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Experiments on the Infectivity for Healthy Calves of Bovine Tubercle Bacilli Discharged in Dung Upon Pasture

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EXPERIMENTS ON THE INFECTIVITY FOR HEALTHY CALVES OF BOVINE TUBERCLE BACILLI DISCHARGED IN DUNG UPON PASTURE

PART I. FROM TUBERCULAR CALVES FED WITH EMULSIONS OF TUBERCLE BACILLI 1934–5.

PART II. FROM TUBERCULAR COWS PASSING TUBERCLE BACILLI IN THEIR DUNG 1935–6

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Part I. From tubercular calves fed with emulsions of tubercle bacilli 1934–5

In a previous paper (Maddock, 1934), it has been shown that healthy calves may be infected with bovine tubercle bacilli when grazed upon pasture infected at intervals with emulsions of the virulent organisms. These infections were designedly heavy, and it was felt that this orientating experiment should be followed by one in which pasture infection was secured in a way more closely approximating to the natural.

Three calves surviving from the previous experiment had reacted strongly to the double intradermal tuberculin test. Microscopic examination of the dung of these animals failed to show the presence of acid-fast bacilli, but to ensure that any natural infection of the dung should be suitably reinforced the three calves were fed on a daily ration of whey heavily infected with emulsions proved to contain virulent tubercle bacilli. When numerous acid-fast organisms appeared in the dung the calves were allowed to graze on the experimental plots for 3 weeks, during which the feeding of infected milk was continued.

The presence of virulent tubercle bacilli was confirmed by the fact that guinea-pigs inoculated with the dung of these calves proved to be tubercular.

After 3 weeks' grazing the calves were removed from the plots and the dung spread as evenly as possible over the area. The calves were slaughtered and post-mortem examinations revealed old tubercular lesions but apparently no active foci either in the intestines or kidneys. Nevertheless the infection of the pasture secured by way of the dung by feeding emulsions of virulent organisms in milk approximated more nearly to the natural than hitherto.

The area of pasture infected in these experiments was 892 sq. yards, approximately 2/11ths of an acre. The infection was even heavier than would occur on ordinary pasture grazed by tubercular animals, or on pasture that had been manured with tubercle-infected manure.

The area which had been infected as described was divided into three plots, and after a good crop of grass had grown two calves proved negative to the tuberculin test were grazed on plot I. A month later two more similar calves were placed on plot II, and at the end of a further month a third pair of calves grazed on plot III. In each case the calves grazed on the plots for 3 weeks and were then taken to a clean paddock. After three separate negative tuberculin tests made at appropriate intervals, the pairs of calves were slaughtered 7, 6 and 5 months after their removal from plots I, II and III respectively. Careful post-mortem examinations failed to reveal the presence of tuberculosis in all cases.

Experimental details

The three tubercular calves used as vectors were confined in a shed adjoining the experimental plots on 10 April 1934. Microscopic examination of the dung on 10, 11 and 12 April revealed no acid-fast organisms.

Source and preparation of tubercular emulsions.

Tubercular lungs were obtained from the abattoir (9 April 1934), and infected portions were dissected out, pulped and stored in the ice-chest (T.B. emulsion No. 9).

Each day about 90 g. of this pulp was emulsified with 1000 ml. of normal saline, freed from large particles by straining and added to 2 gallons of skim milk, itself diluted with 2 or 3 gallons of water, according to the prevailing temperature. No difficulty was experienced in getting the calves to drink the whole of their "ration".

On 17 April the original stock of infected material was reinforced by the addition of further portions of tubercular lungs from another district, and the glands of nine tubercular guinea-pigs which had been inoculated with five bovine strains of tubercle bacilli from infected milk (T.B. emulsion No. 10). On 30 April a new batch of material was prepared from portions of the lungs of two young cows and the glands of two guinea-pigs (T.B. emulsion No. 11). The bovine nature of each strain was proved by the usual methods. In this way twelve different strains of virulent bovine tubercle bacilli were ingested by the calves.

Some estimate of the virulence of the emulsions used was made by inoculating guinea-pigs with 1 ml. of dilutions at the levels shown in Table I.

Direct microscopic counts of acid-fast organisms in the emulsions prepared each day were made by the Breed method. The mean figure per ml. for each day was about 10×10^8, and each calf therefore received about $3{\cdot}3 \times 10^9$ tubercle bacilli daily.

Preliminary microscopic examination of the dung of each calf prior to 12 April when ingestion of the emulsions began, showed no acid-fast bacteria, but on 17, 23 and 30 April and on 7 May numbers of acid-fast organisms were observed. To confirm the probability that these were tubercle bacilli, guinea-pigs were inoculated with dung from each animal on 23 and 30 April and on

Table I

Date	Emulsion	Amount injected	Date guinea-pig D. = died of T.B. K. = killed and proved tubercular
23. iv. 34	T.B. Em. 9 and 10	0·000,1 ml.	D. 9. vi. 34 K. 22. vi. 34
23. iv. 34	T.B. Em. 9 and 10	0·000,000,7 ml.	D. 30. vi. 34 K. 30. vi. 34
30. iv. 34	T.B. Em. 9 and 10	0·000,1 ml.	D. 21. vi. 34 K. 22. vi. 34
30. iv. 34	T.B. Em. 9 and 10	0·000,000,02 ml.	D. 13. vii. 34 K. 20. vii. 34
1. v. 34	T.B. Em. 11	0·000,000,01 ml.	K. 30. vi. 34 K. 30. vi. 34
7. v. 34	T.B. Em. 11	0·000,000,6 ml.	D. 23. vii. 34 K. 25. vii. 34

7 May. The animals died of generalized tuberculosis or were killed and proved to be positive. Attempts to prove kidney infection (3. v. 34) by inoculation of the centrifuged deposit from urine failed.

On 8 May 1934 the three calves which had been used to infect the pasture were slaughtered and careful post-mortem examinations made. The findings are recorded in Table II.

Table II

Submaxillary and salivary glands	Normal
Pharyngeal	Enlarged
Prescapular	Enlarged
Lungs	Normal
Bronchial glands	Enlarged and hard in two, the third caseating
Mediastinal glands	Enlarged, caseating, calcareous
Mesenteric glands	Enlarged throughout the length of gut
Small intestine	Inflamed, thickened Petechial haemorrhages. No ulceration
Kidneys	Shrunken, hard with pale areas surrounded by fibrous tissues
Suprarenal glands	Enlarged
Iliac glands	Enlarged

The tubercular nature of the lesions was confirmed by microscopic examination and animal inoculation.

After the removal of the infecting animals the dung was spread evenly over the surface of the grass and all buildings thoroughly disinfected. The grass was allowed to grow (assisted because of drought by artificial sprinkling of water), and after 1 month a pair of tubercle-free (tuberculin tested) Shorthorn calves were allowed to graze on plot I for 3 weeks from 12. vi. 34.

Similarly after a further month's interval (12. vii. 34) a tuberculin-tested Shorthorn and a tuberculin-tested Guernsey calf were allowed to graze on plot II for 3 weeks.

Again after a further interval of a month, two tuberculin-tested calves grazed on plot III for 3 weeks. In spite of examination by guinea-pig inoculation of soil, dung and grass from each plot immediately prior to the admittance

of the calves, the presence of living tubercle bacilli could only be demonstrated in the case of plot I and even here only one guinea-pig (dung) out of four used in each case became infected. Throughout the experiment the weather was hot and dry. After 3 weeks' grazing the calves were removed to a distant clean paddock and segregated in pairs.

Six weeks after removal from the plot all the calves were tested with tuberculin and again after an interval of 6 weeks. None gave a positive reaction.

Two calves, one from plot III after passing the first test and one from plot II after passing both tests, died from enteritis. No clear signs of tuberculosis were found at post-mortem, but as a precaution material from slightly enlarged bronchial and mesenteric glands was inoculated into guinea-pigs which were killed after appropriate intervals and found to be negative.

On 1 February 1935 all the remaining calves were tested with tuberculin, found to be negative and slaughtered. They were found at post-mortem to be completely free from lesions of any kind.

A synopsis of the facts appears in Table III.

Table III

Three calves passing virulent tubercle bacilli in their dung grazed down the pasture from 17 April to 8 May, and after removal the dung was raked evenly over the pasture.

The pasture was divided into plots and the grass allowed to grow.

	Plot I	Plot II	Plot III
Two calves turned on to graze	12. vi. 34	12. vii. 34	13. viii. 34
Calves removed 3 weeks later	3. vii. 34	2. viii. 34	3. ix. 34
Passed tuberculin test	17. viii. 34	16. ix. 34	18. x. 34
,, ,,	28. ix. 34	28. x. 34	28. xi. 34
,, ,,	1. ii. 35	1. ii. 35	1. ii. 35

Under the conditions of this experiment healthy calves could not be shown to be infected with tuberculosis as a result of grazing on land previously heavily infected with virulent bovine tubercle bacilli.

The organisms which had been deposited on the pasture had passed through the alimentary tract of the infecting animals, and although they were shown to be virulent when introduced into guinea-pigs by subcutaneous inoculation it is possible that their capacity to cause infection by the alimentary route in calves was much reduced and perhaps not comparable with the virulence of organisms from animals discharging bacilli from natural lesions in the alimentary canal. Although it is possible that some natural infection of the dung took place this cannot be asserted with confidence, as no infection of the dung prior to the feeding of the emulsions could be demonstrated. It is also likely that the excessive heat and drought during the experimental period had their effect in reducing the weight of infection at the time of grazing the healthy calves.

Part II. From tubercular cows passing tubercle bacilli in their dung 1935–6

Having failed to infect healthy calves with tubercle after grazing them on pasture which had been contaminated by the dung of calves containing tubercle bacilli which had been given by the mouth, it was thought advisable to attempt to infect the pasture naturally as on the farm. For this purpose a cow suffering from tuberculosis of the udder giving 2 gallons of tubercular milk daily and passing virulent tubercle bacilli in her dung was allowed to graze for $9\frac{1}{2}$ weeks on the pasture.

For the last 3 weeks of the grazing a second tubercular cow was added to augment the infection. Unfortunately this cow, although she was sent to us as one in a very advanced tubercular condition, proved, when a post-mortem examination after slaughter was made, to have belied her appearance.

After the cows were removed the dung was raked evenly over the pasture, the grass allowed to grow, and then divided into two equal plots. When the condition of the pasture was deemed satisfactory three healthy calves were turned on to plot I to graze, and a month later three more healthy calves were turned on to plot II.

The calves on plot I were Shorthorns and those on plot II Friesians. The latter ate much more grass than the former, so that in spite of the fact that the Shorthorns grazed for a month longer than the Friesians, the two plots were exhausted of grass at the same time and both groups were removed after grazing for 53 and 24 days respectively. These calves were kept on clean pasture rather more than 1 year. At post-mortem all the animals were found to be in perfect health.

In an attempt to reveal any residual infection in the pasture, the dividing fence was removed and the whole thrown into one. When the grass had grown three calves were introduced and allowed to graze for more than 9 months, after which they were slaughtered and examined. No infection could be detected.

Experimental details

After examination of a large number of animals a tuberculous cow was obtained. This cow yielded $1\frac{1}{2}$–2 gallons of tubercular milk daily and showed clinical signs of advanced tuberculosis of the lungs. On 25 January 1935 she was turned on to the experimental pasture and remained there until 1 April 1935. During that time eleven samples of dung were taken and all proved tubercular by guinea-pig inoculation. On each of thirty-four occasions when the milk was examined microscopically, acid-fast organisms indistinguishable from tubercle were found in the left forequarter. On two occasions the mixed milk was injected into guinea-pigs and found to be tubercular.

It is of interest to note that of twenty-nine microscopical examinations of the right hindquarter, only fourteen showed the presence of acid-fast organ-

isms. This fact is given merely to emphasize the danger of accepting microscopic examination as evidence of freedom from infection with tubercle bacilli.

On 9 March a second cow not in milk, stated to be an advanced case of tuberculosis, was allowed access to the pasture. Six samples of its dung were taken at intervals, but only one was found to contain virulent tubercle.

The cows were slaughtered on 1 April, and the first cow was found to be suffering from advanced generalized tuberculosis. The intestines were thickened and inflamed but no definite ulceration was evident. The mesenteric and iliac glands were all caseous and enlarged, as were the mammae and supramammary glands. The lungs contained many large tubercular cavities, and all the glands in the thorax were caseous. Microscopically acid-fast organisms were present in slides made from all these sites, and bovine tubercle bacilli were recovered by guinea-pig inoculation. An emulsion made from a portion of the mucous membrane of the small intestine also was proved by cultural methods to contain bovine tubercle bacilli.

The second cow at post-mortem did not show the signs of generalized tuberculosis which had been expected, but the small intestine was thickened and red and contained small nodules scattered throughout its entire length. The mesenteric glands were enlarged, watery and contained small caseating areas. The iliac glands were also enlarged and caseated.

Microscopically acid-fast organisms were seen in the iliac glands and the intestinal nodules.

Emulsions of portions of the iliac and the mesenteric glands and the intestinal nodules were made and injected into guinea-pigs. The iliac glands and intestinal nodules proved to be tubercular.

It is therefore evident that the pasture was infected by cows which would be likely to be found on any farm containing tubercular cattle, and the condition of infection could be regarded as entirely natural.

After the removal of the cows on 1 April the dung was raked evenly over the surface of the pasture. The pasture was then divided into two equal plots. A luxuriant growth of grass had appeared by 13 May 1935. Three healthy Shorthorn calves about 14 weeks old which had passed the intradermal tuberculin test were turned out to graze on plot I on this date.

On 11 June 1935 three healthy Friesian calves about 13 weeks old, also with negative tuberculin reactions, were turned out to graze on plot II.

By 7 July the calves in both plots had eaten all the grass. The Friesians grew much quicker than the Shorthorns and consumed their grass in a much shorter time, i.e. 24 against 53 days. The calves were then moved to clean paddocks and kept separated by fences 13 yards apart. All the calves were tuberculin tested on 6 October 1935, 9 January, 9 April and 26 June 1936, and on all occasions gave no reaction. They were slaughtered on 12 July, 371 days after removal from the infected plot, and a careful post-mortem of each was made with Mr W. L. Little, F.R.C.V.S. The calves were found to be perfectly healthy in all respects.

Owing to the fact that the very dry and hot spell of the summer months continued well on into August 1935 the grass in the pasture had not grown sufficiently to allow three new healthy calves to be turned out to graze until 21 September 1935. These calves were used in an attempt to reveal any infection which might have survived during the growth of the second crop of grass. Therefore on 21 September 1935 the dividing fence was removed and three healthy tuberculin-tested Friesian calves (averaging 13 weeks in age) were turned out to graze on the pasture and remained there for 9 months until slaughtered. They were tuberculin tested on 21 December 1935, 21 March and 26 June 1936. On post-mortem they were found to be perfectly healthy in every respect, 295 days from the date they were turned out to graze on the infected pasture.

(*a*) In an attempt to estimate the time of survival of tubercle bacilli in the naturally infected dung deposited on the pasture preparations for inoculation were made from material collected from many different areas in the plots.

The infected cows were removed on 1 April 1935, and on 13 May 1935 and 11 June 1935 samples of grass, dung and soil were collected from plots I and II respectively and preparations inoculated into guinea-pigs (Maddock, 1933). On 11 September samples of grass and soil were similarly treated. Post-mortem examination of the inoculated guinea-pigs was delayed for not less than 9 months to allow any latent infection to appear, but in no case was a positive result obtained.

(*b*) On 1 April 1935 immediately before the removal from the pasture of the cow which had regularly passed tubercle bacilli in her dung one of her dung pads was covered with a shallow wire cage whilst still fresh.

A sample of this dung was taken on 13 May and tubercle bacilli were recovered from both guinea-pigs inoculated. On 11 June, 10 July and 7 August 1935 samples were taken and inoculated into guinea-pigs, but in these cases no tubercle bacilli were recovered. Samples of the soil under the dung were taken on 10 July, 7 August and 11 September 1935 and were injected after preparation into guinea-pigs, but no tubercle bacilli could be recovered.

Similar negative results were obtained when the grass which had grown on the spot on which the dung had been deposited was tested by inoculation. In all these cases delayed infections were ruled out as the guinea-pigs were kept until June 1936.

Discussion

The foregoing series of experiments shows clearly that the pasture land must have been heavily infected with tubercle bacilli which at the time of their deposition were virulent when introduced subcutaneously into guinea-pigs.

For 42 days after deposition, the dung of the infecting cow was shown to contain living tubercle bacilli, but by the technique used their survival was shown to be of comparatively short duration compared with the survival times shown for artificially infected dung.

It is possible that mere numbers may account for the difference.

The area used for the experimental grazing was only 2/11ths of an acre, and the weight of infection must have been far heavier than would normally take place on a farm. In spite of this fact none of the experimental healthy calves was shown to be infected.

It seems, therefore, that in pasture which has been previously naturally infected, so long as no contact with the infecting animals is possible, healthy animals may be expected to escape infection from this source. It therefore appears that the danger of infection being picked up from pasture manured with farmyard manure is remote. The most likely mode of spread of the disease is by contact of animal with animal either in the open or in cowsheds.

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