THESIS

THE CULTIVATION OF BACTERIUM ABORTUS

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By

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THE CULTIVATION OF BACTERIUM ABORTUS.

INTRODUCTION

The isolation of Bact. abortus presents certain difficulties. The early investigators, although convinced that abortion was due to an undetermined but specific agent, were unable to isolate the organism. It was not until suitable biological requirements were provided that the organism was obtained in pure culture.

To obtain successful results in the primary isolation of Bact. abortus, it is advisable to use specially prepared media having a suitable pH concentration and necessary to incubate under certain conditions peculiar to this organism alone.

Several different media are used for the growth of Bact. abortus and several investigators have described methods for the cultivation of this organism. While these methods have been successful to a certain degree, they are not always wholly satisfactory, due to the amount of labor involved and the results obtained.

It is the purpose of this study to compare the growth of Bact. abortus when grown on different media and when placed under different conditions of incubation in an effort to obtain the most satisfactory method for the cultivation of this organism.

REVIEW OF LITERATURE.

The fact that animals cast their young before the full period of gestation was recorded in ancient times, and that abortion in cattle was contagious in many instances, and might be spread from animal to animal in the herd, was known to farmers and breeders in the early part of the nineteenth century. In 1826 Hutrel d'Arboval, a Frenchman, and in 1834 Youatt, an Englishman, concluded that abortion was often contagious and not due to environment or accidents. In 1878 Lehnert demonstrated this contagiousness by introducing into the vagina of pregnant cows the vaginal discharge and placental tissue of aborted cases, and thus causing abortion. (1).

In 1886 Nocard (2), investigating for the French government, made extensive bacteriological investigations in an effort to isolate a definite organism causing abortion. He obtained a bacillus and a micrococcus in pure culture, but with neither of these was he able to produce the disease.

In 1897 Bang (3) published the results of his study of the etiology of epizootic abortion in Denmark, in which he discovered the organism now known as Bact. abortus. He examined the uterus of a cow which had been slaughtered while showing premonitory symptoms of abortion. Between the uterine mucous membrane and fetal envelopes an abundant

yellow, odourless exudate was found. In cover glass preparations from the exudate stained with methylene blue he observed a very small bacterium, apparently in pure culture. These were found singly and in clumps, free, and often intracellular. The following medium, which was originally designed by Stribolt, was the one on which Bang first obtained an artificial culture of Bact. abortus: A solid medium containing 3/4 percent agar and 5 percent gelatin is prepared and put into culture tubes in the ordinary way. Before sowing, the solid gelatin agar should be liquefied by heat and cooled to 45° C., after which about half its volume of liquid sterile serum in the raw state (species of animal not stated) is to be added, and mixed by shaking. The liquid medium is then inoculated with the seed material, and the tubes again gently shaken, and plunged into cold water to bring about rapid solidification.

According to Bang, "At the end of from two to four days' incubation there appeared a great number of very small colonies which developed only in a definite zone of the tubes. This zone lay about half a centimeter under the surface of the nutritive medium, and it had a thickness of from 1 to 1 1/2 centimeters; colonies were not present above or below this. We had thus not to do with an aerobic bacterium which would have pushed its growth as far as the surface of the nutritive medium, and still less had we to do with an anaerobic form which would have grown as far as the bottom of the tube. The under limit of the zone of growth lay exactly where the limit

of the growth of a strictly anaerobic bacterium shows itself, the necrosis bacillus for example. This highly peculiar behavior of the abortion bacillus towards oxygen made it at once apparent that we had to do with a distinct species."

In speaking of the cultural characters of this bacillus, Bang stated that his observations "appeared to show beyond any doubt that for the abortion bacillus in its behavior towards oxygen there are two optima, first, a degree of oxygen tension in the nutritive medium less than that of the atmospheric air, and, second, the presence in the nutritive medium of a very high tension of oxygen, which, however, lies somewhat under 100 percent. Between these two optima there is an intermediate zone in which the abortion bacillus grows badly or not at all."

In 1901 Preisz (4) at Budapest isolated a similar organism from the vaginal discharge of a case of abortion. He made his inoculation directly on slanted ordinary agar made of meat infusion, peptone and salt, and after passing oxygen into these tubes he sealed them with wax and obtained a growth visible with a hand lens in three days. Inoculated into deep dextrose agar, the zonal growth appeared lying seven to fifteen millimeters beneath the surface of the medium. Stab cultures of the organism rarely reached the surface after prolonged incubation. He considered that his medium was as favorable as the A.G.S. of Bang. He was able, by using alkaline pyrogallol, to produce a growth, and also by using acetylene gas. He believed that in these two instances growth resulted because oxygen

was not wholly absent. He compared the organism to an anaerobe, but it differed in that it would also grow in pure oxygen.

In 1908 Nowak (5), at the University of Krakau in Austria, (Fabyan) took up the study of this organism, being attracted by its very interesting biological characteristics. In studying pure material he found the method of Bang satisfactory, but where contaminations were present the growth might easily be killed out or masked. The broad surface which Petri dishes afforded was desirable to obtain isolated colonies. Nowak sought to obtain an oxygen tension less than air by the use of a closed chamber containing the actively growing culture of an organism like B. subtilis, a method which had already been employed in the removal of oxygen in tetanus cultures. He placed tubes inoculated, some with B.subtilis and others with Bact. abortus, in a glass chamber with paraffin and placed this in the incubator. Satisfactory results were obtained when the relation of the surface of B. subtilis was in proper proportion to the volume of the chamber. If too little of B. subtilis was present, no growth resulted; if too much, all the oxygen was absorbed and Bact. abortus checked. He found one square centimeter of surface growth of B. subtilis to fifteen cubic centimeters of volume the best proportion. Different varieties of B. subtilis gave similar results. The tubes inoculated with suspected abortion material were first incubated twenty-four hours in the large chamber of the incubator in order to develop any contaminations, and then incubated under the influence of B. subtilis, when typical colonies developed

in the clear areas. By gradually using less of B. subtilis, he trained Bact. abortus to grow in a normal atmosphere. In pure oxygen two out of six cultures grew. Under compression he obtained good results from three atmospheres but no growth at six atmospheres, although when this latter tube was placed under the influence of B. subtilis colonies rapidly developed. By his technic he was able to obtain many cultures from the fetus and vaginal discharge where other methods failed.

In the United States the disease has been known for some time but it remained for MacNeal and Kerr (6) in 1910 to isolate and identify Bact. abortus as being responsible for a part at least of the abortions in cattle in this country. By the Novak plate method they were able to obtain cultures of Bang's organism in two out of four cases of abortion studied.

In 1911 Holth (7) demonstrated that material from abortion cases inoculated on slanted serum agar gave no growth in two weeks' incubation, but if similar tubes were sealed with paraffin growth might sometimes be observed after six days. The explanation offered was that the organism by using the oxygen over and over again reduced it to the proper tension.

In 1912 Giltner (8) called attention to the use of media prepared from pregnant uterine wall, fetal membranes, fetus, and amniotic fluid separately.

In 1912 Smith and Fabyan (9) reported that Bact. abortus inoculated into guinea pigs produces a disease with characteristic lesions, and that the organism can be recovered from these pigs after a period of 11 weeks or more. By this method it was

often possible to isolate Bact. abortus from contaminated material when direct cultural methods failed.

The greatest number of organisms were found to be contained in the spleen and lymph nodes of the inoculated animal, while the bone marrow, liver, kidney and lung, in order named, followed in this respect.

The Nowak method of culturing was employed to recover the organism. In addition to B. subtilis, cultures were grown in symbiosis in a closed chamber with B. coli, B. megantherium and a staphylococcus. It was found that B. coli, B.megantherium, and the staphylococcus gave results similar to B. subtilis.

In 1918 Smillie (10) reported that cultures of Bact. abortus could be regularly recovered from inoculated guinea pigs within 3 to 4 weeks. The figures he obtained show that the number of living bacteria in the spleen of the guinea pig was larger at that time than later, although the macroscopic lesions tend to become more prominent as the number of bacteria decreases.

Ordinary veal peptone agar tubed and slanted was the medium employed. Bits of tissue were rubbed over the entire surface of the agar, and the tubes were sealed with wax and incubated at 37° C. Colonies of Bact. abortus were observed on the agar slant after 5 to 10 days' incubation.

Fabyan (11) and Schroeder (12) working independently demonstrated the presence of Bact. abortus in milk by guinea-pig inoculation. In 1918 Evans (13) endeavored to isolate the organism

from milk by the use of direct plating methods. She succeeded in isolating the organism from milk of cows which had been inoculated with strains of that organism, but was unable to isolate the organism from animals which had aborted as a result of natural infection.

In 1920 Stafseth (14) found that excellent growth of Bact. abortus could be obtained by employing spleen or liver media in place of beef. It was found that the addition of 1 percent dextrose or of 1 percent starch improved the spleen media, while the liver agar did not require the addition of carbohydrates.

In 1920 Huddleson (15) reported his results on the isolation of Bact. abortus from milk. He found that the medium and its proper preparation, the proper H-ion concentration, the employment of an agent which eliminated fast-growing organisms, and the method of incubation were the factors which must be considered when isolating Bact. abortus directly from milk.

Liver agar as described by Stafseth was employed; glass wool was used in filtering, and excessive heating was avoided in its preparation and sterilization, for he (Huddleson) states "Huntoon (16) has shown that about half of the initial growing value of the media is removed by over-heating, and the use of cotton, cloth or paper in filtration."

Optimum growth was obtained when the pH concentration of the media was between 6.6 and 6.4. Gentian violet in a saturated aqueous solution was incorporated in the media in sufficient quantity to give the dye a final dilution of 1:10,000

as an agent to eliminate fast growing organisms. The growth of a large percentage of gram positive and a small percentage of a gram negative organisms is inhibited while the growth of Bact. abortus was not in the least affected.

From experiments conducted, Huddleson concluded that the initial growth of Bact. abortus was due to an increased carbon dioxide tension, and, by incubating the inoculated medium in a closed chamber in which 10 percent of the air had been displaced by carbon dioxide gas, obtained growth in 24 to 72 hours.

The results obtained from the direct plating method were identical with the guinea-pig inoculation method for determining the presence of Bactl abortus in milk. The chief advantage of the direct plating method is that it requires only four days to determine the presence of the organism, whereas the animal isolation method requires three to four weeks.

In 1922 Fitch (17) reported that beef infusion agar adjusted to a pH concentration of 6.8 to 7.2 plus 10 percent naturally sterile horse serum was excellent for cultivating Bact. abortus; the cultures developing in either an atmosphere of 10 percent carbon dioxide or 10 percent hydrogen.

In 1922 Hagan (18) prepared cultures of Bact. abortus as follows: The edges of the lower half of a Petri dish containing the inoculated agar were dipped into molten paraffin and sealed to a sterile glass plate. Growth appeared within 3 to 4 days when incubated at 37° C.

METHOD OF INVESTIGATION

The literature reviewed gives several methods for the cultivation of Bact. abortus, but while these methods have been successful to a certain degree, they are not always wholly satisfactory, due to the amount of labor involved and the results obtained. However, the literature does not give a comparative value of the methods now in use for the cultivation of this organism, so this investigation was designed to give definite knowledge regarding this point, and to perfect the technic.

It was necessary at the beginning of the study to eliminate those methods which required a great amount of labor in comparison with other methods which required less labor and gave equally satisfactory results. Bang's original method required too long an incubation period so was not used; Nowak's method of growing in a closed chamber in symbiosis with a culture of B. subtilis, and Hagan's method of plating on Petri dishes which were afterwards sealed separately, were not used because of the labor involved.

Material from aborted feti, milk from animals which had aborted, and organs from guinea pigs which had been inoculated with material containing Bact. abortus, was plated on various media and placed under different conditions of incubation in order to study the effect of these various factors upon the isolation of the organism. The cultivation of laboratory strains

under the same conditions was also studied.

A limited number of experiments were conducted to obtain data on the value of centrifuging milk as an aid in the isolation of the organism by direct plating methods.

SOURCES OF MATERIAL

Material for culturing was obtained from three sources:

(a) In the diseased animal the specific bacteria are found in the placenta and amniotic fluid, within the fetal intestine, sometimes in the tissues of the fetal organs, and in the wall of the maternal uterus. In abortion cases the material studied was obtained from the stomach of the fetus. The abdomen was seared with a heated knife, an incision made through the wall, and the stomach contents were removed and placed in sterile test tubes by means of sterile pipettes.

(b) Various investigators have shown that Bact. abortus is often present in milk which is drawn from apparently normal cows, and if injected in guinea pigs will produce characteristic lesions. Milk was used as a means of supplying the organism Bact. abortus for this study, both for guinea-pig inoculation and direct plating methods.

The samples of milk were collected in sterile test tubes under conditions tending to exclude as far as possible all outside contamination. After washing the udder and flanks, approximately 10 c.c. of milk was collected, having discarded the first milk.

(c) Organs of guinea pigs, which had been inoculated with material containing Bact. abortus, were also used as a source of material in this study.

PREPARATION OF MEDIA.

Liver agar. The liver agar used was prepared as described by Stafseth (14) and improved by Huddleson (15). An infusion was made from beef liver and 2 percent agar, 1 percent peptone, with 0.5 percent sodium chloride added. The medium was cleared by egg albumin, filtered through glass wool and adjusted to a pH concentration of 6.6.

Gentian violet liver agar. Gentian violet liver agar was prepared as described by Huddleson (15). It consisted of the liver agar described above with the addition of gentian violet in a saturated aqueous solution in sufficient quantity to give the dye a final dilution of 1:10,000.

Beef infusion agar. Beef infusion agar was prepared containing 2 percent agar, 1 percent peptone, and 0.5 percent sodium chloride; it was cleared by egg albumin and adjusted to a pH concentration of 7.0. At the time of sowing, approximately 10 percent naturally sterile horse serum was added to the melted agar after cooling to 50° C.

Veal infusion agar. Veal infusion agar was prepared in the same manner as the beef infusion agar described above.

Uterine wall and fetal membrane infusion agar. An infusion was prepared from the uterine wall and fetal membranes of a pregnant uterus, as mentioned by Hiltner(8): 2 percent agar, 1 percent peptone, 0.5 percent sodium chloride was added and the medium adjusted to a pH concentration of 7.0. This medium is referred to in the following tables as "uterine agar".

Peptic Digest agar. Two percent agar was added to peptic digest broth which had been prepared according to directions given by Dubvosky and Lyers (19). The medium was adjusted to a pH concentration of 7.0.

METHOD OF PLATING MILK

One-tenth cubic centimeter of the milk sample to be cultured was placed on the surface of a solidified agar plate and evenly distributed by holding a sterile glass rod, bent at an angle of 90 degrees, against the surface of the medium and at the same time rotating the plate.

METHOD OF PLATING STOMACH CONTENTS OF FETI

The method used to plate the stomach content of feti was the same as followed in plating milk.

METHOD OF PLATING ORGANS FROM INFECTED GUINEA PIGS

Using sterile instruments, the spleen and liver of infected guinea pigs were removed and cut in sections which were streaked over the surface of the solidified agar plates.

METHODS USED FOR INCUBATING CULTURES

The cultures were incubated at 37° C. under the following conditions: In closed jars after 10 percent of the contained air had been displaced by carbon dioxide; in closed jars after 10 percent of the contained air had been displaced by hydrogen; in closed jars after 10 percent of the contained air had been displaced by nitrogen; in closed jars from which 10 percent of the air had been extracted; in jars which were sealed only, nothing being added or subtracted.

The carbon dioxide used was obtained from cylinders of commercial carbon dioxide; the same was true of the nitrogen used. The hydrogen used was prepared in the laboratory by the action of hydrochloric acid on zinc.

EXPERIMENTAL DATA

(1) The Isolation of Bact. abortus from Fetal Material When Plated on Different Media and Placed under Different Conditions of Incubation.

The stomach contents of a fetus aborted in the College experimental herd was plated on veal agar, beef agar, and gentian violet liver agar. Plates in duplicate of each of these media were placed in closed chambers containing 10 percent carbon dioxide, 10 percent nitrogen, and 10 percent hydrogen; in a closed jar from which 10 percent of the contained air had been extracted; and in a sealed jar to which nothing had been added or subtracted.

Table I shows the amount of growth recorded at the end of 60 hours' incubation at 37° C.

Growth had not appeared upon any of the veal plates at the end of this incubation period. The beef agar plates showed growth when incubated in carbon dioxide, hydrogen, or in a 10 percent vacuum. The growth on gentian violet liver agar was very abundant, as is shown by the table.

TABLE I

Shows the Growth of Bact. abortus from Fetal Material When Plated on Different Media and Placed under Different Conditions of Incubation.

Media	10% CO_2		10% N_2		10% H_2		10% Vacuum		Sealed Jar		Aerobic Check	
Veal Agar plus 10% Horse Serum	-	-	-	-	-	-	-	-	-	-	-	-
Beef agar plus 10% Horse Serum	+	+	-	-	+	+	+	+	-	-	-	-
Gentian Violet Liver Agar	++++	++++	++++	+++	+++	++++	++++	++++	+++	+++	-	-

- = No Growth

+ = Amount of Growth Corresponding to Number Recorded.

(2) The Isolation of Bact. abortus from Milk When Plated on Different Media and Placed Under Different Methods of Incubation. Part I.

Milk from Animal 805B1 was plated on a series of gentian violet liver agar plates, beef plates, and peptic agar plates, and placed under conditions of incubation as in Experiment (1). At the end of 60 hours' incubation the average number of colonies of Bact. abortus which had developed on duplicate sets of plates is shown in Table II.

TABLE II

Shows the Isolation of Bact. abortus from Milk When Plated on Different Media and Placed under Different Methods of Incubation.

R.R.Q. 805B1	10% CO_2	10% N	10% H_2	10% Vacuum	Sealed Jar
G.V. Liver Agar	44	6	0	0	0
Beef agar and Horse serum	9	0	0	0	0
Peptic digest agar	0	0	0	0	0
R.F.Q. 805B1					
G.V. Liver agar	50	10	0	0	0
Beef agar and Horse serum	30	0	0	0	0
Peptic Digest agar	32	0	0	0	0

R.R.Q. = Right Rear Quarter
R.F.Q. = Right Front Quarter
G.V. = Gentian Violet

Colonies had developed only in the jars containing carbon dioxide and nitrogen, with the greatest number developing in the presence carbon dioxide. The gentian violet liver agar plates in all cases showed the greatest number of colonies.

(3) The Isolation of Bact. abortus
From Milk When Plated on Different Media and Placed
Under Different Methods of Incubation.
Part II.

An experiment similar to that under (2), except that different media were employed, was conducted in an effort to obtain additional data. Gentian violet liver agar, plain liver agar, uterine agar and peptic digest agar were employed. The average number of colonies of Bact. abortus which had developed on three sets of duplicate plates at the end of 60 hours' incubation is shown in Table III.

TABLE III

Shows the Isolation of Brct. abortus from Milk when Plated On Different Media and Placed under Different Methods of Incubation.

R.R.Q. 805B1	10% CO_2	10% N	10% H_2	10% Vacuum	Sealed Jar
G.V.Liver Agar	280	190	0	0	0
Liver Agar	210	180	0	0	0
Uterine Agar	Contaminated	100	0	0	0
Peptic Digest agar	200	0	0	0	0
R.F.Q. 805B1					
G.V.Liver agar	350	300	0	0	0
Liver agar	460	380	0	0	0
Uterine Agar	330	115	0	0	0
Peptic Digest Agar	310	155	0	0	0

R.R.Q. = Right Rear Quarter

R.F.Q. = Right Front Quarter

G.V. = Gentian Violet

As in the case of the previous experiment, colonies of Bact. abortus developed only in the jars containing carbon dioxide and nitrogen, and, as before, the greatest numbers developed on the plates under the influence of carbon dioxide. Milk from the right rear quarter gave the highest number of colonies on the gentian violet liver agar plates in each jar, while milk from the right front quarter gave the highest number of colonies on the plain liver agar.

(4) The Isolation of Bact. abortus From Guinea-Pig Organs When Plated on Different Media and Placed Under Different Methods of Incubation. Part I.

Spleens from 3 guinea pigs showing characteristic lesions of abortion disease were plated on gentian violet liver agar, beef agar, and peptic digest agar. The plates were incubated under conditions similar to those given under experiment (1). The growth recorded at the end of 60 hours' incubation is shown in table IV.

TABLE IV

Shows the Isolation of Bact. abortus from Guinea Pigs When Plated on Different Media and Placed Under Different Methods of Incubation.

	10% CO_2	10% N	10% H_2	10% Vacuum	Sealed Jar
Spleen G.P. 944					
G.V.Liver Agar	++++	-	+++	++++	+
Beef agar and Horse serum	++	-	-	-	-
Peptic digest agar	++++	-	+++	++++	++++
Spleen G.P. 995					
G.L.Liver agar	+++	-	-	-	+++
Beef agar and Horse serum	-	-	-	-	-
Peptic digest agar	+++	-	-	++++	+++
Spleen G.P. 996					
G.V. Liver agar	-	-	-	-	-
Beef agar and Horse serum	-	-	-	-	-
Peptic digest agar	-	-	-	+	++++

- = No Growth
+ = Amount of Growth Corresponding to Number Recorded.

G.P. = Guinea pig
G.V. = Gentian Violet.

Each of the guinea pigs had been inoculated with material from an abortion case in the College Experimental Herd which was caused by the injection of a laboratory strain of Bact. abortus. As was to be expected, the organism was recovered with greater ease than in the case of abortions caused by natural infection.

Colonies of Bact. abortus developed on plates in all jars except that containing nitrogen. The average number of colonies developing in the jars containing carbon dioxide, in the jar with a 10 percent vacuum, and in the sealed jar being nearly equal. However, the colonies developing in the jar containing carbon dioxide were larger in diameter than those in the other jars.

The peptic digest agar proved a very good medium for the isolation of Bact. abortus, it being the only medium to show growth from the spleen of guinea pig number 996. In other cases the colonies, although as numerous, were not as large as those developing on the gentian agar plates.

(5) The Isolation of Bact. abortus From Guinea-Pig Organs When Plated on Different Media And Placed Under Different Methods of Incubation.

Part II.

Spleens from 2 guinea pigs showing characteristic lesions of abortion disease were plated on gentian violet liver agar, plain liver agar, uterine agar, and peptic digest agar and placed under conditions of incubation as given above. The growth recorded at the end of 60 hours' incubation is shown in table V.

TABLE I

Showing the Isolation of Bact. abortus from Guinea Pigs When Plated on Different Media and Placed Under Different Methods of Incubation.

	10% CO_2	10% N	10% H_2	10% Vacuum	Sealed Jar
Spleen G.P. 1002					
G.V.Liver agar	++	++	-	-	-
Liver agar	++	++	-	-	-
Uterine Agar	++	++	-	-	-
Peptic digest agar	+	+	-	-	-
Spleen G.P. 1007					
G.V.Liver agar	+++	++	-	-	-
Liver agar	++	+	-	-	-
Uterine Agar	+++	++	-	-	-
Peptic digest agar	++	+	-	-	-

- = No Growth

+ = Amount of Growth Corresponding to Number Recorded

G.V. - Gentian Violet

The guinea-pigs in this case had been inoculated with 5 c.c. of milk and the organism was not recovered as easily as in the case of the previous experiment. Colonies of Bact. abortus developed in the jars containing carbon dioxide and nitrogen; the other jars failed to give growth. The best growth in regard to number and size of colonies appeared on the gentian violet liver agar plates which were incubated in 10 percent carbon dioxide.

(6) The Cultivation of Bact. abortus on Different Media When Placed Under Different Methods of Incubation.

In order to obtain the comparative value of the different media and the different methods of incubation on the cultivation of strains of Bact. abortus after isolation , three laboratory strains were cultured on liver agar slants, beef agar slants, uterine agar slants and peptic digest agar slants. These were placed under conditions of incubation similar to those of the previous experiments. The growth recorded at the end of 24 hours' incubation is shown in Table VI.

TABLE VI

Shows the Growth of Laboratory Strains of Bact. abortus in 24 Hours on Different Media When Placed Under Different Methods of Incubation.

	10% CO_2	10% N	10% H_2	10% Vacuum	Arabic
Strain No.200					
Liver agar	++++	++++	++++	++++	++++
Beef Agar	+	++	+	+	+++
Uterine Agar	++	++	+	++	++
Peptic digest agar	++	++	++	+	++
Strain No.1659					
Liver Agar	++++	+++	+++	+++	++++
Beef Agar	+	+	++	++	++
Uterine Agar	++	++	+	+	+
Peptic digest agar	+++	++	++	+	++
Strain No.805B1					
Liver Agar	++++	++	++	++	++
Beef Agar	+	+	+	+	+
Uterine Agar	+	+	+	+	+
Peptic Digest Agar	++	+	+	+	+

\- = No Growth

\+ = Amount of Growth Corresponding to Number Used.

A study of table VI shows that liver agar gives the most luxuriant growth in comparison with the other media; and that incubation in carbon dioxide gives the greatest growth in comparison with the other methods of incubation.

It is also interesting to note that the slants incubated aerobically gave better growth than those incubated under all other conditions excepting those incubated in the jar containing carbon dioxide.

(7) The Value of Adding Serum to Liver Agar For the Isolation of Bact. abortus.

The addition of blood serum is a means used by many workers to enrich their media, and, for the cultivation of Bact. abortus on veal or beef infusion agar, 10 percent of serum is generally added.

In order to determine the value of the addition of serum to liver agar the following experiment was conducted. Petri dishes were poured with gentian violet liver agar, and gentian violet liver agar plus varying amounts of fresh naturally sterile bovine serum. Milk from the right rear and right front quarters of animal L.O. 1 was spread on these plates after which the plates were incubated under 10 percent carbon dioxide at 37° C. for 60 hours. At the end of this period the number of colonies of Bact. abortus developing on each plate was counted and recorded; table VII shows the average count per plate for a series of six incubations.

TABLE VII.

Shows the Value of Adding Bovine Serum to Gentian Violet Liver Agar for the Cultivation of Bact. abortus.

Media	Average count per plate
G.V. liver agar + 5% Serum	5 colonies
G.V. liver agar + 10% Serum	8 colonies
G.V. liver agar + 20% Serum	15 colonies
G.V. liver agar + 30% Serum	Contaminated
G.V. liver agar (no Serum)	38 colonies

G. V. = Gentian violet.

With the addition of 30 percent serum the plates became contaminated, probably due to the fact that the gentian violet was in too high a dilution to inhibit the growth of the contaminating organisms; for with the increase in the amount of serum added per plate the dilution of the dye became correspondingly higher.

The data show that the addition of bovine serum to gentian violet agar does not give an increase in the number of colonies developing from milk; on the other hand, the plain gentian violet liver agar plates give a much higher count.

(8) The Effect of pH Concentration of Liver Agar on the Growth of Bact. abortus.

In an endeavor to learn the effect which the reaction of liver agar has on the growth of Bact. abortus a number of flasks of media were prepared having a pH concentration varying from 6.0 to 7.6. Plates were poured from each flask and milk from the right rear quarter of animal 805B1 was plated. In three series of plates the average number of colonies of Bact. abortus developing per plate is shown in table VIII.

TABLE VIII

Shows the Effect of pH Concentration of Liver Agar on the Isolation of Bact. abortus from Milk.

pH Concentration	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6
Average number of Colonies	5	2	3	6	6	3	5	5	2

As shown by the table, colonies developed over the entire range of pH values, the highest counts occurring at 6.6 and 6.8, the difference in count being very small.

Slants were also prepared from the above flasks and several strains of Bact. abortus were grown under different conditions in an effort to obtain additional data on the effect of the pH concentration.

Strain D-1 had been recently isolated from a fetus aborted as a result of natural infection; strain 1665 had been recently recovered from an abortion case in the College Experimental Herd; strain AE was taken from the first transplant after isolating from milk; strain GP was taken from the first transplant after isolating from the spleen of a guinea pig; and strains LUCAS, 670, and 200 had been grown in the laboratory for some time.

These different strains were incubated at 37° C. under the following conditions: aerobically, with tubes sealed with sealing wax, and under 10 percent carbon dioxide.

Table IX shows the amount of growth recorded at the end of 24 hours' incubation.

TABLE IX

Shows the Effect of pH Concentration of Liver Agar

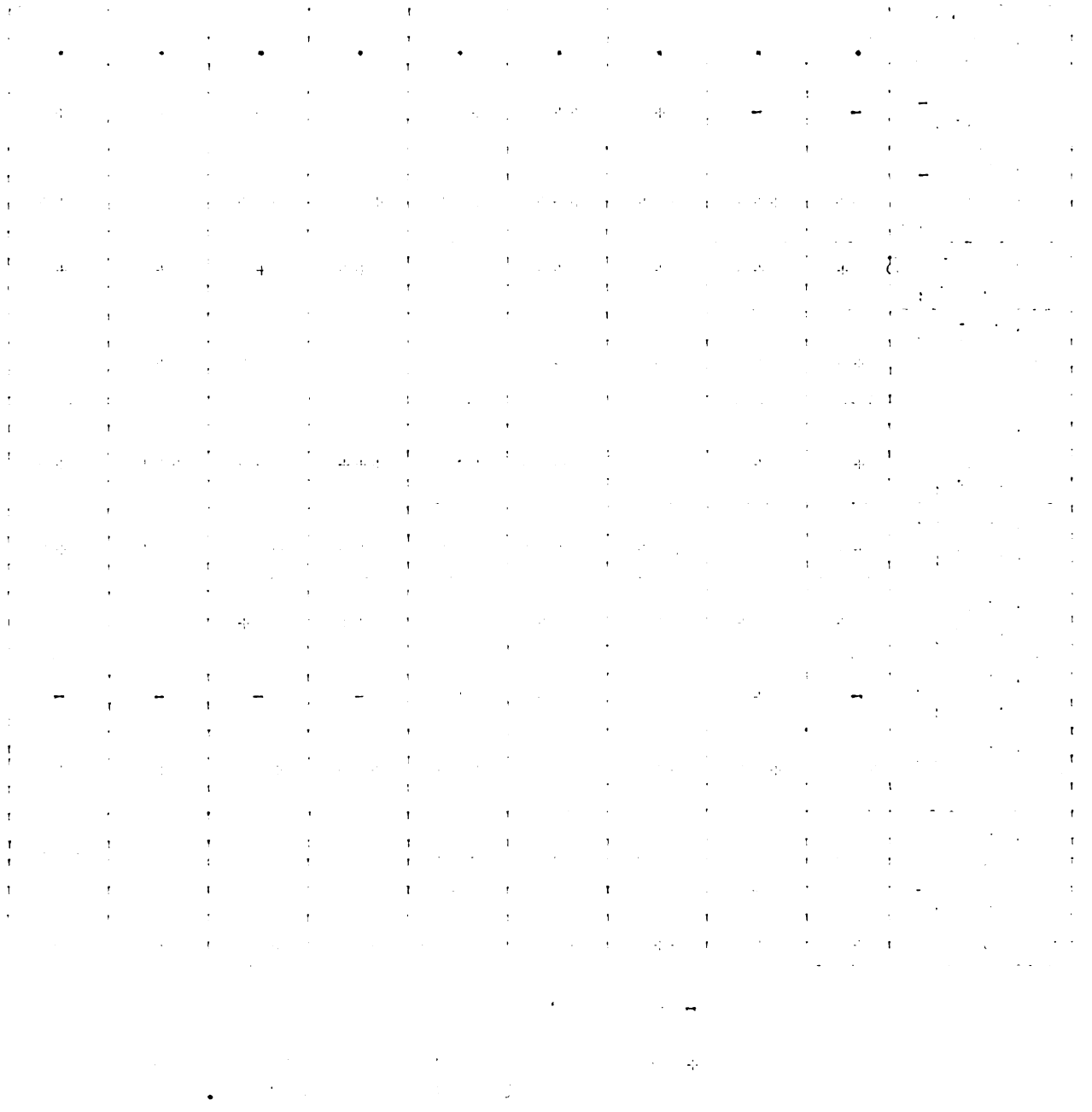
On the Cultivation of Laboratory Strains.

of Bact. abortus

pH Concentration	6.0	6.2	6.4	6.6	6.8	7.0	7.2	7.4	7.6
Strain D-1 Aerobic	-	-	+	++	++	++	+	+	+
Strain D-1 Sealed test tubes	+++	+++	+++	+++	+++	+++	+++	++	++
Strain 1665 Aerobic	+	++	++	++	++	++	+	+	+
Strain 1665 Sealed test tubes	++	++	++	+++	++	++	++	++	++
Strian LUCAS Aerobic	++	++	++	+++	+++	+++	+++	+++	++
Strain 670 Aerobic	++	++	+++	+++	+++	+++	+++	++	++
Strain 200 Aerobic	+	++	++	++	++	++	++	++	++
Strain AE Aerobic	-	+	++	++	++	-	-	-	-
Strain AE 10% CO_2	+++	+++	+++	+++	+++	+++	+++	++	++
Strain X 10% CO_2	+++	+++	+++	+++	+++	+++	+++	+++	+++
Strain GP 10% CO_2	+	+	++	++++	++++	++++	++++	++++	+++

- = No Growth

+ = Amount of Growth Corresponding to Number Recorded.



Growth appeared over the entire range of pH concentration in all cases except the recently isolated strains when grown aerobically. However, the best average growth for all cases seems to occur at the pH concentrations of 6.6 to 6.8.

(9) The Value of Clearing Liver Agar with Egg Albumin.

In order to know the value of clearing liver agar with egg albumin two flasks of media were prepared, one of which was cleared with 10 percent powdered egg albumin before filtering, while the other was not cleared. Plates were poured from each flask and milk from the right rear and right front quarters of animal No. 2 was plated in duplicate. Table X shows the count of Bact. abortus per plate after 60 hours' incubation in an atmosphere of 10 percent carbon dioxide.

TABLE X

Showing the Value of Clearing Liver Agar for the Isolation of Bact. abortus from Milk.

Milk 805B1	Liver Agar Not Cleared		Liver Agar Cleared with Egg Albumin	
R.R. Quarter	0	0	0	0
R.F. Quarter	0	0	92	170

Slants were also made from the above flasks and seeded with different laboratory strains and placed under different conditions of incubation.

Strain D-1 had been recently isolated from a fetus aborted as a result of natural infection; strain 805B1 had been recently isolated from an aborted fetus from the College Experimental Herd; and strains LUCAS and 200 had been grown in the laboratory for some time.

The slants were incubated aerobically and in an atmosphere of 10 percent carbon dioxide. Table XI shows the amount of growth recorded at the end of 24 hours' incubation.

TABLE XI

Shows the Value of Clearing Liver Agar with Egg Albumin for the Cultivation of Laboratory Strains of Bact. abortus.

	Liver agar Not Cleared	Liver Agar Cleared With Egg Albumin
Strain D-1 aerobic	+	++
Strain 805B1 aerobic	+	++
Strain LUCAS aerobic	++	++
Strain 200 aerobic	+++	+++
Strain D-1 10% CO_2	+++	++++
Strain 805B1 10% CO_2	+++	++++

+ = Amount of Growth Corresponding to Number Recorded

The above tables indicate that liver agar is much improved for the isolation and cultivation of Bact. abortus by clearing with egg albumin. This may be due either to the fact that beneficial products are added or that injurious substances are subtracted from the media by the egg albumin.

(10) The Effect of Incubating Aerobically Before Placing in An Atmosphere of 10 Percent Carbon Dioxide on the Growth of Bacterium abortus.

It has been the custom of investigators to incubate, aerobically, for a period of 12 hours, plates cultured with Bact. abortus material before placing under conditions more favorable for the growth of this organism. Contaminating colonies which had developed at the end of this period were marked, thereby showing clear areas upon which the colonies of Bact. abortus would develop when incubated again.

In an effort to determine the effect of this preliminary incubation period upon the growth of Bact. abortus, milk plates were prepared from the right rear and right front quarter of animal No. 2 Plates in duplicate from each quarter were placed directly in an atmosphere of 10 percent carbon dioxide and a corresponding number of plates from each quarter were incubated aerobically for a period of 36 hours before placing in an atmosphere of 10 percent carbon dioxide. Both sets of plates were incubated in carbon dioxide for a period of 3 days. The number of colonies obtained per plate is shown in table XII.

TABLE XII

Shows Effect of Holding Plates Aerobically before Placing in an Atmosphere of 10 percent Carbon dioxide on the Isolation of Bact. abortus.

	R.R. Quarter		L.R. Quarter	
Direct in CO_2	73	97	213	264
Aerobic 36 hrs.	0	0	92	170

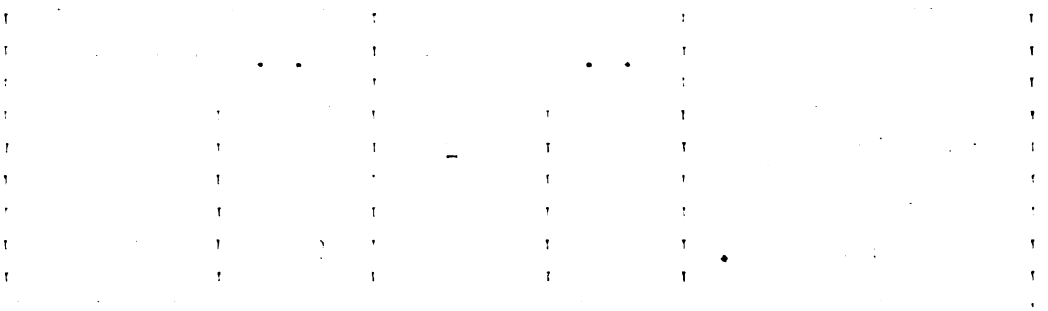
Following the same procedure as given above, a second series of plates was made. One set of plates was placed directly in an atmosphere of 10 percent carbon dioxide, and a second set was incubated aerobically for a period of 12 hours before being placed in carbon dioxide. The number of colonies developing per plate is shown in table XIII.

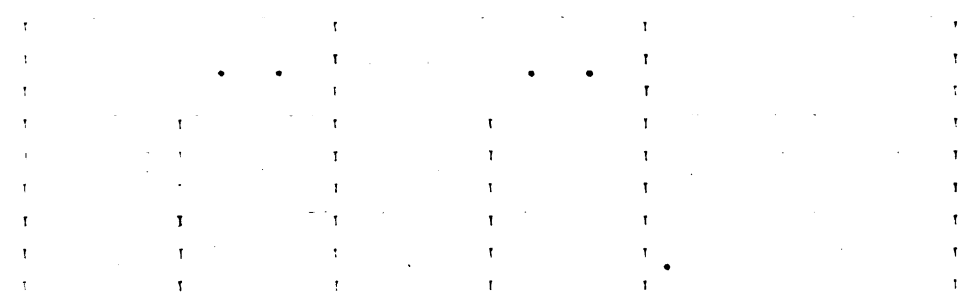
TABLE XIII.

Shows Effect of Holding Plates Aerobically before Placing in an Atmosphere of 10 percent Carbon Dioxide on the Isolation of Bact. abortus.

	R. R. Quarter		L. R. Quarter	
Direct in CO_2	1	12	97	144
Aerobic 12 hrs.	0	2	3	20

In order to obtain additional data, eight samples were collected from the right rear quarter of animal No. 2 and plated and





incubated as above. Table XIV shows the results obtained.

TABLE XIV.

Shows Effect of Holding Plates Aerobically before Placing in an Atmosphere of 10 percent Carbon Dioxide on the Isolation of Bact. abortus.

Sample No.	1	2	3	4	5	6	7	8	Total	Av.
Direct in CO_2	1	1	18	0	150	120	0	60	350	43
Aerobic 12 hrs.	6	13	0	0	0	0	2	14	35	4

The above results show that, on the average, a greater number of colonies of Bact. abortus develop on plates which are immediately placed in an atmosphere containing 10 percent carbon dioxide than on those plates which are first incubated aerobically and later placed under the influence of carbon dioxide.

(11) The Value of Centrifuging as an Aid in the Isolation of Bacterium abortus from Milk.

In an attempt to determine the value of centrifuging as an aid in the isolation of Bact. abortus from milk, the following experiment was undertaken:

One tenth cubic centimeter of milk was plated from each sample collected, after which the same sample was centrifuged for two hours at 2000 revolutions per minute. After centrifuging, plates were made from the cream, middle milk, and sediment from

from each sample. The four series of plates from each sample were then incubated in the same closed chamber in order to give identical conditions of incubation to each plate. The medium used was gentian violet liver agar; the plates were incubated under the influence of 10 percent carbon dioxide.

The milk was obtained from three animals, selected because of the difference in numbers of Bact. abortus per cubic centimeter in their milk. From 1 to 200 colonies per plate were isolated from the right rear quarter of animal No. 1; from 1 to 400 colonies per plate were isolated from the right front quarter of the same animal; from 1 to 8 colonies per plate were isolated from animal No. 2; while no colonies developed on any of the plates from animal number three.

The count of Bact. abortus per plate is shown in tables XV, XVI, XVII, and XVIII.

TABLE XV.

Shows the Number of Colonies of Bact. abortus developing from Milk from the Right Rear Quarters of Animal No. 1 before and after Centrifuging.

	Before Centrifusing	After Centrifusing		
	Whole Milk	Cream	Middle Milk	Sediment
Animal #1 R.R.quarter	19	0	0	4
"	23	0	0	13
"	113	3	3	3
"	158	17	23	2
"	183	0	3	0
"	140	0	7	13
"	10	0	0	0
"	25	5	1	0
"	5	0	21	4
"	21	0	0	0
"	24	0	0	0
"	8	0	0	0
"	1	0	0	0
"	0	0	0	0
"	6	0	0	0
"	13	0	0	0
"	0	0	0	1
"	0	0	0	0
Total	749	25	58	40
Average	42	1.4	3.2	2.2

TABLE XVI.

Shows the Number of Colonies of Bact. abortus developing from the Right Forward Quarter of Animal No. 1 before and after centrifuging.

	Before Centrifuging		After Centrifuging	
	Whole Milk	Cream	Middle Milk	Sediment
Animal #1 R.F.quarter	87	0	0	3
"	151	0	2	21
"	250	0	30	1
"	275	33	3	17
"	360	40	0	33
"	77	13	0	3
"	113	0	3	0
"	203	36	26	0
"	87	3	4	0
"	2	0	0	0
"	136	0	0	0
"	0	0	0	0
"	0	0	0	0
"	0	3	0	0
"	0	0	0	0
"	2	0	0	0
"	14	0	0	0
Total	1,757	128	68	78
Average	103	7.5	4.0	4.5

TABLE XVII

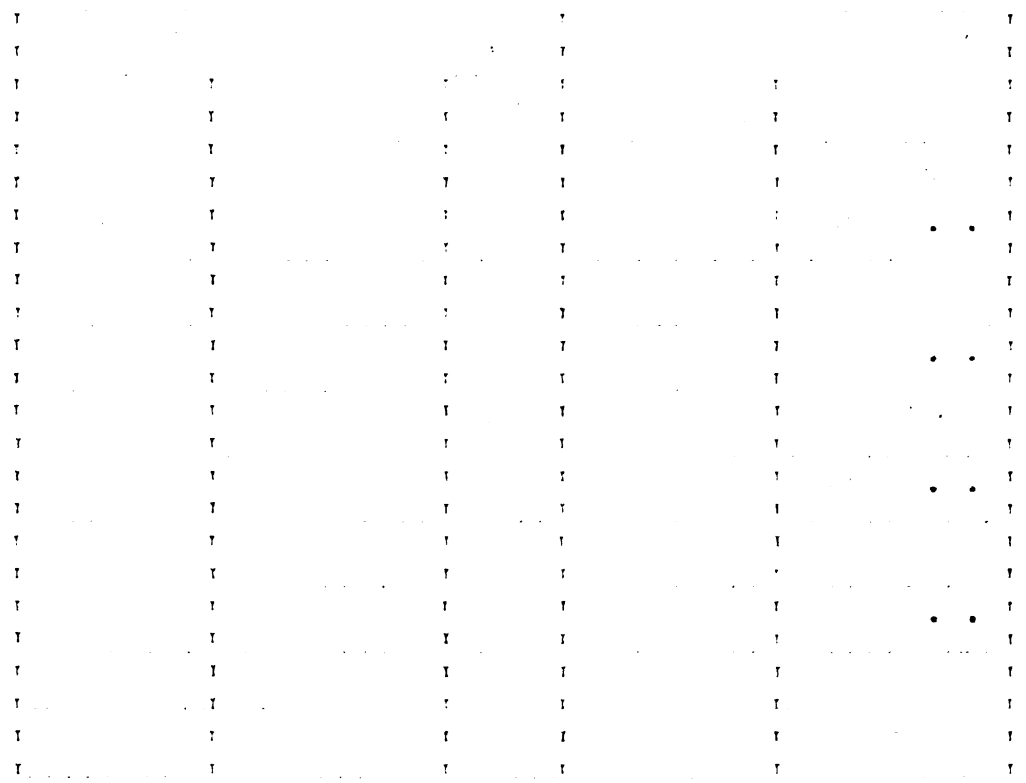
Shows Number of Colonies of Bact. abortus developing from Milk from Animal No. 2 before and after Centrifuging.

	Before Centrifuging	After Centrifuging		
	Whole Milk	Cream	Middle Milk	Sediment
Animal #2 R.R.quarter	2	0	0	0
"	8	0	0	0
R.F.quarter	0	0	0	0
"	0	0	0	0
L.R.quarter	0	0	0	0
"	0	0	0	0
L.R.quarter	1	0	0	0
"	1	0	0	0
Total	12	0	0	0

TABLE XVIII

Shows Number of Colonies of Bact.abortus developing from Milk from Animal No. 3 before and after Centrifuging.

Before Centrifuging	After Centrifuging			
	Whole Milk	Cream	Middle Milk	Sediment
Animal #3 R.R.quarter	0	0	0	0
"	0	0	0	0
R.F.quarter	0	0	0	0
"	0	0	0	0
L.R.quarter	0	0	0	0
"	0	0	0	0
L.F.quarter	0	0	0	0
"	0	0	0	0
Total	0	0	0	0



As is shown in table XV, 18 samples from the right rear quarter of animal No. 1 gave a total of 749 colonies on the plates from milk not centrifuged; 25 colonies on the plates streaked with cream; 58 colonies on the plates from the middle milk; and 40 colonies on the plates streaked with sediment.

Table XVI shows 17 samples from the right front quarter of the same animal with a total of 1,757 colonies on the plates from milk not centrifuged; 128 colonies on the plates streaked with cream; 68 colonies on the plates from the middle milk; and 78 colonies on the plates streaked with sediment.

Table XVII shows 8 samples from animal No. 2 with a total of 12 colonies on the plates from milk not centrifuged, while all plates made after centrifuging failed to show growth.

Table XVIII shows that 8 samples from animal No. 3 failed to show growth either before or after centrifuging.

From the above data it appears that centrifuging milk does not aid in the isolation of Bact. abortus. The total number of colonies developing on plates from milk not centrifuged was 2,518, and the total number of colonies developing on the three series of plates made after centrifuging was but 397. From each individual sample the tables show that in 50 out of 51 cases the number of colonies developing from the milk before centrifuging was far greater than the total number developing on the three series of plates from the milk after centrifuging.

Figure 1.

Figure 1 represents a photographed Petri dish showing the growth of Bact. abortus on peptic digest agar when incubated in an atmosphere of 10 percent carbon dioxide. The colonies were isolated from the spleen of a diseased guinea pig.

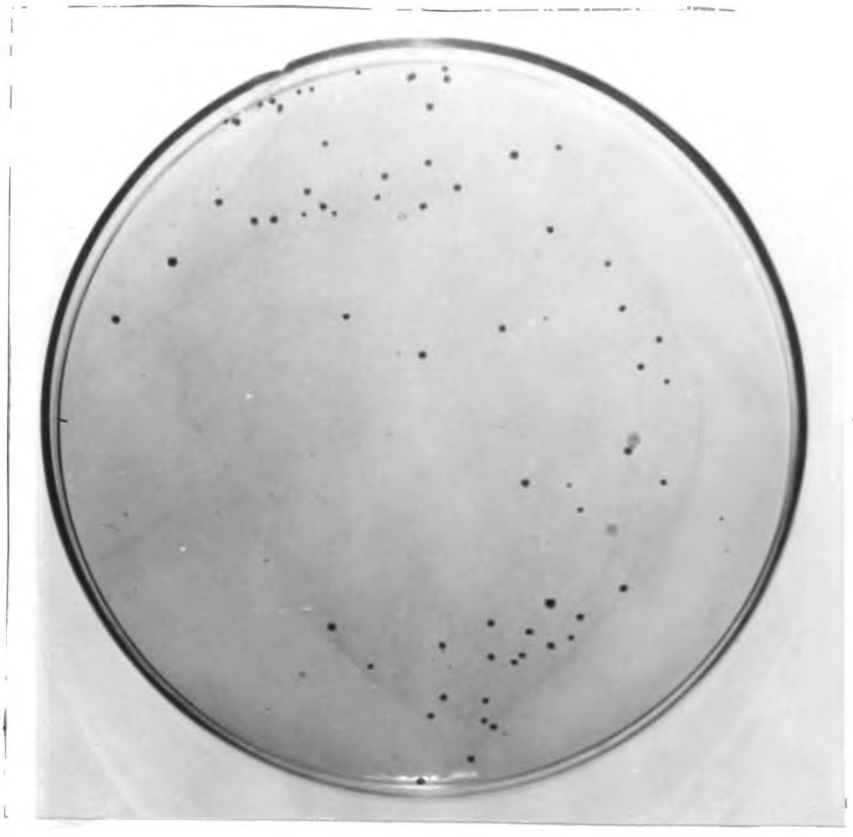


Figure 2.

Figure 2 represents a photographed Petri dish showing the growth of Bact. abortus on gentian violet liver agar, when incubated in an atmosphere of 10 percent carbon dioxide. The colonies were isolated from milk.

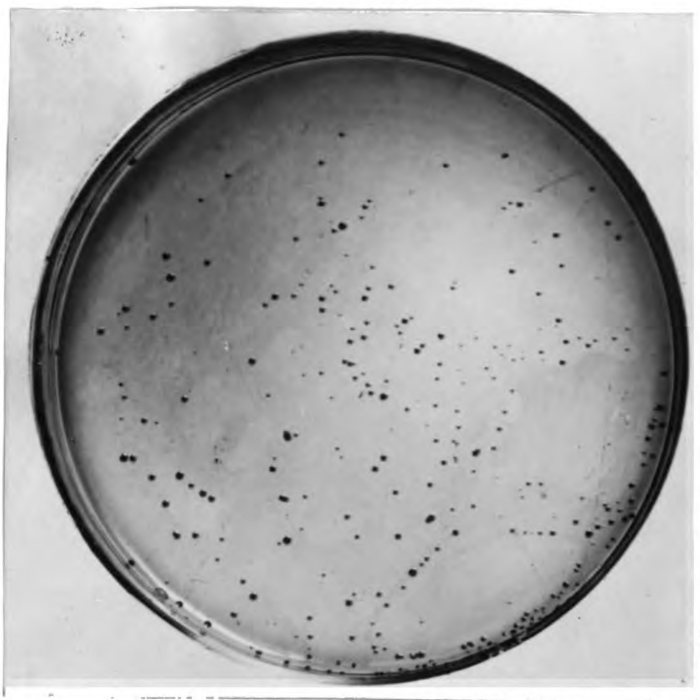


Figure 3.

Figure 3 represents a photographed Petri dish showing the growth of Bact. abortus on gentian violet liver agar when incubated in an atmosphere of 10 percent carbon dioxide. The colonies were isolated from milk.

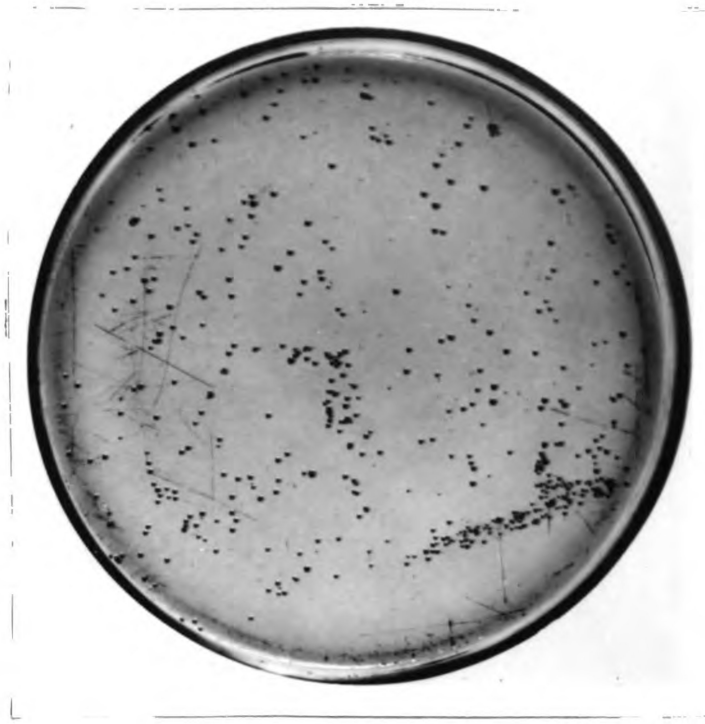


Figure 4.

Figure 4 represents a photographed Petri dish showing the growth of Bact. abortus on gentian violet liver agar when incubated in an atmosphere of 10 percent carbon dioxide. The colonies were isolated from milk.

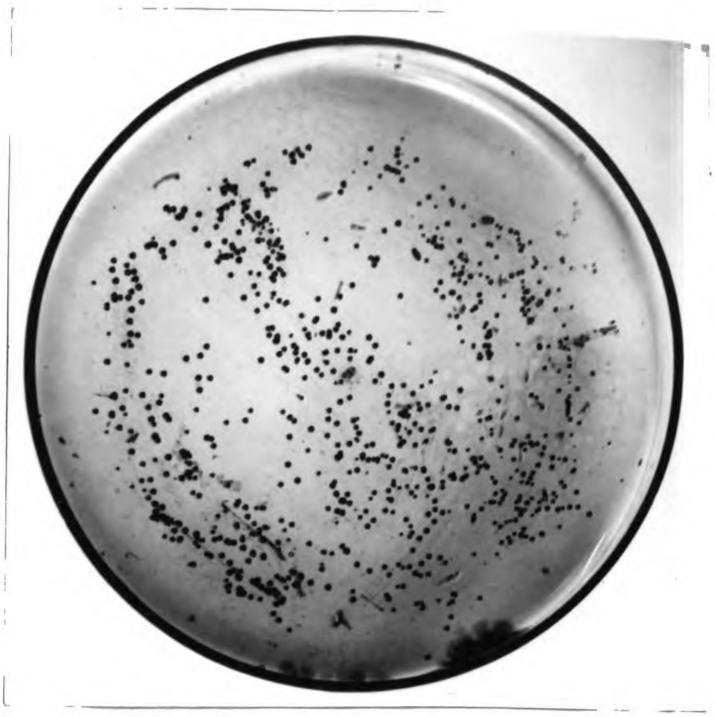


Figure 5.

Figure 5 represents a photographed Petri dish showing the growth of Bact. abortus on gentian violet liver agar when incubated in an atmosphere of 10 percent carbon dioxide. The colonies were isolated from the spleen of a diseased guinea pig.

GENERAL DISCUSSION.

Experiments have been outlined dealing with different methods of isolating and cultivating Bact. abortus. The first part of the experimental work gives data in regard to the isolation and cultivation of Bact. abortus from infected material by means of various media and different methods of incubation. The second part of the experimental work gives data showing, (a) the effect the manner of preparation of liver agar has on the isolation and cultivation of Bact. abortus; and (b) the number of colonies of Bact. abortus isolated from milk under different methods of plating.

The first five tables give a comparative value of several media for the isolation of Bact. abortus as well as a comparative value of various methods of incubation. From a study of these five tables it appears that gentian violet liver agar surpasses other media used for the isolation of Bact. abortus. Peptic digest agar gave satisfactory results, but the method of preparation of this medium is more complicated than is that of liver agar. Plates incubated under the influence of 10 percent carbon dioxide gave the best growth.

The sixth table gives a comparative value of several media and the comparative value of various methods of incubation in regard to the cultivation of previously isolated strains of Bact. abortus. Liver agar gives the

best growth in comparison with the other media. In the case of the recently isolated strains, the use of carbon dioxide gives the maximum growth; for the older strains satisfactory results were obtained by culturing on liver agar and incubating aerobically.

Tables VII to XI inclusive give data in regard to the preparation of liver agar. These results show that the addition of serum to liver agar is of no value in the isolation of Bact. abortus; that the organism develops over a range of pH concentration of 6.0 to 7.6 with the best results obtained at a concentration of 6.6 to 6.8; and that in the preparation of liver agar it is necessary, to obtain satisfactory results in the isolation and cultivation of Bact. abortus, to clear the media with egg albumin before filtering. These results confirm the work of Huddleson. (15)

Tables XII to XIV inclusive give data in regard to methods used in incubating plates in carbon dioxide. The results show that the greatest amount of growth is obtained when the cultured plates are placed directly under the influence of carbon dioxide; the older method was to place the plates under aerobic conditions for a period of 12 hours before placing under a carbon dioxide tension.

Tables XV to XVIII inclusive give data in regard to the technic of isolating Bact. abortus from milk. Fifty-one samples of milk obtained from four animals

were plated in an effort to determine the effect of centrifuging milk as an aid in isolating. The total number of colonies developing on the plates made before centrifuging being 2,518 while the total number of colonies developing on the three series of plates made after centrifuging being but 397. These results indicate that milk should not be centrifuged when one is endeavoring to isolate Bact. abortus, but that direct platings should be made in order to obtain the most successful results.

SUMMARY.

Gentian violet liver agar proved the best medium studied for the isolation of Bact. abortus.

Cultures incubated in a closed jar in which 10 percent of the contained air had been displaced by carbon dioxide gave the most luxuriant growth in comparison with other methods of incubation. The cultured plates should be placed immediately under the influence of this gas.

For the cultivation of strains of Bact. abortus which have been in the laboratory for some time and grow aerobically, slants of liver agar give the best growth in 24 hours' incubation. In the case of recently isolated strains it was found that liver agar incubated in a closed jar containing 10 percent carbon dioxide gave the best growth in 24 hours' incubation.

For the most successful growth of Bact. abortus, liver agar should be adjusted to a pH concentration of 6.6 to 6.8; it should be cleared with egg albumin during its preparation; and the serum should not be added.

In the isolation of Bact. abortus from milk by direct plating methods, the milk should be plated directly as obtained and not centrifuged in an effort to throw the organisms out of suspension.

ACKNOWLEDGMENT.

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