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DEPARTMENT OF THE ARMY
Fort Detrick
Frederick, Maryland

On the practical usefulness of the urea method after Dold for the isolation of bacterial spores, especially for determination of anthrax spores.

by H. Dold and F. Weyrauch

From the Institute for Experimental Therapy "Emil von Behring,"
Marburg, Lahn. Zeitschrift für Hygiene und Infektionskrankheiten, 1924,
Vol. 103, No. 1, 3 Jul 24, p. 150-156.

In the isolation of bacterial spores from bacteria mixtures which consist of both spore-containing and sporeless types of bacteria, to date two methods have been considered: The heating method and the antiformin method (Uhlenhuth and Kylander).

The heating method: The bacterial mixture is heated in a water bath after soaking in water or physiological sodium chloride solution, if necessary. The data given by the various authors concerning temperature and duration fluctuate considerably. Klimmer (1) states that heat should be applied 5 minutes to 1 hour at 70-85°C. Meyer (2) recommends $\frac{1}{2}$ -1 hour at 80°C. These differences can probably be explained by the fact that the individual writers were dealing with bacteria of different types, amounts and resistance. Their reports may apply to the material tested by them, but have no general validity. On the other hand, if the rule is given much leeway in order to satisfy all possible cases in respect to temperature as well as duration, for example heating for 5 minutes (!) up to 1 hour (!) at 70-85°C (Klimmer), then it can be easily seen that very little could be gained in practice from such a rule.

The antiformin method: The bacterial mixture is treated for a specific length of time with antiformin solution, whereby the material that contains

no spores is dissolved, while bacterial spores are said to resist the effects of antiformin. Again there are widely differing data on the required and suitable antiformin concentration, as well as on the required and suitable time element involved. For instance, according to Uhlenhuth (cited by Klimmer) anthrax spores endure the effects of 7 hours in 10% antiformin, while the just mentioned antiformin concentration will kill anthrax spores in ca. 4 hours according to Kade (cited by Klimmer), and finally, as stated by Tuchler (cited by Klimmer), a 24-hour effect of 2½-5% antiformin solution is not sufficient to kill anthrax spores. According to Sobernheim (3), 5-10% antiformin will shortly cause complete dissolution of anthrax spores. The reasons for these extraordinary differences in the observations and reports of the authors should be the same as mentioned in connection with the heating method. One should not equate anthrax spores with anthrax spores, rather one should consider the extraordinary variability in resistance of anthrax spores (cf. Esmarch), even to antiformin. During the course of our own tests we have observed that even the lowest antiformin concentration (5%) reported by Klimmer in the shortest time (1 hour) destroyed the anthrax spores in a material that provably contained anthrax spores.

These reports, which differ strikingly in important details, are to be explained not only by the different resistance of the test material, but also by the different amounts of germs with which the analyser was dealing. In a dense bacterial suspension the damaging chemical and physical influences make themselves felt in a different way, probably less so than in a weak bacterial concentration. Besides, we know from the tests of Reichenbach (4) that even within one bacterial culture the individual germs react differently toward external influences. Each culture contains only a very few highly resistant germs, while the killing of the main mass of germs succeeds

relatively more easily and faster. With respect to the usual "terminal methods" which determine the time required to kill all germs, data concerns the disinfectant values applied to those resistant germs which would as a rule be present in larger amounts in a greater bacterial mass than in one that is smaller.

To date known and customary methods for the isolation of bacterial spores from bacterial mixtures lack clear data that could be used in practical work, for instance in the finding of anthrax germs in the outside world.

The finding of anthrax spores in the manure of infected stalls and on the ground of infected pastures is connected with special difficulties, not only on account of the small number of germs, but also because of the accompanying mass of other bacteria. An animal test usually does not succeed, since other pathogenic bacteria present in such material, for example pathogens of malignant edema or tetanus, often multiply more rapidly and bring about death more speedily than the anthrax bacilli. Therefore all that remains is the culture method, in which a process that eliminates the numerous saprophytic germs is invaluable, since the isolation of anthrax germs otherwise becomes practically impossible due to the luxuriant growth of accompanying saprophytic bacteria.

It became worthwhile, therefore, to test the method recently described by Dold (5) also in respect to its practical utility in comparison with known processes. The method progresses as follows:

If a deposit of bacterial mixture containing both sporiferous and non-sporiferous bacterial types is saturated with urea and allowed to stand at room temperature for 2-4 hours; or incubated for $\frac{1}{2}$ hour at 37°C ; or for 5 minutes at 37°C in a water bath, followed by inoculation of the material on agar, then only sporiferous bacterial types will grow.

First of all we determined the degree to which the three methods were injurious to our test object (anthrax spores).

For the antiformin method we chose, for the sake of simplicity, the lowest concentration found to be effective (0.5%), for the heating process a medial temperature of 80°C (data varied between 70 and 85°C). In connection with the urea method we tested the execution of the method at 37°C (incubator) as well as at room temperature.

It was established that the urea method was least harmful to spores. Even after 14 days germs were found in the concentrated urea held at 37°C, while heat had killed the germs after 4 hours, and antiformin after 6 hours.

In order to approximate the conditions found in practice, we determined in our tests the minimal amount of anthrax spores that could be identified, using our test method (streaking of one loop of 3 mm diameter on agar).

It was established that a streak of one loopful of 1/4,000 dilution of one platinum needle pointful (see footnote) of agar culture (8 days old) on an agar slant results on the average in 1-5 colonies. One loopful of 1/4,000 dilution of 1 needle pointful of an 8 days old anthrax culture could be considered the weakest anthrax spore sowing identifiable by means of our method. Since this amounted to ca. 2,500 spores in 1 ccm, counted by Wright's method, and 1 ccm equals ca. 220 loopfuls, 1 loopful contained approximately 10 germs.

We now proceeded to sow this minimal amount in liquid manure and then first to establish the most favorable conditions under which our material could be subjected to the antiformin and heating methods.

With respect to the antiformin method it was found that a 0.5% concentration and an effective duration of 5 minutes were just enough to kill the vegetative accompanying bacteria in the manure. This concentration and

duration could therefore be considered as most favorable conditions.

In the heating process we similarly tried to ascertain which temperature and time element would be just sufficient to kill asporogenous bacteria. These conditions were found to be met with respect to our test material, in a heat application of 5 minutes at 80°C.

Regarding the urea method, an effect of 5 minutes in a concentrated urea solution in a water bath of 37°C (or 2 hours at room temperature; or ½ hour incubation at 37°) can pass as the optimal condition.

a) Comparative tests of the utility of the three methods under arbitrarily chosen conditions.

Technique: In producing the dilution of our anthrax material we started with a platinum needle pointful of anthrax culture. We are aware of the fact that we are dealing here with a concept that is due to fluctuations, precisely as is the case with the platinum loop usually utilized as an initial measure. However, since the same starting material was used in each of the comparative test series, the comparison value of our results within each test series is not depreciated thereby. On the other hand, this situation explains the diversity in the results of the individual test series in respect to the established absolute numerical values. Nevertheless the relative difference of the 3 methods must be expressed numerically in a summary of the individual test series.

After we had determined 1 loopful (3 mm Ø) of 1/4,000 of one needle pointful of anthrax culture (8 days old) to be the smallest amount of anthrax spores identifiable by our method, it would have been the easiest way to add this minimal amount to 1 ccm liquid manure each in 3 tubes, then to subject the contents of the 3 tubes to methods using urea, heat, and antiformin, whereby the contents of the 3 tubes, separately spread on agar

plates, would have established the number of anthrax bacteria still viable and thereby would have furnished a yardstick with which the relative usefulness of the 3 methods could be measured. We made such tests and obtained in one test in which the entire material (1 ccm NaCl each, into which 1/10,000 of one needle pointful of an 8-day culture had been sown) after treatment was streaked onto 4 agar plates: 69 colonies by the urea method, 31 through heat, 33 by use of antiformin, and 134 colonies in the control.

Regarding this procedure the objection may be raised that since the entire material is streaked, the presence of relatively large amounts of antiformin or urea will hinder the development of still viable spores.

For this reason we later proceeded differently and used a method which avoided the seeding of a large amount of antiformin or urea, and which made sowing of only one loopful possible. Fluctuations in bacterial content of the individual loops due to coincidence could be equalized by means of frequent repetitions.

We triturated one needle pointful of anthrax culture in 1 ccm sodium chloride solution and diluted 1:1,000. By mixing this 1/1,000 dilution with the liquid manure we obtained a dilution of 1/2,000 of a needle pointful per ccm. We now had to proceed differently in individual methods.

1. Antiformin method: 1 ccm of 1% antiformin was added to 1 ccm of the liquid manure containing 1/2,000 needle pointful of anthrax. We thus obtained 1/4,000 needleful of anthrax per ccm in 0.5% antiformin solution.

2. Urea method: Upon addition of urea crystals to the liquid manure containing 1/2,000 needleful of anthrax, crystal water escapes in considerable amounts. We therefore proceeded by introducing 1 ccm of the liquid manure containing 1/2,000 needleful of anthrax, then made a mark on the test tube corresponding to a volume of 2 ccm, now added urea crystals until

oversaturation was reached (at 37°C), and finally filled to the mark with physiological sodium chloride, whereby care was taken to see that a small remnant of undissolved urea remained at the top of the tube.

3. Heating method: Here a total volume of 2 ccm fluid and thereby the desired 1/4,000 needful of anthrax was reached by addition of 1 ccm physiological sodium chloride to 1 ccm of liquid manure containing 1/2,000 needful of anthrax.

4. As control 1/4,000 needful of anthrax in 1 ccm physiological sodium chloride was used.

After meeting the test conditions found optimal for each method, 1 loopful (3 mm Ø) each of the treated material as well as 1 loopful of the control solution were streaked on 1 agar tube; after 4 days the colonies were counted. Concerning the developed anthrax germs in the control solution it should be noted that these could be derived not only from spores, but also from possibly still existant vegetative anthrax germs, that therefore the amount of spores actually present probably was smaller than the anthrax colonies found on the control tubes.

The numerical result of 3 comparative test series: In the control 101 colonies, urea method 68, heat method 61, antiformin 26. This shows a certain superiority of the urea method.

b) Comparative tests of the usefulness of the 3 methods under natural conditions.

We now proceeded to test the three methods comparatively under natural conditions of practice. Opportunities therefor were present in the form of stall manure of animals that had been sprayed with live anthrax bacilli for the purpose of serum extraction.

Technique: Production of stall manure dilution. A plum-sized piece

of stall manure was soaked in 50 ccm of sterile physiological sodium chloride and then filtered. The filtrate was placed in 3 sterile test tubes in amounts of 1 ccm each.

In these tests under natural conditions, too, the differences in degree of dilution arising from addition of antiformin, or from escaping crystal water due to urea, were equalized by the addition of a corresponding amount of sodium chloride solution (see technique under a).

Following completion of treatment necessary for each method, the 3 tubes were shaken, and 0.4 ccm was withdrawn from each by means of freshly sterilized pipettes and distributed on 4 agar plates each. Inoculation was accomplished by smearing with a bent glass rod.

During withdrawal of fluid from the tube, especially during removal by means of a pipette, care should be taken not to touch the sides of the test tube, since material may adhere to them that hasn't been treated or has been treated insufficiently. It is recommended to transfer not more than 0.1 ccm fluid to the agar plate, since there is a possibility that a hindrance to growth may result from the transplanting of large amounts of urea or antiformin.

The results of 4 or 5 such test series were:

In the urea test 10 anthrax colonies (total of 5 separate tests) were obtained, in the heat test 6 (total of 4 separate tests), in the antiformin test 0 (total of 4 individual tests). The fact that we were dealing with anthrax bacilli and not with pseudo-anthrax bacilli was proved in each case by means of animal tests.

This shows that here too, working under natural conditions, the urea method proved its superiority over the two other processes, corresponding to earlier results.

Finally, tests will be reported that proposed to create a selective nutrient (see footnote) for sporiferous germs by the addition of urea to the medium (agar), and thereby to further simplify the isolation method by means of urea. It was noted, however, that there is no urea concentration which would only destroy or at least retard asporogenic accompanying bacteria, without hindering the development of spores. We found, for instance, in a mixture of hay bacilli and staphylococci, that 10% urea agar allowed both bacterial types to grow, while both were retarded in their development beginning at a urea concentration of 12.5%.

Footnote: Jaenish (6) and Meyer (cited elsewhere) recommend endo agar (containing 4% agar and 1-10% peptone) as selective nutrient media for anthrax bacilli. On such an endo agar only anthrax and pseudo-anthrax bacilli are supposed to grow, and no other sporeformers, such as hay bacilli. We had no success with this method. Anthrax and hay bacilli grew uniformly on the endo agar. Also, we could not convince ourselves of the great need for such media. A fairly experienced observer can differentiate anthrax colonies from hay bacilli and other sporeformers after 24 hours, since anthrax colonies are less compact, and show more delicate curl formations and more extensions. Microscopic examination also affords differentiation of anthrax bacilli, which are characterized by their definite chain formations and their more sharply terminated ends. In older colonies, however, these differences are no longer so clearly marked.

Discussion of Results

In all tests it was found that the urea method is relatively least harmful to spores, although thereby all vegetative forms are completely destroyed. For this reason the urea method caused relatively more spores to

appear out of the same start material than the heat process and the anti-formin method.

In this connection it must be considered, in respect to the heat and antiformin systems, that we first determined the conditions most favorable to our test material by means of preliminary tests. In practice this will not be possible, and one will therefore be forced to leave to chance the choice of temperature in the heat process and the concentration and time element in the antiformin method, since we have seen above how widely data on these points differ. It is certain that one cannot work under optimal conditions when the occasion arises, causing results that will be less favorable than our tests. On the other hand, the urea method has the advantage of a concise and clear rule that is applicable to every material.

If fluid test material in large amounts is to be handled, then, in order to limit the expenditure of urea, the material should be rigorously and extensively centrifugalized and only the deposited sediment should be worked with. If a solid substance, for instance fish meal, is to be dealt with, the fish meal should first be agitated in physiological sodium chloride, next the wash water is centrifugalized vigorously and protractedly, and the resultant sediment is subjected to further treatments.

Summary

1. All procedures which propose the isolation of bacterial spores from bacterial mixtures are also more or less harmful to the spores contained in the mixture.
2. The urea method, according to Dold, is less harmful to bacterial spores than the heating process and the antiformin system.
3. In comparative tests of the usefulness of the three methods in

isolating anthrax spores the urea method was superior to the other two procedures under arbitrarily chosen as well as under natural conditions. It offers the advantage of a clear, definite rule, applicable to every test material; while data concerning the other two methods fluctuate within a wide range, so that a preliminary determination of optimal conditions would be necessary in every case.

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