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THE PURIFYING EFFECT OF RAIN IN FREEING THE SOIL OF ANTHRAX SPORES

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(Report presented to the Royal Serum Institute at Rotterdam, then under the direction of Dr. H. E. Reeser.)

In connection with the fact that during the occupation years and shortly thereafter anthrax was able to spread on a number of the small Soenda islands, such as Soemba, Soembawa, and Timor, the question occurred to me whether one could find out how long the soil on which animals suffering from anthrax have deposited their excreta (dung, urine, blood) remains contaminated and to what extent climatological influences affect the presence of anthrax spores and/or bacteria in the uppermost layer of soil or on the surface, where they can be picked up by animals.

It was known from the course of the epizootics on Timor that the occurrence and spread of the disease took place chiefly in the second half of the dry season and the beginning of the rainy season. On Savoe, which is very dry, there was an outbreak, quickly overcome by the way, in August 1946, but on that island the dry season was then quite far advanced. That the monsoon exerts an influence on the course of the epizootic can surely be assumed for those regions.

In the Netherlands and elsewhere a connection has long been assumed between the occurrence of anthrax and the level of the ground water. Kuipers saw the disease completely disappear, in a district where it had prevailed among the cattle for a long time, after the draining of a polder, which caused the ground water to drop. In Russia, in a study published in 1933, Pokshchikhevchiy and Golovin treated anthrax as a soil epizootic. They reported that it appeared from their investigation that a stationary infection finds the most favorable conditions for its development in soil that is rich in organic substances and that is sufficiently moist. After drainage projects are accomplished the soil usually becomes free of anthrax germs. Western Europe may serve as an example, where as a result of drainage and the lowering of the ground water level anthrax as a stationary disease has completely disappeared. But this general principle, which is usually simply taken as a fact, ordinarily finds no experimental explanation. The authors mentioned, after their investigation, which apart from study of the statistical data comprised investigation of the pH of the soil, the soil moisture, the soil temperature and the precipitation (the last two items being obtained from the meteorological institute), and a bacteriological study, came to the following hypothesis:

"When the germs multiply greatly the soil absorbs decomposition products of the vegetative forms of anthrax, and these are adsorbed to soil particles and have a retarding effect on the process of further multiplication of the soil bacteria, just as e.g. no further multiplication can take place in the filtrate of very old cultures. For the future only a certain reserve of highly resistant spores remains in the soil."

The possibility does not seem to be ruled out that in Indonesia, where there are many porous types of soil with a very low humus content, and where the heavy rains make the downward movement of the water much more pronounced than in Europe, the process is different, and the soil is cleansed of anthrax spores by being washed out, so to speak.

With this in mind an attempt has been made to imitate in vitro the downward movement of the water and to study what influence this exerts on anthrax spores present in the soil. As influences other than mechanical had to be excluded, the work was always done with sterilized soil and sterile water.

For safety reasons, in this investigation a non-virulent but completely viable anthrax strain was used which originally came from South Africa and which is also used for the vaccine with which many tens of thousands of animals are immunized in this country and elsewhere.

Seven agar tubes inoculated with this strain were incubated for three days at 37° C and afterwards kept for four days at room temperature (± 15° C). At the end of that time sporulation appeared to be about 3/4 complete. The spores were washed out with a physiological NaCl solution and a very thick suspension obtained in that way.

A number of test tubes were now made leaky by heating the lower end red hot and blowing a hole in it. The opening made was ± 2 mm in diameter. A bit of sterile gauze was laid over it to prevent the soil from getting out.

In the tubes was put:

Tube I	sand	to a depth of 2 cm	(Z2)
Tube II	sand	to a depth of 4 cm	(Z4)
Tube III	garden mold	to a depth of 2 cm	(T2)
Tube IV	garden mold	to a depth of 4 cm	(T4)
Tube V	infusorial earth	to a depth of 2 cm	(I2)
Tube VI	infusorial earth	to a depth of 4 cm	(I4)

On the earth (sand, garden mold, and inf. earth) was put four drops of the spore suspension. The tubes were set in Erlenmeyer flasks and fixed in the neck of the flasks with a layer of cotton wool, but in such a way that the tubes could be readily taken out. These Erlenmeyers served to catch the moisture that dripped through.

Rain was imitated by dripping a quantity of sterile (boiled and cooled) water into the tubes, the quantity corresponding to 5 cm of rainfall. (For the tubes of 14 mm diameter this was 7.7 cc, for those of 15 mm diameter, 8.85 cc.)

After the water had filtered through, one loopful of it was inoculated on agar (filtrate 1 = F1). This gave the following result:

F1 Z2 + abundant growth, colonies almost joined.
F1 Z4 +
F1 T2 +
F1 T4 + } somewhat less abundant growth than F1 Z2, but not much difference among themselves.
F1 I2 -
F1 I4 + 4 colonies.

The colonies were typical anthrax colonies, viewed either with the naked eye or with the magnifying glass (Medusa locks). As a control a streak preparation stained with Giemsa was examined, so that it was absolutely established that in fact anthrax spores had been present in the filtrate. From F1 I2 another inoculation was done, this time with a larger quantity, viz. 3 loopfuls. This produced a growth of 20 colonies.

The conclusion could be drawn that sand, garden mold, and infusorial earth are pervious to spores, and that infusorial earth worked as a filter better than garden mold and sand.

It was now the intention to investigate whether repeated flushing could make the earth free of anthrax spores. The flushing was therefore repeated with the same quantities of water with the following result. (F2 Z2 = 2nd filtrate through a layer of sand 2 cm thick, etc.)

F2 Z2 +
F2 Z4 +
F2 T2 +
F2 T4 + } All these showed abundant growth.

F2 I2 + A few colonies.
F2 I4 + Abundant growth.

Since the water ran through the infusorial earth very slowly and this caused delay in conducting the experiment, we proceeded from this point only with soil and sand.

We continued washing Z2, Z4, T2, and T4 and inoculated with F3 to F18 inclusive; this last represents the filtrate after a total rainfall of 90 cm.

Inoculation with a loopful of filtrate from the last drop to run through after each artificial shower always produced an abundant growth on agar. The conclusion could be drawn from this that a rainfall of 90 cm, which is more than the average annual rainfal in the Netherlands, is not capable of getting rid of all spores present in a layer of sand 2 cm thick.

The tests were now repeated with greater quantities of water and only with 2 cm of sand as a filter. An entirely different tube was also used here. A test tube was melted to a conical shape and provided with a very small opening. The bit of sterile gauze could now be dispensed with, since the sand could not run out through the fine opening.

We worked with artificial showers equivalent to 75 cm. Inoculation was again done with a loopful taken from the last drop to run through after each shower. The result of the inoculations was as follows:

F1	(after 75 cm of rain)	Z2 +	± 60 colonies.
F2	(" 150 " " ")	Z2 +	5 colonies.
F3	(" 225 " " ")	Z2 -	
F4	(" 300 " " ")	Z2 -	
F5	(" 375 " " ")	Z2 -	

In the filtrate derived from the last three showers, then, no anthrax spores showed up, -- at least not with the method used.

The presumption was now that a quantity of rain between 150 and 225 cm was sufficient to eliminate all anthrax spores from a layer of sand 2 cm thick. As a check, inoculations were done from the sand itself. This produced a strong growth on agar. There were thus plenty of anthrax spores left in the sand, and the above presumption turned out to be quite incorrect.

It might be concluded that a part of the spores had been carried away by the water, but that another part had remained behind in the sand and was held so firmly by it that no spores showed up in the filtrate with the method used.

The last experiment was repeated in a somewhat different manner. On a layer of sand 2 cm thick three drops of spore suspension was put down. An artificial rain of 37.5 cm of water each time was supplied. As before, the last drop trickling through after each shower was used for inoculation. At the same time, however, an inoculation was done from the sand itself, the sand being stirred a little with the wire loop.

Inoculation from the filtrate is represented by F1 to F13 inclusive, inoculation from the sand by G1 to G13 inclusive (G = gemengt [mixed, stirred]).

F1	(after	37.5	cm	of	rain)	a great many	colonies;	G1	completely	overgrown.
F2	"	75	"	"	"	± 14	"	G2	"	"
F3	"	112.5	"	"	"	± 60	"	G3	"	"
F4	"	150	"	"	"	± 28	"	G4	"	"
F5	"	187.5	"	"	"	± 14	"	G5	a great many	colonies.
F6	"	225	"	"	"	2	"	G6	" "	"
F7	"	262.5	"	"	"	3	"	G7	completely	overgrown.
F8	"	300	"	"	"	± 50	"	G8	"	"
F9	"	337.5	"	"	"	2	"	G9	"	"
F10	"	375	"	"	"	2	"	G10	"	"
F11	"	412.5	"	"	"	9	"	G11	"	"
F12	"	450	"	"	"	7	"	G12	"	"
F13	"	487.5	"	"	"	9	"	G13	"	"

It appears from this that a number of spores are carried along by the water that is streaming through. A very great number, however, stick very fast to the grains of sand and are not washed away even by very great quantities of water (corresponding to a total rainfall in seven years in the Netherlands). The mechanical effect of rain can therefore only be slight with regard to freeing the top layer of soil from anthrax spores. That in this test, in contrast to the preceding test, the inoculations from the filtrate always turned out positive (although the number of colonies generated amounted in one case to only two) can be explained by the fact that in the process of inoculation from the sand a number of spores were jarred loose from the grains of sand to which they had attached themselves, so that these spores could be carried along with the water and showed up in the filtrate.

An experiment was then set up to find out whether the very topmost layer of sand could be freed of spores.

Into a test tube that had been made leaky a layer of sand 5.5 cm in depth was poured in. Three drops of the same spore suspension was put on this sand. The artificial rain consisted of five showers of 60 cm each. After each shower an inoculation was done just from the topmost layer of sand. The result was as follows. Inoculation from the surface is represented by O1 to O5 inclusive.

O1	(after	60	cm	of	rain)	very	abundant	growth.		
O2	("	120	"	"	")	"	"	"		
O3	("	180	"	"	")	"	"	"		
O4	("	240	"	"	")	"	"	"		
O5	("	300	"	"	")	"	"	"		

It thus appeared that even 300 cm of rain was not capable of making the surface free of spores.

To find out to what extent a difference may exist between spores and bacteria, some filtration tests were made with hog cholera bacteria. Hog cholera bacteria were chosen for this because they show no motion whatever and hog cholera is regarded as a soil disease.

A suspension of hog cholera bacteria was obtained by centrifuging a broth culture and washing out the sediment with physiological NaCl solution. This was repeated three times, so that it could be assumed that the suspension contained little else than hog cholera bacteria and physiological NaCl solution.

As a control an inoculation with this suspension was done on serum agar, which showed that no contamination was present. In the same way as had been done with anthrax spores, the bacteria suspension was applied drop by drop to the sand layer in a test tube that had been made leaky at the bottom. Water was dripped on the sand by means of a burette and an inoculation done with the moisture that ran through. These artificial showers were chosen of such a size that each quantity of water applied corresponded to a rainfall of 7.5 cm.

F again represents inoculation with the last drop to run through after each shower. The outcome was as follows:

F1	(after	7.5	cm	of	rain)	Fair number of colonies grown.
F2	("	15	"	"	")	Large " " "
F3	("	22.5	"	"	")	A few colonies grown.
F4	("	30	"	"	")	No growth.
F5	("	37.5	"	"	")	" "
F6	("	45	"	"	")	A few colonies developed.
F7	("	52.5	"	"	")	No growth.
F8	("	60	"	"	")	" "
F9	("	67.5	"	"	")	" "
F10	("	75	"	"	")	" "

After this inoculations were done not only from the filtrate but also from the surface of the sand. This is represented by O. O1 is the first inoculation from the surface, which was done after the passage of 75 cm of rain through the layer of sand. The quantity of water was set higher from here on, so that each shower corresponded to 25 cm of rainfall. The outcome of the inoculations was as follows:

	O1 (after 75 cm rain) a great many colonies.
F11 (after 100 cm rain) no growth;	O2 (" 100 cm ") many colonies grown.
F12 (" 125 cm ") " "	O3 (" 125 cm ") " " "
F13 (" 150 cm ") " "	O4 (" 150 cm ") " " "

It thus appeared that a number of bacteria were also carried away by the water, but that many bacteria remained stuck to the sand. There was no difference in behavior between anthrax spores and hog cholera bacteria.

An attempt was then made to show to what extent anthrax spores situated at some distance below the surface are carried along with rising ground water.

In nature rising ground water is supposed to occur frequently, for instance when water from higher places reaches a basin-shaped bit of terrain.

A tube was melted and bent in such a way that it consisted of two legs, joined at the bottom by a narrow connecting piece. In one leg (A) was placed a layer of sterile sand to a depth of 2 1/2 cm.

In the other leg (B) three drops of anthrax-spore suspension was dropped. This thus reached the bottom of the sand via the connecting piece. In leg B such an amount of water was then dripped in that the water rising in leg A could just moisten the surface of the sand (principle of communicating vessels). Inoculations were then done from the moist surface of the sand. The moisture seeping up from below appeared to be very rich in anthrax spores.

This experiment was repeated with somewhat thicker layers of sand and garden mold, namely

Sand layers of 4, 6, and 8 cm depth and
Layers of garden mold of 4, 6, and 8 cm depth.

The outcome was always the same, namely that a number of anthrax spores could always be found in the moisture that seeped up.

Because of my departure from the Netherlands this experiment could not be continued, so that it did not become known how deep the layer of sand, garden mold, or infusorial earth must be to prevent the anthrax spores from reaching the surface.

From the seepage tests it appeared that although the soil holds a certain number of spores, it still lets a number through. The smaller the number of spores the greater the chance will be that they will get caught somewhere and not be carried further by the rising or falling water, and the greater the number of spores present the greater the chance that finally some will e.g. in the case of rising water reach the surface. Among other things it appeared from the experiments of the Russian investigators mentioned earlier that under favorable circumstances as to temperature, O_2, moisture content, and the presence of organic matter multiplication of anthrax germs can take place in the soil. If those favorable circumstances prevail, then because of the multiplication of the number of germs the chance is greater still that of this larger number a certain part will reach the surface with rising water. It is also conceivable that this arrival at the surface of the ground may only come to pass after the water has risen several times. If e.g. anthrax spores are present at a depth of 25 cm, then it is possible that with rising water some will come up 10 cm higher. If at that level after the water goes down the circumstances (temperature, O_2, and humus) are favorable, then multiplication will take place, and when the water rises again a part of the germs will come loose and rise a bit higher. It is thus conceivable that several periods of high water between which lie periods of favorable weather for the multiplication of anthrax germs will finally cause these germs to be present in the uppermost layer of soil and on the surface, where they can be picked up by cattle.

The possible correctness of this line of thought should be checked in the laboratory by effecting a rise of water in leg A of the tube described (which in this case must be long enough to contain a layer of earth thick enough to serve as an adequate filter, at least for the passage of water), placing the apparatus in a nutrient medium, and later again effecting the rise of water, and testing whether or not after repetition of the rising of the water, followed by placing in the nutrient medium, the anthrax germs will finally show up in the uppermost layer of soil.

Summary [printed in Dutch and English]

An attempt ~~has been~~ made in several experiments to imitate in vitro the influence of descending and rising water (rainfall) on anthrax spores in the soil. The result is that although a part of the total quantity of spores was washed out, still a considerable number, even after an artificial rain corresponding to the natural rainfall of several years in the Netherlands, could be detected in the surface layers. These spores seemed fairly strongly attached to the soil particles.

The conjecture is expressed that when the circumstances are favorable for multiplication of anthrax germs in the soil, a repeated rising of water will bring the germs to the surface even when a single rising is not sufficient to do so.

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