THE PERSISTENCE OF AVIAN TUBERCLE BACILLI IN SOIL AND IN ASSOCIATION WITH SOIL MICRO-ORGANISMS[1, 2]

CHESTER RHINES

Agricultural Experiment Station, New Brunswick, N. J.

Received for publication August 5, 1934

The purpose of these investigations was to obtain information on the death rate of tubercle bacilli in soil. Undoubtedly many tubercle bacilli become lodged in soil by animal contamination. It is possible, therefore, that tubercle bacilli surviving for a long period in soil may play a rôle in the spread of disease. The amount of infection possible would be proportional to the time of survival. Schalk (1928) has presented evidence of soil transmission of avian tubercle bacilli.

METHOD OF ISOLATING TUBERCLE BACILLI FROM HIGHLY CONTAMINATED MATERIALS

The method of study used to obtain the information in this paper has involved infecting the soil with tubercle bacilli and obtaining a plate count of the tubercle bacilli periodically. A plating method satisfactory for isolating Mycobacteria from many contaminated materials was developed. Tubercle bacilli can be isolated from among Gram-negative bacteria by treatment with alkali, acid or other chemical agents to which Gram-negative bacteria are sensitive. On the other hand, they can be separated from Gram-positive bacteria by culturing on crystal violet medium. Crystal violet retards Gram-positive bacteria. When a mixed culture containing Mycobacteria is treated with sodium hydroxide and plated on glycerol agar containing crystal violet,

[1] Journal Series Paper, New Jersey Agricultural Experiment Station, New Brunswick, N. J.

[2] These investigations were supported by a grant from the National Research Council and carried out under the auspices of Committee on the Microbiology of the Soil.

the Mycobacteria may thus develop in colonies and the plates remain relatively free from other forms. The combination of alkali treatment and culturing on crystal violet medium has been found exceedingly effective in retarding development of most common soil-microörganisms other than certain soil acid-fast bacteria. All acid-fast bacteria cannot be isolated by this method. Several strains worked with were too sensitive to alkali. Pathogenic and non-pathogenic strains may or may not be isolated by this procedure, the success depending upon the physiology of the organism dealt with. Certain soil Mycobacteria have been easily isolated by the method. *Avian 531* was easily isolated but the *H37* human strain and the *Ravenel* bovine strain of tubercle bacilli were found more difficult to deal with because they were not so markedly alkali-resistant.

An important factor in the success of this plating method has probably been the fact that the inoculum was always put on the surface of an agar plate in 1 cc. of a very dilute agar. The bacteria were thus always at the surface of the agar imbedded in a shallow layer of nutrient medium. Satisfactory plates were obtained only after the use of a dilute agar blank was adopted.

The medium used was varied somewhat during the course of the experiments. Slight changes in composition were not observed to give very different results. The addition of yeast, egg yolk, and potato extracts was found to promote growth. One batch of medium was used for each experiment to avoid danger of variation.

Medium used for plating

Glycerol	100	cc.
Peptone	5	grams
Beef extract	3	grams
Yeast extract	2	grams
Extract of two egg yolks		
Extract of 200 grams potato		
K_2HPO_4	0.6	gram
KH_2PO_4	0.2	gram
Agar	40	grams
0.6 cc. of 1 per cent alcoholic crystal violet		
Water to make 2 liters of medium		

Dilution blanks

One part of above medium
Four parts of water

The egg and potato extracts were prepared by boiling the egg yolk and sliced potato in a liter of water for fifteen minutes and filtering off the solids. The agar medium and dilution liquid were sterilized in the autoclave. The melted agar was cooled to 45°C. and poured into sterile Petri dishes. After the agar solidified, the Petri dishes were placed in an autoclave and the temperature maintained at about 70°C. for an hour. The autoclave was not opened until the plates had cooled to room temperature. This partial sterilization of the poured plates eliminated the majority of contaminating organisms which were sucked into the plates while the agar was cooling. The partial sterilization of poured plates probably would be unnecessary in a laboratory protected from dust. The dilution blanks were heated to liquefy and cooled to 40°C. The thoroughly shaken material containing the tubercle bacilli was alkali-treated, neutralized, and diluted as for the ordinary plate count, the dilution agar being used as a sterile dilution medium. One cubic centimeter of a desired dilution was placed on the surface of a prepared agar plate and the dish quickly rotated to spread the inoculum over the surface. The plates were inverted in about half an hour, placed under bell jars and incubated at 37°C. The colonies were counted after three weeks of incubation.

EFFECT OF PURE CULTURES OF SOIL MICROÖRGANISMS ON AVIAN TUBERCLE BACILLUS 531 IN SOIL

Fifty-gram samples of soil were placed in 250 cc. flasks and sterilized in the autoclave for one hour; also, soil containing 20 per cent of horse manure was similarly sterilized. Calcium carbonate was added to all soil. All flasks were equally inoculated with *Avian 531*. The following outline indicates the additional inoculations made into some of the flasks: (1) Pure culture, *Avian 531*, tubercle bacillus; (2) *Avian 531* and *B. megatherium*;

(3) *Avian 531* and *A. aerogenes*; (4) *Avian 531* and soil anaerobe; (5) *Avian 531* and the fungus, *Verticillium chlamydosporum.*

The water content of the soil was adjusted to about 25 per cent and stoppers were inserted above the cotton plugs to prevent evaporation. The flasks were kept at room temperature which ranged about 25°C. The soil was treated for five minutes with 2 per cent sodium hydroxide before plating.

TABLE 1

Plate count of Avian 531 in soil associated with soil microörganisms

DAYS OF INCUBATION	PURE	WITH B. MEGATHERIUM	WITH A. AEROGENES	WITH ANAEROBE	WITH FUNGUS
0	68,000	68,000	68,000	68,000	68,000
17	181,000	525,000	46,000	430,000	10,000
67	980,000	340,000	310,000	400,000	3,000
97	5,750,000	2,950,000	810,000	2,300,000	39,000

TABLE 2

Plate count of Avian 531 in manured soil associated with soil microörganisms

DAYS OF INCUBATION	PURE	WITH B. MEGATHERIUM	WITH A. AEROGENES	WITH ANAEROBE	WITH FUNGUS
0	68,000	68,000	68,000	68,000	68,000
17		450,000	475,000	225,000	12,000
67	1,250,000	660,000	750,000	35,000	3,500
97	21,000,000	11,000,000	18,000,000	50,000	3,000

The pure culture count increased so notably that it seems probable that multiplication occurred. The observed increase is about 100 fold after three months. The soil containing *A. aerogenes* and *B. megatherium*, maintained approximately the same tubercle bacillus population as the pure culture. No significant difference between the pure culture count and the count of *Avian 531* was noted associated with the anaerobe in soil, but the count of the tubercle bacilli in manured soil was low in the presence of the anaerobe. The manure probably made conditions more suitable for the anaerobe. The fungus was decidedly inhibitory to *Avian 531* under the conditions of this experiment.

The plate counts of *Avian 531* after culturing with various pure

cultures of bacteria and actinomycetes in soil for one month are given in table 3. Fifty-gram samples of soil were sterilized and inoculated with equal amounts of an *Avian 531* suspension. Calcium carbonate had been added to the soil. Some of the flasks were also inoculated with a pure culture of another microörganism as indicated in table 3. All the flasks were stoppered with cotton plugs and were kept in a very humid incubator at 28°C. The plate count of tubercle bacilli at the time of inoculation was 870,000 per gram of soil. At the end of one month all samples were plate-counted.

No plate counts were made of any organism other than the tubercle bacillus. It is quite certain that the soil microörganisms

TABLE 3

Avian 531 with soil bacteria and actinomycetes

MICROÖRGANISMS IN SOIL	PLATE COUNT OF AVIAN 531 AFTER ONE MONTH (DUPLICATE COUNTS)	
Avian 531 and *Azotobacter vinelandi*	5,000,000	6,200,000
Avian 531 and *Serratia marcesens*	11,000,000	3,200,000
Avian 531 and *Pseudomonas fluorescens*	1,200,000	600,000
Avian 531 and *Proteus vulgaris*	3,600,000	3,600,000
Avian 531 and *Bacillus subtilis*	3,800,000	2,500,000
Avian 531 and *Actinomyces Lipmanii*	1,400,000	1,900,000
Avian 531 and *Actinomyces violaceus-rubes*	2,100,000	1,600,000
Avian 531 and *Actinomyces pheochromogenus*	5,400,000	4,300,000
Avian 531 (pure culture)	8,600,000	6,300,000

developed abundant populations. Inoculations were large and were made from fresh growths on agar slants. The counts of *Avian 531* from duplicate flasks checked satisfactorily. These counts were not depressed markedly by any of the soil microorganisms. The pure culture count is slightly higher than the others, but the difference is not of a significant order.

Table 4 gives the plate counts of *Avian 531* after being cultured for one month with pure cultures of fungi and with known mixtures of fungi and bacteria. The study was conducted with soil and with soil enriched by 2 cc. of nutrient broth and 0.02 gram of sucrose per 50 grams of soil. Calcium carbonate was added to all soil to control the reaction. The sterile soil samples were also

inoculated with *Avian 531* as in the previous experiments and the additional inoculations made from pure cultures of fungi and bacteria suspended by shaking with sterile gravel and water. The infected soil was kept at 28°C. in a humid incubator. The flasks were plugged with cotton.

TABLE 4

Avian 531 with fungi and with known mixtures of fungi and bacteria

ORGANISMS INOCULATED INTO SOIL	PLATE COUNT OF AVIAN 531 AFTER ONE MONTH	
	In soil enriched by 2 cc. of nurient broth and 0.02 gram of sucrose per 50 grams of soil	In soil
Avian 531 (pure culture)	21,000,000	600,000
Avian 531 and *Humicola*	1,200,000	2,400,000
Avian 531 and *Aspergillus niger*	9,600,000	13,000,000
Avian 531 and soil penicillium	2,200,000	3,900,000
Avian 531 and *Trichoderma 105*	340,000	2,200,000
Avian 531 and *Mucor Rouxii*	*	4,500,000
Avian 531 and *Rhizopus 120*	6,900,000	5,800,000
Avian 531 and *Aspergillus flavus*	*	4,000,000
Avian 531 and *Verticillium chlamydosporum*	900,000	3,200,000
Avian 531, Aspergillus flavus, A. aerogenes and *B. megatherium*		3,000,000
Avian 531, Trichoderma 105, A. aerogenes and *B. megatherium*		3,100,000
Avian 531, A. aerogenes and *B. megatherium*		5,400,000
Avian 531, all fungi listed above, *A. aerogenes*, and *B. megatherium*		400,000
Avian 531 and all fungi listed above	*	*
Avian 531, soil penicillium, *A. aerogenes*, and *B. megatherium*		1,500,000

* Fungi overgrew plates so badly that no counts were obtained.

Macroscopic examination of the flasks revealed that the enriched soil had a greater abundance of fungus mycelium. The presence of *A. aerogenes* and *B. megatherium* reduced the amount of fungus mycelium.

The soil was treated for ten minutes with 2 per cent sodium hydroxide before plating. The plate count of *Avian 531* at the time of inoculation was 10,000,000 per gram of soil.

Considerable variation occurred in the plate counts, but in general, the numbers of *Avian 531* were not markedly reduced. At least, the data showed conclusively that a large percentage of the tubercle bacilli survived, even when associated with complex mixtures of fungi and bacteria. While the data as a whole indicate conclusively the long viable period of *Avian 531*, they have less value in indicating the effect of a single organism on the death rate. The plate count does not warrant conclusions from a single determination.

AVIAN 531 IN TOLUOL STERILIZED SOIL

Extensive studies were made of the death rate of *Avian 531* in soil partially sterilized by toluol. Plate-counting of tubercle bacilli in toluol-sterilized soil was less difficult than in unsterilized soil because the fungi which overgrew many plates were partially eliminated and the soil acid-fast bacteria, difficult to distinguish from tubercle bacilli, were destroyed. Five cubic centimeter portions of toluol were added to 250 cc. flasks containing 50 grams of soil. The flasks were allowed to remain stoppered for twenty-four hours. The stoppers were then replaced by sterile cotton plugs and aeration was permitted for one week at 37°C. A heavy suspension of *Avian 531* was then prepared by shaking the bacteria from a glycerol agar slope with glass beads. All the flasks were then equally inoculated with the same amount of suspension and the water content adjusted to about 25 per cent. Rubber stoppers were used to retain the moisture. Control flasks were similarly treated except that inoculation with tubercle bacilli was omitted. The acid-fast bacteria were counted by plating at various intervals. The data are presented in table 5. At some of the intervals the total bacterial count of the soil was also determined by plating on nutrient agar.

The soil was treated with 2 per cent sodium hydroxide for thirty minutes in the *Avian 531* count procedure. No acid-fast bacteria appeared on the plates from the control soil. Undoubtedly the death rate of the tubercle bacillus was not rapid in this soil. The slight rise in count after the twenty-nine days of incubation scarcely indicated that an actual increase in numbers

occurred. Recovery of the tubercle bacilli became more and more difficult as the number of tubercle bacilli decreased and the soil bacteria increased. Although the observations made with the toluol-sterilized soil are not equivalent to what might have occurred in unsterilized soil, the results prove that tubercle bacilli are capable of existing in soil in competition with many soil microörganisms for at least two months.

TABLE 5

AVIAN 531 IN TULUOL STERILIZED SOIL		TOTAL NUMBER OF BACTERIA IN SOIL
Days after inoculation	Number of Avian 531 per gram of soil	
0	53,000	
7	160,000	8,500,000
29	272,000	
67	3,000	16,000,000

The results of table 5 are quite typical of those from several similar experiments.

TABLE 6

Survival of Avian 531 in toluol sterilized soil under various conditions

NUMBER OF DAYS AFTER INFECTION	NUMBER OF TUBERCLE BACILLI PER 1/1000 GRAM OF SOIL											
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12
0	78	78	78	78	78	78	78	78	0	16	78	0
17	2	17	6	24	16	22	38	69	0		88	0
30	5	125	150	16	0	13	12	20		240	9	0
48	22	16	90	1	40	1	18	28		325	1,900	
99	5	2	0			21	0	3	0	40	*	0

* Bad contamination of plates.

EFFECT OF VARIOUS ORGANISMS AND VARIOUS CONDITIONS ON SURVIVAL OF AVIAN 531 IN TOLUOL-STERILIZED SOIL

Toluol-sterilized soil was prepared as in the previous experiment. All flasks except the controls, 9 and 12, which were not infected, and 10, which received 1 cc. of *Avian 531* suspension,

received 5 cc. of *Avian 531* suspension. Modifications are indicated in the following list which gives the various treatments.

1. Five cubic centimeters of *Avian 531*.
2. Five cubic centimeters of *Avian 531* and inoculated with *Colpoda cuculus*.
3. Five cubic centimeters of *Avian 531* and inoculated with *Colpoda Steinii*.
4. Five cubic centimeters of *Avian 531* and inoculated with mixed soil ameba culture.
5. Five cubic centimeters of *Avian 531* and incubated at 37°C.
6. Five cubic centimeters of *Avian 531* and 0.05 gram of ammonium sulfate added per 50 grams of soil.
7. Five cubic centimeters of *Avian 531*; 4 cc. less water than in first flask.
8. Five cubic centimeters of *Avian 531*; 5 cc. more water than in first flask.
9. Control, no *Avian 531* added.
10. *Avian 531* in sterile soil. Soil sterilized in autoclave. One cubic centimeter of *Avian 531* added.
11. Five cubic centimeters of *Avian 531* per 50 grams of soil. This infected soil was placed in a pot in the field.
12. Control (no *Avian 531*) placed in pot in the field.

The water content of the soil was about 25 per cent in all the flasks except 7 and 8. The soil of series 7 had about 17 per cent water and the soils of series 8 contained about 35 per cent water. All the flasks were stoppered throughout the course of the experiment to prevent loss of water. Samples 11 and 12, however, were exposed to field conditions. The weather was excessively rainy during the first few weeks of the experiment and then became hot and dry. Toluol sterilization eliminated protozoa, which were, therefore, present only in the soil to which cultures were introduced. All samples but 5, 11, and 12 were kept at room temperature.

Table 6 gives the results of the *Avian 531* plate counts. The soil samples were treated with 2 per cent sodium hydroxide for thirty minutes before plating. Counts are given in thousands per gram of soil.

The data are much too variable for detection of differences in death rate as a result of the different treatments. None of the protozoa altered the death rate sufficiently for detection. Varying moisture, temperature and nitrogen content did not alter the viability. The results with *Avian 531* in the field are most irregular but it is certain that large numbers survived six weeks. The results as a whole leave no doubt that *Avian 531* is able to survive for a considerable period under a variety of conditions. The pure culture, sample 10, had a quite significant increase in numbers.

SURVIVAL OF AVIAN 531 IN UNSTERILIZED SOIL

Although the study of the survival of *Avian 531* in unsterilized soil presents serious difficulties, some degree of success has been

TABLE 7

Survival of Avian 531 in unsterilized soil

DAYS AFTER INOCULATION	NUMBER OF AVIAN 531 PER GRAM OF SOIL
0	56,000,000
3	65,000,000
13	67,000,000
23	14,000,000
31	11,000,000
44	3,500,000
59	2,700,000

achieved. Early attempts failed because the amounts of *Avian 531* added to the soil were too small and the medium was not suitable for the best plating results with tubercle bacilli. The death rates of large inocula of tubercle bacilli have been determined by plating the infected soils on the medium described in this paper. The soil was treated with 2 per cent sodium hydroxide for thirty minutes before being plated. The count of *Avian 531* was so high in these studies that there was no danger of soil acid-fast bacteria being counted as tubercle bacilli. Control samples of soil inoculated with heat-killed tubercle bacilli yielded only about 10,000 acid-fast bacteria per gram of soil. The soil was moist. Fifty gram samples were kept in 250 cc. flasks. The

flasks were kept in a very humid incubator at 28°C. Conditions were favorable for great activity of soil microörganisms.

Another experiment has given results verifying the death rate observed in table 7, and has added a few observations on the death rate under somewhat varied conditions. Tubercle bacilli were inoculated into flasks of soil as in the previous experiment and the plate count determined periodically. The soil was treated with 2 per cent sodium hydroxide for five minutes before plating.

TABLE 8

Survival of Avian 531 in unsterilized soil

DAYS AFTER INOCULATION	A	B	C	D	E
0	6,000,000	6,000,000	6,000,000	6,000,000	6,000,000
5	8,500,000				
12	3,500,000				
19	1,200,000				
33	800,000	660,000	530,000	10,000	20,000

A. Incubated at 28°C.; water content about 25 per cent.

B. Incubated at 37°C.; water content about 25 per cent.

C. Incubated at room temperature (about 20°C.); water content about 25 per cent.

D. Samples allowed to air dry at 28°C. in an incubator with no humidifier.

E. Incubated at 28°C. in a test tube, depth of soil being about 5 inches. Water was added until the water level was above the soil surface.

Table 7 and column A of table 8 represent counts made under very similar conditions. The numbers of tubercle bacilli decreased consistently with time of culture in the soil. The rate of decline in numbers was higher than that noted generally in pure culture associations. The rate is so slow, however, that it seems justifiable to conclude that the tubercle bacilli would survive for many more months. According to the figures of table 8 the death rate is about the same at 37°C. and at room temperature (about 20°C.) as at 28°C. This organism has an optimum growth at 37° or slightly higher and will grow fairly well at 28°C. The growth rate at room temperature is extremely slow. At room temperature, survival must be due chiefly to the inoculated

cells maintaining their viability rather than to the production of new cells at a rate sufficiently high to maintain the observed population.

In soil allowed to air-dry at 28° and in soil covered with water, the death rate was much accelerated. Even these unusual treatments did not completely destroy the tubercle bacilli. Control plates showed that soil acid-fast bacteria were not confused with the tubercle bacilli at the low dilutions.

DISCUSSION

Avian 531 increased in population when inoculated into sterile soil and when grown in soil in association with some pure cultures of bacteria. In association with other pure cultures of microorganisms a slow death rate has been noted. The difference in death rates caused by different organisms was not extensively studied. The plate count of tubercle bacilli decreased consistently in soil sterilized by toluol and in unsterilized soil. This death rate is due to the presence of the complex microbiological population but no evidence points to particular organisms as chiefly responsible for the destruction of the tubercle bacilli or to the mechanism of destruction.

SUMMARY

1. A method suitable for plate-counting of *Avian 531* tubercle bacilli in the presence of complex microbiological populations was developed.

2. The *Avian 531* tubercle bacillus was found to multiply in sterile soil and when associated with pure cultures of some soil bacteria. Under the conditions of this same experiment, a fungus checked development of *Avian 531*, especially in manured soil.

3. In general, bacteria, fungi and actinomycetes did not markedly depress the number of tubercle bacilli in soil when cultured in association with the tubercle bacilli.

4. *Avian 531* tubercle bacilli survived in toluol-sterilized soil for long periods under a variety of conditions. Recovery from toluol-sterilized soil was made after three months. The death rate was slow.

5. *Avian 531* tubercle bacilli were slowly destroyed in soil which had not been sterilized. The plate count numbers were reduced to about one-sixth of the original counts in one month. If the same death rate continued, the tubercle bacilli inoculated into the soil would survive for many months.

REFERENCE

SCHALK, A. F. 1928 Jour. Am. Vet. M. Asso., **72**, 852.