A COMPARATIVE BACTERIOLOGICAL STUDY OF BOVINE ABORTION AND UNDULANT FEVER.

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PART II.

In a previous communication on this subject (Khaled, 1922) the most important conclusions arrived at were these:

1. *Brucella abortus* Bang and *B. melitensis* Bruce, are morphologically identical and cannot be differentiated by cultural, biochemical or staining methods, nor by the agglutination reaction.

2. From absorption-agglutination experiments, *B. melitensis* appears to be a substrain of *B. abortus*.

3. Dose for dose, *B. abortus* is much less virulent for guinea-pigs (injected intraperitoneally) than *B. melitensis*.

4. One monkey vaccinated intravenously with killed suspensions of *B. abortus* was found to have become immune to a subsequent infection with living *B. melitensis* whilst the control monkey showed signs of the disease.

Repetition of cross-immunisation experiment. The importance of the cross-immunisation experiment made it necessary to have it repeated with a larger series of monkeys. Four *Macacus* monkeys were chosen; three for vaccination and infection, and one for infection only (to act as control). The following is a typical experiment:

(1) *Macacus rhesus* "A" ♀, received the following inoculations:

3. ii. 22. 1/4 slope of killed *B. abortus*, strain "900," suspended in 2 c.c. saline.

12. ii. 22. 1/2 slope of same in 2 c.c. saline.

20. ii. 22. Do.

Serum tested on 28. ii. 22 agglutinated *B. abortus* in 1 : 3200 and *B. melitensis* in 1 : 800.

(2) *Macacus rhesus* "B" ♂, not previously immunised, to act as control.

Final test. Both monkeys were inoculated on March 3rd with 1/2 slope (intravenously) of living *B. melitensis* "893."

After 18 hours *B. melitensis* was recovered from both monkeys, but at the end of the first week it was possible to recover it only from the control and not from the blood of the immunised monkey.

On the eighth day the titre of the serum of "A" was 1 : 6400 for *B. abortus* and 1 : 1600 for *B. melitensis*, while the serum of "B" agglutinated *B. abortus* in 1 in 100 and *B. melitensis* in 1 in 200 only.

From the tenth day onwards signs of illness (emaciation, weakness, irregular undulating fever and diminished appetite) showed themselves in the control but not in the immunised monkey. In this the only effect of injecting living *B. melitensis* was to cause a rise in the agglutination-titre of its serum (for *B. melitensis* and *B. abortus*) and an initial loss of weight which was soon regained. The following table records the weights of the two monkeys so that they may be compared:

	M. rhesus "A"	*M. rhesus* "B" (control)
Before infection	3800 gm.	3700 gm.
10. iii. 22	3700	3590
15. iii. 22	3650	3540
22. iii. 22	3610	3500
30. iii. 22	3630	3460
6. iv. 22	3690	3430
24. iv. 22	3750	3400
4. v. 22	3790	3360

Post-mortem examination. On June 4th, *i.e.* about three months after infection, both animals were killed. No naked-eye changes were seen except enlargement and congestion of the spleen of "B" (control). From this as well as from the heart's blood a pure culture of *B. melitensis* was recovered.

No organisms could be recovered from the tissues of monkey "A" (immunised).

The same procedure was adopted in immunising and infecting the other two monkeys with the exception that other strains of *B. abortus* were used for vaccination. The same results were also obtained except that in one monkey there was no increase in the agglutination-titre of its serum after infection with *B. melitensis*.

From this it seems that emulsions of killed *B. abortus* inoculated intravenously can protect monkeys against a subsequent infection with living *B. melitensis*.

Cross-Immunisation of Goats.

Vincent (1918), by using ether-killed polyvalent vaccine (*B. melitensis* and *B. paramelitensis*), was able to immunise a series of goats sufficiently to withstand massive doses of the living virus given subcutaneously, intravenously and intraperitoneally. No germs could be recovered from milk or post-mortem.

Marich, Sultana and Mifsud (1921), working under Prof. Zammitt, tried to repeat Vincent's experiments on the Island of Malta, but they failed to corroborate his results, getting practically as many infections in the "immunised" goats as in the control animals. On reading the papers of these authors one is struck by the circumstance that the toxicity of *B. melitensis* necessitated the use of moderate doses in immunisation and that the resulting

immunity was therefore very small as judged by the low agglutination-titre and by the fact that the immunised animals contracted the disease. Burnet (1923) also failed to immunise goats against *B. melitensis* by vaccinating the animals with the killed organism.

In 1921 the writer inoculated two goats with a single slope of living *B. abortus* and found that the agglutination response to both *B. melitensis* and *B. abortus* was very distinct; yet because the goats were not in milk at the time, it was not thought of much use trying to infect them with living *B. melitensis*. The success of the monkey cross-immunisation experiments detailed above and in a previous communication suggested that it would be well worth while to see if it could be applied to goats, *i.e.* to see whether immunisation with living *B. abortus* would prevent the excretion of *B. melitensis* in milk after infection of the animal with this latter organism.

TECHNIQUE OF EXPERIMENT.

A. *Milk:*

1. *Collection:*

The udders were first cleaned with soap and water, then with lysol solution (1 in 1000) and afterwards with alcohol, special care being paid to the nipples. When dry, they were milked with clean, sterile-gloved hands. The first few cubic centimetres of milk (from each nipple) were rejected. The rest was received in sterile Erlenmeyer flasks, which were plugged when full and marked with the number of the goat and the date and hour of the milking. By using the above precautions very few extraneous organisms could find their way into the milk.

2. *Culture:*

Two methods were used in parallel so as to obtain more trustworthy results:

(*a*) *Direct plating:* 1–2 c.c. of the milk, drawn with a sterile pipette, were allowed to spread on a glucose-agar plate. After the milk film had dried the plates were inverted and incubated at 37° C. Examination for suspicious colonies was made after one, two, three, and five days.

(*b*) *Indirect plating:* 0·5, 1, and 2 c.c. of milk were added, each to a glucose-broth tube containing 10 c.c. of media. After shaking, the tubes were incubated for three days and loopfuls were plated out each day and the plates examined for suspicious colonies. This method apparently favours the multiplication of *B. melitensis*, the goat's milk seemingly acting as a favourable adjunct to the media and thus increasing the chances of detecting the organism if the initial infection of the milk is small.

3. *Agglutination:*

Whey was used in preference to whole milk as a sharper end point could be obtained. To 10–15 c.c. of the milk, a few drops of acetic acid were added and the milk allowed to stand. The separated whey was filtered off and the filtration repeated until a limpid fluid was obtained. This was neutralised with

N/10 NaOH. The neutral fluid was put up for agglutination, dilutions of 1 : 20 to 1 : 1280 being used.

B. *Blood:*

1. *Culture:*

The neck was shaved in the region of the external jugular vein. After painting the space with tinct. iodi., 10 c.c. of blood are drawn aseptically and placed in 100 c.c. of glucose broth in an Erlenmeyer flask and incubated. Glucose agar plates were inoculated and the plating and incubation continued for seven days.

2. *Agglutination:*

The agglutination was carried out in small tubes 5 cm. long by 0·5 cm. diameter. The serum dilution was made up with normal sterile saline in series 1 : 50 to 1 : 12,800, while the bacterial emulsion was made up in sterile distilled water to which formalin (1 : 1000) was added. After two hours' incubation at 37° C., the tubes were placed at room temperature and read next day (*i.e.* 18 hours later). Positive tubes usually showed sedimentation.

The two goats selected for cross-immunisation were healthy looking and were giving a very good output of rich milk. They had kidded 12 and 21 days previously.

Samples of their blood and milk were cultured for *B. melitensis* (and *B. abortus*) and their sera and whey tested for *B. melitensis* and *B. abortus* agglutinins. All these tests were negative.

Immunisation was started on February 23rd, 1923, each goat receiving intravenously the following doses:

	Date: day and month	
	Feb. 23	One slope of *B. abortus* "900" (72 hours old growth).
	,, 28	Three slopes *B. abortus* "900" emulsified in 10 c.c. saline.
	Mar. 5	Six slopes *B. abortus* "900" in 15 c.c. saline.
Final test	,, 12	*Infected intravenously* with three slopes of *B. melitensis* "893," 72 hours old, emulsified in 10 c.c. saline.

The response of the two goats to these inoculations was as follows (Table I):

Throughout the experiment the animals were quite happy and partook freely of their food, but the output of milk gradually diminished until at the end it was reduced to about 150 c.c. per diem. No local signs or symptoms of inflammation of the udders or adnexa were noticeable.

Table I.

Date 1923: day and month	Blood culture Goats I and II	Milk culture Goat I	Milk culture Goat II	Serum agglut. Goat I	Serum agglut. Goat II	Whey agglut. Goat I	Whey agglut. Goat II
Feb. 24	*B. abortus*	Negative		Negative		Negative	
27	Negative	*B. abor.*	Neg.	v. *ab.* 1 : 100	Neg.	„	
				v. *me.* 1 : 50			
Mar. 3	„	Neg.	*B. abor.*	v. *ab.* 1 : 800	1 : 400	v. *ab.* 1 : 40	Neg.
				v. *me.* 1 : 400	1 : 200	v. *me.* 1 : 20	
4	„	Negative		v. *ab.* 1 : 1600	1 : 800	v. *ab.* 1 : 40	1 : 20
				v. *me.* 1 : 800	1 : 400	v. *me.* 1 : 20	1 : 20
7	„	„		v. *ab.* 1 : 3200	1 : 1600	v. *ab.* 1 : 80	1 : 40
				v. *me.* 1 : 1600	1 : 800	v. *me.* 1 : 80	1 : 40
8	„	„		Same		Same	
10	„	„		v. *ab.* 1 : 6400	Same	v. *ab.* 1 : 160	1 : 80
				v. *me.* 1 : 3200		v. *me.* 1 : 80	1 : 40
12	„	„		v. *ab.* 1 : 12800	Same	v. *ab.* 1 : 320	1 : 160
(Inoculation with *B. melitensis*)				v. *me.* 1 : 6400		v. *me.* 1 : 160	1 : 80
13	*B. melitensis*	„		Same		Same	
15	Negative	„		„		„	
18	„	„		v. *ab.* 1 : 12800	—	v. *ab.* 1 : 320	1 : 160
				v. *me.* 1 : 12800		v. *me.* 1 : 160	1 : 80
24							
26							
27							
28	„	„		Same		Same	
29							
30							
Apr. 2							
3	—	—		—		v. *ab.* 1 : 160	1 : 160
						v. *me.* 1 : 160	1 : 80
4	Negative	Negative		v. *ab.* 1 : 12800	1 : 12800	—	
				v. *me.* 1 : 12800	1 : 6400		
5–13	„	„		Same		Same	

CONTROL GOAT.

A healthy well-fed goat that had kidded four days previously, giving daily about two pints of very rich milk.

The blood and milk were cultured for *B. melitensis* and the serum and whey tested for *B. melitensis* and *B. abortus* agglutinins. All these tests gave a negative result.

On March 15th it was infected intravenously with three slopes of *B. melitensis* "893" (72 hours old), emulsified in 10 c.c. sterile saline. Table II shows the response to the inoculation.

The output of milk diminished slightly but the most marked phenomenon was the swollen, red, hot and tender state of the udders whilst the general condition showed apparent depression, inactivity and emaciation.

Post-mortem examination. On April 13th, the three goats were anaesthetised by chloroform, after tying their legs. They were then bled to death.

In the two immunised goats no distinct naked-eye change was noticed in the spleen, liver or the mammary gland, nor any microscopic change detected in these organs or in the sections of one of the deep glands. Cultures taken from all these tissues and from the heart's blood were negative.

In the control goat the noteworthy change was the actue inflammatory condition of the mamma and the enlargement of the spleen. Cultures from

the mamma, spleen and heart's blood gave a growth of *B. melitensis*, those from the liver and a deep lymph gland were negative.

The number of experimental animals is small but the results as seen in Tables I and II are marked. *B. abortus* appeared in the milk during immunisation only once or twice and then died out and was eliminated; a high degree of immunity was obtained and no clinical signs followed an intravenous dose of three slopes of living *B. melitensis*, nor could anything be recovered from repeated blood and milk cultures nor from the spleen or liver after death.

Table II.

Date 1923: day and month	Blood culture	Milk culture	Serum agglutination		Whey agglutination	
			v. *B. abortus*	v. *B. melitensis*	v. *B. abortus*	v. *B. melitensis*
Mar. 15 (Infected with *B. melitensis*)	—	—				
16	*B. melitensis*	Negative	Nil		Nil	
18	,,	,,	1 : 100	1 : 100	,,	
19	,,	,,	1 : 200	1 : 200	,,	
20	,,	,,	Nil		,,	
21	,,	,,	,,		,,	
22	,,	,,	1 : 400	1 : 800	,,	
23	—	,,	Nil		,,	
24	—	*B. melitensis*	1 : 800	1 : 1600	,,	
25	—	,,				
26	—	,,				
27	—	,,				
28	—	,,				
29	*B. melitensis*	Negative	1 : 1600	1 : 6400	1 : 20	1 : 40
30	—	,,				
31	—	Positive				
Apr. 2	—	,,	—	—	1 : 80	1 : 80
3	—	,,				
4	—	,,	1 : 1600	1 : 6400	1 : 80	1 : 160
5–12	*B. melitensis*	,,	Same		Same	

In the control goat, on the other hand, an acute mammitis set in and the organism was recovered repeatedly from the blood and milk during life and from the spleen at autopsy.

The results of this experiment are comparable with the finding of Pratt-Johnson (1921) in the production of immunity against *B. cholerae suis*. He showed the possibility of securing solid immunity against a highly virulent organism by vaccination with living members of a related type of low virulence rather than with the killed antigen of a homologous strain.

Attempts to Apply these Experimental Results to the Treatment of Undulant Fever in Man.

A survey of recorded attempts to treat undulant fever with *B. melitensis* antigen reveals several common features, whether the antigen used has been of the ordinary or sensitised type or whether it has been autogenous or stock:

(1) Very small doses, 50–200 millions, have to be used.

(2) Long intervals of ten days have to intervene between the injections.

(3) The vaccine should be used only when the temperature has fallen or is falling.

The vaccine is not indicated in the acute cases owing to the great toxicity of the virus.

What appears to be desirable therefore in a disease like undulant fever is a vaccine of such low virulence as will permit its usage in all cases, acute and mild, at any period of the disease, at frequent intervals and in such doses (if possible living) as will produce sufficient immunity to cut down the fever. It was therefore thought worth while to test whether *B. abortus* vaccine might satisfy these requirements. Three cases of undulant fever have been treated in this way.

Case I. M. H., 35 years old, suffering from a severe fever, the temperature reaching 104° F. in the evening and little less in the morning. Widal test carried out on the 40th day of the disease was negative for *typhoid* and *paratyphoid* but positive for *melitensis*; the serum agglutinating the laboratory strain up to 1 : 800. Blood culture on the 45th day of the disease gave a pure *B. melitensis* growth. The condition of the patient being rather bad, it was not felt advisable to treat him with *melitensis* vaccine. The physician, therefore, agreed to try the much less toxic, killed, *abortus* vaccine. A first dose of 300 millions was given. This produced no ill-effect and was followed, five days later, by a second of 600 millions. The subsequent doses were 800, 800, 1000, 1500 and 2000 millions at five-day intervals. The temperature began to show a drop after the fifth injection and came down to normal two days after the eighth injection, *i.e.* 37 days from starting the treatment.

Case II. W. H., 27 years old. Blood sent for culture on the tenth day of the fever, on suspicion of enterica. Culture gave pure growth of *B. melitensis*. Serum agglutinated laboratory *melitensis* strain in 1 : 200 dilution. A dose of 500 millions of killed *B. abortus* was given subcutaneously on the 16th day of the disease. Five days later a second dose of 500 millions was given, followed by 800, 800, 1000 and 1500 millions at five-day intervals. The body reaction was bearable. The case could not be classified as an acute, severe one. The temperature became normal 26 days from the commencement of vaccine treatment.

Case III. H. A., 30 years old, suffering from fever for five months, with occasional afebrile periods. His serum agglutinated *B. melitensis* in 1 : 1600 dilution. Blood examined culturally on three weekly occasions gave a negative result. The fever was rather mild, but the emaciation was marked, sweating intense and joint pains severe. The initial dose given was 600 millions. There being not much reaction, a second injection of 800 millions was given after five days and repeated after another five days. These three doses did not have much effect on the temperature but the patient began to feel a general well-being, the joint pains ameliorated a little, and the sweating definitely diminished. Subsequent doses of 1000, 1500, 1500 and 2000 millions were given. The last dose was repeated three times before the temperature dropped to normal. With each dose the general condition of the patient got better and his weight increased.

As seen from these three cases higher doses of *abortus* vaccine could be used in the treatment than is possible with *melitensis* vaccine. The temperature charts (not here detailed) show curtailment of the course of the fever, especially in Case II, whilst in all three cases the patients felt and looked better under the vaccine treatment. The number of cases, however, is very small and the method requires repetition before a definite conclusion can be formed[1].

Summary.

1. Certain cross-immunisation experiments (on monkeys) with *B. abortus* and *B. melitensis* carried out in 1921 were repeated with the same effective result.

2. It was found that goats vaccinated intravenously with *B. abortus* in massive doses are protected from subsequent infection with a virulent *B. melitensis* strain, and fail to pass that organism in the milk.

3. The record of the effect of *B. abortus* vaccine in treating three cases of undulant fever is given.

In conclusion I wish to express my thanks and gratitude to the staff of the Lister Institute for the hospitality of the laboratories and for many valuable criticisms.

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Pratt-Johnson, J. (1921). Further studies on the production of immunity in rabbits against an organism of high virulence for the species. *Brit. Journ. Exp. Path.* ii. 239.

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[1] Nicolle, Burnet and Conseil (1923) have also recently shown that living *B. abortus* may be given with impunity to healthy subjects (five tested). 800–900 millions of the living 24 hr. culture were given subcutaneously without causing fever or other troublesome symptoms and they also suggest the possibility of employing *B. abortus* in the prophylaxis and vaccine-therapy of undulant fever. *Compt. rend. Acad. Sci.* clxxvi. 1034. [Editor's note.]

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