

FURTHER STUDIES ON THE SURVIVAL TIME OF THE BOVINE TUBERCLE BACILLUS IN SOIL, SOIL AND DUNG, IN DUNG AND ON GRASS, WITH EXPERIMENTS ON FEEDING GUINEA-PIGS AND CALVES ON GRASS ARTIFICIALLY INFECTED WITH BOVINE TUBERCLE BACILLI

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In a previous paper (Maddock, 1933) it was reported that when soil, soil and dung mixtures and dung were infected by adding fine emulsions of bovine tubercular material, virulent tubercle bacilli could be recovered after 178 days' exposure under field conditions. The experiments reported in the aforesaid paper were carried out in 1931, but it was felt that weight would be added to the results if the experiments could be repeated in a year of differing weather conditions.

During the year 1932, therefore, the experiments described in the first part of the present paper were made.

TECHNIQUE

The technique for the recovery of *Mycobacterium tuberculosis* was the same as that reported in my previous paper. Briefly it consisted of a refinement of the usual method by adding amounts of NaOCl/NaOH mixtures calculated to satisfy the requirements of the organic matter present and kill the majority of saprophytic organisms whilst leaving the tubercle bacilli as far as possible unimpaired. (It may be noted that cultures of B.C.G., when incorporated in various mixtures containing organic matter, were found to withstand concentrations of the chlorine preparations equal to or greater than those tolerated by the bovine tubercle bacilli used.) As before, emulsions of tubercular material, proved in all cases by culture and inoculation to contain virulent bacilli of the bovine type, were used. The emulsions were added in such quantities as to give a final concentration in the various mixtures of about 100,000 tubercle bacilli per gram.

PART I

VIABILITY OF BOVINE TUBERCLE BACILLI IN SOIL, DUNG AND SOIL MIXTURES, AND IN DUNG EXPOSED IN THE OPEN DURING 1932

The weather of 1932 differed from that of 1931 in that it was, on the whole, warmer. The average mean temperature for the months of July, August and September, 1932, was 2.9° F. higher than that of the same period in 1931. The maximum temperature in 1932 was 94.6° F. as compared with 76.0° F. for 1931.

The plots were laid down on May 4, 1932—some weeks earlier than in 1931—and samples were taken at monthly instead of fortnightly intervals. The results are shown in Table I.

Table I

| No. of exp. | Date of sampling | No. of days infected plot exposed | Plot I soil only | Plot II soil 75 % dung 25 % | Plot III soil 50 % dung 50 % | Plot IV soil 25 % dung 75 % | Plot V dung only |
|-------------|------------------|-----------------------------------|------------------|-----------------------------|------------------------------|-----------------------------|------------------|
| 1 | 4. vi. 32 | 31 | + 59 | + 56 | + 59 | + 59 | + 49 |
| 2 | 4. vii. 32 | 61 | + 133 | + 112 | + 64 | + 82 | + 60 |
| 3 | 3. viii. 32 | 91 | - 210 | - 481 | - 481 | - 199 | - 481 |
| 4 | 7. ix. 32 | 126 | - 422 | + 74 | + 55 | - 446 | - 446 |
| 5 | 3. x. 32 | 152 | - 420 | - 420 | + 154 | - 420 | + 112 |
| 6 | 3. xi. 32 | 183 | - 389 | - 389 | - 237* | - 246 | - 298 |
| 7 | 5. xii. 32 | 215 | - 319 | - 319 | - 319 | - 312 | - 319 |
| 8 | 18. i. 33 | 259 | - 275 | - 275 | - 275 | Died | - 275 |
| 9 | 6. ii. 33 | 278 | Died | - 52 | - 256 | - 256 | - 256 |

+ =died or killed and proved tubercular.

- =non-tubercular.

Note. The figures after the + or - sign indicate the number of days after inoculation the guinea-pigs survived or were killed.

* Acid-fast bacilli were found in the lungs of one of the guinea-pigs which died of pneumonia. An emulsion of the portion of the lung containing these bacilli was injected into two guinea-pigs. They remained perfectly healthy and on being killed 111 days later showed no trace of tubercular infection.

Discussion of results

In 1931 it was found that virulent organisms could be recovered from infected material exposed in the open for 178 days. Table I shows, however, that in 1932 when the weather was characterised by longer spells of heat and drought the maximum time after which recovery was possible proved to be 152 days. After 61 days from the time of first exposure had elapsed, the tests in which virulent organisms were recovered were much more irregular than in 1931. Indeed the test made at 91 days showed negative results throughout. Thereafter, positive results occurred irregularly up to 152 days. It is possible that the weather or factors correlated with it had, in fact, an effect on the viability, but attempts to correlate meteorological conditions with recovery of organisms were unsuccessful.

The figures for survival for the two years, having in mind the nature of the experiments, are substantially of the same order, and it may be inferred that material infected with tubercle bacilli must be exposed for about six months before it can be considered to have lost its virulence, at least when introduced by the subcutaneous route. The retention of the infectivity of the organisms seems to run roughly parallel to the amount of organic matter remaining. These experiments were made with organisms derived from naturally infected material under conditions which reproduced as closely as possible those obtaining when dung is naturally infected from lesions in the alimentary tract. Nevertheless the conditions were not entirely natural and experiments are now in progress to make them so by using the dung from naturally infected animals.

PART II

VIABILITY OF BOVINE TUBERCLE BACILLI ON GRASS GROWING IN A PASTURE DURING 1932 AND 1933

This experiment was carried out as previously described (Maddock, 1933). Three grass plots were infected with emulsions of tissue containing bovine tubercle bacilli, No. 1 with approximately 120,000, No. 2 with 1,200,000, and No. 3 with 120,000,000 per sq. ft. respectively. The results for 1932 are given in Table II.

During the very hot summer of 1933 two attempts to recover tubercle bacilli directly from infected grass were made. On June 12 the grass had been infected with 25,000,000 tubercle bacilli per sq. ft., and with samples taken on July 4, *i.e.* 22 days after infection, the result of guinea-pig inoculation was negative. On July 10 the grass had been infected with 5,000,000 tubercle bacilli per sq. ft., and with samples taken on July 31, *i.e.* 21 days after infection, the result of guinea-pig inoculation was also negative.

On September 28 a patch of grass was infected with 7,000,000 tubercle bacilli per sq. ft., and it was hoped to have a series of results throughout the winter.

Table II

| No. of exp. | Date of sampling | Rainfall since infection of grass in. | No. of days after infection | Plot I 120,000 T.B. per sq. ft. | Plot II 1,200,000 T.B. per sq. ft. | Plot III 120,000,000 T.B. per sq. ft. |
|-------------|------------------|---------------------------------------|-----------------------------|--|---|--|
| 1 | 28. iv. 32 | 0.16 | 3 | +14 | +21 | +28 |
| 2 | 2. v. 32 | 1.70 | 7 | +61 | +68 | +23 |
| 3 | 9. v. 32 | 2.50 | 14 | +70 | +65 | +53 |
| 4 | 16. v. 32 | 2.86 | 21 | -603 | -603 | +61 |
| 5 | 23. v. 32 | 4.81 | 28 | -582 | +87 | +103 |
| 6 | 30. v. 32 | 5.56 | 35 | -546 | -245 | +105 |
| 7 | 13. vi. 32 | 5.98 | 49 | -428 | -532 | +43 |
| 8 | 27. vi. 32 | 6.02 | 63 | Died | -414 | -518 |
| 9 | 11. vii. 32 | 8.16 | 77 | -400 | -400 | -504 |
| 10 | 25. vii. 32 | 9.33 | 91 | -386 | -43 | Died |
| 11 | 12. ix. 32 | 13.26 | 140 | -337 | -159 | -337 |

+ =died or killed and proved tubercular.

- =non tubercular.

Note. The figures after the + or - sign indicate the number of days from inoculation which the guinea-pigs survived or were killed. In No. 1 experiment the animals were killed as the glands caseated.

Unfortunately owing to damage by calves only a small portion of the original patch remained available and the last portion was cut on January 12, 1934. The results secured by guinea-pig inoculation are as follows:

Table III

| No. of exp. | Date of infection of grass | No. of T.B. per sq. ft. | No. of days after infection | Result |
|-------------|----------------------------|-------------------------|-----------------------------|--------|
| 1 | 12. vi. 33 | 25,000,000 | 22 | -218 |
| 2 | 10. vii. 33 | 5,000,000 | 21 | -190 |
| 3 | 28. ix. 33 | 7,000,000 | 33 | -133 |
| 4 | 28. ix. 33 | 7,000,000 | 49 | -133 |
| 5 | 28. ix. 33 | 7,000,000 | 63 | +55 |
| 6 | 28. ix. 33 | 7,000,000 | 83 | -151 |
| 7 | 28. ix. 33 | 7,000,000 | 105 | -124 |

+ =tubercular. - =non-tubercular.

Summary

The results are too few and the methods for recovery of tubercle bacilli from grass too uncertain to permit of more than tentative remarks, but both experiments during the heat of the summer were negative after three weeks. During the autumn and winter tubercle bacilli were shown to survive for 63 days after infection of the grass.

PART III

(a) FEEDING EXPERIMENTS WITH GUINEA-PIGS ON PASTURE INFECTED WITH BOVINE TUBERCLE BACILLI IN 1932

In the experiments previously described resort has been had to the method of subcutaneous inoculation of guinea-pigs, in order to prove the survival or destruction of virulent tubercle bacilli in the material dealt with. It is generally accepted that very small numbers of organisms introduced by this route may

set up infection, but this is no measure of the ability of similar numbers or of any number of the organisms to initiate infection when introduced by the alimentary route. In view of the well-known susceptibility of the guinea-pig to tuberculosis, an attempt was made to estimate the infectivity of tubercle bacilli after exposure on grass for various periods by feeding pens of these animals on grass.

The whole of the available area of pasture was evenly infected with a fine emulsion of tubercular bovine lungs (proved to contain virulent organisms of the bovine type) which gave an infection of about 4,000,000 bacilli per sq. ft.

The guinea-pigs, usually in batches of twelve, were confined in wire runs (8×3 ft.) and allowed access to the grass after a varying number of days from the time of infection of the whole area. Every day for 21 days the pens were moved to a fresh patch of grass. After grass feeding the animals were kept on the grass as it grew in the open.

By beginning feeding at various intervals after infection of the grass it was hoped to estimate the effect of exposure on the infectivity. The general plan of the experiment involving the use of 125 guinea-pigs is shown in Table IV.

Table IV

| Date of sampling | No. of days after infection | No. of guinea-pigs put down | No. of plot |
|------------------|-----------------------------|-----------------------------|-------------|
| 21. v. 32 | 1 | 6 | 1 |
| 24. v. 32 | 4 | 12 | 2 |
| 28. v. 32 | 8 | 12 | 3 |
| 4. vi. 32 | 15 | 12 | 4 |
| 15. vi. 32 | 26 | 12 | 5 |
| 18. vi. 32 | 29 | 12 | 6 |
| 2. vii. 32 | 43 | 12 | 7 |
| 16. vii. 32 | 57 | 11 | 8 |
| 30. vii. 32 | 71 | 12 | 9 |
| 13. viii. 32 | 85 | 12 | 10 |
| 3. ix. 32 | 106 | 12 | 11 |

Summary

Autopsies on all the guinea-pigs kept for as long as 20 months failed to reveal any trace of tuberculosis, although the grass grazed by the animals was proved by subcutaneous inoculation to bear virulent organisms, which might have been expected to reach the alimentary tract in considerable numbers, at least in the earlier experiments.

(b) EXPERIMENTS IN 1933. EFFECT OF ENVIRONMENT

The fact that the animals used in the 1932 experiment were kept outdoors suggested that environment might have contributed to the totally negative result reported in the previous section. It was also likely that the capacity of the organisms to cause infection particularly by the alimentary route was seriously affected by exposure and/or by the fact that the animals themselves were living in the open.

In an endeavour to elucidate these points the repeat experiment in 1933 was planned as follows.

Two groups of twelve guinea-pigs were used—one was kept outdoors and allowed to graze on infected grass, the other confined in a shed and fed on cut infected grass from a similar area at the rate of 2 oz. per pig per day. The pen containing those in the open was moved as the grass was consumed—usually every second day.

The first infection of grass was carried out on 12. vi. 33 at the rate of about 25,000,000 tubercle bacilli per sq. ft.

Three further infections over the same area were made at intervals as shown in Table V, which also shows the approximate weight of the infections.

Table V

| Date grass infected | No. of T.B. per sq. ft. of grass |
|------------------------|-------------------------------------|
| 12. vi. 33 | 25,000,000 |
| 10. vii. 33 | 5,000,000 |
| 21. viii. 33 | 11,000,000 |
| 21. ix. 33 | 7,000,000 |

Discussion of results

(a) Guinea-pigs kept in the open

Seven of the original twelve guinea-pigs kept outdoors survived from 12. vi. 33 to 20. x. 33 when they were removed indoors. During this time five animals had died and three were replaced by new guinea-pigs. All deaths up to October 3 were from pneumonia, and no sign of tubercular infection of any of them could be found in spite of careful examination of the whole animal, including the gut.

On October 20, 1933, two animals died, one of tuberculosis, and tuberculin tests, made on November 23 on the remaining eight animals, showed six to be infected. Of these two died of advanced tuberculosis on December 6 and 10 respectively. The remaining four reactors were killed on December 11 and proved to be tubercular. This left two animals which were killed on March 19, 1934—one proving to be tubercular.

In brief it was found that eight out of ten animals fed in the open on grass infected four times with tubercle bacilli during a very hot summer at monthly intervals contracted tuberculosis. This is in contrast to the 1932 experiment where, with only one infection of the grass, no animals of a large number contracted tuberculosis although the organisms used had been proved to be infective by inoculation.

(b) Guinea-pigs kept indoors

Nine of the twelve animals in this group survived from 12. vi. 33 to 20. x. 33, when feeding with cut grass ceased. Two of the original twelve had died of pneumonia and were replaced early in the experiment. A third died of pneumonia on 31. viii. 33. One guinea-pig was born on 17. ix. 33. On November 23,

all were tested with tuberculin and six gave reactions. One died of tuberculosis on 30. xi. 33 and the other five reactors, killed on 14. xii. 33, proved to be tubercular. By 19. iii. 34 the remaining six animals had died or had been killed but none were tubercular. In short six animals out of twelve which were fed on cut infected grass contracted tuberculosis.

Summary

By repeated infection of a grazing area it has been possible to induce tuberculosis in guinea-pigs grazed in the open and in those fed indoors on cut grass. In the 1932 experiment a total of thirty guinea-pigs were allowed access to plots of grass which had been infected, one, four and eight days previously and all failed to become infected. The combined experiments (1932 and 1933) suggest that under some weather conditions, animals, although admitted to pasture within a very short time of its infection may escape infection with tuberculosis, but that repeated infection of pasture results in a high incidence of disease in animals grazing on it.

PART IV

FEEDING EXPERIMENTS WITH CALVES IN 1933. FED AS IN

PART III (*b*)

The experiments described in this section were planned when it was known that the experiment on the feeding of guinea-pigs in 1932 had proved to be completely negative. If the suggestions of these experiments were to be accepted, it seemed important to establish at once whether or not it was possible to infect calves from pasture which had been infected repeatedly.

The technique of infection was as described in Part III, and the dates of reinfection as in Table V.

Nine calves from tuberculin-tested herds were used in the experiments and shown to give no reaction to a double intradermal inoculation with tuberculin. They received approved well-balanced rations throughout the experiment. Three remained in the herd of the National Institute for Research in Dairying as controls, three (two heifers and a steer) were run on infected pasture from June 13 to November 22 (Table V), and three, two heifers and a steer, were kept in confinement and fed on cut infected grass. No contact between the groups was possible. On December 16, all the animals were retested with tuberculin and all the controls proved to be negative. The three calves fed outside and two of those fed indoors were positive. One (a steer) of the indoor group was negative. Three and a half months later, after living in contact with a reacting animal, this steer was again negative to the double intradermal tuberculin test and was obviously in rude health.

Two reactors were slaughtered, one from the outside calves and one from the inside, and carefully dissected. A typical autopsy report is given in Table VI.

Table VI. *Autopsy report on two calves infected by feeding on grass*

| | |
|--|--|
| Lungs | Edges limp. No visible tubercles |
| Pleura | A few tubercles at base of right lung (in one calf only) |
| Superficial glands of neck and cervical lymphatics | No enlargement |
| Mediastinal glands | All enlarged and patent. No caseation |
| Abdominal glands | Hepatic suprarenal and mesenteric glands along the whole length of the intestine to the rectum were grossly enlarged |
| Small intestine | Thickening but no ulceration |
| Liver, kidney and spleen | Normal |

Virulent tubercle bacilli were recovered from the lesions which, as shown in Table VI, indicate that infection probably took place by the alimentary route.

Discussion

It has been possible to infect calves by feeding on grass which had been repeatedly infected (four times at monthly intervals) with emulsions of tubercle bacilli. Without making inferences, it seems that the case of the calf which escaped the comparatively massive dosage of organisms is interesting. The records of the herd from which it came (Reading University herd) show that in 23 years no reactor to tuberculin has been known in the family which, however, has given markedly less milk and fewer calves on the average than other families.

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REFERENCE

MADDOCK (1933), *J. Hygiene*, **33**, 103-117.

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