert an essential influence on the specificity and sensitivity of the method. They make it possible to

distinguish individual anthrax bacilli among the numerous capsule-less saprophytes. The quality of the conjugate is raised significantly after it is saturated with the help of the capsule-less anthrax strain 34F.

A common opinion still does not exist in respect to the possibility of obtaining antibodies to capsular γ-, d-glutamyl polypeptide of anthrax bacilli. Leonard and Thorne (1961) were not able to obtain true antibodies to γ-, d-glutamyl polypeptide in immunized rabbits; they consider the precipitation of the latter with serum proteins as a nonspecific reaction. On the other hand, Levina and Arkhipova (1964) maintain that by the immunization of rabbits with capsular anthrax bacilli it is possible to obtain antibodies, causing a specific reaction. We did not conduct special investigations in this direction; however, the tests conducted do not confirm the opinions of Leonard and Thorne that capsular anthrax bacilli are stained under the influence of normal and heterologous fluorescent sera, and the data of Levina and Arkhipova that for the staining of capsules of anthrax bacilli it is necessary that they be preliminarily treated with acid solutions.

Regardless of which of the opinions is correct, the importance of the combined method proposed by us, its sensitivity, specificity, rapidity and ease of accomplishment speak for themselves. The significant advantages of the method can be considered that laboratory animals are not required for it and it is specially suited for field conditions.

Conclusions

1. A combined method (selective capsule-forming medium and fluorescent antibodies) is proposed for the detection of anthrax spores in the soil.

2. The method is characterized by a high degree of sensitivity - anthrax spores in the soil may be detected at an intensity of contamination of 100-210 spores in 1 gram of soil.

3. The method is simple and speeds up diagnosis. The soil suspension is inoculated in only one Petri dish with the selective capsule-forming medium, and in all cases the answer can be obtained no later than in 20 hours after the onset of the investigation. ( )

Literature

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GRAPHIC NOT REPRODUCIBLE

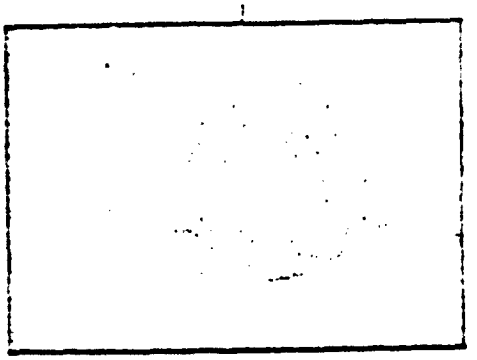

Figure 1. Numerous encapsulated anthrax bacilli in a soil preparation contaminated with 2000 spores per 1 gram.

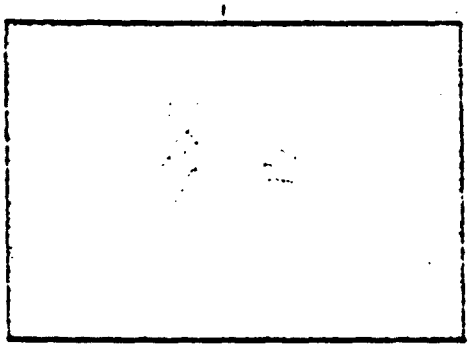

Figure 2. Individual encapsulated anthrax bacilli in a soil preparation contaminated with 200 spores per 1 gram.

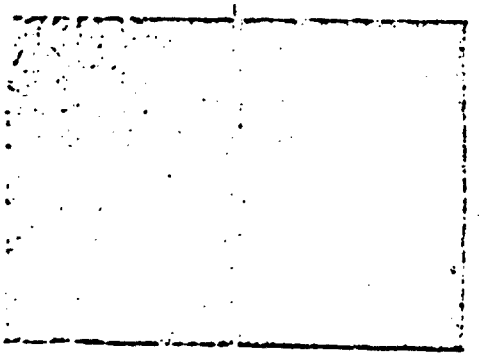

Figure 3. Control preparations - seedings from samples of non-contaminated soil

Table 1

Sensitivity of the combined method for detecting anthrax spores in the soil by the direct seeding of a soil suspension.

Amount of soil	Total number of anthrax spores in sample	Number of spores in 1 gram of soil	Number of investigated samples	Result	
				Test	Control
10	20 000	2 000	5	5/0	0/5
10	2 000	200	13	13/0	0/13
10	1 000	100	16	14/2	0/16
10	200	20	10	3/7	0/10

Table 2

Sensitivity of the combined method for detecting anthrax spores in soil samples, treated by the method of Conradi. (Headings are the same as in table 1)

10	20 000	2 000	6	6/0	0/6
10	4 000	400	17	13/4	0/17
10	2 000	200	5	1/4	0/7

Legend (for tables 1 and 2): Numerator - number of samples in which anthrax bacilli detected; denominator - number of samples in which anthrax bacilli not found.