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#157
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The bacterial differential diagnosis of anthrax by means of a new specific test (The string of pearls test "Perlschnurtest.")

By J. Jensen and H. Kleemeyer. From the Hygiene Institute of Gottingen Univ. Special printing from the Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, I Orig. 159. VEB Gustav Fischer Verlag, Jena. Printed in Germany. Published under license #2211-1 of the Bureau for Literature and Publication of the German Democratic Republic. 1953. With 1 table. Received on 12 December 1952. Pages marked 494 through 500.

The differentiation of the true *B. anthracis* from the anthracoid types is, as the pertinent literature shows, not always possible without difficulties. The variability of *B. anthracis*, which can be extended to many of its "classic" properties, already became obvious in Robert Koch's laboratory. Since then, an abundance of works has been published which on one hand deal with this variability and on the other hand with the properties and behavior of the anthracoid species or the so-called transitional forms. (See the detailed literature in GILLISSÉN, Ref. 1) However, for the purposes of the extremely important problem of transformation, which is intimately connected with these intermediate forms (and that not only theoretically), it is of similarly decisive significance that the *B. anthracis* can be clearly differentiated from other similar species of bacilli on the basis of its unique properties.

It is true that anthrax infections in humans at present scarcely exist in Germany. In other countries, however, as a few figures will show, they are not so rare: In the year 1946 Bulgaria had 1911 (55), Italy 1762, Turkey 1607 (94), Roumania 1179 (85) and Iran 1764 (40) cases (fatalities in parenthesis) (Ref. 2.)

The special biological properties of the anthrax bacillus and its spores, as well as the manner in which it spreads, offer possibilities enough for the occurrence of the disease in man and animal in every conceivable locale. Each effort is justified, therefore, that has the aim of improving or simplifying the bacteriologic diagnosis of anthrax in some direction, including efforts made with a view to control of epidemics.

However, he who makes such an attempt will immediately be confronted with the question of just what should be referred to. After all, the difficulties of bacteriologic differential diagnosis arise out of the fact that the numerous "classic" properties of typical *B. anthracis* are not met completely in every strain and that they can also be found in part in bacilli resembling anthrax. It follows that attempts toward specificity of a new method must use the "classic" methods, notwithstanding doubts ascribed to them.

In order to build on as sound a foundation as possible we have examined 50 anthrax strains and 43 more or less anthrax-like strains for some of their most important "classic" properties. We were forced to forego numerous other biochemical and serologic types of tests due to the relatively high number of strains. We then compared the new method discussed here with these test results. We are aware of the fact that errors of traditional diagnostic (even if reduced) are inherent in our results.

Principles and Method of the "String of Pearls Test"

Our test is principally based on the fact that living anthrax bacilli in penicillin-containing culture take on characteristic forms. The type of the various forms is strictly dependent on the concentration of penicillin. Especially striking is the formation of large evenly rounded globules. Since every viable bacillus develops into such a globule, under certain conditions

(according to the growth formation) chains resembling strings of pearls are formed. The conditions for the creation of the globular form and the string of pearls, respectively, are: 1. Maintenance of favorable conditions for growth, since the formation is an expression of misguided growth and should not be confused with swelling activities (Reference 3), and 2. A specific range of penicillin concentrations: Between 0.5 and 0.05 I.E./ccm. With higher concentrations the globules are relatively small and less apparent; with lower ones ellipsoids or cylindrical shapes are formed instead.

The vegetative bacille therefore act in this respect just like the germinating anthrax spores, which have proven themselves in our laboratory for years in connection with the determination of penicillin levels, due to their constant form change coupled with strict dependency on concentration. (Ref. 4.)

We have already previously mentioned that out of numerous tested substances only penicillin causes such a form change of *B. anthracis* (Ref. 5.) The test at hand aims at proving that only *B. anthracis* reacts in the described fashion to these effects.

The procedure for testing a questionable type of bacillus is as follows: First of all a blood agar culture is established, from which a bouillon tube is injected with a plentiful sowing. After the incubation of this bouillon culture for three hours at 37° one loop each is brought to three agar wafers; the first contains 0.5, the second 0.05 I.E./ccm., the third is free of penicillin. The wafers which are suitably cut from penicillin agar plates with a cork drill (1 cm diameter) and placed on slides, should not be thicker than 2 mm, as far as that is practicable. The wafers, thus prepared, are incubated in a moist chamber for three hours at 37°. After this time the form changes that possibly have appeared are observed microscopically by viewing with the strong

dry system or with oil immersion. The more or less lengthy anthrax chains on the penicillin-containing wafers resemble strung-up pearls, and that usually more completely so at 0.05 I.U. (see fig. 1 a) than at the higher concentration (see Fig 1 b.) On the third wafer which contains no penicillin and thus serves as growth control, there are found only slim and on the average longer chains of bacilli of the usual shape, size and arrangement (see Fig. 1 c.)

In this fashion laboratory strains can be tested, as well as those that are isolated from remitted material or from the patient himself. Of course one could also inoculate the test material directly on the agar wafers or be content with its prior enrichment in bouillon -- we would like to recommend the previously described three-stage method, however, because it is most easily suitable to the exposure of large amounts of young, quickly growing bacilli to the effect of penicillin. Besides, the blood plate can serve at the same time for further bacteriologic diagnostic, in the event *B. anthracis* are not involved.

However, it may be useful, especially if speed is essential, to apply the direct method additionally; for if it is successful, depending on whether or not the number of transferred germs is sufficient for observation, then the result is already available after 3 hours. The inclusion of the blood plate in the test process only means a slight delay, however, since the diagnosis can always be made within 24 hours.

If anthracoid types or other gram positive sporeformers resembling *B. anthracis* are tested in the same manner, one of the following three reactions will always be observed:

1. Uniformly favorable, undisturbed growth on all three plates, consequently no reaction to penicillin.
2. More or less strong retardation of growth without or with slight

change in form at both penicillin concentrations or only at the higher one (see Fig 2 b.)

3. Same as 2., but with form changes, which however are completely different from anthrax shapes (club-shaped swellings or "balled-up" growth) (Fig. 2 a.)

Only in one of the 43 anthrax-like strains tested by us did we find forms that distantly resemble the globular form of *B. anthracis*, and that only during hasty observation, yet, as Fig. 3 a, b show, these can be differentiated unequivocally.

Since we used the familiar methods in testing the remaining properties, their description can be dispensed with.

Origin and Properties of Tested Anthrax Strains (See Footnote 1)

Our total of 50 anthrax strains originates from 16 different German Hygiene Institutes. Whether or not, and to which degree, some of these strains have common ancestors could not be established. Aside from the labeling as anthrax bacilli and the usual laboratory and collection numbers and origin data, only twelve showed additional remarks: Two strains were designated as "avirulent," five as "fully virulent," four as "pathogenic for mice," and one as "pathogenic for man."

The testing of these 50 strains had the following result:

1. Colony form on agar: All typical ("Medusa head")

Footnote 1: A detailed rendering of the results has been decided against, in favor of overall perspective, as well as discussion of variable properties (for instance: encapsulation under diverse conditions; influence of animal passages, etc.), since no aspects did not result in this connection.

4. Motility: Motionless 10, in motion 33.
5. Growth in bouillions: all cloudy, partly with surface film.
6. Growth in gelatin stab: all shapes unlike anthrax. Liquefaction between 1 and 5 days 34 strains; no liquefaction 9.
7. Hemolysis on blood agar: after 24 hours negative 7; positive 36.
8. Splitting of dextrose and saccharose: Not tested.
9. Pathogenicity: Not tested.
10. Thermo precipitation after ascoli (see footnote 1 page 6): 19 positive; 2 doubtful; 22 negative.
11. "String of pearls test": All unlike anthrax (no globules).

Worth mentioning at this time is the behavior of an anthrax mutant which was kindly relinquished to us by Prof. Tomcsik, and which was not included in the above record. Contrary to its original strain it does not produce typical penicillin forms, thus acts negatively in the "string of pearls test."

Concerning the origin and the serologic and biochemical properties of the mutant see the diverse publications of Tomcsik (References 6, 7, 8.)

While the original strain, in our experience, was virulent for mice and guinea pigs and behaved typically in every respect, the mutant is apathogenic, morphologically atypical and noticeably mobile!

The negative result of the "string of pearls test" with the mutant is of special interest because we are dealing in this case with two strains which, while they are provably closely related, nevertheless are so basically different in their appearance that the mutant could not even be considered as anthrax-like without knowledge of its origin. In this case the loss of several typical properties of *B. anthracis* would also have an effect on the result of the string of pearls test, while, for instance, the sole loss of virulence might

not affect it (See the strains that have remained apathogenic in spite of manifold animal passages; see also GIBBSON (ref. 1.)

Discussion of Results

The testing of 50 anthrax strains consequently revealed that two strains grow atypically in bouillon, that five are pathogenic neither for mice nor for guinea pigs, and that eleven behave negatively in thermo precipitation. Five of the lastly mentioned eleven strains three reappear among the apathogenic ones, all other exceptions concern only one property. Since further one of the strains designated as avirulent is found among the apathogenic, we can summarize as follows regarding the anthrax strains: Of 50 strains, each tested for eleven properties, eleven (bouillon 2; pathogenicity 1; Ascoli 8) acted atypically relative to one test method, and three strains (pathogenicity, Ascoli) relative to two methods.

Assuming, in view of the origin of the strains (collective strains of bacteriological institutes) and the overwhelming majority of typical properties — which should be justified — that all 50 strains are genuine anthrax strains, then this test result points to two items:

1. that the Ascoli reaction is only slightly dependable and
2. that bacterial anthrax diagnostic can be difficult even under such favorable starting conditions.

The diagnostic difficulties, however, become much more clearly evident, if one similarly considers the other sporeforming types and the results of their testing. However, at this time we do not want to direct our attention to the typical properties of the various types, but only on those properties they share with *B. anthracis*. For in the similarity lies the problem of mutual destruction.

All 43 strains are gram positive like the anthrax bacillus; 27 of them resemble it in regard to their colony form, the morphology of the individual bacilli and their arrangement in chains. Ten strains are motionless like *B. anthracis*; like the latter eleven do not cause hemolysis within 24 hours, and 30 liquefy gelatin, without showing the typical growth formation, however. Likewise, a certain precipitation, 19 strains act like *B. anthracis*.

It follows, without considering the gram positivity, that numerous strains coincide with the typical anthrax bacillus in several properties. That is, four strains have one each, 23 strains two each, 13 strains three each, and one strain four properties in common with it. By the inclusion in this consideration of atypical anthrax strains the overlapping areas are naturally extended.

If we now ask ourselves in summing up, which properties are positively delineated by all 43 sporeformers in relation to typical as well as atypical anthrax bacilli, then only two are left, in which no overlapping can be ascertained.

1. The peculiar growth of the anthrax bacillus in gelatin ("reversed fir tree"), which, however, can be approximated (imperfectly) by some types of anthracoids (brush shape)(see Footnote 1).

2. Formation of globules or strings of pearls under influence of penicillin.

The fact that we did not test the pathogenicity of the anthrax-like strains does not change this result, since, no matter how the test would have turned out, the nonconformity of the anthrax strains would have led to overlaps in this case also. The same applies to sugar fermentation (ref. 9.)

Footnote 1: However, the reliability of this growth characteristic suffers by its dependency on the composition of the gelatin.

In view of the foregoing, the string of pearls test is absolutely specific when applied to the strain-material tested by us, since it identifies every anthrax strain unmistakably, and besides allows a positive differentiation of more or less anthrax-like strains.

Yet we are quite aware of the fact that a final decision on the specificity and practical efficiency of the method described must be won by means of much more copious material. Therefore we would like to encourage its practice and testing not only on laboratory strains, but above all also in areas where anthrax is less rare as a disease in man or animals. We were able to experience, on the occasion of a laboratory infection, that in principle this method can also be applied to practice. With the help of our method the infection was recognized within a few hours, at a time when the local findings (check pasture) did not as yet give cause to any kind of specific suspicion. In this case our early diagnosis by means of the string of pearls test was confirmed by a later, thorough bacteriologic examination as well as by the clinical development (see Fig. 4.)

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Explanation of Plates

Fig. 1: *B. anthracis*, strain #50. a) 0.05 I.E. Penicillin/ccm. b) 0.5 I.E. penicillin/ccm. c) Growth control.

Fig. 2: *B. subtilis*, strain #5. a) 0.05 I.E. penicillin/ccm. b) 0.5 I.E. penicillin/ccm.

Fig. 3: *B. mesentericus*, strain #154. a) 0.05 I.E. penicillin/ccm. b) 0.5 I.E. penicillin/ccm.

Fig. 4: See text.

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