

THE VALUE OF HEAT-KILLED CULTURES FOR THE
PREVENTION OF THE BACILLUS ABORTUS
INOCULATION DISEASE OF GUINEA PIGS.

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The great economic importance of infectious abortion in cattle and swine, particularly in cattle, has provided the stimulus for many experiments aimed at the prevention of the disease through the use of preparations made from the *Bacillus abortus* and its products. Naturally the use of killed cultures of the organism was among the first of these procedures attempted and it has, perhaps, been the method most extensively tried. In spite of this the exact immunizing value of the killed cultures is not known though it is quite generally thought at the present time to be slight. The interpretation of results of immunization experiments against infectious abortion in cattle is attended with certain inherent difficulties which have left nearly all such experiments open to question.

The disease of guinea pigs due to inoculation with *Bacillus abortus*, first described by Smith and Fabyan (1912), seemed to offer a more favorable opportunity for studying the mechanism of the process of immunity than the cattle disease, since it is not attended with the difficulties found in the latter. Such a study was taken up, therefore, not only for the light which might be cast by it on the general biology of the organism but for the possible assistance which it might render toward solving the cattle problem. It was realized, of course, that results obtained in working with one species of animal cannot safely be applied to another species, by inference, unless there is strong corroboratory evidence.

HISTORICAL.

Previous attempts to immunize guinea pigs to *B. abortus* by the use of killed cultures have been made by Ascoli (1915), and by Stafseth (1920). Stafseth found that no immunity was produced by the procedure, but his work is of little value because the size of his infecting dose was so enormous as to overwhelm any but the strongest of immunities.

Ascoli's work may be criticized similarly, although it is evident that he appreciated the importance of a reasonably small infecting dose and made an effort to attain it. His immunizing cultures were killed with ether. Both single large doses of the immunizing suspension and small repeated doses were tried. The infecting doses varied from one series of animals to another, but the smallest used was 1:100,000 of an agar slant growth. The animals were killed after periods of time varying from a few days to several months. The agglutinin titer of the blood was determined when the animals were killed, and cultures were made from the spleen. The nature and extent of the lesions apparently were not considered.

The animals previously subjected to the immunization process as well as normal animals used as controls developed agglutinins for *B. abortus* and the organism was recovered from the spleens of all. It was concluded, therefore, that the use of killed cultures of *B. abortus* was ineffectual in preventing subsequent infection by inoculation.

Plan of Experiment.

Since previous work, supported by similar observations dealing with the disease in cattle, indicated that the degree of immunity produced in the animals probably would be slight, if not nil, it was planned to carry on observations upon a quantitative basis in an effort to detect immune processes which might not be of sufficient degree to afford complete protection.

A group of twenty-four female animals was selected from the stock, uniformity of age and size being sought. All were young, half grown animals weighing in the neighborhood of 350 gm. Agglutination tests with *Bacillus abortus* antigen showed the blood of all to be negative in a dilution of 1:10. One-half of the animals (twelve) were subjected to preliminary injections of killed cultures and finally all, with the exception of two animals from each group which were preserved as controls, were inoculated with living culture. Throughout the experiment all animals were kept together in a single large pen.

Evidences of immunity in the group of animals receiving the pre-

liminary treatment were sought by comparing the group with the untreated in the following respects: (a) changes in body weight; (b) differences in the agglutinin curves; (c) extent and character of the lesions, with especial reference to the size and condition of the spleen; and (d) number of *Bacillus abortus* cultivable from the spleen.

Immunization.

The culture used throughout this work was a typical strain of *B. abortus* isolated from a bovine placenta, Case A 898, by passage through Guinea Pig 1865. It grew readily on plain veal infusion agar, provided the cultures were hermetically sealed. For antigenic material this strain was grown 48 hours on plain agar in a Blake flask which had been sealed with wax. A suspension of the resulting growth was made in physiological salt solution and heated at 60°C. for 1 hour. Both cultural and animal inoculation tests proved the suspension to have been sterilized. It was diluted to a reading of 2.4 cm. on the gauge devised by Gates (1920) and stored, without preservation, in the refrigerator until ready for use.

The twelve animals selected for immunization were given intraperitoneal injections of 1 cc. of the killed bacterial suspension weekly. The manner in which the animals were reacting to the injections was gauged by the agglutination curve. The weekly injection of rather large doses of killed bacterial suspension appeared to exert little effect on the health of the animals, although reference to Table I will show that the group did not gain in weight so well as did the untreated group. The gain in weight during the period of immunization for the treated group averaged 162 gm., while for the other group the average was 231 gm.

The curve in Text-fig. 1 is typical of the agglutinin response in all of the treated animals. After a sudden rise following the first injection there was a slow rise until an average titer of 1:640 was reached, beyond which point it appears that there is no stimulation by the use of dead cultures. After six weekly injections the process was stopped and some time was allowed to elapse before the test inoculations were made, in order to allow the body to recover its equilibrium which it was thought might have been upset by the stress of the immunizing process. At the end of the 3rd week following the last dose of killed culture, the agglutinin titers began to show evidence of falling and the time was considered ripe for inoculation with the living culture.

The Infecting Dose.

The strain of *Bacillus abortus* which had been used in the preparation of the immunizing suspension was also used for infection.

The culture had been passed through Guinea Pig 2106 just prior to use. The growth from a 24 hour sealed slant agar culture was washed off with physiological salt solution and the resulting suspension diluted to make a reading of 2.4 cm. on the Gates loop gauge. This makes a suspension somewhat denser than a 24 hour typhoid culture in bouillon. Dilutions of 10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} , and 10^{-6} were made from the original suspension. The last three dilutions were used for inoculating, 1 cc. being injected intraperitoneally in each case.

In the matter of dosage, it was sought, in some of the animals, to approach rather closely to the minimum amount necessary to produce infection; in others to give a moderate dose; and in a few to give what might be considered a comparatively large dose. The question of the minimum infective dosage of suspensions of *B. abortus* is discussed elsewhere (Hagan, 1922). Reference to the communication cited will show that the smallest dose administered was probably several times the amount actually needed to produce infection. The individual variation in resistance is so great that smaller doses than the minimum amount given in this experiment could not safely be depended on to infect all normal animals, although $\frac{1}{100}$ of this amount probably would have infected most guinea pigs.

The animals were killed and examined in pairs consisting of one animal from each group. Some were killed within 3 weeks after the test inoculation; others in 6, 9, and 12 weeks. All were bled regularly at weekly intervals and the agglutination curves obtained.

RESULTS.

Body Weight.—A complete weight record will be found in Table I. Analysis of this shows that during the entire experiment the average gain in body weight was approximately the same in the two groups; *i.e.*, immunized group 308 gm., non-immunized 304 gm. At the same time two animals used as controls, which were not subjected to any experimentation except periodic bleeding for agglutination work, made an average gain of 430 gm. It has already been stated that during the immunization process the treated animals were outstripped in the amount of weight gained by some 70 gm. on the average for the group. From the time of inoculation to the time of death the position was reversed, the immunized animals gaining 109.5 gm. while the non-immunized gained only 53 gm. This margin undoubtedly would have been widened if the animals had been allowed to live longer, as

is indicated by the comparatively great weight losses suffered by the non-immunized animals which were allowed to live the longest.

The Agglutination Curves.—At the time of administering the living culture, the agglutinin titer of the immunized group averaged about 1:320, while the bloods of the non-immunized were negative at a dilution of 1:10. Text-fig. 1 shows the behavior in the immunized control animals. Following the last injection of killed culture there is a gradual decline in the curve lasting some 13 weeks before it reaches the base-line. Text-figs. 2 to 4 show the curves in the inoculated animals receiving different amounts of infective material. The latter halves of these curves show that there is practically no difference in the agglutinin response to the infecting dose in the two groups, and that the size of the infecting dose has made little difference in the agglutinin response. In Text-figs. 3 and 4 the smaller doses have produced a delay in the agglutinin production, and the curve for the immunized group falls appreciably before the effect of the test inoculation becomes operative. In the case of the comparatively heavy inoculation represented by Text-fig. 2 this fall does not occur.

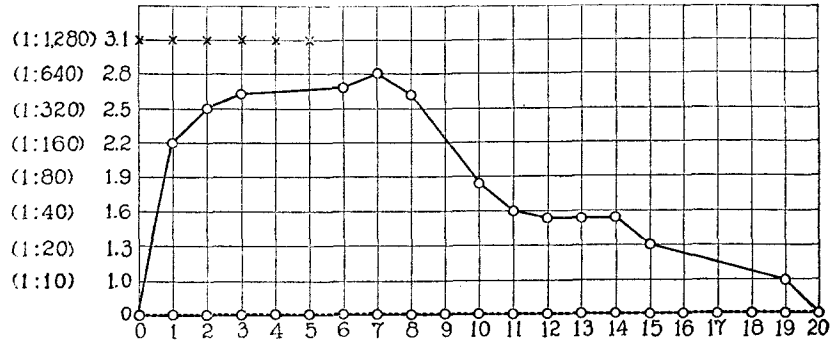
Differences in Extent and Character of Lesions.—All of the inoculated animals became infected, thus showing that the treatment was ineffectual in preventing the disease. Certain differences in the character of the disease process have been noted, however, as a result of the preliminary treatment. These have to do with the spleen, lymph glands, and kidneys.

The spleen presented the most striking lesion of the disease in all cases, immunized or non-immunized. Reference to the spleen weights in Table I shows that enlargement occurred in all cases except in Guinea Pigs 2083 and 2071, both of which were immunized animals which had received small doses of the infecting culture and were killed early. The splenic enlargement, with one exception, was greater, however, and usually much greater in the non-immunized than in the immunized group. The spleen weight for the former group averaged 4.87 gm., while for the latter it was only 3.1 gm., a difference of more than 50 per cent. There is no evidence that the character of the process in the spleen is different in the two groups. It is probable that the difference is only a matter of degree; *i.e.*, that in the immunized group the process is only delayed.

TABLE I.

Guinea pig No.	Immu- nized.	Injected.	Length of time lived.	Initial weight.	Maximum weight.	Weight when killed.	Loss in weight from maximum.	Change in weight during immunization.	Change in weight during disease.	Spleen weight.	Agglutinin titer when killed.	Spleen.	
												Bacteria per gm.	Bacteria per organ.
2073	+	Control.	24	410	720	720	0	+190	+100	1.07	1:40	0	0
2081	-	"	24	405	740	740	0	+215	+95	1.06	0	0	0
2074	+	10 ⁻³	24	390	670	670	0	+155	+90	4.12	1:5,120	?	?
2082	-	10 ⁻³	24	410	745	725	20	+245	+70	7.34	1:1,280	807,000	5,900,000
2083	+	10 ⁻⁵	24	370	690	690	0	+170	+110	1.07	1:160	83,000	88,000
2090	-	10 ⁻⁵	24	430	680	680	0	+190	+70	3.09	1:640	43,000	124,000
2071	+	10 ⁻⁶	24	335	710	710	0	+185	+130	1.19	1:320	615,000	731,000
2076	-	10 ⁻⁶	24	465	755	720	35	+250	-10	6.01	1:320	367,000	2,205,000
2070	+	10 ⁻⁶	42	340	645	645	0	+75	+110	3.00	1:2,560	123,000	369,000
2077	-	10 ⁻⁶	42	395	640	530	110	+200	-85	5.49	1:5,120	196,000	1,078,000
2067	+	10 ⁻⁶	42	370	660	630	30	+160	+100	2.48	1:1,280	198,000	491,000
2080	-	10 ⁻⁶	42	385	760	760	0	+235	+130	2.59	1:1,280	42,000	109,000
2086	+	10 ⁻⁶	63	400	750	750	0	+220	+90	2.24	1:1,280	12,500	38,000
2088	-	10 ⁻⁶	63	430	1,000	1,000	0	+240	+300	2.23	1:2,560	?	?
2075	+	10 ⁻⁶	63	410	850	850	0	+180	+240	4.8	1:2,560	33,800	162,000
2079	-	10 ⁻⁶	63	305	725	725	0	+215	+180	2.74	1:5,120	?	?

2068	+	Control.	85	415	800	800	0	+160	+170	1.07	0	0	0
2085	-	"	85	385	915	915	0	+260	+235	1.07	0	0	0
2069	+	10 ⁻³	85	335	560	480	80	+125	- 10	5.92	1:5,120	109,000	645,000
2078	-	10 ⁻³	85	390	710	540	170	+230	-115	8.75	1:5,120	75,500	662,000
2072	+	10 ⁻³	85	365	730	730	0	+185	+155	3.26	1:2,560	11,300	36,900
2087	-	10 ⁻³	85	390	915	670	245	+260	- 30	4.32	1:2,560	38,000	105,000
2084	+	10 ⁻³	85	330	600	570	30	+140	+ 80	2.97	1:2,560	43,000	128,000
2089	-	10 ⁻³	85	400	830	690	140	+230	+ 20	6.17	1:5,120	58,600	361,500

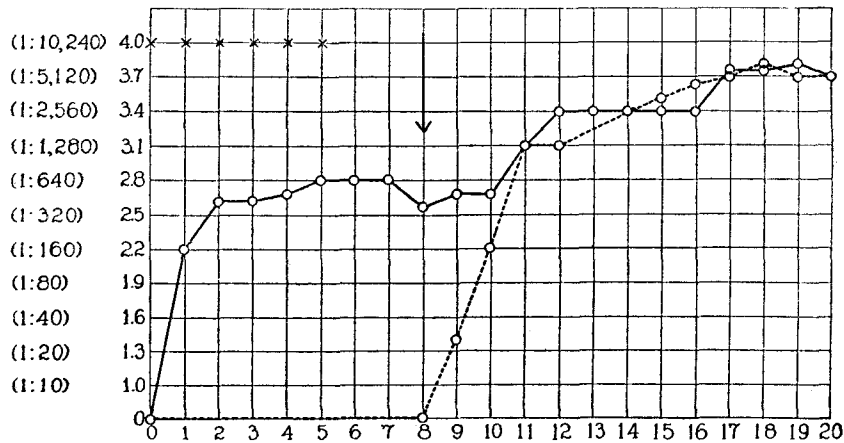


TEXT-FIG. 1. Composite agglutinin curves of normal control, and of immunized but non-infected animals.

○ — ○ Composite curve of Guinea Pigs 2068 and 2073, animals which were immunized but not infected. The times of injecting the killed suspensions of *B. abortus* are indicated at the points x.

○ ····· ○ Composite curve of Guinea Pigs 2081 and 2085, animals which were untreated but allowed to live throughout the experiment in the same pen with the infected animals.

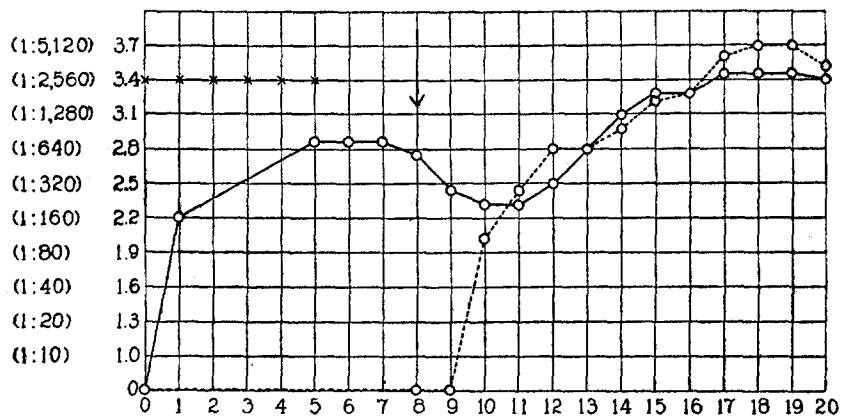
In this and all succeeding agglutinin curves, the abscissæ represent weeks, and the ordinates the logarithms of the dilution of the blood. The actual blood dilutions are given in parenthesis.



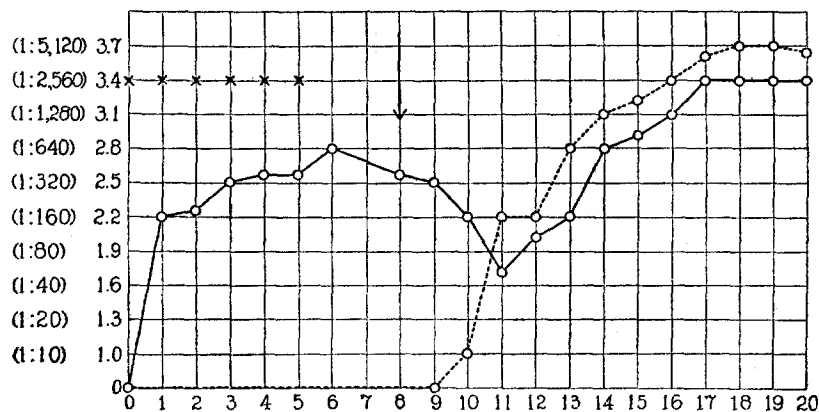
TEXT-FIG. 2. Composite agglutinin curves of immunized and non-immunized animals, infected with 1:1,000 dilution of a standard suspension of *B. abortus*. The times of injecting killed suspension of *B. abortus* are indicated by x, the time of infection by the arrow.

○ — ○ Guinea Pigs 2068 and 2074, immunized.

○ ····· ○ Guinea Pigs 2078 and 2082, non-immunized.



TEXT-FIG. 3. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:100,000 dilution of a standard suspension of *B. abortus*.
 —○ Guinea Pigs 2070, 2072, 2083, and 2086, immunized.
○ Guinea Pigs 2077, 2087, 2088, and 2090, non-immunized.



TEXT-FIG. 4. Composite agglutinin curves of immunized and non-immunized animals, infected with a 1:1,000,000 dilution of a standard suspension of *B. abortus*.
 —○ Guinea Pigs 2067, 2071, 2075, and 2084, immunized.
○ Guinea Pigs 2076, 2079, 2080, and 2089, non-immunized.

Enlargement of the lymph glands occurred, coincident with splenic enlargement, and was more pronounced in the non-immunized animals than in the immunized.

A striking lesion in the non-immunized animals which lived the longest and in several of the non-immunized animals which were killed early occurred in the kidneys. This lesion is frequently found in the later stages of the guinea pig disease and was described by Smith and Fabyan (1912). It consists in cellular infiltration in the early stages, followed later by sclerosis. That the lesion is of great import to the body economy is rendered probable by the fact that as the severity of the sclerosis increases emaciation of the body keeps pace. The condition is always accompanied by a very large spleen, but great enlargement of the spleen is sometimes found without accompanying kidney disease and emaciation. This kidney lesion was found among the immunized animals but once, in Guinea Pig 2069, one of the animals which received a heavy infecting dose.

The Number of Bacillus abortus Present in the Spleen.—The manner of determining the bacterial count of the spleen is described in the appendix. The counts are given in Table I. It will be noted that the bacterial count per gram of splenic tissue is greater in the immunized than in the other group, while the reverse is true when the bacterial content of the entire organ is considered. The explanation for this lies in the greater enlargement of the organ in the latter group. Since this enlargement is largely due to engorgement of the sinuses with blood, the bacterial concentration per unit of volume is decreased by dilution. Taking the entire organ, however, there is an appreciably greater number of cultivable *Bacillus abortus* present in the spleens of the non-immunized than in those of the immunized animals.

SUMMARY AND CONCLUSIONS.

From the data presented it seems permissible to draw the following conclusions.

1. Guinea pigs cannot be rendered immune to the *Bacillus abortus* inoculation disease by treatment with heat-killed cultures of this organism. This agrees with the work of Ascoli, and with the general conclusions of many that dead cultures confer very little or no immunity to infectious abortion in cattle.

2. The progress of the disease can be delayed appreciably by such treatment. This is supported by the following observations: (a) the loss in body weight due to the disease is delayed; (b) the development of splenic enlargement is partially inhibited or delayed; (c) the development of renal lesions is delayed or prevented; and (d) the rapidity of multiplication of *Bacillus abortus* in the splenic pulp is reduced.

APPENDIX.

Technique of Bleeding Guinea Pigs for Agglutination Tests.—The repeated bleeding of guinea pigs for periodic agglutination tests has been accomplished in an easy and fully satisfactory manner. Many animals have been bled ten to twenty times without injury and without difficulty in obtaining the requisite amount of blood for macroscopic tube tests. The method is as follows:

The laboratory should be warm so as to encourage the circulation in the peripheral vessels. One of the ears is rubbed briskly with a pledget of cotton soaked in xylol, and then dried with a clean bit of cotton; the blood vessels are examined and one of them nicked transversely with the point of a scalpel. The vessels can be seen to better advantage if some form of illumination below the ear is used. A vigorous flow of blood generally wells up from the small incision. The blood is taken into a 0.1 cc. serological pipette which is provided, for convenience, with a length of rubber tubing and a mouthpiece. One pipetteful (0.1 cc.) is immediately delivered into 0.9 cc. of citrated physiological salt solution, making a dilution of the blood of approximately 1:10. If necessary to work with low dilutions, two pipettefuls (0.2 cc.) may be delivered into 0.8 cc. of salt solution, making a dilution of 1:5. The tubes are shaken up and placed in the refrigerator over night. The next day as much as 0.7 to 0.8 cc. of a perfectly clear dilution of plasma may be pipetted off.¹ Since only 0.5 cc. is needed for the agglutination tests, the quantity is ample. The citrate-salt solution consists of 0.9 per cent sodium chloride and 2.0 per cent sodium citrate in distilled water. This amount of citrate

¹The dilution figures in this and accompanying papers are given in terms of the whole blood. The serum or plasma dilutions may be roughly computed as twice as great as that of the whole blood.

does not have any effect on the agglutinin titer so far as could be determined by checking against sera obtained by defibrination. The citrated plasma has the advantage over most sera in that it is perfectly clear and untinged with hemoglobin.

Agglutination Technique.—The technique of carrying out the agglutination tests is practically that described by Smillie, Little, and Florence (1919).

The antigen has been freshly prepared and no preservative added, although equally good results appeared to be obtained by using an antigen preserved with 0.25 per cent formalin and kept stored in the refrigerator for several months. Antigen preserved with 0.5 per cent phenol is not satisfactory because it induces a permanent cloudiness of the suspension which renders the reading of partial reactions difficult.

The Determination of the Approximate Numbers of Bacillus abortus Present in the Spleen.

Preparation of Splenic Tissue for Plating.—The following method was used for obtaining a measured and representative sample of splenic tissue for plating purposes.

A portion of the organ representing from 0.5 to 1.5 gm. is removed aseptically to a sterile weighed potato tube and its weight determined. The fragment is then crushed thoroughly in a manner to be described and diluted with nine times its weight of physiological salt solution. After thorough mixing, 0.1 cc. portions are used for plating, unless it is suspected that the bacterial count will be greater than 100,000 organisms per gm. of tissue, in which case 0.1 cc. of a 1:5 or 1:10 dilution of the original suspension is used.

For grinding or crushing the splenic tissue efficient crushers were made from heavy walled culture tubes in the following manner.

Tubes of as nearly perfect form as possible are selected from the stock and arranged in pairs, one tube of which is of such size that it will just slip easily into the other like the plunger of a syringe. The smaller tube is then constricted in its middle part by heating in a blast flame and drawing it out. The larger tube is shortened by cutting it off at such length that its mouth will be about at the level of the middle of the constriction of the smaller tube when the latter is placed within it. The inner tube is used as a pestle while the outer serves as

a mortar. The surface of the pestle is roughened by rotating it in contact with a piece of carborundum paper held in the hand. The inside of the mortar has not been altered although it probably could be improved somewhat by etching with hydrofluoric acid. Great roughening is not to be desired since the mortar is apt to be cut and broken, in the process of grinding, by sharp edges on the pestle. For the same reason abrasives such as fine sand or carborundum powder cannot be used. The apparatus is intended for crushing purposes rather than for grinding.

The crusher is wrapped in paper and sterilized by hot air. The piece of weighed spleen is placed in the larger tube and forced to the bottom with the pestle. It is now compressed and broken up by rotating and exerting gentle pressure on the pestle, the soft pulp being forced up between the sides of the pestle and the inner wall of the mortar. If the fit between the two elements of the crusher is good, by the time the pulp reaches the space in the upper end of the mortar left by the constriction of the shaft of the pestle it will be thoroughly reduced to a homogeneous paste. When the crushing has been completed small portions of salt solution are pipetted into the mortar and the pestle slowly lifted while being rotated. The vacuum in the bottom of the pestle causes the fluid to pass the pestle, thereby cleansing it of the pasty pulp mass. Several repetitions of this process generally leave the pestle clean, after which it is removed and the remainder of the quantity of salt solution needed to make a 1:10 dilution added.

Plating Methods.—With *B. abortus* ordinary plating methods are useless because this organism requires a peculiar gaseous environment which is not satisfied by the free atmospheric exchange of the ordinary Petri dish. For obtaining suitable gaseous conditions for the cultivation of the *B. abortus* in plate culture, there have been available the methods of Nowak (1908) and of Huddleson (1920). Since both of these are somewhat involved, while furthermore simple hermetic sealing of the tubes had proven an entirely satisfactory method of obtaining growth in tube cultures, it was decided to try this method with plate cultures in lieu of the others. A simple and satisfactory method of sealing the plates has been devised and the results in obtaining growth of *B. abortus* have been entirely satisfactory.

Pieces of double strength window glass were cut into 12 cm. squares, and others into pieces 12 by 24 cm. in order to handle two cultures as a unit. The edges of the glass were ground off to remove sharp points and corners. These squares were used as bases upon which the halves of the Petri dishes containing the cultures were sealed. The steps in the method are as follows:

1. Paraffin of a melting point of 55–60°C. is placed in a shallow vessel and heated until it smokes. The high temperature serves to sterilize the paraffin. When it has cooled to 60–70°C., a few degrees above its melting point, it is ready for use.

2. A glass square is placed on the table and its upper surface thoroughly traversed with the Bunsen flame. This not only sterilizes the surface but serves to warm the glass.

3. The lower half of the Petri dish containing the inoculated agar is grasped, open surface downward, with the tips of the fingers and the edges dipped into the molten paraffin to a depth of 5 to 8 mm. The entire margin should not be submerged at one time, otherwise when the plate is lifted the rush of air under the edge is apt to cause spattering of the paraffin on the agar surface. This may be avoided easily by tilting the plate when submerging. Since the temperature of the glass of the Petri dish is lower than the melting point of the paraffin, a layer of the latter in a congealed state adheres to the plate when it is lifted.

4. The plate is placed on the center of the glass square and gently pressed down. Since the square is warm, and there has been no delay between the dipping of the edges of the plate and planting it on the glass surface, an excellent seal between the plate edge and the glass base is formed.

5. To assure a perfect seal, the flame of a micro burner is run quickly around the joint between plate and base. If done properly the surface only of the paraffin layer will be melted and all cracks and crevices will be filled. If too much heat is used the glass will become heated through and will cause the paraffin to spread out in a thin layer on the base, thus forming an imperfect seal, or at best a thin seal which is apt to rupture through when a partial vacuum has formed within the plate.

Even when hermetically sealed, poured plates are not satisfactory for quantitative work when dealing with *B. abortus* for the reason that the organism will not develop in anaerobic or semianaerobic conditions. The deep lying bacilli will, in most instances, fail to develop and those that do grow will produce colonies so minute that counting is difficult or impossible. Surface inoculation has been used exclusively in this work. 0.1 cc. of the splenic suspension is pipetted onto the surface of the agar plate and spread uniformly by means of a bent glass rod. This amount of fluid will spread evenly over the surface of the agar without leaving an excess of fluid. The plate is then tilted, in an inverted position, on the rim of the lid to allow the surface to dry somewhat before it is sealed.

Growth generally appears within 3 to 4 days, though sometimes not for 5 or 6 days. Within 24 hours of the time when colonies can first be seen, they will have reached a diameter of 1 mm. if there is not too much crowding. After this, growth is comparatively slow, but if very few colonies are present they may reach a diameter of from 3 to as much as 6 mm. The colonies have no tendency to spread or coalesce. Because of the discrete character of the growth as many as 1,000 colonies may grow on a single plate without evidence of interference with each other, other than that their ultimate size will not exceed 1 mm.

Generally all colonies appear at the same time and grow at about the same rate of speed, so that they have about the same size. In some instances, however, two crops of colonies may appear. In these cases the second crop will become visible 1 to 3 days after the first as minute colonies lying between the first. The second crop colonies always remain small, seldom reaching a diameter of 0.5 mm. even when they are not crowded and are subjected to prolonged incubation.

Because of this phenomenon, it is always advisable to incubate the plates for 3 or 4 days following the appearance of the first crop of colonies before making the colony count.

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